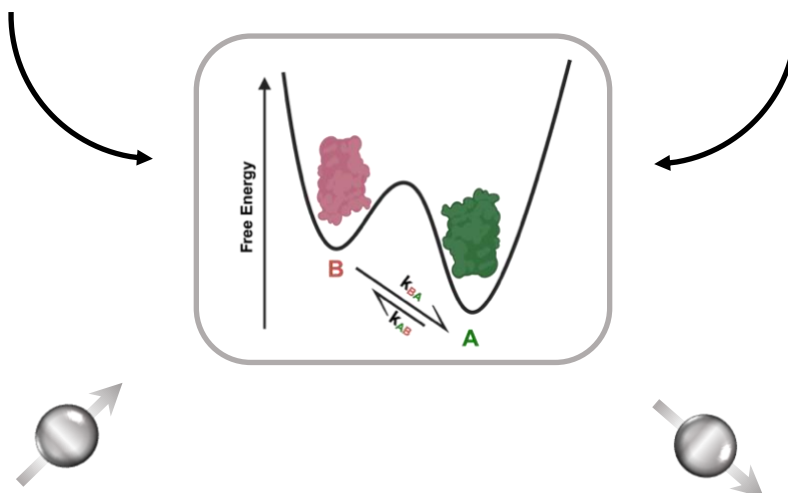
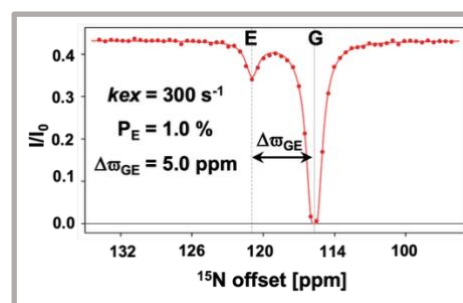
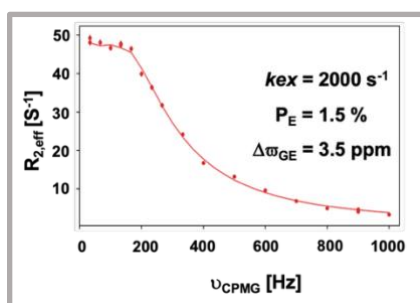


BIOMOLECULAR NMR OF PROTEINS AND PEPTIDES: STRUCTURAL ANALYSIS AND ATOMIC-RESOLUTION FUNCTIONAL DYNAMICS

Alexis Marius WANKO NEMBOT



A dissertation submitted in fulfillment of the degree of Doctor of Philosophy (PhD) in Sciences

Supervisor: Prof. Christian DAMBLON

Academic year 2025-2026

« Oui, c'est le SEIGNEUR qui donne la sagesse, la science et l'intelligence viennent de lui. »

Proverbes 2 : 6 — Bible, version PDV2017

Dedication

Je dédie cette dissertation

À mon épouse Line Kengne

À mes enfants Manuel-Arthur Wanko et Mathéïs Wanko

À mes parents Jacqueline Wanko et François Nembot

Acknowledgement

Au terme de cette recherche, je rends grâce au Seigneur, mon Dieu, pour Son Esprit, pour l'humilité, la force, la sagesse et la persévérance qu'Il m'a accordées afin de mener à terme cette recherche. Je reconnais humblement que sans Lui, rien de ce travail n'aurait été possible. La bible déclare : « ³Je l'ai rempli de l'Esprit de Dieu, de sagesse, d'intelligence et de savoir pour toutes sortes d'ouvrages, ⁴je l'ai rendu capable de faire des inventions, de travailler l'or, l'argent et l'airain, ⁵de graver les pierres à enchâsser, de travailler le bois et d'exécuter toutes sortes d'ouvrages. » Exode 31 : 3-5 — Bible, version LSG. Ces paroles ont été pour moi une source constante d'inspiration et de réconfort tout au long de ce parcours exigeant. À l'image des magiciens de Pharaon qui reconnurent, en leur temps, l'œuvre divine en déclarant : « ... C'est le doigt de Dieu ... » (Exode 8 :19), je déclare aujourd'hui avec foi, force et reconnaissance que cette recherche est, elle aussi, le fruit de la grâce de Dieu. Que toute la gloire Lui revienne, Lui qui donne l'intelligence, éclaire l'esprit et conduit les œuvres humaines à leur accomplissement.

À mon promoteur, Christian Damblon, j'exprime ma profonde et sincère gratitude. Merci de m'avoir accueilli au sein du laboratoire de Chimie Biologie Structurale (CBS) et de m'y avoir ouvert les portes de la Résonance Magnétique Nucléaire (RMN). Tu as été pour moi bien plus qu'un promoteur : un véritable mentor tout au long de ces onze années de recherche. Cinq premières années à mi-temps, alors que je travaillais parallèlement dans le nettoyage industriel ; les trois années suivantes à temps plein, rendues possibles grâce à une bourse accordée par le CBS ; et enfin les trois dernières années à mi-temps, durant lesquelles je suis devenu collaborateur indépendant. Ce parcours atypique n'aurait jamais pu aboutir sans ton soutien constant. Je me souviens encore avec émotion de ce jour d'octobre 2015 où tu as accepté d'être mon promoteur. À cette époque, je ne connaissais rien à la RMN, et pourtant tu m'as fait confiance, tu m'as accueilli et formé avec une patience remarquable. Malgré les difficultés et les incompréhensions qui ont jalonné ce long chemin, tu as toujours été présent, patient, humble et profondément compréhensif. Au fil des années, tu as été bien plus qu'un encadrant scientifique : tu as été un guide, un soutien, un conseiller et un père durant cette aventure scientifique et humaine. Merci infiniment pour tout. Que le Seigneur te bénisse abondamment, toi et toute ta descendance.

J'aimerais également exprimer ma profonde reconnaissance à celles et ceux qui ont contribué directement à ce travail et s'y sont impliqués de manière significative.

Je remercie Alexander N. Volkov pour m'avoir accueilli à la Vrije Universiteit Brussel (VUB) et pour avoir pris le temps de me former à la détermination de la structure tridimensionnelle des protéines par résonance magnétique nucléaire.

J'adresse un remerciement tout particulier à Guillaume Bouvignies et Ulric B. Le Paige (Sorbonne Université) pour leur précieuse expertise, leur soutien et leurs conseils techniques, leur disponibilité et les nombreuses discussions scientifiques enrichissantes autour des phénomènes de relaxation en RMN.

Je remercie également Frédéric Cawez, avec qui j'ai passé de nombreuses heures en production de protéines, parfois jusque tard dans la nuit, et sans qui je n'aurais pas pu étudier les enzymes TEM-1 et TEM-121. J'associe à ces remerciements l'ensemble de l'équipe du CIP, et plus particulièrement Alain Brans, pour la formation dispensée en production de protéines ainsi que pour leur soutien technique.

À la suite de Frédéric Cawez, je tiens à remercier Moreno Galleni pour son implication constante et le suivi attentif visant à finaliser la partie consacrée à la relaxation RMN des enzymes de la famille TEM dans cette recherche. Je le remercie tout particulièrement pour sa disponibilité, ses conseils avisés et son engagement tout au long de cette étape essentielle de ma recherche.

Enfin, j'aimerais exprimer ma profonde gratitude aux personnes proches de moi, en dehors du cadre scientifique, qui m'ont soutenu et encouragé tout au long de cette recherche. J'espère que ce manuscrit vous rendra fiers et vous montrera à quel point votre soutien a été essentiel à l'aboutissement de ce travail.

Je remercie tout particulièrement Hughes Lowe, qui a pris sur lui de me permettre de poursuivre mes études en Belgique en finançant mon voyage ainsi que la première année de mes études. Merci infiniment, grand frère, pour ta générosité et ta confiance. Que le Seigneur te bénisse abondamment, toi et toute ta descendance.

Je remercie de tout cœur mon épouse Line Kengne, qui a payé le prix des sacrifices et des frustrations ayant rythmé mon quotidien tout au long de cette recherche. Merci pour ta patience, ton soutien constant et indéfectible, pour ta présence rassurante dans les moments de doute, et pour l'amour que tu m'as témoigné chaque jour. Merci surtout pour tes prières fidèles, qui ont contribué à ce que l'Esprit du Seigneur m'éclaire davantage et me donne la force d'aller jusqu'au bout.

Je remercie avec une profonde émotion et affection mes deux enfants, Manuel-Arthur et Mathéïs, pour leurs sourires et la force qu'ils me donnent chaque soir à mon retour. Vous avez été une source inépuisable de motivation et de joie.

Enfin, je ne saurais conclure sans remercier mes amis et mes proches, que je n'ai pas nommément cités ici. Merci de m'avoir accompagné tout au long de ce parcours à travers nos échanges constructifs et votre soutien.

Table of contents

Table of Contents

Part I. Introduction	19
Part II. NMR Spectroscopy and Research Objectives	25
Chapter I. NMR Spectroscopy: Principles and Applications.....	27
I.1. Principles	29
I.2. Applications.....	34
Chapter II. Objectives.....	39
Part III. NMR-based Structure Determination of Proteins and Peptides	51
Chapter III. NMR-based structure determination of Proteins	53
Chapter IV. NMR-based structure determination of Peptides.....	81
IV.1. Overview of the standard approach	82
IV.2. Defining disulfide pairings.....	86
IV.3. Structural characterization of non-canonical amino acids.....	87
IV.4. Structural Restraints and Peptide Structure Determination by NMR.....	89
Chapter V. Research Contribution.....	91
Part IV. NMR Relaxation	115
Chapter VI. Introduction	117
Chapter VII. Theoretical foundations of NMR relaxation on the picosecond–nanosecond (ps–ns) timescale	123
VII.1. Longitudinal and transverse relaxation rates and heteronuclear Nuclear Overhauser Effect	129
VII.2. Model Free Analysis (MFA)	139
Chapter VIII. Theoretical Foundations of NMR Relaxation on the Microsecond–Millisecond (μ s–ms) Timescale.....	149
VIII.1. Introduction	151
VIII.2. CPMG relaxation dispersion experiment	154
VIII.3. CEST experiment	163
VIII.4. CPMG and CEST Data Analysis	170
Chapter IX. Research Contributions	173
IX.1. Structural and Functional Insights into CspA (Under Review at Journal of Magnetic Resonance).....	175

IX.2. Antibody Modulation of TEM-1 Conformational Dynamics (manuscript in preparation)	209
IX.3. Resistance Mutation Enhances TEM β -Lactamase Dynamics (manuscript in preparation)	247
Part V. General Conclusions & Perspectives	269
Chapter X. General Conclusions	271
Chapter XI. Perspectives	277
Bibliography	283

List of Abbreviations

- 1D: One-dimensional NMR spectrum (e.g. ^1H spectrum)
- 2D: Two-dimensional NMR spectrum (e.g. 2D-HSQC)
- 3D: Three-dimensional NMR spectrum (e.g. HNCA)
- ADR: Ambiguous Distance Restraints
- AHP: afterhyperpolarization
- AIC: Akaike's Information Criterion
- ARIA: Ambiguous Restraints for Iterative Assignment
- ATB: Automated Topology Builder
- BK: Large conductance calcium-activated K^+
- BLI: Biolayer Interferometry
- BMRB: Biological Magnetic Resonance Bank
- CCD: Chemical Component Dictionary
- CCPN: Collaborative Computational Project for NMR
- CEST: Chemical exchange saturation transfer
- CNS: Crystallography & NMR System
- COSY: COrrrelation SpectroscopY
- CPD: composite-pulse decoupling
- CPMG: Carr–Purcell–Meiboom–Gill
- CSA: Chemical shift anisotropy
- CSDs: cold-shock domains
- CSP: Chemical Shift Perturbation
- CSPs: Cold-shock proteins
- cw: continuous wave
- CYANA: Combined Assignment and Dynamics Algorithm for NMR Applications
- DD: dipole–dipole
- DIANA: Distance Ange Dynamics for NMR Applications
- DQF-COSY: Double Quantum Filtered COSY
- DYANA: DYnamics Algorithm for Nmr Applications
- EFG: electric field gradient
- FLYA: fully automated structure determination algorithm
- GROMOS: GROningen MOlecular Simulation
- HCN: Triple-resonance
- IK: intermediate-conductance calcium-activated K^+
- InChI: International Chemical Identifier
- INEPT: Insensitive Nuclei Enhancement by Polarization Transfer
- MFA: Model-free Analysis
- mmCIF: Macromolecular Crystallographic Information File

- MRI: magnetic resonance imaging
- ncAAs : non-canonical amino acids
- NMR: Nuclear Magnetic Resonance
- NOE: Nuclear Overhauser Effect
- NOESY: Nuclear Overhauser Effect Spectroscopy
- NSR: Nuclear Spin Relaxation
- PADLOC: pattern of disulfides from the local constraints
- PDB: Protein Data Bank
- rc-CPMG: relaxation-compensated CPM
- RDC: residual dipolar couplings
- RF: radiofrequency
- Rf-field: radiofrequency field
- RMSD: root mean square deviation
- RWA: rotating wave approximation
- SMILES: Simplified Molecular Input Line Entry System
- TOCSY: Total Correlation Spectroscopy
- TROSY: Transverse relaxation optimized spectroscopy
- VHH: Variable heavy-chain domain of heavy-chain antibody

Abstract

Proteins are dynamic molecular entities whose biological functions are governed not only by their three-dimensional structures but also by their intrinsic molecular motions. Nuclear Magnetic Resonance (NMR) spectroscopy provides a unique framework for simultaneously investigating protein structure and dynamics at atomic resolution in solution.

This dissertation exploits advanced NMR methodologies to probe both fast internal motions occurring on the picosecond–nanosecond timescale and low-populated conformational states involved in exchange processes on the microsecond–millisecond timescale. In particular, complementary approaches such as ^{15}N CPMG relaxation dispersion and chemical exchange saturation transfer (CEST) experiments were employed and analyzed to extract exchange parameters.

These methodologies were applied to a relatively wide range of protein sizes, including enzymatic systems from the TEM β -lactamase family, studied both in their free form (~ 30 kDa) and in complex with a nanobody (~ 45 kDa). The results reveal that ligand binding induces extensive and long-range dynamical rearrangements, demonstrating that nanobody binding modulates the conformational energy landscape of TEM enzymes and highlighting the central role of coordinated effects and conformational dynamics in enzymatic function.

In addition, NMR-based structural studies of a mesophilic cold shock protein CspA (~ 7.5 kDa), combined with NMR molecular dynamics, further illustrate how structure and dynamics are intricately linked to protein adaptation and function.

Finally, well-defined NMR solution structures of Leiurotoxin I and its synthetic analog Lei-Dab7 were determined. Structural investigations of these two peptides, combined with quantitative evaluation of their binding affinities [measured by Jean-Luc Hayen] toward the three human SK channel subtypes (hSK1–3), provide the first direct structural and functional comparison of Leiurotoxin I and Lei-Dab7, offering new insights into their interactions with human SK channels.

Overall, this research underscores the necessity of adopting a dynamic view of protein structure and function. By combining advanced NMR relaxation techniques with structural analysis, this dissertation contributes to a deeper understanding of biomolecular dynamics and provides methodological and conceptual insights applicable to a broad range of biological systems.

Résumé

Les protéines sont des entités moléculaires dynamiques dont les fonctions biologiques sont gouvernées non seulement par leur structure tridimensionnelle, mais également par leurs mouvements moléculaires intrinsèques. La spectroscopie de résonance magnétique nucléaire (RMN) constitue un outil unique permettant d'explorer la structure et la dynamique des protéines à résolution atomique en solution.

Cette thèse exploite des méthodologies avancées de RMN afin de sonder à la fois la dynamique moléculaire à l'échelle de temps picoseconde–nanoseconde et les états conformationnels faiblement peuplés impliqués dans des processus d'échange à l'échelle microseconde–milliseconde. En particulier, des approches complémentaires telles que les expériences de relaxation dispersion ^{15}N CPMG et de transfert de saturation par échange chimique (CEST) ont été mises en œuvre et analysées afin d'extraire des paramètres d'échange.

Ces méthodologies ont été appliquées à une gamme relativement large de tailles protéiques, incluant des systèmes enzymatiques de la famille des β -lactamases TEM, étudiés à la fois à l'état libre (~ 30 kDa) et en complexe avec un nanobody (~ 45 kDa). Les résultats montrent que la liaison du ligand induit des réarrangements dynamiques étendus et à longue portée, démontrant que la fixation du nanobody remodèle le paysage énergétique conformationnel des enzymes TEM et soulignant le rôle central des effets coordonnés et de la dynamique conformationnelle dans la fonction enzymatique.

Par ailleurs, des études structurales par RMN d'une protéine de choc thermique froid mésophile CspA ($\sim 7,5$ kDa), combinées à une étude de la dynamique moléculaire par RMN, illustrent également comment la structure et la dynamique sont étroitement liées à l'adaptation et à la fonction des protéines.

Enfin, des structures en solution RMN de la Leiurotoxine I et de son analogue synthétique Lei-Dab7 ont été déterminées. L'étude structurale de ces deux peptides, combinée à l'évaluation quantitative de leurs affinités de liaison vis-à-vis des trois sous-types de canaux SK humains (hSK1–3) [réalisée par Hayen Jean-Luc], fournit la première comparaison structurale et fonctionnelle directe entre la Leiurotoxine I et le Lei-Dab7, apportant de nouveaux éclairages sur leurs interactions avec les canaux SK humains.

Dans l'ensemble, ce travail souligne la nécessité d'adopter une vision dynamique de la structure et de la fonction des protéines. En combinant des techniques avancées de relaxation RMN avec des analyses structurales, cette thèse contribue à une meilleure compréhension de la dynamique des biomolécules et apporte des avancées méthodologiques et conceptuelles applicables à un large éventail de systèmes biologiques.

Part I. Introduction

The origins of nuclear magnetic resonance date back to the late 1930s, when Isidor Rabi and colleagues introduced the molecular beam magnetic resonance method to determine nuclear magnetic moments in isolated molecules such as LiCl, LiF, and Li₂ using atomic beams and oscillating magnetic fields (Rabi et al. 1939). In December 1945, Purcell, Torrey, and Pound independently extended these concepts to condensed matter, detecting weak radiofrequency signals arising from the nuclei of atoms in ordinary materials (Purcell et al. 1946). Almost simultaneously, Bloch, Hansen, and Packard carried out an independent experiment in which they detected radiofrequency signals from protons in water (Bloch et al. 1946). Together, these two pioneering experiments mark the birth of Nuclear Magnetic Resonance (Levitt 2013).

In his Nobel Prize lecture, Purcell gave a poetic reflection before turning to a fundamental question. Recalling the excitement of discovery, he said: *“I remember, in the winter of our first experiments, just seven years ago, looking on snow with new eyes. There is the snow lay around my doorstep - great heaps of protons quietly precessing in the earth’s magnetic field. To see the world for a moment as something rich and strange is the private reward of many a discovery. But I am afraid it has little bearing on the sober question we must, as physicists, ask ourselves: What can we learn from all this about the structure of matter?”*

In the years that followed, NMR rapidly evolved into a powerful physical tool for investigating matter. Key advances included the discovery of the chemical shift, the variation in resonance frequency arising from different atomic environments, and spin–spin coupling, which revealed how neighboring nuclei influence one another. These findings, reported by Gutowsky, Slichter, and McCall [early 1950s] (Gutowsky et al. 1951), transformed NMR from a physical curiosity into an indispensable method for elucidating molecular structure.

One of the most instructive ways to illustrate the power of NMR spectroscopy is to compare the first one-dimensional (1D) spectra of small molecules with those of macromolecules. The ¹H NMR spectrum of ethanol (**Figure 1**), for example, clearly displays the fundamental features of NMR: each line comes from a chemically equivalent group of protons in the molecule, the intensity being proportional to the number of hydrogen atoms involved, 3 (CH₃ group), 2 (CH₂ group), and 1 (hydroxyl), respectively, reflecting the relative number of nuclei in each environment (Purcell 1953). This simple spectrum demonstrates how NMR can provide both structural and quantitative information in solution.

In contrast, the ¹H NMR spectrum of a protein appears far more complex (**Figure 2**), with thousands of overlapping resonances distributed over a broader chemical shift range (especially in the amide region, ~7–10 ppm, and the aliphatic region, ~0–5 ppm). Although a 1D spectrum of a protein already conveys information about the folding state,

secondary structure, and dynamics, full structural elucidation requires higher-dimensional experiments to resolve spectral crowding (Wüthrich 1986).

Together, the simple ethanol spectrum and the more complex protein spectrum illustrate the evolution of NMR from a tool for analyzing small organic molecules to one capable of probing the structure and dynamics of large biomolecules in solution.

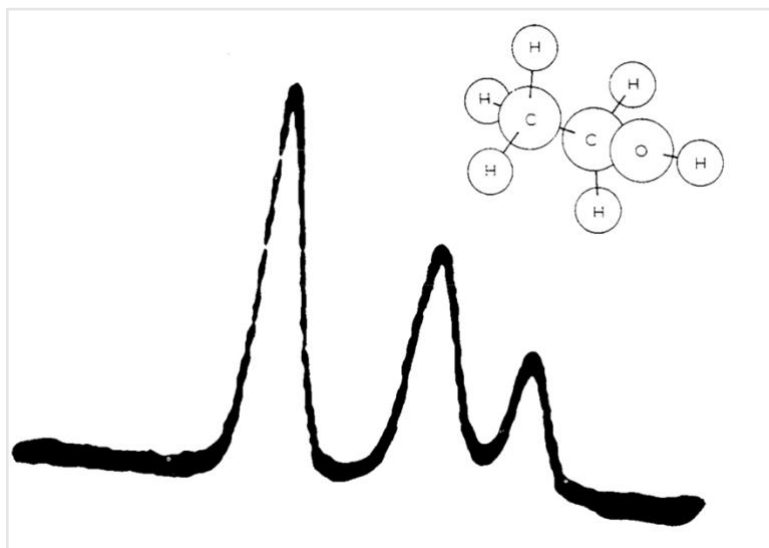


Figure 1. The first “high-resolution” proton NMR spectrum, recorded at 30 MHz, displaying the proton chemical shifts in ethanol. The three lines arise from the CH_3 hydrogens, from the CH_2 hydrogens, and from the OH hydrogen, respectively. Figure from (Purcell 1953)

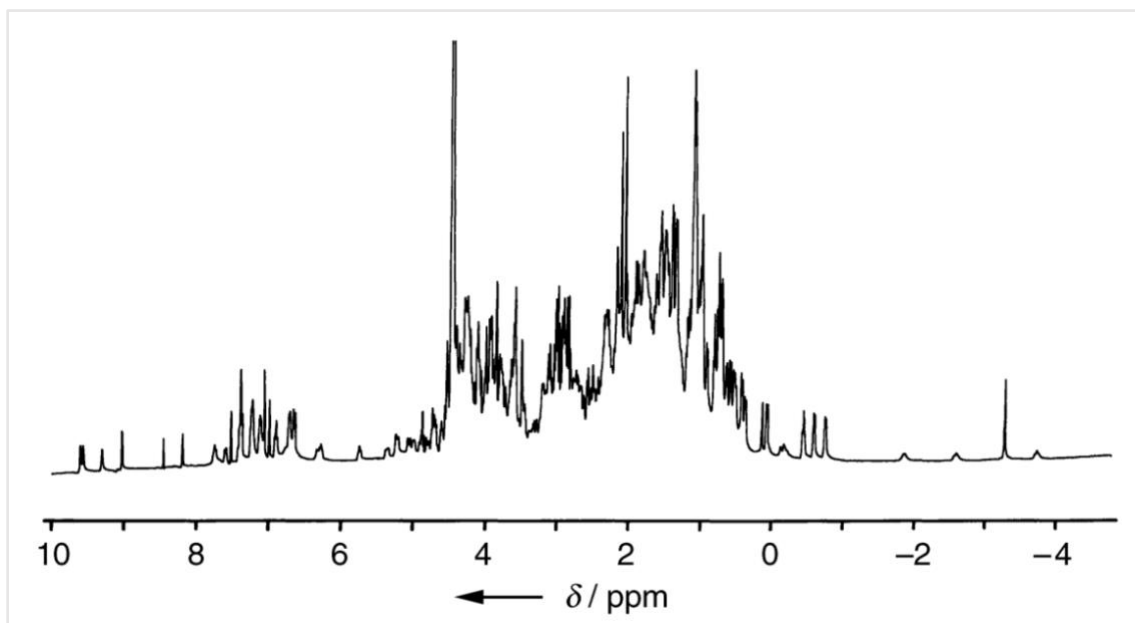


Figure 2. 1D ^1H NMR spectrum (360 MHz, 528C, solvent $^2\text{H}_2\text{O}$) of horse ferrocyanochrome c. Figure from (Wüthrich 2003)

Since its discovery, nuclear magnetic resonance (NMR) spectroscopy has become a powerful tool for investigating the structure and behavior of matter. Advances in magnet technology, pulse sequence design, and Fourier-transform methods have considerably expanded its scope, enabling applications that range from small molecules to complex biomolecular systems. Today, NMR contributes to diverse fields, including chemistry, structural biology, materials science, and medicine, notably through magnetic resonance imaging (MRI). This versatility reflects its unique ability to provide detailed insights into both structure and dynamics across multiple scales, highlighting that proteins and peptides are inherently dynamic rather than static entities. Their biological functions arise not only from their three-dimensional structures but also from their intrinsic motions. These motions span from fast local fluctuations to conformational exchange processes and play a key role in biomolecular function. Processes such as molecular recognition, enzymatic catalysis, and allosteric regulation are increasingly recognized to involve conformational dynamics. Consequently, a complete understanding of biomolecular function requires integrating structural information with a detailed characterization of these dynamic processes. In this regard, NMR spectroscopy is uniquely suited to investigate such dynamics, as it provides site-specific information over timescales ranging from picoseconds to seconds, a capability that is particularly important given that proteins and peptides are inherently dynamic rather than static entities.

Building on these capabilities, the next part introduces the fundamental principles of NMR spectroscopy and defines the research objectives addressed in this dissertation.

Part II. NMR Spectroscopy and Research Objectives

Chapter I.
NMR Spectroscopy: Principles and Applications

I.1. Principles

Atoms are composed of electrons and a central nucleus. NMR spectroscopy is based on the interaction between atomic nuclei and a strong external magnetic field (Levitt 2013). This interaction is made possible by an intrinsic property of certain atomic nuclei known as nuclear spin.

Each nucleus is characterized by a spin quantum number I (**Table 1.1**) which determines whether the nucleus possesses spin angular momentum ($\vec{I}$). Angular momentum describes the rotational motion of an object. Nuclei with a non-zero spin quantum number ($I \neq 0$) exhibit a fundamental property called the magnetic moment ($\vec{\mu}$). The magnetic moment ($\vec{\mu}$) is a measure of the strength and orientation of a magnetic dipole (Sarkar 2014), produced by a particle equipped with spin, such as an electron, proton, or atomic nucleus.

The relationship between the magnetic moment ($\vec{\mu}$) and the spin angular momentum ($\vec{I}$) is given by the equation:

$$\vec{\mu} = \gamma \vec{I} \quad \text{Equation 1.1}$$

where the constant γ is called the gyromagnetic ratio. It determines the resonance frequency (Larmor frequency) of a nucleus in a given magnetic field.

Table 1.1. Nuclear isotopes and their properties

Isotope	Quantum number I	Natural abundance	Gyromagnetic ratio ($\gamma/\text{rad s}^{-1} \text{T}^{-1}$)
^1H	1/2	~100 %	267.522×10^6
^2H	1	0.015 %	41.066×10^6
^{12}C	0	98.9 %	—
^{13}C	1/2	1.1 %	67.283×10^6
^{14}N	1	99.6 %	19.338×10^6
^{15}N	1/2	0.37 %	-27.126×10^6

In liquid NMR spectroscopy, nuclei with a spin quantum number $I = 1/2$ are the most commonly measured. These nuclei, such as ^1H , ^{13}C or ^{15}N , are ideal for NMR analysis because they possess both a non-zero magnetic moment and well-defined spin states, leading to sharp, easily interpretable resonance signals.

In contrast, nuclei with $I = 0$, such as ^{12}C , have no angular momentum and no magnetic moment. As a result, they do not interact with the external magnetic field and are not detectable by NMR.

Nuclei with spin quantum numbers $I > 1/2$ such as ^{14}N , have electric quadrupole moments due to their asymmetric nuclear charge distributions. These quadrupole interactions lead to rapid relaxation of the magnetic states, causing significantly broadened resonance lines and often reduced spectral resolution.

A nucleus with $I = 1/2$, such as ^1H , ^{13}C , or ^{15}N , has two allowed spin states when placed in an external magnetic field. The number of spin states is determined by the formula:

$$\text{Number of spin states} = 2I + 1 \quad \text{Equation 1.2}$$

Thus, for $I = 1/2$, the nucleus exhibits two spin states. These states are characterized by another quantum number m_I , which, according to quantum mechanics, can take values ranging from $-I$ to I in integer steps (Levitt 2013).

For a spin-half nucleus, this results in:

- $m_I = 1/2$ denoted as the α (alpha) state
- $m_I = -1/2$ denoted as the β (beta) state

The relative energies of these states depend on the sign of the gyromagnetic ratio (γ). For nuclei with a positive γ (e.g., ^1H and ^{13}C), the magnetic moment is parallel to the angular momentum, the α state (**Figure 1.1 a**) corresponds to the lower energy (**Figure 1.1 c**) and is aligned with the external magnetic field B_0 , whereas the β state has its magnetic moment opposed to B_0 (**Figure 1.1 b**) and is higher in energy (**Figure 1.1 c**) (Levitt 2013). In contrast, for nuclei with a negative γ (e.g., ^{15}N), the magnetic moment is oriented opposite to the angular momentum, resulting in a reversal of the α ($m_I = 1/2$) and β ($m_I = -1/2$) energy levels. In this case, the β state corresponds to the lower-energy state (magnetic moment aligned with B_0), while the α state is higher in energy (Levitt 2013).

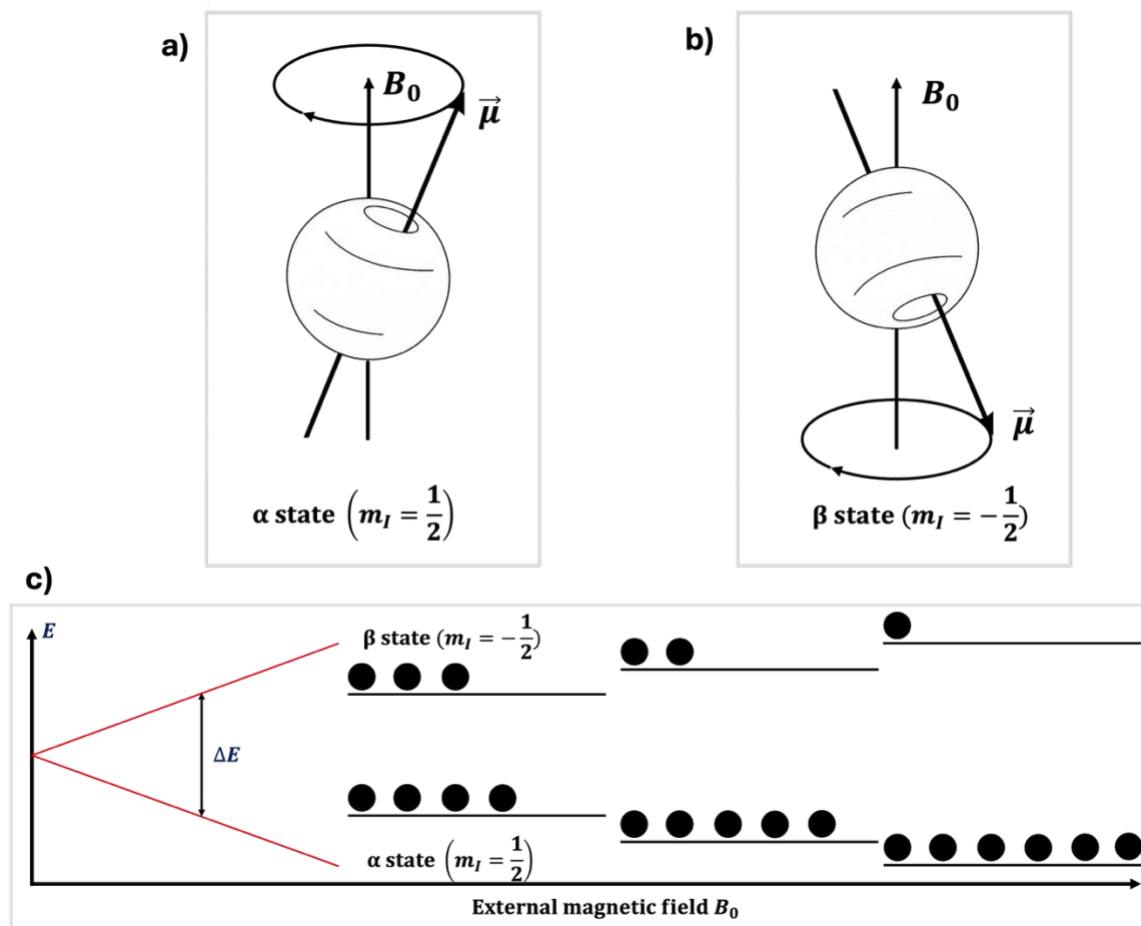


Figure 1.1. Nuclear spin precession, energy level splitting, and population difference in an external magnetic field B_0 for a nucleus with a positive gyromagnetic ratio ($\gamma > 0$).

As we can see in **Figure 1.1**, the magnetic moment of spin is a vector. The direction of the vector indicates the axis of the rotational motion. In this dissertation, the direction of the magnetic moment is called the spin polarization axis.

In general, nuclei with a non-zero spin quantum number such as proton, have a randomly directed spin polarization axes (**Figure 1.2 a**)

When these nuclei are placed in an external magnetic field B_0 , their intrinsic magnetic moments respond by turning around the direction of B_0 , always keeping the same angle between spin polarization axis and B_0 (**Figure 1.2 b**). This motion is called precession. These magnetic moments can adopt only two orientations, either parallel (low-energy state; $\gamma > 0$) or antiparallel (high-energy state; $\gamma < 0$) to the direction of the magnetic field. This phenomenon results from the interaction between the magnetic moment of the nucleus and the applied magnetic field, known as the Zeeman effect (Levitt 2013). The parallel orientation corresponds to a more stable, lower-energy configuration, while the antiparallel orientation is less stable and higher in energy. The difference in energy

between these two spin states enables the absorption of radiofrequency energy at a specific resonance frequency, called Larmor frequency ω_0 .

$$\omega_0 = \gamma B_0 \quad \text{Equation 1.3}$$

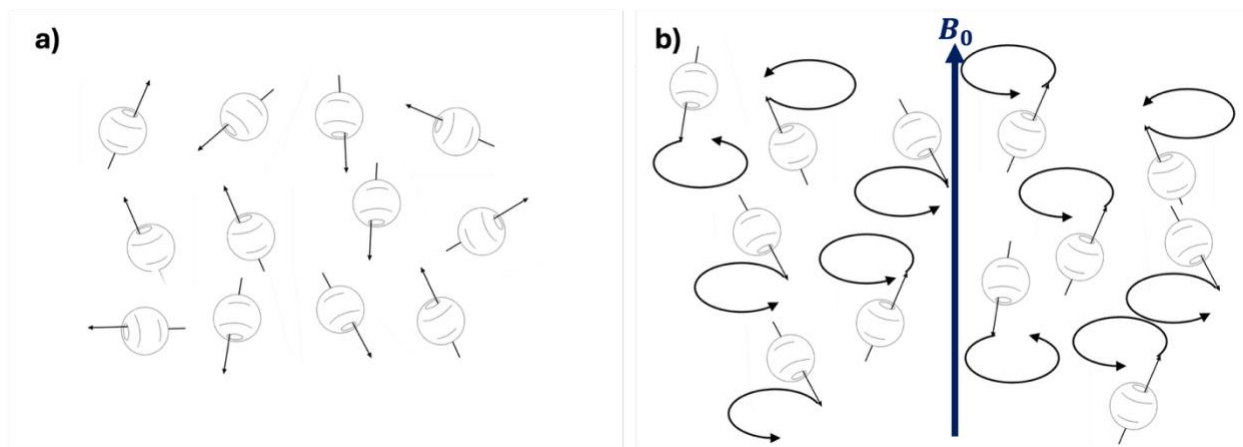


Figure 1.2. Randomly and aligned spins polarizations. a) Randomly directed spin polarizations, b) Spins precession with the same angle between spin polarization axis and the field

Since the population of the α state (N_α) is slightly greater than the β state (N_β), the slight excess of nuclei in the α state creates a net magnetization M_0 (**Figure 1.3**) that can be detected and analyzed to provide structural and chemical information about the sample. The population ratio between α and β states is governed by the Boltzmann distribution, and is relatively small, typically on the order of 10^{-4} under ordinary field ($B_0 \approx 11.74$ T) and room-temperature conditions (Levitt 2013), with its exact value depending on both magnetic field strength and temperature:

$$\frac{N_\alpha - N_\beta}{N_\alpha} \approx 10^{-4} \quad \text{Equation 1.4}$$

The net magnetization M_0 , which lies along the z -axis (the direction of the external magnetic field), cannot be directly detected by the NMR receiver coils. However, when a (RF) pulse is applied at the Larmor frequency and its magnetic component is oriented perpendicular to the z -axis, it leads to a rotation of the net magnetization M_0 into the transverse (xy) plane. This process is referred to as excitation. Once in the xy -plane, the transverse component of the magnetization precesses around the external magnetic field,

inducing an oscillating current in the receiver coil. This transverse magnetization is detectable and forms the basis of the recorded NMR signal (free induction decay).

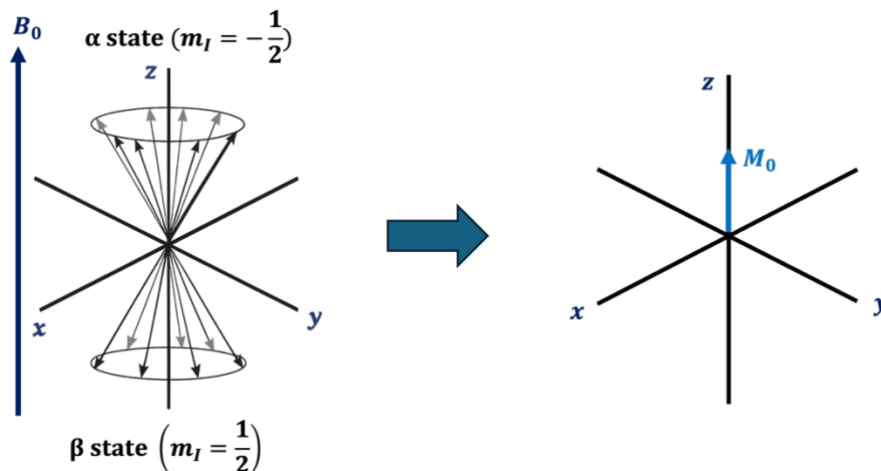


Figure 1.3. Net magnetization at thermal equilibrium for a nucleus with a positive gyromagnetic ratio ($\gamma > 0$).

When a nucleus is placed in an external magnetic field B_0 , electrons surrounding the nucleus begin to circulate, creating tiny local magnetic fields that oppose the applied field. This phenomenon is called diamagnetic shielding (Levitt 2013). As a result, the nucleus feels a slightly reduced magnetic field, known as the effective magnetic field B_{eff} :

$$B_{eff} = B_0(1 - \sigma) \quad \text{Equation 1.5}$$

where:

- B_{eff} is the experienced magnetic field of a nucleus
- σ is the shielding constant, determined by the local electronic environment.

Since the resonance frequency of a nucleus is determined by the local magnetic field it experiences (as indicated by **Equation 1.5**), nuclei of the same type can resonate at slightly different frequencies depending on their electronic environment. These variations in local magnetic fields cause the NMR signals of chemically non-equivalent nuclei to be spread across a range of frequencies. This phenomenon is known as the chemical shift, and it provides crucial information about the molecular structure and electronic surroundings of each nucleus. In addition, neighboring nuclei can influence one another through scalar spin–spin coupling (J-coupling), which arises from the indirect interaction of nuclear spins

mediated by bonding electrons. J-coupling splits NMR signals into multiplets, yielding information about the number and connectivity of neighboring nuclei, and thus adding another powerful dimension to structural analysis.

I.2. Applications

NMR spectroscopy is an essential analytical tool for the atomic-resolution investigation of biomolecules, particularly proteins and peptides. It offers detailed insights into several fundamental properties of macromolecules, including their three-dimensional (3D) structures, molecular dynamics, and intermolecular interactions. Unlike other structural methods, NMR enables the study of biomolecules in solution under near-physiological conditions, making it particularly well-suited for exploring the conformational flexibility, binding behavior, and functional mechanisms of proteins and peptides.

I.2.1. NMR-Based structure determination of Proteins and Peptides

In March 2026, a total of 179,893 protein structures in the Protein Data Bank (PDB), accounting for approximately 83%, were solved using X-ray crystallography, while only 12,793 structures, representing 6%, were determined by solution NMR spectroscopy. The remaining entries were resolved using methods such as cryo-electron microscopy (cryo-EM) and hybrid techniques.

One of the primary reasons for the lower number of protein structures determined by NMR compared to X-ray crystallography is the size limitation inherent to NMR spectroscopy. Unlike crystallography, which can often accommodate large macromolecular complexes, NMR is best suited for small to medium-sized proteins, typically below 35 kDa (Snyder et al. 2005; Yee et al. 2005). This limitation arises because larger molecules exhibit slower rotational tumbling in solution, leading to increased transverse relaxation and line broadening, which reduces signal intensity and spectral resolution. Specifically, the slower motion leads to broadened spectral lines and reduced signal sensitivity and resolution, making resonance assignment and structural interpretation increasingly difficult as molecular weight increases. With respect to small proteins, NMR spectroscopy and X-ray crystallography, the two primary experimental techniques for high-resolution protein structure determination can be considered complementary rather than competing methods (Savchenko et al. 2003; Snyder et al. 2005; Yee et al. 2005). Each technique offers unique advantages and has distinct limitations, particularly in terms of sample requirements. In a comparative study involving 263 proteins targets, their suitability for structural determination by either method was assessed (Yee et al. 2005). Interestingly, only 8% of the proteins were found to be compatible to both NMR and X-ray crystallography, while 16% were compatible exclusively with NMR and another 16% exclusively with X-ray crystallography (Yee et al. 2005). As described by Yee *et al.*, a decision to “stop work” for both methods was made when a structure of the protein, or

that of one of its orthologs is deposited the PDB (Yee et al. 2005). These results show the complementary nature of NMR spectroscopy and X-ray crystallography, demonstrating that each method can provide structural information for distinct subsets of proteins that may not be accessible by the other.

Consequently, there are proteins that can be readily crystallized but for which, high-quality NMR spectra cannot be obtained, as well as proteins that are well-suited for NMR spectroscopy but cannot be crystallized. In the latter case, crystallization is typically hindered by properties such as the presence of conformational heterogeneity, as only well-ordered, soluble proteins with a single dominant conformation tend to form suitable crystals (Gronwald and Kalbitzer 2004; Dobrianov et al. 1999; Smyth and Martin 2000). In contrast, NMR spectroscopy is uniquely capable of analyzing proteins that are partially disordered, intrinsically flexible, or exist in multiple conformational states, making it especially valuable for studying dynamic or non-crystalline protein systems (Gronwald and Kalbitzer 2004; Dobrianov et al. 1999; Smyth and Martin 2000).

Another factor contributing to the greater prevalence of X-ray crystallography is its widespread reputation for producing high-resolution structural data. X-ray crystallography is particularly valued for its ability to determine atomic-level details, often yielding structures with very high spatial resolution. However, the high resolution requires tightly packed crystal that is difficult to obtain with flexible proteins. A key advantage of NMR spectroscopy lies in its capacity to study proteins in solution, under conditions that closely mimic their physiological environment (Gronwald and Kalbitzer 2004; Schroeder and Rosengren 2020; Sun and Wagner 2006). This makes NMR especially suited for examining protein dynamics, flexibility, and conformational variability, aspects that are often less accessible through crystallographic methods.

Peptides, defined as short chains of amino acids linked by peptide bonds, play crucial roles in numerous biological processes, including signaling, immune response, and enzyme regulation. Peptides serve as key biological molecules with roles in signaling, regulation, host defense, and therapeutic development (Kuzmenkov et al. 2022; Armstrong et al. 2021; Dubovskii et al. 2015; Daniele-Silva et al. 2021; Walewska et al. 2008; Norton 2018). In parallel with the extensive use of NMR spectroscopy for protein structural analysis, peptides have emerged as highly suitable targets for NMR-based investigations.

Peptides are generally well suited to solution NMR because of their relatively small size, which often results in narrow line widths, well-resolved spectra, and favorable signal-to-noise ratios. Moreover, peptides that are unstructured, partially folded, or adopt multiple conformations in solution which can present challenges for crystallographic methods can be readily characterized using NMR. This makes NMR a powerful tool not only for elucidating static structures but also for probing conformational flexibility in peptides

(Kuzmenkov et al. 2022; Walewska et al. 2008; Norton 2018). In recent years, the application of multidimensional NMR techniques, often combined with isotopic labeling and advanced computational tools, has enabled the detailed structural characterization of diverse classes of peptides, including peptide toxins, signaling peptides, antimicrobial peptides, peptide hormones, and synthetic therapeutic peptides (Takai et al. 1992; Sharma et al. 2021; Webb 2018; Dubovskii et al. 2015; Armstrong et al. 2021; Kuzmenkov et al. 2022). NMR therefore represents a valuable and complementary method for the structural characterization of peptides, particularly when crystallization is not feasible or when solution-state behavior is of biological interest.

I.2.2. NMR relaxation

NMR spectroscopy is not only a highly informative technique for determining high-resolution structures of biomolecules, but it also plays a unique and critical role in exploring their dynamic behavior (Walinda et al. 2018). A key aspect of such dynamic investigations is the study of relaxation phenomena, which provides insights into molecular motion, conformational exchange, and interactions at atomic resolution (Kleckner and Foster 2011; Lu and Yang 2021; Tiwari et al. 2024; Walinda et al. 2018). Relaxation refers to the process by which nuclear net magnetization returns to equilibrium after being disturbed by a radiofrequency pulse. In structural biology, NMR relaxation measurements are widely used to probe internal flexibility, residue-specific motion, and interactions with ligands or other macromolecules. These dynamic features are often closely linked to biological function, particularly in enzymes, signaling proteins, and intrinsically disordered regions (Mittermaier and Kay 2006; Lu and Yang 2021; Tiwari et al. 2024; Bouvignies and Kay 2012; Khandave et al. 2024).

An illustrative example of the power of NMR relaxation techniques is their application in studying complex protein dynamics involving multiple, low-population intermediate states. In recent studies, NMR relaxation experiments have been employed to investigate millisecond-timescale interconversion between the folded (ground) state and one or more transiently populated intermediate conformations (Khandave et al. 2024, 2023; Knoverek et al. 2021; Madhurima et al. 2023; Rangadurai et al. 2020; Sekhar et al. 2015; Tiwari et al. 2024). These studies demonstrate that NMR relaxation extends well beyond its traditional applications, enabling the characterization of dynamic processes that are otherwise invisible to static structural methods. By employing various NMR relaxation techniques and analyzing the resulting data, researchers were able to derive quantitative kinetic models. These models offer critical insights into the folding pathways, energy landscapes, and functional motions of proteins, highlighting the essential role of NMR relaxation in understanding biomolecular dynamics at atomic resolution.

The principles and applications presented above establish the methodological framework used throughout this work. Based on these foundations, the present study applies NMR-based approaches to selected protein systems in order to investigate their structural properties and dynamic behavior under different functional states. The specific research objectives of this dissertation are presented in the next chapter.

Chapter II.

Objectives

Understanding the mechanisms that govern protein function remains a major challenge in biomolecular science and is often approached through structural characterization. However, static structures alone provide a partial description of molecular behavior. Increasing evidence shows that molecular dynamics play a crucial role in enabling functional processes. Incorporating information on dynamics is therefore essential to achieve a more complete understanding of protein function. NMR spectroscopy provides unique experimental access to both structure and dynamics across multiple timescales, complementing recent advances in structure prediction methods such as AlphaFold.

In this context, this dissertation aims to characterize the structure of biomolecules and, primarily, to investigate how molecular dynamics contribute to their structure–function relationships. To illustrate this across diverse dynamical regimes and molecular sizes, we studied three systems: class A β -lactamases (TEM-1 and TEM-121), both in their free form and in complex with a camelid single-domain antibody (VHH or cAb_{TEM13}) that inhibits these β -lactamases; and a mesophilic cold shock protein.

TEM-1 and TEM-121 β -lactamases

β -lactamases constitute one of the most extensively studied enzyme families due to their central role in antimicrobial resistance and their remarkable evolutionary adaptability (Bush 2018). The history of β -lactamases closely parallels that of β -lactam antibiotics. The first β -lactam antibiotic, penicillin, was discovered by Alexander Fleming in 1928 and rapidly transformed the treatment of bacterial infections. However, even before the widespread clinical use of penicillin, Fleming observed the existence of bacterial enzymes capable of inactivating the drug. The first β -lactamase was subsequently characterized in *Escherichia coli* by Abraham and Chain in 1940, demonstrating that bacterial resistance to β -lactam antibiotics could arise through enzymatic degradation. Since then, β -lactamases have evolved into a highly diverse superfamily comprising thousands of variants distributed among both Gram-positive and Gram-negative bacteria. Their continuous diversification under antibiotic selective pressure has made them a paradigm for studying molecular evolution and the emergence of antimicrobial resistance. β -lactamases are the primary drivers of bacterial resistance to β -lactam antibiotics; a broad and clinically essential class that includes penicillins, cephalosporins, monobactams, and carbapenems (**Figure 2.1**). These antibiotics exert their bactericidal effect by targeting penicillin-binding proteins (PBPs), also known as DD-transpeptidases, which catalyze the final steps of peptidoglycan biosynthesis, a process essential for maintaining bacterial cell wall integrity (Ghuysen 1991). β -lactamases counteract this mechanism by hydrolyzing the β -lactam ring of these antibiotics, thereby rendering them inactive before they can reach their PBP targets (**Figure 2.2**). The widespread dissemination of β -lactamases among bacteria has become one of the major challenges in modern medicine,

significantly reducing the effectiveness of many frontline antimicrobial therapies. The emergence of novel β -lactamase variants with expanded substrate spectra or increased resistance to inhibitors further complicates the treatment of bacterial infections and highlights the need for a detailed understanding of the molecular mechanisms underlying enzyme evolution and antibiotic resistance.

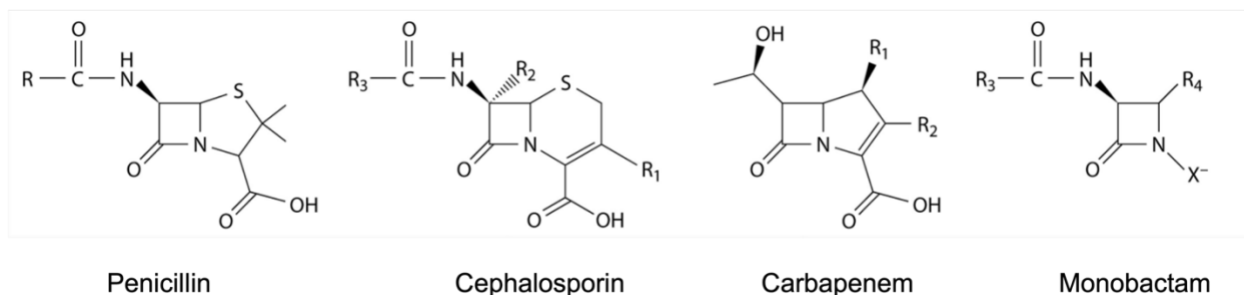


Figure 2.1. Generic structures of the main clinically important β -lactam antibiotics. The β -lactam core is shown for the principal antibiotic families used in clinical medicine. Adapted from (Bush and Bradford 2020).

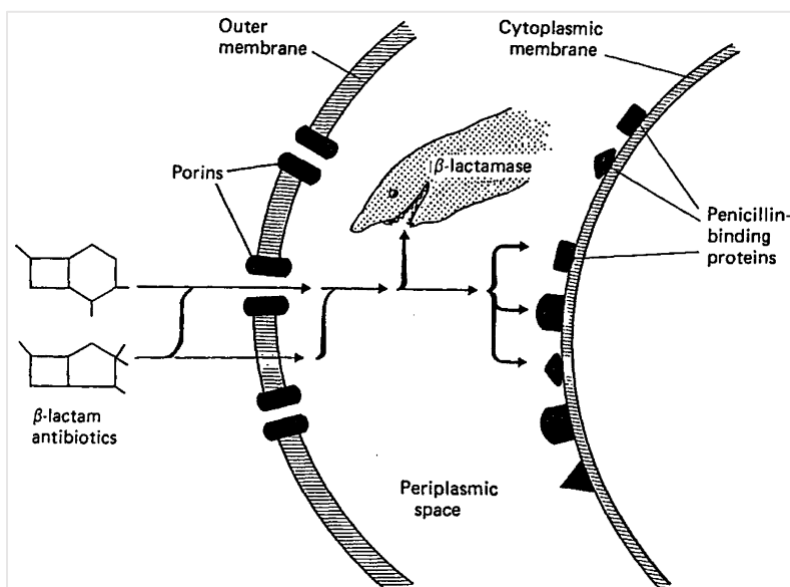


Figure 2.2. β -lactam resistance mechanism in Gram-negative bacteria. β -lactam antibiotics enter the periplasmic space through outer membrane porins and target penicillin-binding proteins at the cytoplasmic membrane. β -lactamases present in the periplasm hydrolyze the antibiotics, preventing their interaction with penicillin-binding proteins. Reproduced and digitally enhanced from Prof. Christian Damblon's course material

Based on their amino acid sequence and catalytic mechanism, β -lactamases are classified into four major classes, designated A to D according to the Ambler classification scheme. Classes A, C, and D are serine β -lactamases that hydrolyze β -lactam antibiotics through the formation of a covalent acyl–enzyme intermediate involving an active-site serine residue. The mechanism of active-site serine β -lactamases begins with the reversible formation of a non-covalent Michaelis complex (ES) between the enzyme and the β -lactam substrate. This is followed by the nucleophilic attack of the catalytic S70 serine residue on the β -lactam carbonyl carbon, leading to opening of the β -lactam ring and formation of a covalent acyl–enzyme intermediate (ES^{*}). In the final deacylation step, a water molecule, activated by the E166 glutamate carboxylate in class A β -lactamases, attacks the carbonyl carbon of the acyl–enzyme ester bond, resulting in hydrolysis of the intermediate, regeneration of the free enzyme, and release of the inactive product (MATAGNE et al. 1998) (**Figure 2.3**). In contrast to PBPs, β -lactamases display a rapid deacylation step, reflected by a high k_3 value. As a result, the acyl–enzyme intermediate is transient in β -lactamases, whereas it is much more stable in PBPs (MATAGNE et al. 1998)

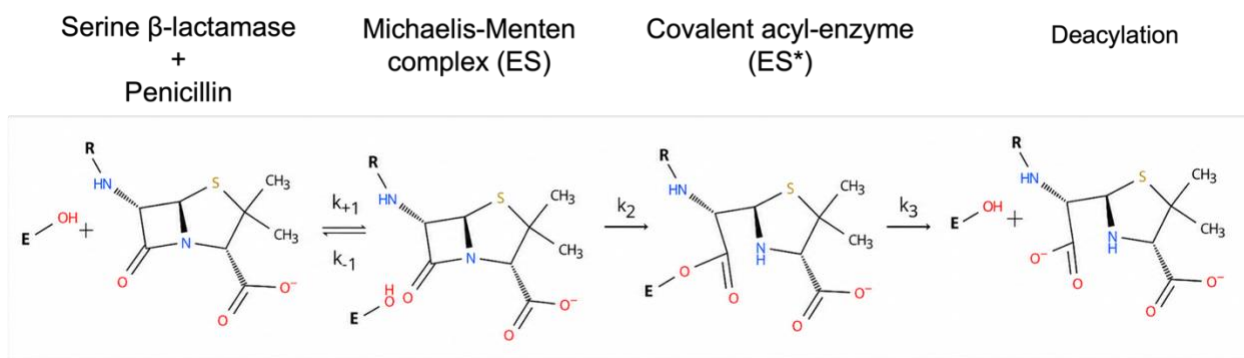


Figure 2.3. Catalytic mechanism of active-site serine β -lactamases. The β -lactam substrate first forms a non-covalent Michaelis complex with the enzyme, followed by nucleophilic attack of the catalytic serine and formation of a covalent acyl–enzyme intermediate. Rapid deacylation by an activated water molecule regenerates the free enzyme and releases the hydrolyzed inactive product. Adapted from (MATAGNE et al. 1998).

Class B enzymes, also known as metallo- β -lactamases, employ one or two zinc ions to activate a water molecule that directly attacks the β -lactam ring.

Among the different classes, class A β -lactamases are particularly widespread and clinically relevant (Maharjan et al. 2017; Ranjbar and Alam 2024; O'Neill 2016). Class A β -lactamases consist of two distinct domains: an all- α -helical domain (the all- α domain)

and an α/β domain composed of both α -helices and β -strands (**Figure 2.4**). The α domain forms a compact helical region and contains the catalytic serine (S70) which is located at the N-terminal end of a central α -helix. The α/β domain contains a central β -sheet flanked by α -helices and is formed by sequence segments that are distant in the primary structure but brought into close proximity in the folded protein. The all- α domain interrupts this α/β -domain region in sequence, yet in the folded protein the separated α/β segments are brought together to form a compact domain, highlighting the extensive chain rearrangement required to assemble the native topology. The active site is located at the interface between the two domains, where several conserved structural elements, including the Ω -loop and catalytic motifs, contribute to substrate recognition and catalysis.

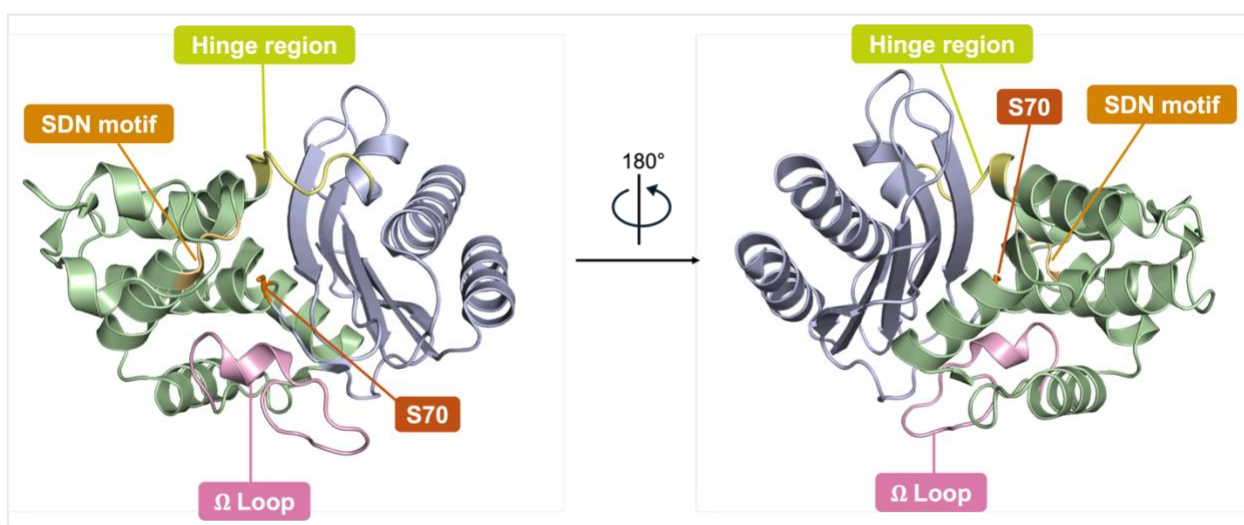


Figure 2.4. Structural representation of a class A β -lactamase (PDB ID: 1M40). The protein is shown as a cartoon and colored according to structural regions: the helical all- α domain is shown in pale green, the α/β domain in light blue, the hinge region in pale yellow, and the Ω -loop in light pink. The active-site serine (S70) is highlighted in orange, while the conserved SDN motif is colored in light orange.

One of these elements is the N-terminal region of the first α -helix of the α domain, which carries the conserved catalytic motif S70XXK73. This motif contains the active-site serine (S70), which initiates β -lactam hydrolysis through the formation of the acyl–enzyme intermediate during catalysis. Another conserved element is the SDN motif, comprising S130, D131, and N132, which forms part of the active-site cavity and contributes to maintaining the proper organization of catalytic residues required for efficient β -lactam hydrolysis. Within this class, the TEM-1 β -lactamase enzyme stands as a model system. First identified in *Escherichia coli*, TEM-1 is one of the most prevalent β -lactamases in

Gram-negative bacteria and has been the subject of extensive structural, kinetic, and biophysical investigations (Bush 2018; Bush and Bradford 2020).

TEM-1 β -lactamase is a valuable model system because it combines a well-characterized three-dimensional structure and catalytic mechanism with a remarkable capacity for evolutionary adaptation. This evolvability is exemplified by the emergence of numerous TEM variants exhibiting altered substrate specificities and resistance profiles. However, the small structural differences observed among variants often do not fully explain their divergent catalytic specificities and inhibitor susceptibilities. Increasing evidence suggests that enzyme function is governed not solely by the ground-state structure, but also by the distribution and interconversion of conformational substates across multiple timescales. In this view, mutations may not simply alter active-site geometry; instead, they may redistribute conformational populations, modulate dynamic exchange processes, and reshape the underlying energy landscape that governs catalysis and molecular recognition. This issue is further amplified by the selective pressure exerted by antibiotic use in clinical, veterinary, and animal-production settings, including the historical use of antibiotics for growth promotion and productivity enhancement in livestock. Such practices have contributed to the broader emergence and dissemination of resistant bacteria, particularly β -lactamase-producing Enterobacterales carrying expanded-spectrum β -lactamase (ESBL), AmpC, carbapenemase, or inhibitor-resistant TEM (IRT) determinants. In this context, ESBL/IRT enzymes such as TEM-121 represent clinically relevant examples of β -lactamase evolution under antibiotic selection pressure (Smet et al. 2020; Van Boeckel et al. 2015).

TEM-121 differs from TEM-1 by six amino acid substitutions (Q39K, E104K, R164S, A237T, E240K, and R244S). The Q39K substitution is not recognized as a canonical determinant of either the ESBL/IRT characteristics of TEM β -lactamases. In contrast, the substitutions E104K, R164S, A237T, and E240K are characteristic of ESBL variants and contribute to the expansion of the substrate spectrum. The R244S substitution, which affects a residue involved in inhibitor recognition, is associated with reduced susceptibility to β -lactamase inhibitors and is frequently observed in IRT enzymes. Together, these substitutions illustrate how a limited number of sequence changes can substantially alter the functional properties of TEM β -lactamases and contribute to the emergence of clinically relevant resistance mechanisms.

The development of β -lactamase inhibitors provided an important strategy to restore the efficacy of β -lactam antibiotics against β -lactamase-producing bacteria. Clavulanic acid, used clinically in combination with amoxicillin as Augmentin (GlaxoSmithKline), was one of the first successful examples of this approach (Geddes et al. 2007). By irreversibly inactivating many class A β -lactamases, clavulanic acid offered an effective therapeutic solution. However, this solution proved to be only temporary, as the subsequent emergence of inhibitor-resistant and extended-spectrum β -lactamase variants reduced

the clinical effectiveness of β -lactam/ β -lactamase inhibitor combinations (Knox 1995). This limitation highlights the need for alternative strategies capable of targeting β -lactamases through mechanisms distinct from classical small-molecule inhibitors. In this context, antibodies represent valuable molecular tools because they can bind specific enzyme surfaces with high affinity and may modulate enzyme activity by stabilizing or perturbing particular conformational states.

Variable heavy-chain domain of heavy-chain antibodies (VHHs) are small, highly soluble single-domain antibody fragments found in camelids that often bind their targets with high affinity, frequently in the nanomolar range (K. Conrath et al. 2005; Hoey et al. 2019; Zavrtanik et al. 2018). Unlike conventional monoclonal antibody fragments, they consist of a single polypeptide chain, facilitating efficient recombinant production in hosts such as *Escherichia coli* and *Pichia pastoris*. Despite their reduced size, VHHs generally retain the binding specificity and affinity of the heavy-chain antibodies from which they are derived. This single-domain architecture also contributes to their high stability, solubility, and robustness, making them easier to produce and manipulate than multichain antibody fragments such as Fab or scFv. In contrast, antibody fragments such as Fab and scFv are composed of two polypeptide chains whose proper assembly and folding may be compromised in the absence of the structural framework provided by the parental antibody. Beyond these practical advantages, VHHs are valuable molecular probes because their small size can allow them to recognize cryptic epitopes or enzyme active sites that are less accessible to conventional antibodies (Conrath et al. 2005; Hoey et al. 2019; Zavrtanik et al. 2018). Furthermore, the production of multichain antibody fragments is generally more challenging than that of single-domain VHHs. These properties make VHHs particularly suitable for both therapeutic applications and biosensing platforms. The TEM-specific VHH (cAb_{TEM-13}) therefore represents a relevant system to explore this alternative mode of β -lactamase inhibition.

In this context, the first objective of this thesis was to investigate the conformational dynamics of TEM β -lactamases and to determine whether changes in dynamic exchange processes can help explain functional differences that are not fully rationalized by static structural comparisons alone. Particular attention was given to two complementary sources of conformational modulation: resistance-associated mutations, examined by comparing TEM-1 and the ESBL/IRT variant TEM-121, and VHH (cAb_{TEM13}) binding, examined through the TEM-1/cAb_{TEM13} and TEM-121/cAb_{TEM13} complexes. This approach aimed to assess how both sequence variation and antibody binding modulate the conformational landscape of TEM β -lactamases and contribute to their altered catalytic, inhibitory, and resistance-related properties.

This part of the thesis builds upon previous work conducted by Dr. Frédéric Cawez (CIP). Specifically, we investigate the interaction between TEM β -lactamases and the VHH (**Figure 2.5**), first reported by Conrath et al. (2001), which has been shown to inhibit beta-

lactamase activity (Conrath et al. 2001). The molecular mechanism underlying this inhibition remains incompletely understood. The X-ray crystallographic structure of the TEM-1/cAb_{TEM-13} complex, determined by Dr Frédéric Cawez, indicates that cAb_{TEM-13} binds outside the catalytic site and does not induce major structural rearrangements in the active-site region compared with the free enzyme. These observations suggest that the inhibitory effect may arise from an indirect mechanism, potentially involving allosteric modulation or changes in the conformational dynamics of TEM-1.

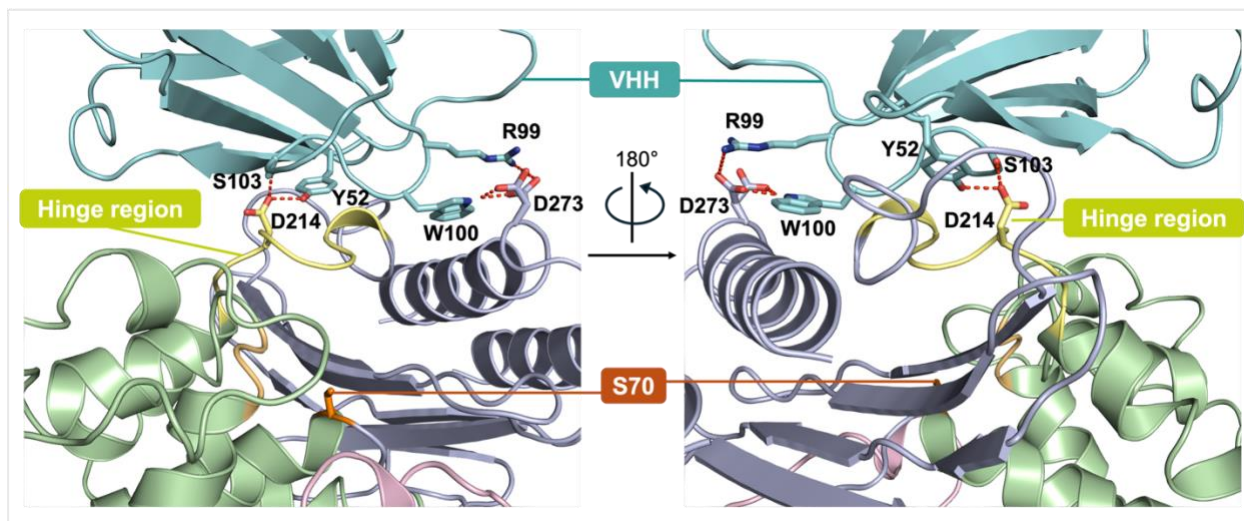


Figure 2.5. X-ray crystal structure of the TEM-1/cAb_{TEM-13} complex determined by Dr. Frédéric Cawez. The structure shows the interaction interface between TEM-1 β -lactamase and the cAb_{TEM-13} VHH, with hydrogen bonds highlighted. The helical all- α domain is shown in pale green, the α/β domain in light blue, the hinge region in pale yellow, and the Ω -loop in light pink. The active-site serine (S70) is highlighted in orange, while the conserved SDN motif is colored in light orange.

Our objective was to gain mechanistic insight into how VHH binding modulates enzyme function through changes in conformational dynamics processes. To this end, a complementary and integrative biophysical strategy was employed, combining X-ray crystallography, molecular dynamics (MD) simulations and NMR relaxation. The investigation of protein dynamics through NMR relaxation measurements represents a core objective of this dissertation, enabling quantitative characterization of motions spanning the picosecond–nanosecond (ps–ns) and microsecond–millisecond timescales (μ s–ms).

Finally, NMR relaxation experiments were employed to compare TEM-1 and the ESB/IRT variant TEM-121 in both their free and cAb_{TEM13}-bound states, in order to elucidate how resistance-conferring mutations exert their effects through modulation of

conformational flexibility. In particular, this analysis aimed to assess potential alterations in fast backbone dynamics (ps–ns timescale) as well as in slower conformational exchange processes (μ s–ms timescale), which are frequently associated with functional transitions, even when only subtle structural differences are observed.

Cold Shock Proteins

While β -lactamases provide a complex and functionally relevant framework to investigate the relationship between dynamics, mutation, and ligand binding, complementary model systems with reduced structural complexity offer a well-suited framework. Cold shock proteins (CSPs) provide such a model, owing to their small size and well-defined structure. Cold shock proteins (CSPs) constitute a family of small, highly conserved nucleic acid-binding proteins (Sinchaikul et al. 2002; Zeeb et al. 2006) that are widely distributed among bacteria and play a central role in the cellular response to temperature downshift. Typically composed of approximately 70 amino acids, CSPs adopt a compact five-stranded antiparallel β -barrel fold belonging to the oligonucleotide/oligosaccharide-binding (OB) fold family. Their nucleic acid-binding activity is mediated by two highly conserved ribonucleoprotein motifs, RNP1 and RNP2, which enable interactions with single-stranded RNA and DNA (*Figure 2.6*).

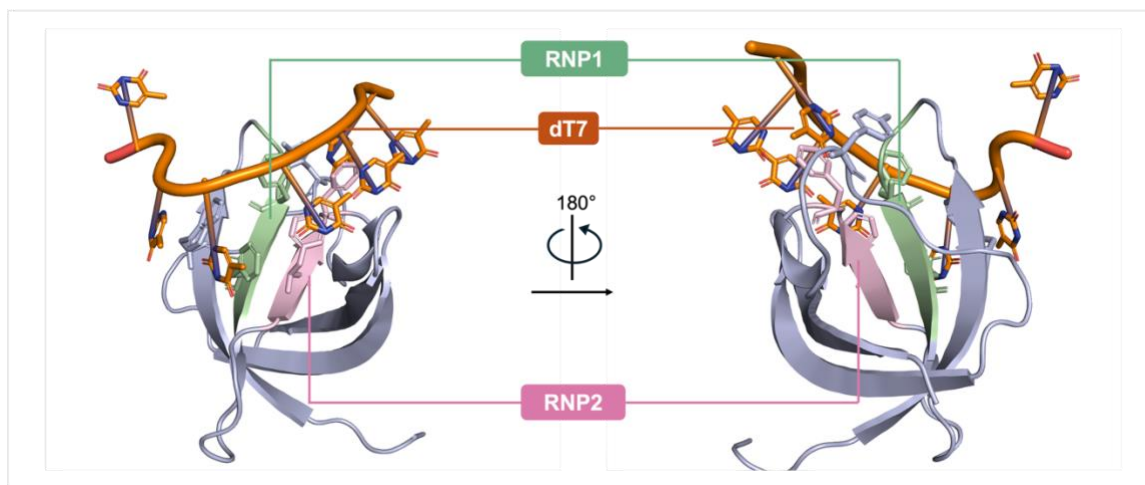


Figure 2.6. Structural representation of the heptathymidine-binding protein CspA showing RNP1 (pale green) and RNP2 (light pink). Reproduced from (Wanko Nembot et al. 2026).

Upon cold shock, CSPs act primarily as RNA chaperones, preventing the formation of stable secondary structures in mRNA that would otherwise impair transcription and translation at low temperatures (Yan Zhang and Gross 2021; Yonghong Zhang et al. 2022). CSPs are found in psychrophilic, mesophilic, thermophilic, and hyperthermophilic

organisms. Psychrophilic CSPs function efficiently at temperatures ranging from -1 to 10°C and are commonly found in organisms inhabiting permanently cold environments, such as sea ice, polar oceans, glaciers, and permafrost soils. Mesophilic CSPs are adapted to moderate environmental conditions, whereas thermophilic and hyperthermophilic homologs remain active at temperatures above 60°C and are typically found in organisms living in high-temperature environments, including hot springs and deep-sea hydrothermal vents. Despite these differences in thermal habitats, CSPs generally retain a highly conserved three-dimensional structure and nucleic acid-binding function. Comparative studies have shown that, despite adaptation to different thermal environments, CSPs retain a highly conserved overall fold, suggesting that thermal adaptation is achieved primarily through subtle changes in flexibility, and conformational dynamics rather than major structural rearrangements.

The second objective of this thesis was therefore to determine a well-defined NMR solution structure of CspA and to characterize its molecular dynamics across multiple timescales using advanced relaxation-based NMR approaches, in order to provide deeper insight into its functional mechanisms.

Leiurotoxin

Peptide toxins are not only valuable pharmacological probes, but have also attracted considerable interest as therapeutic leads, since several venom-derived peptides or peptide-inspired molecules have been developed for medical applications (King 2011; Robinson et al. 2017). Among the peptide toxins known to inhibit SK channels, apamin, a bee venom peptide, is the most extensively studied and has served as a reference compound because of its high affinity and selectivity. Several scorpion toxins have also been identified as potent SK channel blockers, including PO5, TsKapa, Tamapin, and Leiurotoxin I.

Leiurotoxin I is a potent and highly selective peptide toxin isolated from the venom of the scorpion *Leiurus quinquestriatus*. The peptide comprises 31 amino acids, has a molecular mass of approximately 3.4 kDa, and is amidated at its C-terminus. Its three-dimensional structure is stabilized by three disulfide bridges connecting Cys3–Cys21, Cys8–Cys26, and Cys12–Cys28, which confer the characteristic cysteine-stabilized α/β (CS- α/β) fold found in members of the αKTx5 toxin family. Based on structural and functional information derived from Leiurotoxin I and PO5, a synthetic analog designated Leiurotoxin-Dab7 (Lei-Dab7) was subsequently engineered. In this analog, Met7 is replaced by the unnatural amino acid diaminobutyric acid (Dab). Previous functional studies have shown that Lei-Dab7 retains high affinity for the hSK2 subtype. Remarkably, this single amino-acid substitution substantially enhances subtype selectivity, resulting in a strong preference for hSK2 over hSK1 and hSK3 channels.

The third objective of this thesis was to determine a well-defined three-dimensional structure of Leiurotoxin I and its analogue Lei-Dab7 in order to provide structural insights into their recognition of SK channels. This objective aimed to clarify how subtle sequence modifications, such as the substitution introduced in Lei-Dab7, influence toxin structure, toxin–channel interactions, and subtype selectivity. More specifically, these structural studies were undertaken to provide an atomic-level framework for interpreting their binding affinities toward the two human SK channel subtypes (hSK2–3), which were quantitatively characterized by Jean-Luc Hayen.

To achieve these objectives, the dissertation is organized into several parts. First, the necessary background and methodologies for NMR-based resonance assignment and three-dimensional structure determination of proteins and peptides are introduced (Part III). This part includes Chapter III. NMR-based structure determination of proteins; Chapter IV. NMR-based structure determination of peptides, and Chapter V. Research Contributions. Subsequently, the focus shifts to biomolecular dynamics as probed by NMR relaxation (Part IV). As resonance assignment is a prerequisite for NMR relaxation analysis and given that some contributions integrate both structure determination and NMR relaxation, the section on NMR relaxation is presented in Part IV. It begins with an introduction to NMR relaxation (Chapter VI), followed by the theoretical foundations of relaxation processes on the picosecond–nanosecond and microsecond–millisecond timescales (Chapters VII and VIII, respectively), and concludes with the corresponding research contributions (Chapter IX). Finally, the dissertation concludes with a general conclusions and perspectives (Part V).

Part III. NMR-based Structure Determination of Proteins and Peptides

Chapter III.

NMR-based structure determination of Proteins

Achieving high-resolution structure determination of biomolecules such as proteins using NMR spectroscopy typically requires the acquisition of a comprehensive and diverse set of experimental restraints. A process referred to as the standard approach (Billeter et al. 2008; Gronwald and Kalbitzer 2004; Peter Güntert 2009).

Nonetheless, this approach can become significantly more challenging for proteins of high molecular weight or those with complex topologies, where spectral overlap, signal broadening, and relaxation effects limit the quality and quantity of extractable data. In such instances, computational modeling strategies can be employed to augment sparse NMR datasets with additional structural information, thereby facilitating accurate structure determination despite experimental limitations.

The following section provides an in-depth overview of the standard NMR-based structure determination approach.

III.1. Overview of the standard approach

When NMR spectroscopy was first introduced for protein structure determination in solution in the early 1980s, the analysis of two-dimensional (2D) spectra was performed entirely manually, often using large paper plots. As vividly recounted in the memoirs of pioneers published at *Magnetic Resonance in Chemistry* (Vol. 41, Issue S1) (Peter Güntert 2009), rulers were used to verify peak alignment, and assignments, notes, and annotations were recorded manually in notebooks or directly on the printed spectra. At the time, only the initial and final stages of the analysis pipeline were computerized, specifically, the Fourier transformation of raw NMR data and the final calculation of the 3D structure, following unsuccessful attempts at interactive model building guided by NMR constraints.

Today, although numerous computational tools have been developed to automate many steps of the classical NMR-based protein structure determination process (see **Figure 3.1**) the expertise of a skilled operator remains essential. In the remainder of this dissertation, we present the workflow for protein structure determination by the standard NMR approach.

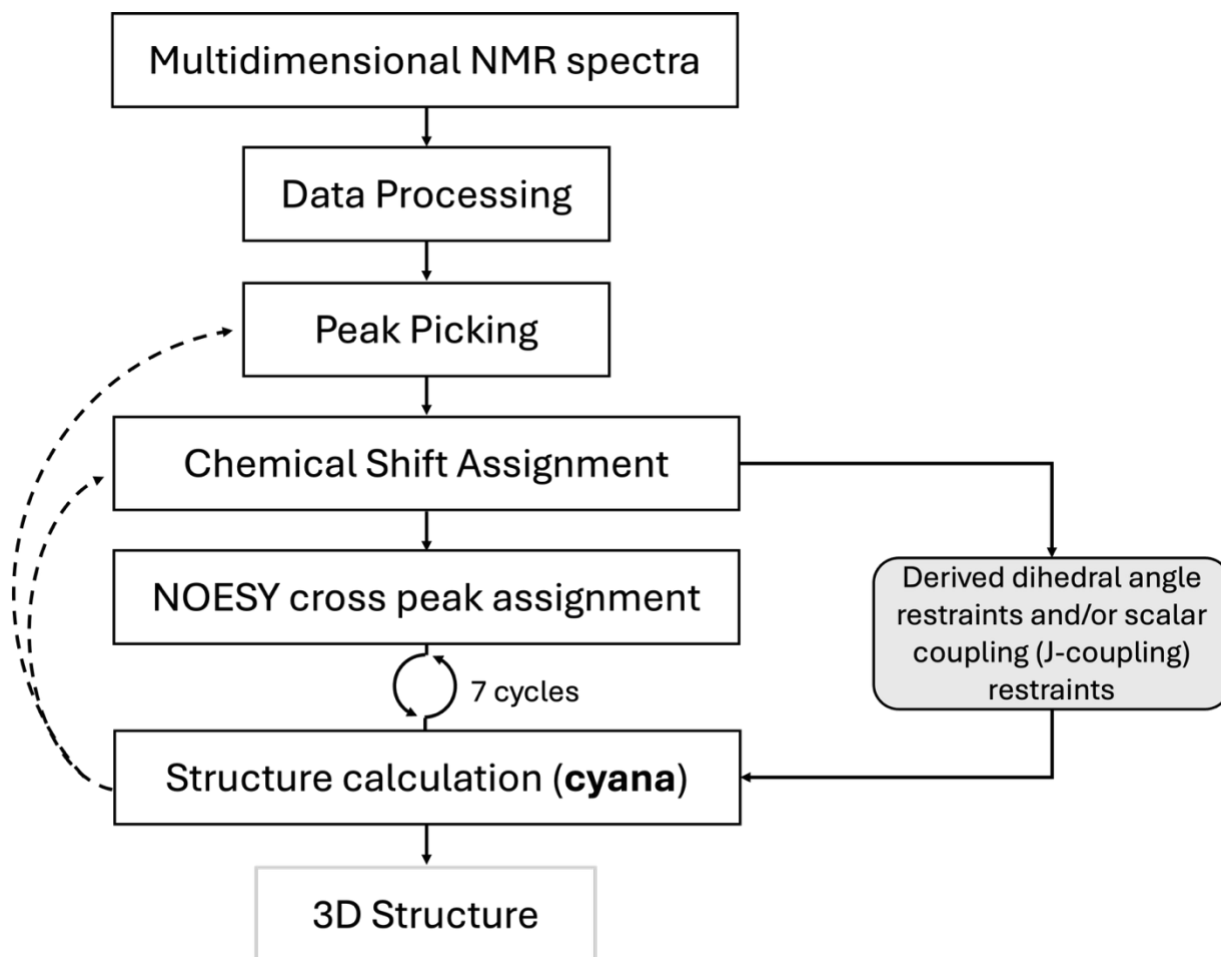


Figure 3.1. Standard workflow for NMR-based protein structure determination. Adapted from (Peter Güntert 2009).

III.1.1. Chemical shifts assignment

Before the recording of multidimensional spectra, the protein NMR sample must be carefully prepared. As with any analytical technique, sample quality is important in NMR. However, the purity requirements are generally less stringent than for X-ray crystallography or cryo-EM. In most cases, uniformly labeled ^{13}C and ^{15}N protein is overexpressed in a bacterial system, typically *Escherichia coli*, which is usually grown on a ^{13}C and ^{15}N isotopically enriched minimal medium. Following expression, the protein is purified and concentrated to obtain an NMR sample of a few hundred microliters, with a concentration in the millimolar range (typically $\geq 500 \mu\text{M}$). To enhance frequency referencing and ensure spectral stability, 10% (v/v) D_2O is commonly added to the sample.

Once the sample is prepared, a series of multidimensional NMR experiments is recorded using the same sample. Each experiment differs by the applied pulse sequence and the

type of correlation to be detected. These spectra produce experiment-specific signals, providing insights into the covalent and spatial connectivities between protein atoms.

The acquired spectra are typically classified into three major categories:

- 1) Triple-resonance (HCN) spectra, used for backbone assignment
- 2) Side-chain spectra (HHC), used to assign chemical shifts of side-chain atoms
- 3) NOESY spectra (HHN & HHC), which provide distance restraints critical for 3D structure calculation.

A. Backbone chemical shifts assignment

Accurate assignment of backbone chemical shifts including $^1\text{H}^{\text{N}}$, ^{15}N , $^{13}\text{C}_{\alpha}$, $^{13}\text{C}_{\beta}$, and $^{13}\text{C}'$ resonances is a crucial first step in protein structure determination by NMR spectroscopy. These chemical shift assignments provide the foundation for identifying secondary structure elements, analyzing protein dynamics, and performing high-resolution 3D structural calculations.

Traditionally, backbone assignments are achieved through a suite of triple-resonance NMR experiments, such as HNCA, HN(CO)CA, HNCACB, HN(CO)CACB, HN(CA)CO, and HNCO, typically recorded in combination with a two-dimensional [^{15}N - ^1H]-HSQC spectrum. These experiments establish through-bond correlations between nuclei within the same residue and adjacent residues, enabling sequential assignment along the polypeptide backbone.

The connection between two sequential amino-acids is determined because of a unique direct coupling interaction between the amide nitrogen, and the C_{α} of the preceding amino-acid although separated by two chemical bonds (see **Figure 3.2**). This unique coupling pattern is a fundamental characteristic of protein architecture, enabling the establishment of a sequential pathway that is crucial for assigning backbone resonances with precision.

Before the assignment process begins, it is essential to identify «real» signals, those arising from protein atoms and distinguish them from noise and spectral artifacts. This step, known as peak picking, is typically initiated with the highly sensitive [^{15}N - ^1H]-HSQC spectrum. The peaks identified in this spectrum serve as root resonances, anchoring the assignment process by providing initial coordinates for further resonance correlations.

Although various software programs offer automated peak picking routines, it remains common practice to manually pick peaks or refine automatically generated lists through manual intervention. Popular tools for peak picking include AUTOPSY (Koradi et al. 1998), ATNOS (Herrmann et al. 2002), SPARKY (W. Lee et al. 2015), NMRView (Johnson 2004), CcpNmr Analysis (Vranken et al. 2005), and XEASY (Bartels et al. 1995).

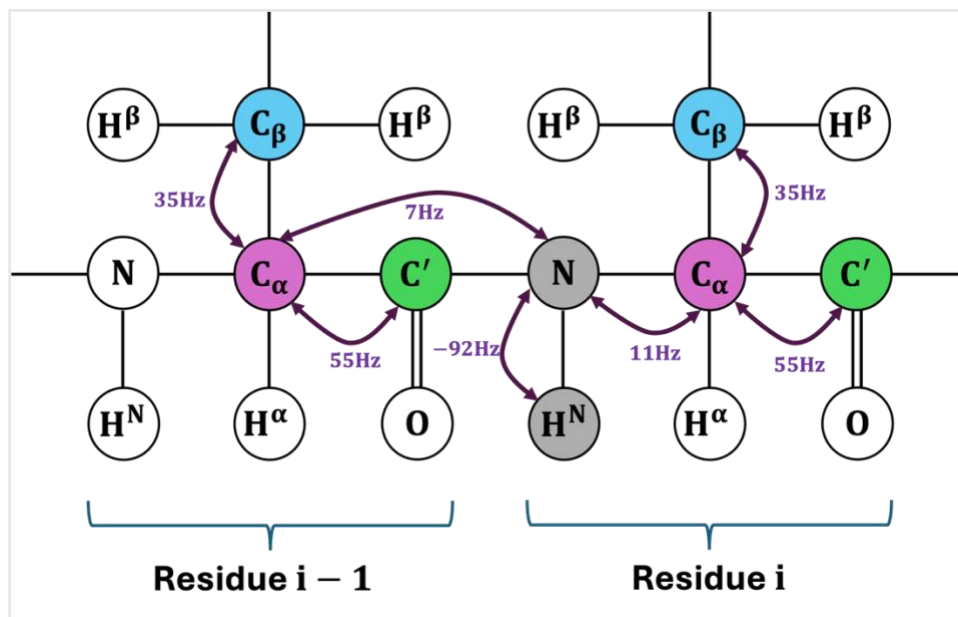


Figure 3.2. Through-bond coupling between amide hydrogen, nitrogen, and key backbone atoms, including $C_{\alpha}(i)$, $C_{\alpha}(i-1)$, $C_{\beta}(i)$, $C_{\beta}(i-1)$, $C'(i)$ and $C'(i-1)$.

The primary challenge in peak picking lies in accurately identifying all true peaks, especially in highly overlapped spectral regions, and in distinguishing real signals, which arise from atomic nuclei, from noise and spectral artifacts. The quality of the resulting peak lists has a direct and significant impact on the success of chemical shift assignments. Missing real peaks from the list may render the assignment of certain atoms impossible, while the inclusion of spurious or noise-related peaks may lead to erroneous assignments.

A.1. $[^{15}\text{N}-^1\text{H}]$ -HSQC spectrum

The $[^{15}\text{N}-^1\text{H}]$ -HSQC experiment correlates the resonances of covalently bound amide hydrogen and nitrogen atoms and typically yields one cross-peak per amino acid residue with the exception of proline, which lacks a backbone amide proton. During the experiment, magnetization is transferred from the amide proton to the directly bonded ^{15}N nucleus via scalar-coupling. The chemical shift then evolves on the nitrogen dimension, after which the magnetization is transferred back to the proton for detection (see **Figure 3.3**). This experiment is the standard entry point for NMR analysis of proteins and provides a comprehensive overview of $^1\text{H}^{\text{N}}\text{-}^{15}\text{N}$ correlations. In addition to backbone amide signals, it also reveals side-chain correlations such as those from tryptophan $^{15}\text{N}_{\epsilon}\text{-}^1\text{H}_{\epsilon}$ groups and asparagine/glutamine $^{15}\text{N}_{\delta}\text{-}^1\text{H}_{\delta_2}$ and $^{15}\text{N}_{\epsilon}\text{-}^1\text{H}_{\epsilon_2}$ groups. Arginine $^{15}\text{N}_{\epsilon}\text{-}^1\text{H}_{\epsilon}$ correlations can also be observed in principle. However, their nitrogen chemical shifts typically fall outside the standard spectral window, resulting in folded or aliased peaks (Ikura and Kay 1990).

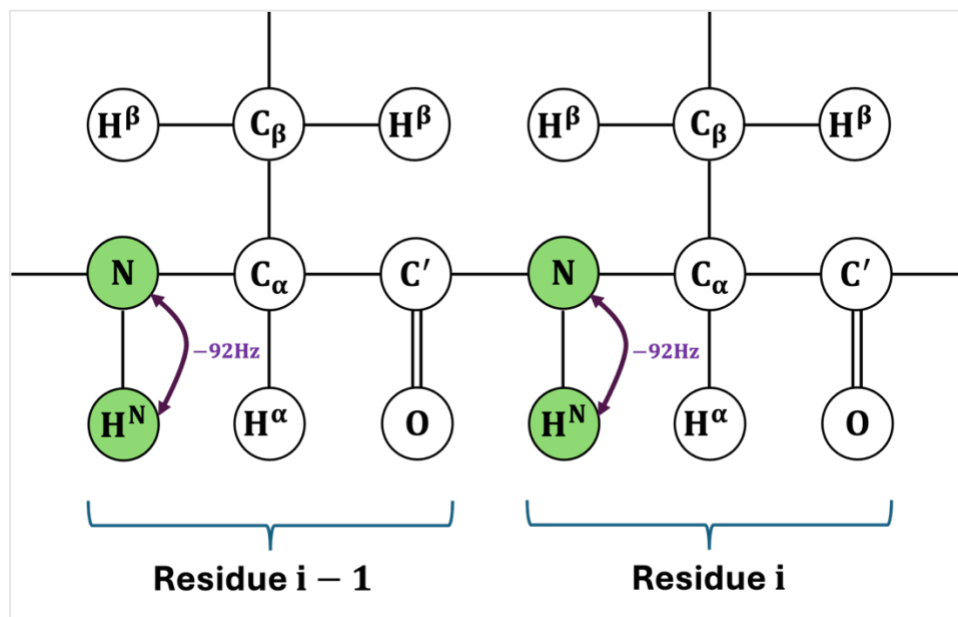


Figure 3.3. $[^{15}\text{N}\text{-}^1\text{H}]$ -HSQC experiments for protein backbone assignment. Atoms that lead to peaks are encircled in green.

A.2. HNCACB and HN(CO)CACB spectra

The HNCACB and HN(CO)CACB (Whitehead et al. 1997; Wittekind and Mueller 1993) experiments are among the most commonly used triple-resonance techniques for the sequential assignment of neighboring amino acid residues. The one-bond and two-bonds scalar-couplings ($^1J_{\text{H}(\text{N})\text{-N}}$, $^1J_{\text{H}(\text{N})\text{-C}\alpha}$, $^1J_{\text{C}\alpha\text{-C}\beta}$ and $^2J_{\text{H}(\text{N})\text{-(C}\alpha-1)}$) between $^1\text{H}^{\text{N}}$, ^{15}N , and $^{13}\text{C}\alpha$ or $^{13}\text{C}\beta$ nuclei in proteins span a broad range, from approximately ~ 7 to 95 Hz (Stephan Grzesiek and Bax 1992; Ikura and Kay 1990; Meissner and Sørensen 2001; Whitehead et al. 1997; Wittekind and Mueller 1993). Particularly important for triple-resonance methodologies are the $\sim 15\text{ Hz}$ one-bond coupling between $^{13}\text{C}'(i-1)$ and $^{15}\text{N}(i)$ and the two-bond coupling between $^{13}\text{C}\alpha(i-1)$ and $^{15}\text{N}(i)$ which varies between 4 and 9 Hz (Stephan Grzesiek and Bax 1992; Ikura and Kay 1990; Meissner and Sørensen 2001; Whitehead et al. 1997; Wittekind and Mueller 1993). These couplings are essential for correlating nuclei of adjacent residues, enabling sequential connectivity along the polypeptide chain.

In the HNCACB experiment, magnetization is transferred from the amide proton ($^1\text{H}^{\text{N}}$) to the directly bonded ^{15}N nucleus and subsequently from ^{15}N to $^{13}\text{C}\alpha$ and then to $^{13}\text{C}\beta$ via scalar-coupling. The magnetization then follows the reverse path (see Figure 3.4) transferring back through $^{13}\text{C}\alpha$ and ^{15}N to the amide proton for detection. As a result, each amide group gives rise to four peaks in the carbon dimension: two $\text{C}\alpha$ and two $\text{C}\beta$ signals,

one set corresponding to the residue at position i , and another to the preceding residue at position $i - 1$.

Because magnetization is partially transferred from $^{13}\text{C}\alpha$ to $^{13}\text{C}\beta$, the chemical shift evolved simultaneously on $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$, but with opposite sign due to the additional transfer for $^{13}\text{C}\beta$. Their resonances appear along a single dimension. The chemical shifts of the other two dimensions correspond to the ^{15}N and $^1\text{H}^{\text{N}}$ nuclei of the amide group.

In contrast to HNCACB, the HN(CO)CACB experiment provides signals exclusively from the preceding residue ($i - 1$). In this experiment, magnetization is transferred from the amide proton ($^1\text{H}^{\text{N}}$) of residue i to the directly bonded ^{15}N , and then only to the carbon atoms ($^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$) of residue $i - 1$ via the intervening carbonyl (^{13}CO) group. This selective transfer occurs because the pulse sequence is designed to pass through the $^{13}\text{CO}(i - 1) - ^{15}\text{N}(i)$ coupling (~ 15 Hz) without involving intra-residue ^{13}C nuclei. This is achieved by using selective ^{13}CO excitation, which ensures that magnetization is routed specifically through the $^{13}\text{CO}(i - 1)$ spin.

As a result, HN(CO)CACB serves as a filtering experiment, suppressing signals from residue i and providing a clear spectral fingerprint of only the preceding residue. This allows for the unambiguous differentiation between intra-residue and inter-residue peaks when the HNCACB and HN(CO)CACB spectra are interpreted together.

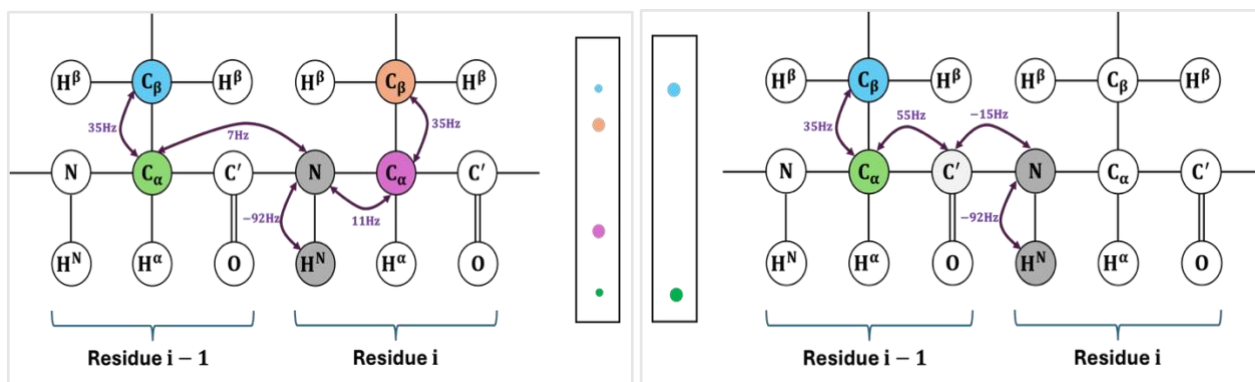


Figure 3.4. HNCACB (left) and HN(CO)CACB (right) experiments. Atoms that contribute to observable peaks in each experiment are color-coded, while the backbone ^{15}N and $^1\text{H}^{\text{N}}$ nuclei common to all correlations are shown in grey. The peaks in the "strip" and their corresponding atoms are highlighted in matching colors.

A.3. HNCO and HN(CA)CO spectra

The HNCO (Whitehead et al. 1997) experiment is widely regarded as the most sensitive among the triple-resonance NMR experiments, primarily due to two key factors: a short and highly efficient magnetization transfer pathway and reduced relaxation losses

throughout the coherence transfer steps. In the HNCO experiment, magnetization is transferred from the amide proton ($^1\text{H}^{\text{N}}$) to the amide nitrogen (^{15}N) and then selectively to the carbonyl carbon (^{13}CO) of the preceding residue ($i - 1$) via the ^{15}N – ^{13}CO scalar-coupling. The magnetization is then transferred back along the same pathway from ^{13}CO to ^{15}N , and finally to $^1\text{H}^{\text{N}}$ for detection. The chemical shift evolved on all three nuclei ($^1\text{H}^{\text{N}}$, ^{15}N , and ^{13}CO), resulting in a three-dimensional spectrum. Importantly, this experiment yields a single ^{13}CO peak corresponding to the carbonyl carbon of residue ($i - 1$) for each amide group.

In contrast, the HN(CA)CO (Whitehead et al. 1997) experiment is less sensitive because the transverse relaxation of the C_α nuclei is relatively rapid, and the pulse sequence requires full transfer of magnetization to C_α , which further contributes to signal loss. It yields signals from both residue (i) and the preceding residue ($i - 1$). Here, magnetization is transferred from $^1\text{H}^{\text{N}}$ to ^{15}N , then via the one-bond ^{15}N – $^{13}\text{C}_\alpha$ coupling to $^{13}\text{C}_\alpha$, and subsequently to ^{13}CO via the $^{13}\text{C}_\alpha$ – ^{13}CO coupling. For detection, the magnetization is transferred back along the same path from ^{13}CO to $^{13}\text{C}_\alpha$, then to ^{15}N , and finally to $^1\text{H}^{\text{N}}$. In this experiment, the chemical shift evolved on $^1\text{H}^{\text{N}}$, ^{15}N , and ^{13}CO , but not on $^{13}\text{C}_\alpha$, resulting again in a three-dimensional spectrum.

Because the amide ^{15}N is scalar coupled to the $^{13}\text{C}_\alpha$ nuclei of both its own residue (i) and the preceding residue ($i - 1$), magnetization is transferred to both carbonyl carbons (^{13}CO), resulting in two ^{13}CO peaks per amide group in the HN(CA)CO spectrum. This dual observation facilitates more comprehensive sequential assignment by complementing the residue-specific information obtained from the HNCO experiment.

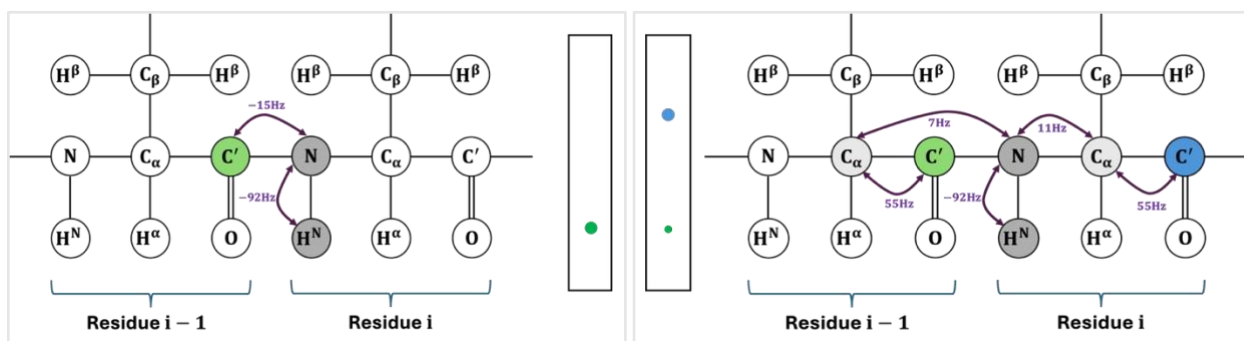


Figure 3.5. HNCO (left) and HN(CA)CO (right) experiments. Atoms that contribute to observable peaks in each experiment are color-coded in blue and green, while the backbone ^{15}N and $^1\text{H}^{\text{N}}$ nuclei common to all correlations are shown in grey. The peaks in the "strip" and their corresponding atoms are highlighted in matching colors.

A.4. Standard backbone chemical shift assignment

Nowadays, the standard protocol for backbone chemical shift assignment in proteins involves a combination of three-dimensional triple resonance NMR experiments and a two-dimensional ^{15}N - ^1H -HSQC spectrum. Since triple resonance experiments correlate the backbone amide proton ($^1\text{H}^{\text{N}}$) and nitrogen (^{15}N) signals with carbon resonances, most commonly $^{13}\text{C}_{\alpha}$, $^{13}\text{C}_{\beta}$, or $^{13}\text{C}'$, the cross-peak positions from the ^{15}N - ^1H -HSQC can be used to extract 2D slices from the 3D spectra for analysis (see **Figure 3.6**).

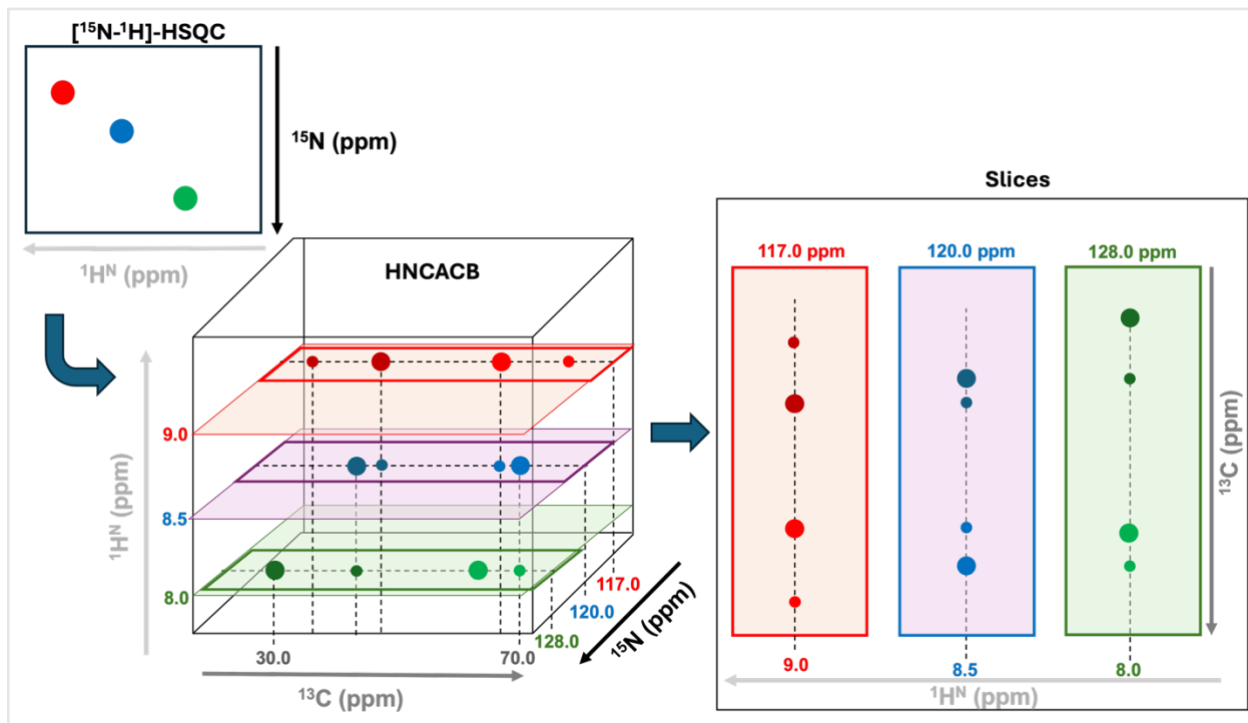


Figure 3.6. Standard 3D triple resonance experiments for protein backbone assignment.

The amino acid type of a residue can often be inferred based on its characteristic C_{α} and C_{β} chemical shifts (see **Figure 3.7**) which are distinct and specific to certain amino acid types. In particular, glycine ($\text{C}_{\alpha} \sim 45$ ppm), threonine ($\text{C}_{\beta} \sim 70$ ppm), serine ($\text{C}_{\beta} \sim 60$ ppm), and alanine ($\text{C}_{\beta} \sim 20$ ppm) exhibit distinct chemical shift signatures and are often used as starting points for the matching process.

The subsequent step is the sequence-specific assignment, where each set of NMR signals (i.e., spin system) is mapped to a unique position in the protein's amino acid sequence. This is accomplished by combining complementary experiments that correlate the $^1\text{H}^{\text{N}}$ - ^{15}N signals of residue i either:

- to carbon resonances of residue $(i - 1)$, as in $\text{HN}(\text{CO})\text{CACB}$ and HNCO ,

- to carbon resonances of both residue ($i - 1$) and residue (i), as in HNCACB and HN(CA)CO.

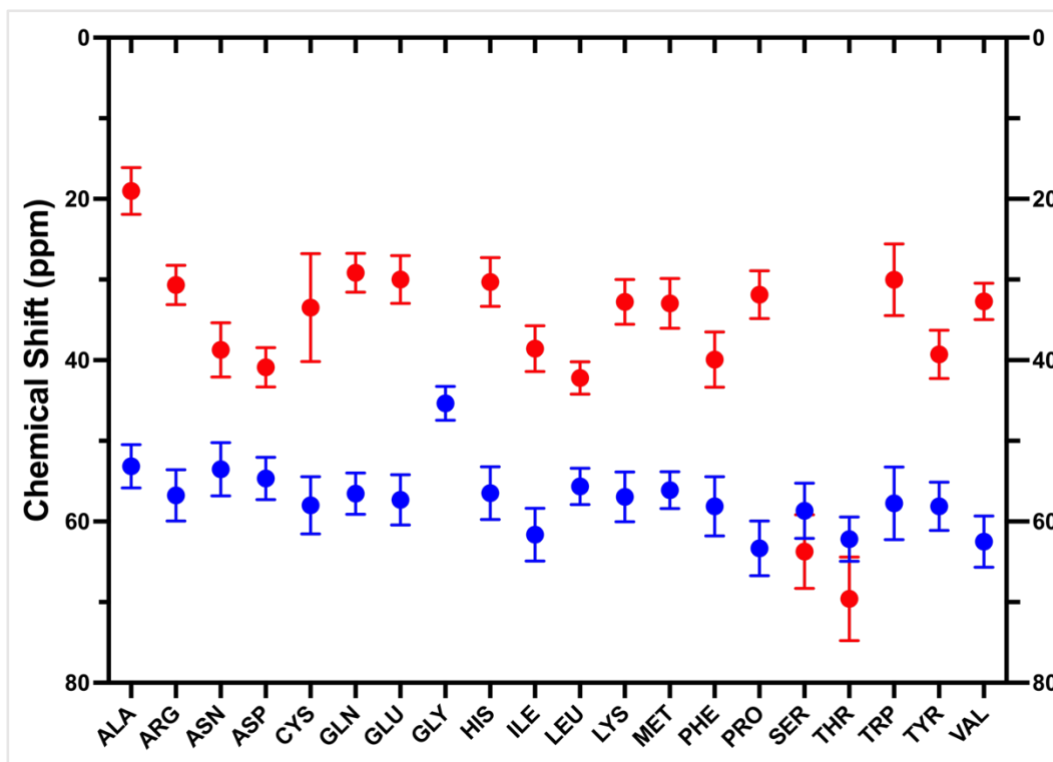


Figure 3.7. $C\alpha$ and $C\beta$ BMRB chemical shift statistics. 29th July 2025.

This pairing allows for the distinction between intra-residue and inter-residue correlations. By comparing two such spectra, one can determine which peaks are shared (intra-residue) and which are unique to one spectrum (inter-residue). This comparative approach enables the linking of sequential spin systems, thereby building stretches of assigned residues along the sequence (see **Figure 3.8**).

Residues are mapped to the sequence by confirming agreement between their chemical shifts and amino acid type. Proline residues, which lack amide protons and are thus absent from $[^{15}\text{N}-^1\text{H}]$ -HSQC-based spin systems, must be assigned indirectly, using inter-residue correlations from adjacent spin systems: assuming that a proline residue is at position $i - 1$, the $C\alpha$ and $C\beta$ atoms of proline will appear at the position corresponding to $(i - 1)$ in the HNCACB and HN(CO)CACB spectra in the correlation of the preceding residue at position i .

For many applications, backbone assignment alone provides sufficient information to address key biological questions. The identification of backbone amide resonances enables chemical shift perturbation (CSP) studies, which are widely used to map protein–

ligand or protein–protein interaction sites with residue-level resolution. Similarly, backbone assignments form the foundation for relaxation measurements, allowing characterization of internal motions, conformational exchange, and overall molecular dynamics. Even in the absence of complete side-chain assignments or a full 3D structure, backbone chemical shifts offer a robust framework for probing conformational changes, binding interfaces, and dynamic properties, making them an indispensable resource in modern biomolecular NMR studies.

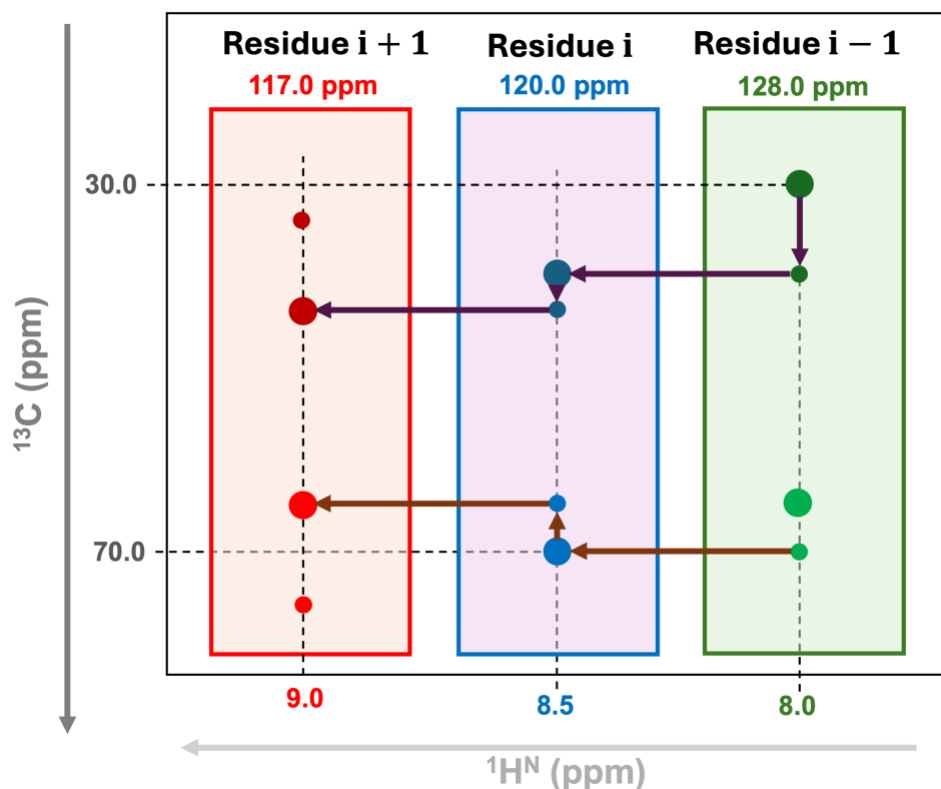


Figure 3.8. Backbone sequential assignment.

After completing the sequence-specific backbone assignment, the next step involves assigning the chemical shifts of the side-chain nuclei, which are essential for full structural characterization and subsequent distance restraint analysis.

B. Side chains chemical shift assignment

The chemical shift assignment of side-chain nuclei is a crucial step toward completing resonance assignments and preparing for structure calculation. The side-chain assignment in NMR spectroscopy refers to the process of identifying the resonances of

the non-backbone atoms of a protein or peptide, namely, the side-chain protons and side-chain carbons. Unlike backbone assignment, which focuses on the core structure (amide protons, C_α , and C_β), side-chain assignment involves detecting and correlating resonances from the functional groups attached to the C_β atoms. Here's an overview of the principle behind side-chain assignment in NMR spectroscopy.

The chemical shift assignment of side-chain nuclei is typically achieved using experiments that correlate the amide $^1H^N$ and ^{15}N signals of residue i with the side-chain nuclei of the preceding residue ($i - 1$), as well as intra-residue atoms.

Two commonly used experiments for this purpose are HBHA(CO)NH (Moy et al. 1995) and C(CCO)NH (S. Grzesiek et al. 1993; Moy et al. 1995). The HBHA(CO)NH experiment enables the identification of $^1H^\alpha$ and $^1H^\beta$ protons of residue ($i - 1$) by transferring magnetization from the amide group to the α and β protons via the intervening $^{13}C'$ nucleus (see **Figure 3.9**). Similarly, the C(CCO)NH experiment provides access to the aliphatic carbon chemical shifts of the preceding residue ($i - 1$) by transferring magnetization through the carbonyl carbon to side-chain carbons.

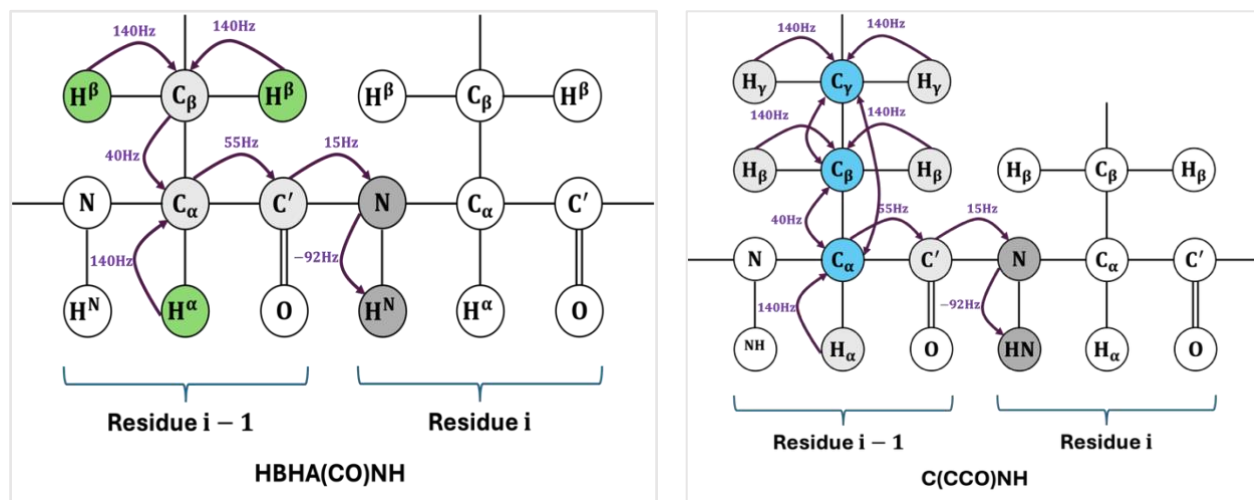


Figure 3.9. HBHA(CO)NH (left) and C(CCO)NH (right) experiments. Atoms that contribute to observable peaks in each experiment are color-coded, while the backbone ^{15}N and $^1H^N$ nuclei common to all correlations are shown in grey.

While the C(CCO)NH experiment is valuable for accessing side-chain carbon resonances, its sensitivity is limited, particularly when magnetization transfer involves a longer pathway across multiple atoms. The presence of many scalar-couplings in such pathways can lead to signal attenuation, weakening the overall sensitivity. Therefore, offers limited coverage of intra-residue side chains.

To overcome these limitations and achieve comprehensive intra-residue side-chain assignments, HCCH-TOCSY-type experiments such as H(C)CH-TOCSY and (H)CCH-TOCSY (Bax et al. 1990; Fesik et al. 1990) are employed. These experiments use TOCSY (Total Correlation Spectroscopy) spin-lock mixing to distribute magnetization among all scalar-coupled aliphatic protons or carbons within the same residue, making them especially effective for assigning branched or extended side chains but not aromatics. Spin-lock fields do not distribute magnetization to aromatic carbons because their resonance frequencies ($\sim 110\text{--}170$ ppm) lie far outside the frequency range addressed by the spin-lock ($\sim 0\text{--}70$ ppm). As a result, aromatic carbons are effectively excluded from the magnetization-transfer pathways.

Spin-lock is an NMR technique in which spin magnetization is kept “locked” away from the influence of the static magnetic field B_0 . Instead of precessing around B_0 , the spins precess around the applied RF-field, where they experience a much weaker effective magnetic field, allowing magnetization to evolve under slow interactions such as scalar-couplings and enabling magnetization transfer.

(H)CCH-TOCSY & H(C)CH-TOCSY are 3D experiments that by transferring magnetization through all carbons within an aliphatic carbon bound chain, allow correlation of all scalarly-coupled CH groups within the aliphatic side chain of the same amino acid. (H)CCH-TOCSY provides ^{13}C chemical shift of all connected CH and H(C)CH-TOCSY provides ^1H chemical shift of all connected CH. These experiments are particularly useful in cases of proton resonance overlap, as they spread the correlations into the carbon dimension, thereby enhancing spectral resolution.

Because TOCSY-based experiments correlate $^{13}\text{C}\text{--}^1\text{H}$ pairs within a residue, they rely on the $[^{13}\text{C}\text{--}^1\text{H}]\text{-HSQC}$ spectrum to provide root resonances as starting points for building full spin systems. The initial assignment of these root resonances (particularly $\text{C}_\alpha\text{--H}^\alpha$ and $\text{C}_\beta\text{--H}^\beta$ cross-peaks) is derived from backbone assignment and HBHA(CO)NH experiments, which provide chemical shifts for the same atoms through $^1\text{H}\text{--}^{15}\text{N}$ -based correlations. Once these entries are matched, sidechain TOCSY experiments can expand from these points to identify the remaining aliphatic resonances within each residue.

Following the complete or near-complete assignment of aliphatic side-chain nuclei, the next step in the resonance assignment workflow involves the identification of aromatic side-chain resonances. Aromatic residues such as phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp), and histidine (His) contain ring systems with characteristic chemical shifts and coupling patterns, which can be exploited using dedicated NMR experiments.

Aromatic sidechain atoms cannot be connected to aliphatic atoms using carbon-carbon TOCSY because TOCSY relies on through-bond carbon-carbon correlations within a single spin system, involving carbons within the same chemical environment. Aromatic

and aliphatic carbons, however, present very different chemical shift that do not permit spin-locking

Aromatic proton and carbon resonances are typically assigned using 2D experiments specifically designed to correlate the protons and carbons within the aromatic rings. The most commonly employed experiments for this purpose are: HB(CB)(CGCD)HD and HB(CB)(CGCDCE)HE (Yamazaki et al. 1993). In these experiments, the atoms in brackets are not observed but are involved in magnetization transfer, allowing the correlation between the observed nuclei. These two-dimensional experiments are extensions of the aliphatic backbone correlation schemes and utilize aliphatic and aromatic carbon selective excitation to achieve a polarization transfer.

- HB(CB)(CGCD)HD experiment correlates the H^β protons to the H^δ protons in the aromatic ring via sequential magnetization transfer through the C_β , C_γ , and C_δ carbon atoms.
- HB(CB)(CGCDCE)HE further extends this pathway to H^ϵ protons, passing through C_ϵ .

These experiments allow for the intra-residue identification of ring protons by exploiting the unique scalar-coupling networks in aromatic systems. Importantly, these experiments benefit from prior aliphatic assignments, particularly of the H^β and C_β nuclei, as starting points for magnetization transfer.

Once the aromatic ring proton and carbon chemical shifts have been identified via the above 2D correlation experiments, they are typically confirmed and cataloged using the $[^{13}\text{C}_{\text{aro}}-^1\text{H}]$ -HSQC spectrum. This two-dimensional heteronuclear experiment provides a direct readout of all CH groups in the aromatic region, offering high resolution and sensitivity. The assignment process involves mapping the peaks observed in HB(CB)(CGCD)HD and HB(CB)(CGCDCE)HE experiments to the $[^{13}\text{C}_{\text{aro}}-^1\text{H}]$ -HSQC spectrum. This mapping ensures that each CH group in the aromatic ring is uniquely assigned and cross-validated.

The successful assignment of side-chain aromatic resonances is heavily dependent on the quality and completeness of aliphatic side-chain assignments, particularly of the H^β and C_β nuclei. These atoms serve as entry points for the magnetization transfer into the aromatic ring. Moreover, combining these 2D aromatic experiments with high-resolution $[^{13}\text{C}_{\text{aro}}-^1\text{H}]$ -HSQC spectra allow for a robust and comprehensive approach to the assignment of aromatic residues, which is essential for interpreting NOE patterns for three-dimensional structures calculation.

C. Assignment of large proteins

Standard triple resonance experiments used for chemical shift assignment are highly effective for proteins up to approximately 100 kDa (Lian and Barsukov 2011; Zhu and Yao 2008; Abramov et al. 2020) in molecular weight. However, as protein size increases, two major limitations arise:

- Relaxation effects due to slower molecular tumbling of larger molecules, which significantly reduce the efficiency of magnetization transfer between nuclei. As a result, relaxation effects reduce signal intensity and increase signal linewidth.
- Spectral overlap, which becomes increasingly problematic in large proteins, making peak identification and assignment challenging.

Due to these constraints, standard assignment protocols become less effective for large proteins. To address these issues, several alternative strategies have been developed and successfully implemented in the study of high molecular-weight proteins (Lian and Barsukov 2011; Zhu and Yao 2008).

In the following section, two common key approaches are presented, each designed to overcome the challenges posed by increased molecular size and to extend the applicability of NMR-based assignments to larger biomolecules.

C.1. Deuteration: isotopic labeling to reduce relaxation losses

The first strategy to mitigate the challenges associated with large protein NMR studies is selective isotopic labeling, and more specifically, protein deuteration. In fully protonated proteins, the high abundance of protons and their large gyromagnetic ratio make them the primary source of relaxation, which affects ^1H , ^{13}C and ^{15}N nuclei. This cross-relaxation, primarily responsible for the Nuclear Overhauser Effect (NOE), accelerates transverse relaxation (R_2) and leads to faster signal decay and ultimately reducing spectral sensitivity and resolution (Pervushin et al. 1997) because of increasing linewidths. To clarify, NMR relaxation reflects the return of nuclear spin magnetization toward equilibrium through interactions with fluctuating local magnetic fields arising from the surrounding molecular environment. Longitudinal relaxation (R_1) describes the recovery of magnetization along the static magnetic field (z -axis), whereas transverse relaxation (R_2) characterizes the decay of magnetization in the plane perpendicular to the field due to dephasing caused by these local field fluctuations. In contrast, the cross-relaxation process involves interactions between two different nuclei, typically those that are spatially close within 5 Å of each other. This is observed in the NOESY experiment, which measures these interactions through population transfer.

To counteract this, proteins can be expressed in deuterated media to yield partially deuterated samples (75-85%), where most non-essential protons for $\{^{15}\text{N}\}$ -based NMR

study are replaced by deuterium. Because deuterium (^2H) possesses a gyromagnetic ratio approximately six times lower than that of ^1H , it contributes significantly less to dipolar coupling interactions. This reduction leads to a six-fold decrease in dipolar relaxation contributions, effectively minimizing R_2 relaxation and improving overall signal lifetimes and resolution (decreasing of the linewidth).

While deuteration significantly reduces relaxation losses and improves spectra for larger proteins, it may not, by itself, fully address the problem of spectral overlap or guarantee sufficient signal resolution. Therefore, complementary strategies are often required to further enhance the quality of NMR data for high molecular-weight proteins.

C.2. Transverse relaxation optimized spectroscopy (TROSY)

The second strategy is using an experiment known as Transverse Relaxation Optimized Spectroscopy (TROSY) which was developed to ease the study of large proteins with NMR. The name of this experiment originates from the fact that it optimizes transverse relaxation by using the destructive interference of two phenomena that lead to relaxation: the chemical shift anisotropy (CSA) and dipole–dipole coupling (DD). This is possible for proteins because CSA and DD coupling give contributions of similar amplitudes but of opposite signs for the ^1H - ^{15}N of the peptide bond and can in some conditions cancel relaxation out (Zhu and Yao 2008). To understand how DD and CSA are averaged out during relaxation in a TROSY experiment, let's consider an isolated ^1H - ^{15}N spin pair for simplicity.

In the case of a small protein undergoing fast tumbling, the conventional $[^1\text{H}$ - $^{15}\text{N}]$ -HSQC spectrum, recorded without decoupling of these spins, will typically show four signals, each corresponding to the individual multiplet components, as illustrated in **Figure 3.10. (a)**. In this scenario, the CSA and DD couplings do not contribute significantly because they are averaged out to zero due to the rapid tumbling of the molecule in solution (Cavanagh, J et al. 2006; Bax and Grzesiek 1993).

For simplicity and clearer analysis of heteronuclear correlations, the conventional $[^1\text{H}$ - $^{15}\text{N}]$ -HSQC spectrum is often recorded with decoupling, which removes the splittings caused by coupling between proton and nitrogen (Freeman and Kupče 1997). Additionally, decoupling enhances sensitivity by consolidating the intensity of a multiplet into a single peak (see **Figure 3.10. (b)**).

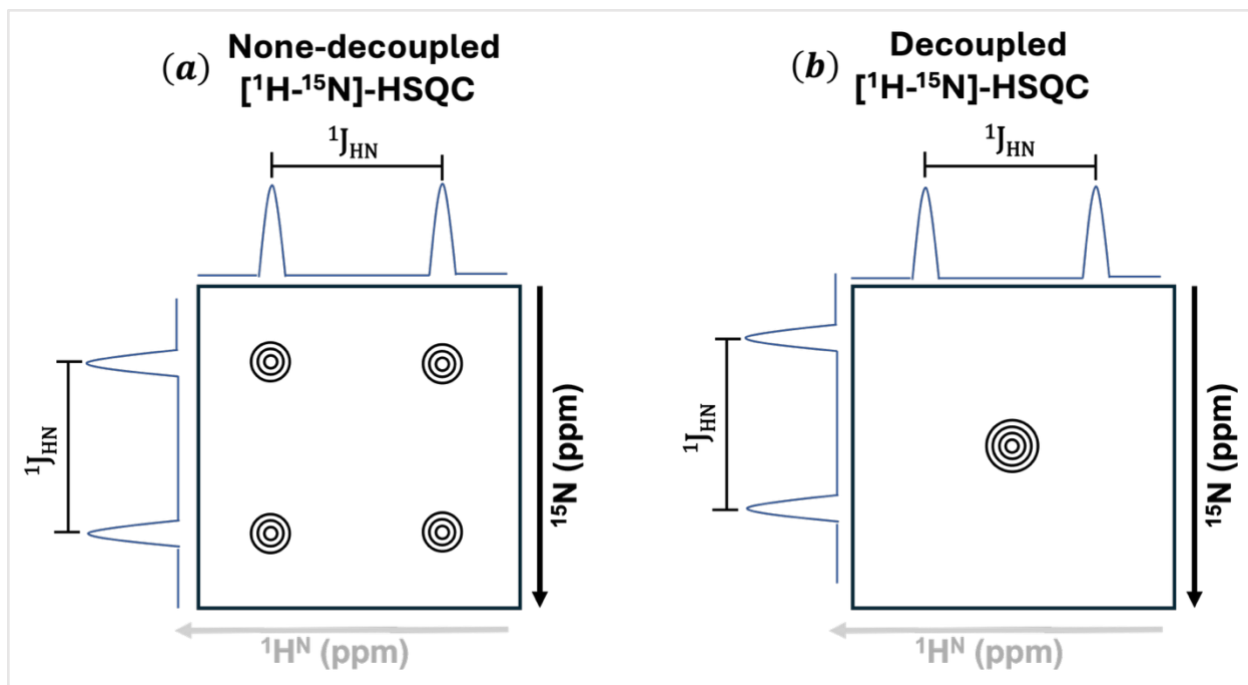


Figure 3.10. None-decoupled and decoupled $[^1\text{H}-^{15}\text{N}]\text{-HSQC}$ of an isolated spin pair. (a) None-decoupled $[^1\text{H}-^{15}\text{N}]\text{-HSQC}$. (b) Decoupled $[^1\text{H}-^{15}\text{N}]\text{-HSQC}$. Adapted from (Fernandez 2003; Pervushin et al. 1997)

In the case of large proteins (> 25 kDa) (Loria et al. 1999; Salzmänn et al. 1998) that undergo slow tumbling, DD and CSA have a dominant impact (Pervushin et al. 1997), increasing transverse relaxation rates and leading to peak broadening and loss of resolution. The interference between DD and CSA can be either additive ($\text{CSA} + \text{DD}$) or subtractive ($\text{CSA} - \text{DD}$). In the latter case, the transverse relaxation is reduced (Fernandez 2003; Loria et al. 1999; Pervushin et al. 1997; Salzmänn et al. 1998; Zhu and Yao 2008). More specifically, in a conventional $[^1\text{H}-^{15}\text{N}]\text{-HSQC}$ spectrum without decoupling for such large proteins, the four peaks from an isolated $^1\text{H}-^{15}\text{N}$ spin pair will have different linewidths, directly demonstrating the relaxation interference (see **Figure 3.11 (a)**).

In $[^1\text{H}-^{15}\text{N}]\text{-HSQC}$, decoupling removes $^1\text{H}-^{15}\text{N}$ coupling by inverting passive spin state faster than the scalar-coupling. As a result, each $^1\text{H}-^{15}\text{N}$ pair appears as a single resonance in the 2D spectrum, located at the chemical shift of the proton and that of the nitrogen, with a linewidth that reflects the average of the individual linewidths of the coupled spins. However, even in the presence of decoupling, spectral resolution remains limited for large molecular systems due to increased transverse relaxation rates (see **Figure 3.11 (b)**), as discussed above.

The TROSY technique selects the least-relaxing component by exploiting subtractive interference ($CSA - DD$), thereby reducing the transverse relaxation rate and improving spectral resolution (see Figure 3.11 (c)).

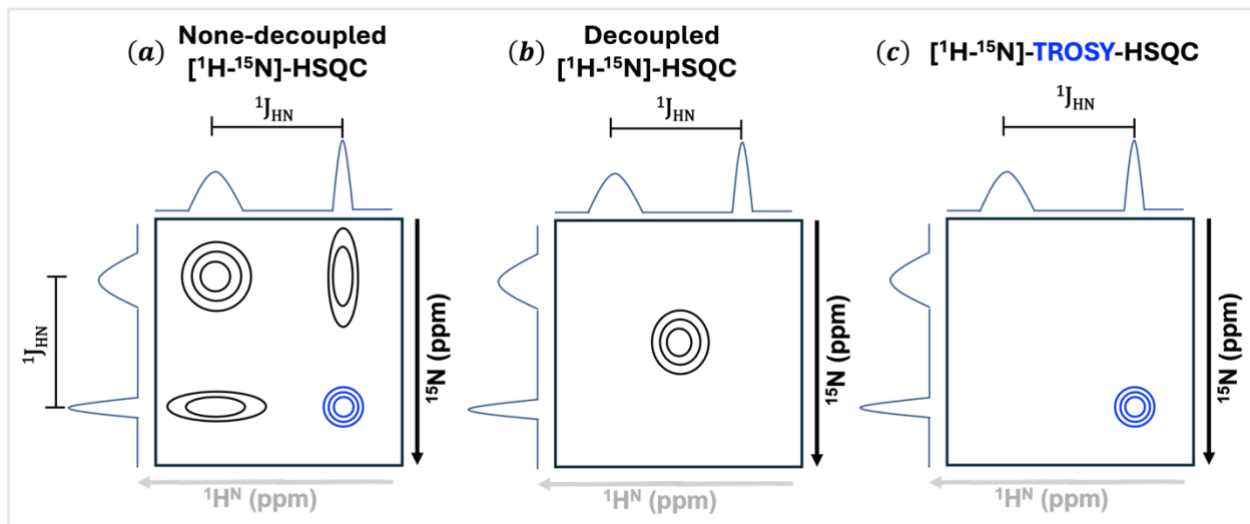


Figure 3.11. $[^1\text{H}-^{15}\text{N}]\text{-TROSY-HSQC}$ spectrum for an isolated spin pair in large protein. (a) None-decoupled $[^1\text{H}-^{15}\text{N}]\text{-HSQC}$ spectrum. (b) Decoupled $[^1\text{H}-^{15}\text{N}]\text{-HSQC}$ spectrum. (c) $[^1\text{H}-^{15}\text{N}]\text{-TROSY-HSQC}$ spectrum.

TROSY reduces the transverse relaxation in multidimensional experiments and hence enables the assignment of proteins of more than 100 kDa (Lian and Barsukov 2011; Zhu and Yao 2008; Abramov et al. 2020). A set of TROSY-based experiments (Zhu and Yao 2008), based on the same assignment strategy as the standard triple-resonance experiments for backbone assignment as well as TOCSY-TROSY are available (Zhu et al. 1999).

III.1.2. NMR restraints for structure calculation

Once the chemical shifts of nuclei have been accurately assigned, several types of structural restraints can be derived to aid in NOESY peak assignment and structure calculation such as angular and J-coupling restraints.

A. Angular restraints

Angular restraints are particularly valuable for defining the local geometry of the protein backbone. Backbone chemical shifts are highly sensitive to the local conformation, particularly the ϕ (phi) and ψ (psi) backbone torsion angles. This correlation allows for the extraction of angular restraints based on observed chemical shifts. Programs such as TALOS, TALOS+, and TALOS-N (Cornilescu et al. 1999; Shen and Bax 2013; Shen et al.

2009) implement this approach by predicting backbone torsion angles using the chemical shifts of C α , H α , C β , C', N, and H^N atoms.

The prediction process relies on a database of tripeptide fragments derived from high-resolution 3D protein structures with experimentally assigned chemical shifts. The query residue's chemical shifts and sequence context are matched against this database. Subsequently, a neural network-based filtering step is applied to refine the matches and assess their consistency. When the predictions from multiple database matches converge, reliable estimates of the ϕ and ψ torsion angles are extracted for the residue in question. These predicted torsion angles are then used as angular restraints during structure calculation.

Importantly, such angular restraints significantly enhance the accuracy and reliability of NOESY assignment. By restricting the conformational space in which NOEs are interpreted, they help disambiguate cross-peaks and improve convergence during 3D structure determination.

B. J-coupling restraints

The local magnetic field experienced by a nucleus is influenced by nearby nuclei to which it is covalently bonded. For scalar-coupled nuclei, each spin-1/2 nucleus can adopt two spin states (α and β) (*Protein NMR Spectroscopy* 2006) giving rise to four possible combinations ($\alpha\alpha$, $\alpha\beta$, $\beta\alpha$, $\beta\beta$). These states correspond to slightly different energy levels, leading to the splitting of NMR signals into multiplets (Macomber 1995). The magnetic interaction responsible for this splitting, known as J-coupling (or scalar coupling), is transmitted through the bonding electrons and arises primarily from the Fermi contact interaction, with smaller contributions from other mechanisms such as interaction between electron spin magnetic moments and nuclear spins. J-coupling manifests itself as a splitting of the resonance signal of the nucleus being observed. When a nucleus is coupled to multiple covalently bonded neighbors, each additional coupling causes further splitting of the signal. The magnitude of this splitting is characterized by the coupling constant, denoted as $^nJ_{AX}$, where n refers to the number of bonds between nuclei A and X. These coupling constants are measured in Hertz (Hz) and are notably independent of the external magnetic field strength (G. A. Morris 1997).

Among the different types of couplings, three-bond (^3J) couplings are especially informative in protein NMR, as they are sensitive to the dihedral angles between the interacting nuclei. For instance, the $^3J_{H^N, H\alpha}$ coupling constant is directly correlated with the ϕ torsion angle of the protein backbone through the Karplus equation (Martin Karplus 1959). However, due to the multivalued nature of this relationship, a single coupling constant may correspond to multiple possible ϕ angles. Measurement of J-coupling constants is achieved through specialized NMR experiments, such as IPAP (In-

Phase/Anti-Phase) (Ottiger et al. 1998), which allows accurate quantification of scalar-couplings by separating components of doublets into different phases. However, $^3J_{H(N)-H\alpha}$ couplings are more commonly obtained from HNHA-type experiments (Stephan Grzesiek and Delaglio, n.d.), where the coupling is derived from peak-intensity ratio.

These J-coupling-derived angular restraints, when combined with those obtained from chemical shift-based prediction (e.g., TALOS), provide complementary and reinforcing constraints that improve the accuracy of structure calculations and help guide NOESY peak interpretation.

III.1.3. NOEs assignment and structure calculation

The determination of protein structures by NMR spectroscopy relies on distance restraints derived from NOESY (Nuclear Overhauser Effect Spectroscopy) experiments. These restraints are based on the detection of the Nuclear Overhauser Effect (NOE), a physical phenomenon resulting from dipolar interactions between spatially proximate hydrogen nuclei (^1H), typically within 5 Å.

Unlike scalar-couplings, which reflect through-bond connectivity, NOEs provide through-space information that is essential for mapping the three-dimensional spatial arrangement of atoms in a protein. This makes NOE-derived restraints the most direct source of interatomic distances in solution-state NMR structure determination.

In NOESY spectra, these interactions manifest as cross-peaks, with intensities approximately proportional to the inverse sixth power ($1/r^6$) of the distance between the interacting protons (r), based on the isolated two-spins. However, due to phenomena such as spin diffusion (magnetization transfer across the molecule), relaxation, and spectral overlap, the quantitative interpretation of NOE intensity is limited. As such, each cross-peak is interpreted as providing an upper bound on the inter-proton distance (Levitt 2013).

To translate NOESY cross-peak intensities into usable distance restraints, a calibration constant specific to the spectrum must be determined. This enables the conversion of NOE intensities into semi-quantitative spatial constraints, which are then used in computational structure calculation algorithms.

To optimize NOE detection across different proton environments within the protein, three types of 3D heteronuclear-edited NOESY experiments are commonly employed:

- ^{15}N -NOESY-HSQC (Marion, Driscoll, et al. 1989; Marion, Kay, et al. 1989; Zuiderweg and Fesik 1989): targets interactions involving backbone amide protons and nearby protons; essential for mapping long-range contacts involving the protein backbone.

- ¹³C α -NOESY-HSQC (Marion, Driscoll, et al. 1989; Marion, Kay, et al. 1989; Zuiderweg and Fesik 1989): focuses on aliphatic side-chain proton interactions through ¹³C editing; provides detailed information about sidechain packing and methyl group environments.
- ¹³C α -NOESY-HSQC (Stanek et al. 2013): designed to detect NOEs involving aromatic to aliphatic side-chain protons; particularly useful for assigning Phe, Tyr, Trp, and His residues and understanding their role in tertiary structure.

Together, these experiments provide comprehensive coverage of NOE interactions throughout the protein structure, enabling accurate and convergent 3D structure calculations when integrated with chemical shift assignments and angular restraints.

Once NOESY spectra have been recorded, the next critical step in NMR structure determination involves the assignment of NOE cross-peaks to specific atom pairs. Each assigned cross-peak provides an inter-proton distance restraint, which constitutes a central input for three-dimensional structure calculations. During this NOE assignment process, observed cross-peaks are correlated with atom pairs based on previously determined chemical shifts. Ideally, the position of each cross-peak in the spectrum would allow for a straightforward and unambiguous assignment. However, in practice, chemical shift overlap especially in larger or structurally complex proteins frequently leads to multiple possible assignment candidates for a given peak.

To address this ambiguity, several computational strategies have been developed and implemented in automated NOE assignment algorithms. Among the most effective and widely adopted solutions are the following three core concepts:

A. Network Anchoring

Network anchoring (Herrmann et al. 2002; Peter Güntert 2004; Peter Güntert and Buchner 2015) is employed to evaluate the likelihood of each assignment based on its consistency with neighboring, previously assigned atoms. For instance, if residue i has been reliably assigned, then plausible cross-peaks for residues $i - 1$ and $i + 1$ will support and reinforce its assignment. This strategy is a key component of the CYANA program.

B. Ambiguous Distance Restraints (ADR)

Originally proposed by Michael Nilges and first implemented in the software ARIA (Linge, Habeck, et al. 2003; Rieping et al. 2007), ambiguous distance restraints (Herrmann et al. 2002; Peter Güntert 2004; Peter Güntert and Buchner 2015) allows multiple assignment possibilities to be retained rather than prematurely discarded. In this framework, a cross-

peak is treated as a superposition of potential assignments, and a distance restraint is applied to the sum of their NOE intensities. As long as the correct assignment is present among the candidates, the resulting structural model will still satisfy the distance constraint.

C. Constraint Combination

Constraint combination (Herrmann et al. 2002; Peter Güntert 2004; Peter Güntert and Buchner 2015) generates composite ambiguous restraints for groups of peaks that could result from multiple assignments. These constraints are fulfilled if at least one of the assignments is correct, thereby reducing the risk that incorrect or overlapping signals will distort the final 3D structure.

By applying these strategies, automated NOE assignment becomes feasible, even for large proteins with high spectral complexity. Programs such as FLYA (Peter Güntert 2009), CYANA (Peter Güntert 2004; Peter Güntert and Buchner 2015), ARIA (Linge, Habeck, et al. 2003; Rieping et al. 2007), and Xplor-NIH (Schwieters et al. 2003) incorporate at least one of these techniques to enhance robustness, minimize user intervention, and increase the accuracy of the derived structures.

Once a comprehensive list of interproton distance restraints has been established from NOESY spectra, the three-dimensional structure of the protein can be computed. This process involves the generation of spatial models that satisfy all experimental constraints, particularly the distances inferred from NOE cross-peaks. Several software packages have been developed for NMR-based structure calculation, including:

- FLYA (fully automated structure determination algorithm)
- CYANA (Combined Assignment and Dynamics Algorithm for NMR Applications), formerly known as DYANA (DYnamics Algorithm for Nmr Applications) (P. Güntert et al. 1997) and DIANA (Distance Ange Dynamics for NMR Applications) (Peter Güntert et al. 1991),
- ARIA (Ambiguous Restraints for Iterative Assignment) (Linge, Habeck, et al. 2003; Rieping et al. 2007),
- Xplor-NIH (Schwieters et al. 2003)
- CNS (Crystallography & NMR System) (Brünger et al. 1998), an evolution of X-PLOR ("X-Plor Version 3.1: A System for X-Ray Crystallography and NMR" 1993).

Among these, CYANA has become one of the most widely used tools for NMR structure determination, largely due to its integrated pipeline for automated NOE assignment and iterative structure refinement.

In CYANA, the structure determination is carried out through seven iterative cycles that alternate between NOE assignment, structure calculation and NOE assignment evaluation (**Figure 3.12**). In each cycle, an initial structural model is generated using the current set of distance restraints derived by the CANDID algorithm (Herrmann et al. 2002). A crucial step involves the initial assignment of NOESY cross-peaks to candidate atom pairs (cycle 1). For each observed cross-peak, CYANA compares its spectral coordinates with all possible proton-proton pairs for which chemical shifts have been assigned and network-anchoring, retaining those that fall within a user-defined chemical shift tolerance window.

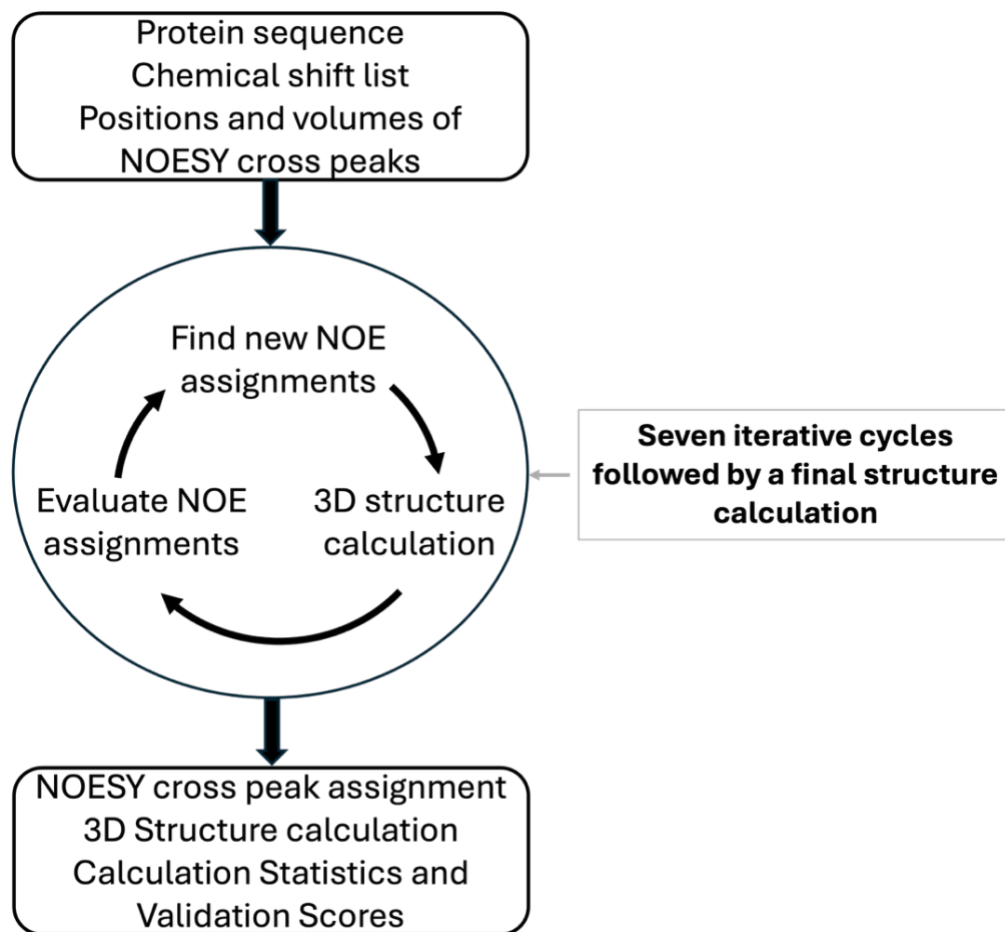


Figure 3.12. General workflow of automated NOESY assignment combined with structure calculation using CYANA. Adapted from (Peter Güntert 2004)

This procedure forms the basis of the initial peak list annotation. Angular restraints are very helpful at this stage. This stage is critical for the success of the iterative NOE assignment and structure calculation process in CYANA. A well-chosen tolerance range ensures that:

- True NOE interactions are included among the candidate assignments,
- False-positive matches due to overlapping chemical shifts are minimized, and
- The algorithm retains enough flexibility to resolve ambiguities in subsequent cycles using internal validation strategies like network anchoring.

If the tolerance is set too narrowly, many genuine interactions may be excluded from consideration, leading to insufficient structural information and failure to fold the protein correctly. On the other hand, overly broad tolerances increase assignment ambiguity, which can degrade structure quality by allowing incompatible or incorrect restraints to influence the optimization process.

The CANDID module within CYANA handles this initial assignment and is designed to update and refine NOE assignments over multiple cycles of structure calculation. During each cycle, intermediate structures are used to evaluate the plausibility of each candidate assignment based on geometric consistency with previously validated restraints. This iterative refinement improves both the accuracy of the NOE assignment and the convergence of the final structure ensemble.

In summary, the proper definition and handling of initial NOE cross-peak assignments in CYANA constitute a cornerstone of successful NMR-based structure determination, balancing the need for completeness and specificity in a chemically and geometrically consistent manner. The resulting structure is then used to refine NOE assignments and improve the selection of relevant cross-peaks in the next iteration. This iterative approach greatly enhances the consistency and convergence of the final structural ensemble.

The core of the structure calculation process involves simulated annealing using molecular dynamics in torsion angle space. This method explores conformational space by minimizing a potential energy function, which incorporates:

- Distance restraints from NOESY-derived interactions,
- Dihedral angle restraints
- Hydrogen bond restraints, and
- A repulsive term to prevent atomic overlap.

Additional orientational restraints, for instance, from residual dipolar couplings (RDCs) may also be integrated to further refine the model. RDCs arise when molecules experience partial alignment in the static magnetic field, leading to incomplete averaging

of DD interactions and providing long-range angular information about internuclear vectors relative to the static magnetic field.

Together, these restraints guide the folding of the protein into a structure that is both physically plausible and in agreement with the experimental NMR data.

After the iterative NOE assignment and structure calculation cycles, the final structure calculation is carried out in CYANA using the refined and validated list of unambiguous and ambiguous distance restraints. At this stage, no further NOE assignments are made; instead, the program focuses on generating a high-quality ensemble of 3D conformers that satisfy all experimental constraints as closely as possible.

The final calculation relies on simulated annealing in torsion angle space, a robust optimization method that minimizes a target function composed of:

- NOE-derived distance restraints,
- Dihedral angle restraints (e.g., from TALOS-N predictions),
- Van der Waals repulsion terms to prevent atomic clashes.

Typically, CYANA generates 100–200 trial structures, from which the 20 conformers with the lowest target function values (i.e., best agreement with the input restraints) are selected to form the final structural ensemble (**Figure 3.13**). These conformers are considered equally valid representations of the protein's solution-state structure and together illustrate the conformational precision and flexibility inherent in NMR-derived models.



Figure 3.13. Ribbon representation of NMR solution Structure of Cold Shock Protein CspA.

Each structure in the ensemble is evaluated based on several quality criteria, including:

- The number and magnitude of NOE violations,
- RMSD (Root Mean Square Deviation) within the ensemble,
- Ramachandran plot statistics for backbone geometry,

- Clash scores and torsion angle outliers, and
- Consistency with experimental data (e.g., completeness of assignments and restraint satisfaction).

The final structure ensemble produced by CYANA represents a set of conformations consistent with the experimental restraints and the precision of the structure determination. Ultimately, NMR-based structure determination with CYANA exemplifies the synergy between experimental precision and computational rigor, enabling the elucidation of atomic-resolution structures of proteins in their native environments

Chapter IV.

NMR-based structure determination of Peptides

Peptides have attracted considerable interest over several decades as powerful and often highly selective probes of the structure and function of ion channels and receptors. These properties have made them valuable leads for drug development (Verdes et al. 2016; Sanchez-Campos et al. 2022), and indeed, several venom-derived peptides have been successfully developed into therapeutics for the treatment of conditions such as hypertension, type 2 diabetes, neuropathic pain, and others (King 2011; Schmidtke et al. 2010).

NMR spectroscopy has played a key role in elucidating the structures of peptide toxins in solution, under near-physiological conditions, and in probing their structure and function relationships. Unlike X-ray crystallography, which requires crystallization, a process that remains a significant bottleneck, solution-state NMR enables structural analysis without this constraint, making it particularly well-suited for the study of small, flexible peptides.

This chapter focuses on the application of solution-state NMR to the structural characterization of peptides. It discusses the requirements for sample preparation, the types of NMR experiments commonly used to obtain structural restraints, and methodological considerations specific to peptides containing non-canonical amino acids.

IV.1. Overview of the standard approach

Peptides are typically composed of 10 to 60 amino acids and are often stabilized by multiple disulfide bonds. They sometimes also incorporate non-canonical amino acids. They represent a structurally diverse and biologically potent class of molecules. Due to their small size, high rigidity, and good solubility, these peptides are well-suited for structural elucidation using solution-state NMR spectroscopy. Since isotopic labeling of peptides is often cost-prohibitive (Desai et al. 2022), their structures are typically determined at natural isotopic abundance. Therefore, the main differences between NMR-based protein structure determination and peptide structure determination lie in three key areas:

- The assignment of chemical shifts,
- The structural characterization of non-canonical amino acids
- The identification and pairing of disulfide bonds.

II.1.1. Chemical shifts assignment

Peptides are obtained either by chemical synthesis (Cepeda et al. 2019) or purification from natural sources, including organisms such as bacteria, plants, animals, and venoms from various sources (Siigur and Siigur 2022; Fosgerau and Hoffmann 2015). A prerequisite for structure determination is a sample that is highly pure, soluble, and conformationally stable. NMR spectroscopy is particularly sensitive to impurities and

minor conformational states. Therefore, it is essential to assess the purity and homogeneity of the sample quantitatively, as even low-abundance impurities or alternate conformations can give rise to additional resonances that complicate spectral interpretation (Norton 2018).

Due to the natural isotopic abundance of the peptide sample, standard triple-resonance experiments and sequence-specific backbone assignment strategies typically employed for isotopically labeled proteins are not applicable. As a result, resonance assignment must rely primarily on the analysis of two-dimensional homonuclear ^1H NMR spectra, such as COSY (COReLation SpectroscopY), TOCSY, and NOESY, complemented by heteronuclear ^{13}C - ^1H -HSQC spectrum acquired at natural abundance. Assignment follows a systematic approach involving the identification of spin systems and the establishment of sequential connectivities, most commonly using the strategy originally developed by Wüthrich, Wagner and coworkers (Schubert et al. 2002; Wüthrich et al. 1984; Wuthrich et al. 1982). Eventually, if the sample concentration is sufficiently high (typically in the millimolar range), a ^{15}N - ^1H -HSQC spectrum may also be recorded, even at natural abundance, to assist in the identification of backbone amide protons.

For the assignment process, CCPN Analysis is commonly used to visualize, compare, and annotate multiple spectra. Synchronized display of 2D spectra (e.g., TOCSY, NOESY, COSY, HSQC) within CCPN allows the user to track correlations across experiments efficiently and maintain consistent atom labeling. This software environment significantly enhances the reliability and speed of resonance assignment, especially in complex or overlapping spectral regions.

The assignment procedure typically begins with a ^{13}C - ^1H -HSQC experiment. It is preferable to use an edited ^{13}C - ^1H -HSQC, as it allows differentiation of signals based on the number of protons attached to each carbon atom. In this experiment, CH, CH₃, and CH₂ groups are displayed with opposite phases or intensities, making them readily distinguishable. When combined with knowledge of characteristic chemical shift ranges, the edited ^{13}C - ^1H -HSQC facilitates the visual identification of key carbon types, such as C α , methyl, and methylene groups within side chains.

The assignment strategy consists of identifying amino acid residues by tracing connectivity from the side chain toward the backbone. Starting with the ^{13}C - ^1H -HSQC spectrum, a signal corresponding to a methyl group is selected. This resonance serves as an anchor point. The COSY spectrum is then used to identify the scalar (J) couplings, allowing one to follow the spin system (an amino acid here) from the side chain back to the backbone protons.

The COSY is one of the simplest two-dimensional NMR experiments. It correlates the chemical shifts of protons that are scalar coupled, typically, through two or three bonds (^2J or ^3J couplings). The informative part of the COSY lies in the cross-peaks (non-

diagonal peaks), which appear symmetrically off-diagonal (see **Figure 4.1**). These cross-peaks represent correlations between ^3J coupled protons and provide the basis for spin system identification within an amino acid residue.

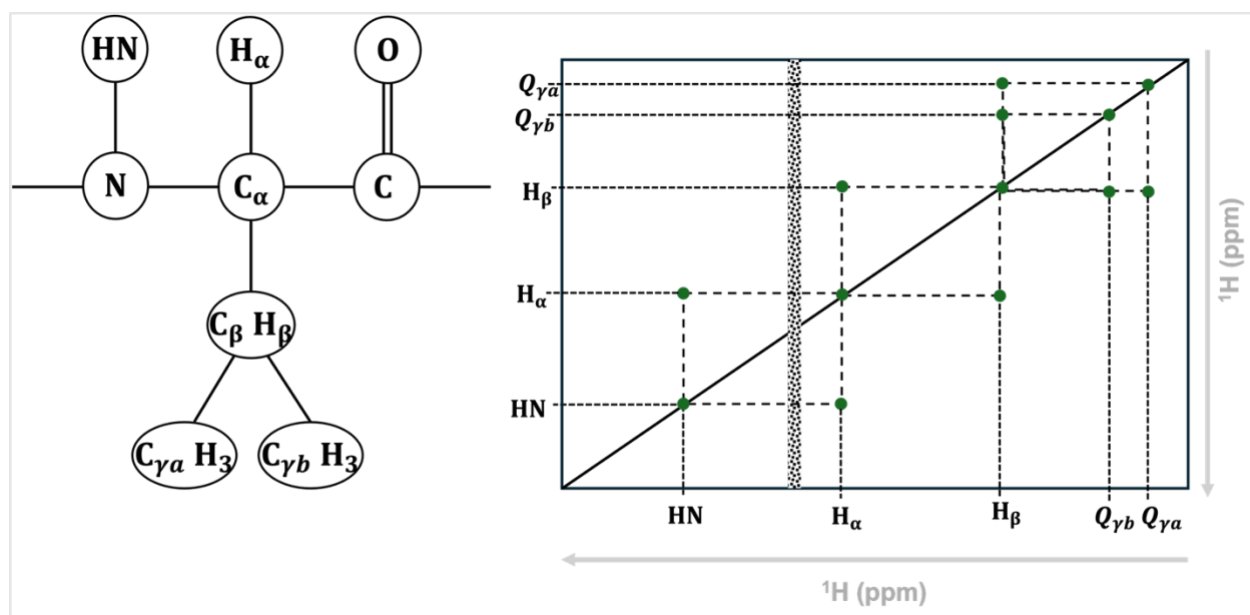


Figure 4.1. Identification of Valine spin system via COSY NMR.

Once a residue spin system is identified with COSY, a 2D TOCSY experiment is used for validation. 2D TOCSY is almost the same as regular COSY. However, instead of just showing that two protons are coupled, it creates correlations between all protons within a given spin system, not just between geminal or vicinal protons as in COSY. Correlations are seen between distant protons as long as there are couplings between every intervening proton. This is extremely useful for identifying amino acids: all protons on a given amino acid will have a correlation with all other protons (see **Figure 4.2**). In 2D TOCSY, magnetization is transferred successively over up to 5 or 6 bonds. Transfer is interrupted by weak or absent proton-proton couplings as well as hetero atoms, such as oxygen. The number of transfer steps can be adjusted by changing the spin-lock time. A short time lower than 10 ms may give only one-step transfers and its TOCSY spectrum will be very similar to its COSY spectrum. More usefully, a long spin-lock time such as 80 ms will give up to 5 or 6-step transfers, resulting in a complete connectivity map, in which, each proton of a residue shows cross-peaks with all other protons in the same residue (**Figure 4.2**). This feature is especially valuable for residue type identification, particularly in peptides analyzed at natural abundance.

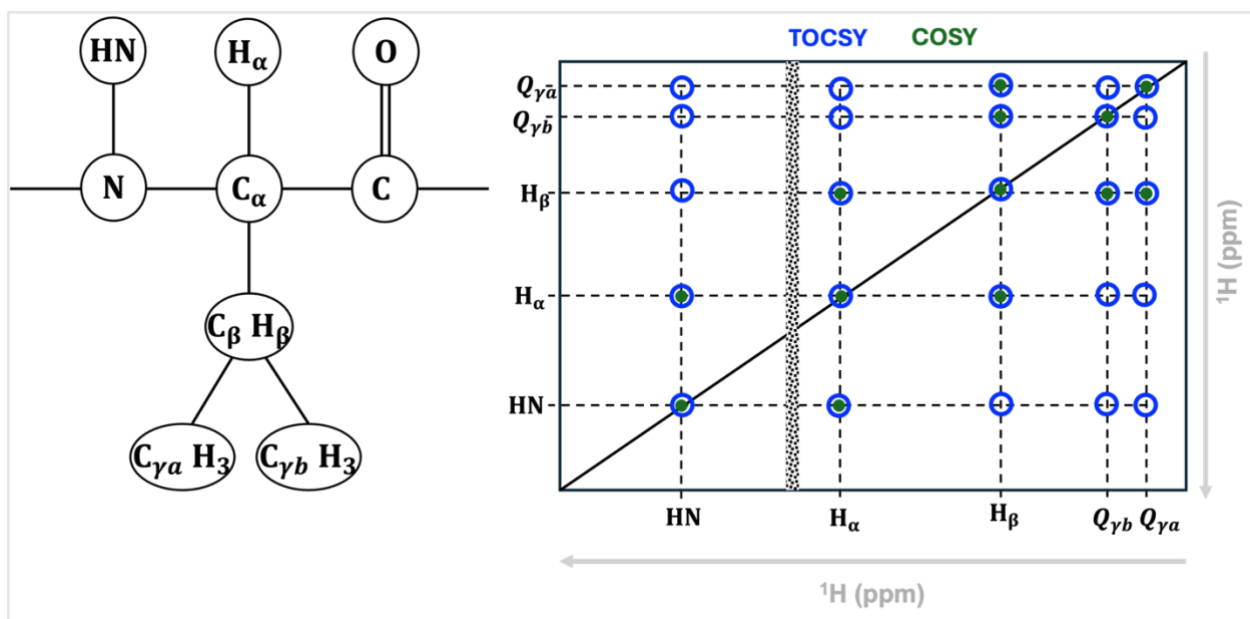


Figure 4.2. Validation of Valine spin system via TOCSY NMR.

Following the identification of residue types via homonuclear spectra, sequential connectivities are determined using 2D [^1H - ^1H]-NOESY.

In the context of peptide resonance assignment, NOESY plays a crucial role in establishing sequential connectivities along the peptide backbone. Specifically, NOE cross-peaks are expected between the amide proton (H^{N}) of residue i and protons from neighboring residues, typically, the α -protons (H_α) of residues $i - 1$. These are referred to as sequential NOEs, and they are essential for determining the order of residues in the amino acid sequence and for building the peptide's backbone connectivity map.

In a typical NOESY spectrum:

- Strong intra-residue NOEs (e.g., between H^{N} and H_α of the same residue) help confirm spin system identity (Michael P Williamson et al. 1985).
- Sequential NOEs (e.g., between $\text{H}^{\text{N}}[i]$ and $\text{H}_\alpha[i - 1]$) indicate proximity between adjacent residues (Michael P Williamson et al. 1985).
- Medium and long-range NOEs (e.g., $\text{H}^{\text{N}}[i]$ to side chains of distant residues) provide crucial structural information, especially for defining secondary and tertiary structure elements (Michael P Williamson et al. 1985).

The mixing time in a NOESY experiment significantly affects the intensity and detectability of cross-peaks. Short mixing times (e.g., 100–150 ms) favor detection of short-range NOEs and reduce spin diffusion, thereby enhancing the reliability of sequential

assignments. Longer mixing times (e.g., >250 ms) may increase sensitivity but can introduce ambiguity due to spin diffusion.

Ultimately, the NOESY spectrum, when combined with COSY and TOCSY spectra, enables a complete resonance assignment by confirming the residue type (via spin system) and position in the sequence (via NOE connectivity). It also forms the foundation for extracting distance restraints, which are used in structure calculation.

IV.2. Defining disulfide pairings

Disulfide bonds play a crucial role in stabilizing the three-dimensional structures of many peptide and small cysteine-rich proteins. Accurate identification of disulfide pairings is essential for reliable structure determination and meaningful functional interpretation, particularly in peptides derived from animal venoms, which often contain complex disulfide bonding patterns. However, this task remains challenging and is often accompanied by ambiguities, as disulfide bonds are poorly defined in most NMR datasets, especially when peptides are studied at natural isotopic abundance (Norton 2018).

A fundamental limitation arises from the lack of measurable scalar couplings across the S–S bond, which limits the direct identification of disulfide connectivity and leads to scarce experimental restraints in these regions.

One commonly used NMR-based approach involves the observation of NOESY cross-peaks between the β -methylene protons (H^β) of two cysteine residues, which may indicate spatial proximity consistent with a disulfide bond. However, this method suffers from two major limitations (Mobli and King 2010) :

- 1) Spectral overlap: in cysteine-rich peptides, the chemical shifts of cysteine H^β protons often overlap, making NOE cross-peaks ambiguous or uninterpretable.
- 2) False positives: in folded peptides, especially those with multiple adjacent disulfide bonds, H^β protons of a particular cysteine can be spatially close to one another cysteine residue to which it is not connected than it is to the H^β proton of the cysteine residue in the same disulfide bond. This can lead to spurious NOE correlations between cysteine residues that are not actually disulfide-linked (see **Figure 4.3**).

To address these issues, isotopic labeling strategies have been developed, allowing for more precise determination of disulfide connectivities through tailored NMR experiments (Walewska et al. 2008). While effective, these methods can be cost-prohibitive or impractical for small synthetic peptides studied at natural abundance.

As an alternative, computational approaches have been introduced. One notable example is the PADLOC method (Poppe et al. 2012), which applies Bayesian inference to local cysteine topology and uses probabilistic rules to predict the most likely disulfide pairings. This technique circumvents the need for isotopic labeling and provides a statistically supported framework for disulfide assignment. In addition, since the distance between the C β atoms of two cysteine residues involved in a disulfide bond typically falls within a range of 3.5 to 4.3 Å, with an average distance of 3.8 Å (Harrison and Sternberg 1996; Hazes and Dijkstra 1988; Srinivasan et al. 1990), geometric restraints can be applied to infer disulfide connectivity during structure calculation. For this purpose, CYANA offers built-in libraries that allows users to define disulfide bond restraints explicitly, either based on prior biochemical knowledge.

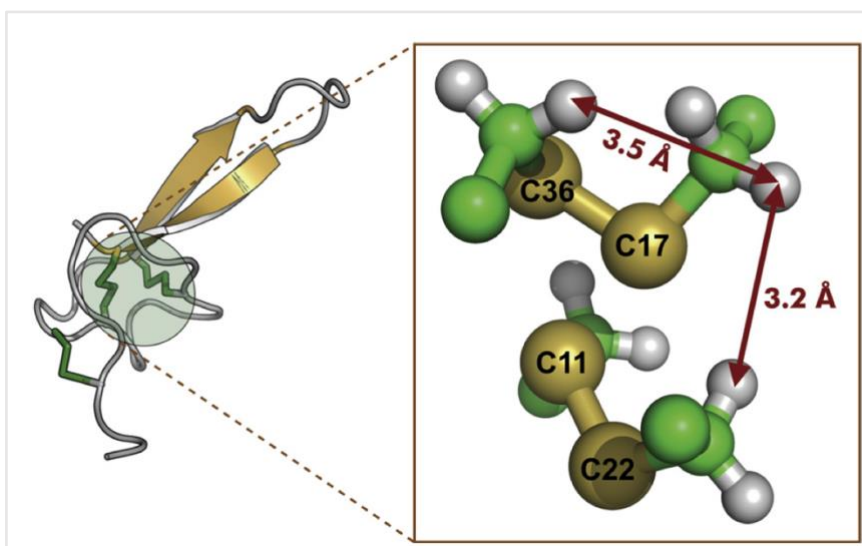


Figure 4.3. Highlighting a pair of spatially proximal disulfide bonds. Figure from (Mobli and King 2010)

Taken together, these approaches combining experimental NOEs, computational inference, and disulfide restraints enable the determination or validation of disulfide connectivities, even in the absence of direct spectral evidence. However, caution must be exercised, and whenever possible, multiple sources of evidence should be integrated to ensure accurate assignment.

IV.3. Structural characterization of non-canonical amino acids

The 20 canonical amino acids encoded by the universal genetic code are translated by the ribosomal machinery, which decodes nucleotide sequences into proteins or peptides. This process enables the programmable biosynthesis of peptides and proteins directly from genetic templates. In recent years, efforts to expand the genetic code have enabled

the incorporation of non-canonical amino acids (ncAAs) into peptides and proteins, providing a powerful means of probing and engineering structure function relationships, as well as gaining insights into information flow between nucleic acid and protein levels (Budisa 2025).

Despite the increasing use and biological relevance of ncAAs both in naturally occurring peptides (e.g., post-translationally modified toxins) and in synthetic analogues designed for enhanced activity or stability, their structural characterization remains a significant challenge. This is largely due to the fact that ncAAs are not included in standard force fields or residue libraries used by most molecular modeling and structure refinement programs. Consequently, the use of ncAAs in NMR-based structure determination requires the manual and/or automated creation of custom molecular topology and parameter definitions.

A critical step in handling ncAAs for NMR structural analysis is to define their molecular topology in a Protein Data Bank (PDB) compatible format. This involves specifying the chemical structure, bond connectivity, atom types, and, when necessary, stereochemistry and partial charges. These definitions must be compatible with both structure calculation software (e.g., CYANA, Xplor-NIH) and structural databases such as the PDB which requires submission of detailed mmCIF (Macromolecular Crystallographic Information File) or CCD (Chemical Component Dictionary) files for any non-standard residues. The correct representation of these residues is essential not only for accurate structure calculation and refinement but also for data reproducibility and transparency during public deposition. One publicly available platform that facilitates this process is the Automated Topology Builder (ATB) (Kuschert et al. 2023; Malde et al. 2011).

The ATB is a web-accessible server that generates topologies and force field parameters for a broad range of molecules. Originally developed for use with the GROMOS family of force fields, it is now widely applied in molecular simulations, computational drug design, and X-ray refinement workflows. Its flexibility and accessibility make it a valuable tool for researchers working with novel chemical entities, including modified or synthetic amino acid residues.

The ATB serves three main functions (Malde et al. 2011) :

- 1) Topology repository: it maintains a curated library of molecules that have been parameterized within the GROMOS framework, including amino acids, ligands, and small molecules.
- 2) Simulation ready systems: it provides pre-equilibrated configurations for complex systems (e.g., solvent mixtures, membrane bilayers), which can be directly used as starting points for molecular dynamics simulations.
- 3) Force field generation: it allows users to upload custom molecules and automatically generate force field compatible topologies and parameters in various

formats, including GROMOS, GROMACS, and CNS, which is particularly relevant for NMR structure refinement.

Alongside computational tools like ATB, the CCD maintained by the Worldwide Protein Data Bank (wwPDB) plays a critical role in the standardization and deposition of macromolecular structures. The CCD provides comprehensive chemical definitions for all components found in PDB entries, including non-canonical amino acids, small molecules, ligands, and post-translational modifications. Each entry includes:

- Atom and bond connectivity
- Chemical and idealized 3D structures
- Stereochemistry and chirality
- Formal charges and bond orders
- Simplified Molecular Input Line Entry System (SMILES) and International Chemical Identifier (InChI) representations
- A unique 3-letter residue code for PDB compatibility

Together, the ATB and CCD enable the seamless incorporation of ncAAs into structure determination pipelines ensuring both modeling accuracy and compliance with data deposition standards.

Once the molecular topology and chemical parameters of ncAAs have been defined, the CYLIB algorithm (Yilmaz and Güntert 2015) can be used to convert this information into CYANA compatible residue library entries. These entries are required for the automated structure calculation process in CYANA, as they define the atomic connectivity, geometry, and stereochemistry of the ncAA in a format that can be interpreted by the software's internal force field and structure calculation engine.

IV.4. Structural Restraints and Peptide Structure Determination by NMR

As with protein structure determination, the precision and accuracy of peptide structures derived from NMR data are highly dependent on both the number and spatial distribution of experimental restraints across the molecule. The reliability of the final structural model improves significantly with the inclusion of diverse and well-distributed restraints. The primary categories of restraints used in NMR-based peptide structure determination include:

1. Distance Restraints

Distance restraints inferred from the intensity of NOEs are the most critical for defining the three-dimensional fold of the molecule. These restraints are particularly important in small peptides, where long-range NOEs are often limited but can still define key structural elements such as turns or compact motifs.

2. Dihedral Angle Restraints

Dihedral (torsion) angle restraints provide complementary information about local backbone conformations, particularly ϕ and ψ angles. These can be:

- Predicted from backbone chemical shifts using computational tools such as TALOS, TALOS+ or TALOS-N.
- Directly derived from scalar-couplings observed in Double Quantum Filtered COSY (DQF-COSY) spectra recorded in both H₂O and D₂O (Norton 2018).
- In some cases, for structurally rigid peptides, dihedral angles can even be estimated from high-resolution 1D proton spectra (Norton 2018).

3. Disulfide Bond Restraints

When present and accurately defined (as described previously), disulfide bond restraints contribute significantly to the structural integrity of cysteine-rich peptides. These covalent linkages act as both topological constraints and stabilizing features and are often essential to correct folding and biological function.

Once a sufficient set of experimental restraints has been assembled, structure calculation can be performed using CYANA, following the same general protocol as used for proteins. CYANA integrates NOE-derived distance restraints, dihedral angles, and any fixed covalent linkages (e.g., disulfide bridges) to generate an ensemble of low-energy structures that best satisfy the input data. Validation metrics such as RMSD, NOE violation statistics, and Ramachandran distributions are then used to assess the quality of the calculated ensemble.

A validated peptide structure that satisfies all experimental restraints becomes a powerful tool for exploring structure–function relationships, mechanistic insights, and molecular interactions. In particular, such structures are critical for understanding the behavior of bioactive peptides, including toxins, inhibitors, or peptide therapeutics.

Chapter V.

Research Contribution

V.1. Structural and Functional Insights into Leiurotoxin-Dab7 (manuscript currently in preparation)

This section presents the results obtained as part of my doctoral research, representing my contribution to a collaborative study with Jean-François Liégeois, Jean-Luc Hayen, and Frédéric Kerff, with additional support from the Mass Spectrometry Laboratory. These results will be included in a joint manuscript currently in preparation.

Structural and Functional Insights into Leiurotoxin-Dab7, a Highly Selective Inhibitor of Human Small-Conductance Ca^{2+} -Activated Potassium Channels

Marius Wanko Nembot¹, Hayen Jean-Luc², Kerff Frédéric³, Christian Damblon¹ and

Liégeois Jean-François²

¹Structural Biological Chemistry Laboratory, MolSys Research Unity, University of Liege, Liège, Belgium.

²Department of Pharmacy, University of Liège, Liège, Belgium

³InBioS-Center for Protein Engineering, Laboratory of Crystallography, University of Liège, Liège 4000, Belgium

To whom correspondence should be addressed: Prof. Jean-François Liégeois, University of Liège, Department of Pharmacy, 4000 Liège, Belgium; orcid.org/0000-0002-6100-9493; Email: JF.Liegeois@uliege.be

INTRODUCTION

In many neurons of the central nervous system, action potentials are followed by a transient increase in intracellular Ca^{2+} concentration, which in turn triggers a prolonged afterhyperpolarization (AHP) of the membrane potential (Lancaster and Adams 1986; S. H. Williams and Constanti 1988; Stocker et al. 1999). This AHP is mediated primarily by small-conductance, voltage-insensitive, Ca^{2+} activated K^+ channels (SK channels). These channels play a crucial role in regulating neuronal excitability, shaping firing patterns, and modulating neurotransmitter and hormone release in both neuronal and endocrine cells (Ghanshani et al. 2000; Grissmer et al. 1992; Tuteja et al. 2005). In humans, SK channels are encoded by three homologous genes KCNN1 (SK1), KCNN2 (SK2), and KCNN3 (SK3) which give rise to distinct but closely related channel subtypes (Köhler et al. 1996; Stocker and Pedarzani 2000; Tuteja et al. 2005). These isoforms share high sequence similarity, particularly within their six transmembrane domains and the pore-forming region, where they display approximately 80–90% sequence identity. In contrast, the cytoplasmic N- and C-terminal domains show greater divergence, contributing to differences in subcellular localization, regulation, and pharmacological sensitivity among subtypes (Brown et al. 2020; Butler et al. 2023; Köhler et al. 1996; W.-S. Lee et al. 2003; Saljic et al. 2024; Stocker and Pedarzani 2000; Xu et al. 2003). Despite these structural differences, all human SK channels share a voltage-independent gating mechanism that relies on intracellular Ca^{2+} binding to calmodulin (CaM) constitutively associated with their C-terminal CaM-binding domain (C.-H. Lee and MacKinnon 2018; W.-S. Lee et al. 2003; Schumacher et al. 2001). Functionally, SK channels are broadly expressed throughout the central nervous system, as well as in cardiac, smooth muscle, and endocrine tissues, where they play essential roles in regulating membrane excitability, firing frequency, and Ca^{2+} -dependent feedback signaling (Köhler et al. 1996; W.-S. Lee et al. 2003). Beyond the central nervous system, SK channels are also expressed in the peripheral nervous system (Bahia et al. 2005), the heart (Tuteja et al. 2005), the vascular endothelium (Brähler et al. 2009), and the liver (Barfod et al. 2001), where they contribute to the modulation of electrical activity, vascular tone, and secretion.

Pharmacological modulation of SK channels has provided valuable insights into their physiological and pathological roles (Brown et al. 2020). Among these, selective SK channel blockers have been invaluable tools for defining the role of SK mediated currents in regulating neuronal excitability, synaptic transmission, and cardiac electrophysiology (Bond 2004; W.-S. Lee et al. 2003; P. Pedarzani and Stocker 2008; Sah 1996). Apamin binds with high affinity to the extracellular vestibule of SK channels, blocking the pore and thereby inhibiting the Ca^{2+} -activated K^+ current without affecting large-conductance (BK) or intermediate-conductance (IK) channels (Ishii et al. 1997; Strøbæk et al. 2000).

The bee venom peptide toxin apamin has been shown to be a highly sensitive and selective blocker of SK channels, exhibiting high affinity for both native and recombinant

SK channel subtypes. In addition to apamin, several other peptide toxins have been identified as potent SK channel inhibitors, including scorpion toxins PO5 (Zerrouk et al. 1993), TsKapa (Blanc et al. 1997), Tamapin (Paola Pedarzani et al. 2002), Leiurotoxin I and Leiurotoxin-Dab7 (Auguste et al. 1990; Mpari et al. 2005; Zerrouk et al. 1993), a non-natural mutant of Leiurotoxin I. These toxins, isolated from various venoms, share a common structural motif that enables them to interact with the extracellular pore region of SK channels, thereby blocking Ca^{2+} -activated K^{+} currents with high selectivity.

Among these channel blockers, Leiurotoxin-I stands out as a potent and highly selective peptide isolated from the venom of *Leiurus quinquestriatus*. Based on its structure, and using Leiurotoxin-I and PO5 as templates, an analog designated Leiurotoxin-Dab7 (Lei-Dab7) was previously engineered, in which the methionine residue at position 7 of Leiurotoxin I was replaced by the unnatural amino acid diaminobutanoic acid (Dab) (Possani et al. 2001; Shakkottai et al. 2001). Lei-Dab7 was designed to investigate the functional importance of residues at positions 6 and 7, which form part of the conserved RXCQ motif reported to be critical for SK channel recognition and binding (Auguste et al. 1990; Abia et al. 1986). Accordingly, Lei-Dab7 has been reported to recognize the SK2 subunit with high affinity (Mpari et al. 2005; Shakkottai et al. 2001).

While the NMR structure of Leiurotoxin-I has been determined and deposited in the PDB, the overall quality of this structure could be further improved to enable more reliable molecular docking and structure-based modeling. For instance, the Cys3–Cys21 disulfide bridge was poorly defined, likely due to the absence of nuclear overhauser effect (NOE) constraints between the residues Phe2–Cys3–Asn4 and Lys20–Cys21–Ile22 (José C. Martins et al. 1995). In contrast, no three-dimensional structure of Lei-Dab7 has yet been reported. Surprisingly, data supporting the NMR structure and detailed pharmacological characterization of these peptides are still missing, limiting our understanding of their structure–function relationships. Moreover, as discussed below, previous activity studies have shown that a single amino-acid substitution at position 7 of Leiurotoxin-I is sufficient to confer SK2 selectivity to the modified peptide Lei-Dab7, whereas the native Leiurotoxin-I preferentially targets SK3 (Shakkottai et al. 2001). During these studies, key channel residues responsible for the divergent activity profiles were identified: His521 on SK3 and Asn367 on SK2.

Given these reported studies, we sought to understand how a minimal structural change, specifically a single amino-acid substitution in SK channel inhibitor, can profoundly alter subtype selectivity. To address this, we undertook the determination of a refined NMR solution structures of Leiurotoxin I and its synthetic analog Lei-Dab7 and to quantitatively evaluate their binding affinities toward the two human SK channel subtypes (hSK2–3). This work provides the first direct structural and functional comparison of Leiurotoxin I and Lei-Dab7, offering new insights into their interactions with human SK channels.

MATERIALS AND METHODS

Sample Preparation and NMR Data Acquisition. Lyophilized Leiurotoxin I and Lei-Dab7 peptides were each dissolved in 50 mM sodium phosphate buffer pH 5.9 to obtain a final peptide concentration of 500 μ M. To each sample, 5% (v/v) deuterium oxide (D_2O) was added for spectrometer field-frequency locking and chemical shift referencing. The solutions were transferred into 5 mm Shigemi NMR tubes (Shigemi Inc., Japan) for data acquisition.

All NMR experiments were performed at 25 °C on a Bruker Avance III HD 700 MHz spectrometer equipped with a helium-cooled CryoProbe™. One-dimensional (1D) ^1H and two-dimensional (2D) ^1H – ^1H correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), and nuclear Overhauser effect spectroscopy (NOESY) spectra were recorded for structural analysis. TOCSY spectra were acquired with a mixing time of 80 ms, and NOESY spectra with a mixing time of 350 ms.

Additionally, a 2D ^1H – ^{13}C heteronuclear single quantum coherence (HSQC) spectrum was acquired at natural ^{13}C abundance at 25 °C. The HSQC experiment was collected with 256 (t_1) and 1024 (t_2) complex data points and 32 scans per increment.

All NMR datasets were processed using TopSpin version 3.6 (Bruker BioSpin) and analyzed with CCPNMR Analysis v2 (Vranken et al. 2005) for resonance assignment and structure determination.

Structural restraints and structure calculation. The NMR structures of Leiurotoxin I and Lei-Dab7 were calculated using distance restraints derived from NOE cross-peak intensities obtained from 2D ^1H – ^1H NOESY spectra. Structure calculations were performed using CYANA version 3.98.15 (P. Güntert et al. 1997; Peter Güntert 2004; Peter Güntert and Buchner 2015). Backbone dihedral angles (φ and ψ) were predicted from assigned chemical shifts using the TALOS+ program (Shen et al. 2009).

After seven iterative cycles of simulated annealing and torsion angle dynamics, a total of 100 conformers were generated. The resulting structures were ranked according to the final CYANA target function values, and the 10 lowest-energy conformers were selected for further analysis. Structural quality and stereochemical parameters were assessed using the Protein Structure Validation Software Suite (PSVS) (Bhattacharya et al. 2007).

Disulfide bond connectivity, established during peptide synthesis and confirmed by mass spectrometry, was enforced during structure calculation using a procedure relative to the default approach implemented in CYANA for defining disulfide bonds. All molecular representations and structural figures were prepared using PyMOL (Schrödinger, LLC; <http://www.pymol.org>).

RESULTS AND DISCUSSION

Leiuorotoxin I spatial structure in solution. NMR chemical shifts and the resulting three-dimensional structure were deposited in the BMRB and PDB databases under accession codes 34995 and 9R5M, respectively. Backbone chemical shift assignments were obtained for approximately 60% of residues within the well-defined regions (A1–H31), while 77% of side-chain resonances were assigned, resulting in an overall assignment completeness of 68%. This level of assignment completeness is considered fully adequate for small, disulfide-rich peptides studied by two-dimensional NMR, where extensive resonance overlaps and conformational constraints often limit complete assignment. The resulting structure is consistent with the experimental restraints and is characterized by a low backbone RMSD of 0.11 Å, as reported in the wwPDB validation. Structural statistics for the 10 lowest-energy conformers of Leiuorotoxin I are summarized in **Table 1**. For clarity, we refer to the previously determined NMR structure as 1SCY (José C. Martins et al. 1995; J. C. Martins et al. 1990) (PDB ID).

Table 1. Summary of structural statistics for Leiuorotoxin I NMR structures.

	9R5M structure ^a	1SCY structure ^b
Distance restraints		
Total	495	332
Intra-residue ($ i - j = 0$)	134	155
Sequential ($ i - j = 1$)	137	80
Medium range ($ i - j > 1$ and $ i - j < 5$)	101	40
Long range ($ i - j \geq 5$)	120	32
Total dihedral-angle restraints	46	-
Number of restraints per residue	16.9	11.1
Number of long-range restraints per residue	3.8	1.0
RMSD from average coordinates (Å) ^{c,d,e}		
Backbone atoms	0.10 ± 0.05	0.83 ± 0.18
Heavy atoms	0.67 ± 0.13	1.56 ± 0.14
Ramachandran statistics ^{c,d}		
Most favored regions (%)	100.0	92.2
Additional allowed regions (%)	0.0	7.8
Generously allowed (%)	0.0	0.0
Disallowed regions (%)	0.0	0.0
Global quality scores ^d		
	Raw/Z-score	Raw/Z-score
Verify 3D	-0.09/-8.83	-0.06/-8.35
ProsaII	-0.29/-3.89	-0.12/-3.18
Procheck (phi-psi)	-0.28/-0.79	-0.14/-0.24
Procheck (all dihedrals)	-0.82/-4.85	-0.33/-1.95
Molprobrity clash score	10.08/-0.20	1.45/1.28

Statistical analyses were performed for residues 3–30 of 9R5M and residues 3–21 and 24–29 of 1SCY.

^a The NMR structure determined in this work (PDB ID 9R5M)

^b Previously reported NMR structure (PDB ID 1SCY)

^c Order residue ranges [$S(\phi) > S(\psi) 1.8$].

^d Generated using PSVS 1.5 program (Tang et al. 2010) with respect to mean and standard deviation for a set of 252 X-ray structures < 500 residues, of resolution ≤ 1.80 Å, R-factor ≤ 0.25 and R-free ≤ 0.28

^e RMSD analyses were performed over residues 1–31 for both structures.

The two NMR structures differ in the number and distribution of experimental restraints used for structure calculation. The 9R5M structure (**Figure 1**) is based on a larger set of distance restraints (495 vs 332), corresponding to a restraint density of 16.9 restraints per residue. A notable difference is observed in the number of long-range restraints, which play a key role in defining the global fold. The 9R5M structure includes 120 long-range restraints (3.8 per residue), whereas 1SCY contains 32 (1.0 per residue). This difference indicates that the 9R5M structure is more constrained at the level of tertiary structure and may contribute to its improved definition relative to 1SCY. Moreover, the summary of experimentally observed NMR restraints further indicates that the 9R5M structure benefits from a broader sampling of medium-range contacts, whereas the 1SCY structure contains comparatively fewer such restraints. Such differences may contribute to variations in the degree of structural definition.

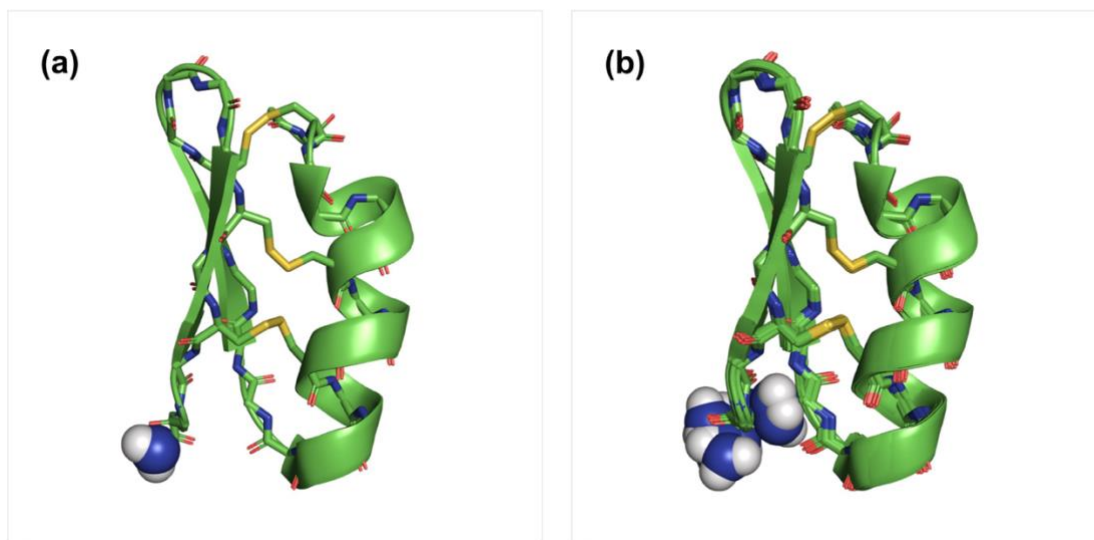


Figure 1. NMR solution structure of Leiurotoxin I (PDB ID: 9R5M). (a) Lowest-energy conformer. (b) Superposition of the ten lowest-energy conformers illustrating the well-converged structural ensemble. Disulfide bonds are shown in yellow, and C-terminal amide ($-\text{NH}_2$) groups are represented as spheres.

The overall quality of both structures was assessed using PSVS which integrates several widely used validation tools, developed by independent research groups. Each program computes a raw score based on specific structural features. Verify3D (Bowie et al. 1991) evaluates the compatibility between the amino acid sequence and its three-dimensional structure and gives a score based on probability of finding a particular residue type in the environment observed in the structure. Prosall (Sippl 1993) calculates a knowledge-based statistical potential based on pairwise interactions as a function of the spatial separation of C β atoms. PROCHECK (Laskowski et al. 1996) assesses stereochemical quality through analysis of backbone dihedral angles (ϕ/ψ) and overall geometry. MolProbity (Lovell et al. 2003) evaluates steric interactions, with the clash score quantifying the number of unfavorable atomic overlaps per 1000 atoms. Raw values correspond to the direct outputs of these validation metrics, calculated for a given structure without normalization. To enable comparison, a reference set of 252 "high-quality" X-ray structures (≤ 500 residues, resolution ≤ 1.80 Å, R-factor ≤ 0.25 , and R-free ≤ 0.28) was used to derive statistical parameters for each validation tool (Bhattacharya et al. 2007). The mean and standard deviation of the score distributions were then used to calculate Z-scores for each metric according to

$$Z - score = \frac{X - \mu}{\sigma}$$

where X is the observed value for the structure (raw value), μ is the mean, and σ is the standard deviation of the reference dataset. The Z-score thus reflects the number of standard deviations by which a given raw value deviates from the mean of the reference set. Therefore, absolute Z-score values close to zero indicate good agreement with typical "high-quality" structures.

These validation metrics indicate that both structures show trends toward agreement with stereochemical parameters (Prosall and PROCHECK), while the MolProbity assessment highlights differences depending on whether raw or normalized scores are considered. The apparent discrepancy between raw and Z-scored MolProbity values may reflect the distinction between absolute clash counts and their normalization relative to a reference dataset. These observations suggest that the two structures exhibit complementary features and should be interpreted alongside restraint-based metrics when assessing overall structural quality.

Overall, both structures are supported by experimentally derived restraints; however, the broader distribution of restraints, and in particular the greater number of long-range contacts in 9R5M, are consistent with a more well-defined global fold. Importantly, the

increased number of restraints in 9R5M reflects a larger set of independent experimental observations rather than redundant constraints, as evidenced by the substantial contribution of long-range contacts. The larger number of restraints in 9R5M is mainly due to an increased number of long-range contacts, which provide independent information on the global fold. Differences in experimental conditions may contribute to this observation, as the 9R5M measurements were performed at 25 °C and pH 5.9 (phosphate buffer), whereas the 1SCY data were acquired at 37 °C and pH 4.3 (in H₂O/²H₂O (9:1) or in ²H₂O). Global quality assessments based on PSVS validation metrics indicate no substantial differences between the two structures at this level. However, the revised structure is supported by a higher density of experimental restraints, particularly long-range and side-chain restraints, which are generally associated with improved structural definition in NMR structure determination (Bhattacharya et al. 2007). Together with the improved side-chain packing illustrated in **Figure 2**, these observations support a more precisely defined structural ensemble.

In addition, two localized regions that deviate beyond the intrinsic ensemble variability were identified (**Figure 3**). These deviations are confined to: (i) Region I, encompassing the N-terminal loop and the adjacent α -helix (residues A1–R6); and (ii) Region II, comprising β -strand β 1 and the β 1– β 2 loop (residues K20–D24). These regions are more precisely defined in the 9R5M ensemble, reflecting the higher density and improved distribution of long-range NMR restraints.

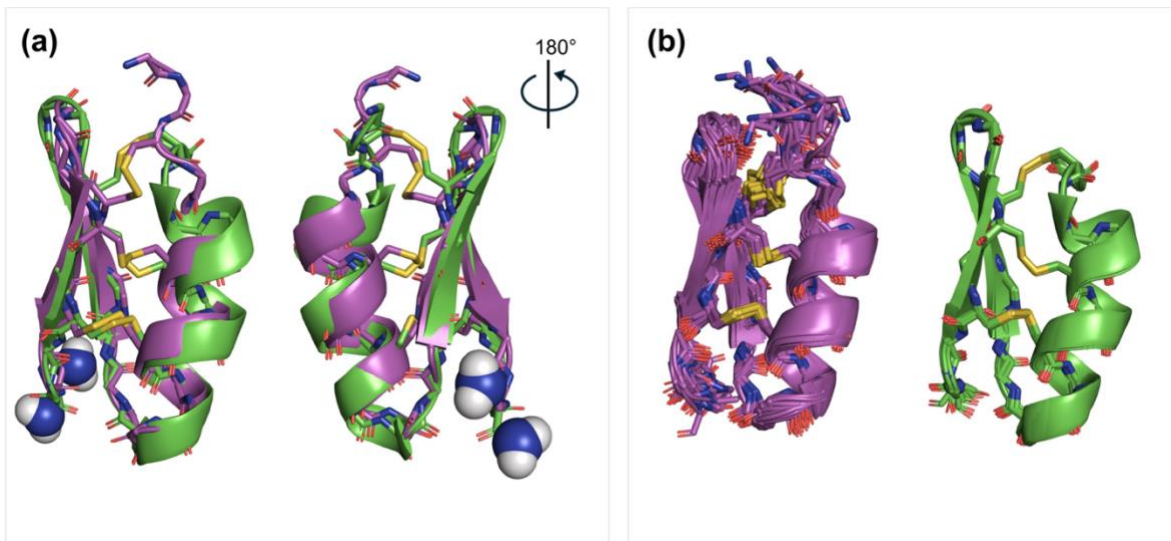


Figure 2. Comparison between the refined NMR solution structure of Leiurotoxin I (PDB ID: 9R5M) and the previously reported NMR solution structure (PDB ID: 1SCY). (a) Lowest-energy conformer. (b) Superposition of the ten lowest-energy conformers illustrating the well-converged structural ensemble. Color code: 9R5M (green), 1SCY (magenta). Disulfide bonds are shown in yellow, and C-terminal amide (–NH₂) groups are represented as spheres.

Region I (**Figure 3a**) adopts a highly reproducible conformation within the 9R5M ensemble compared with the corresponding region in the 1SCY model. This improved definition is supported by 17 long-range NOE restraints with an average upper-bound distance of 5.28 ± 0.16 Å. In addition, 16 medium-range restraints contribute to the structural definition of this region, with an average upper-bound distance of 4.46 ± 0.47 Å, approaching the long-range distance regime. Together, these restraints form a dense and internally consistent network that robustly defines the conformation of Region I.

Region II deviates slightly from the conformations observed in the 1SCY model but remains highly reproducible within the 9R5M ensemble (**Figure 3b**). This region is supported by 20 long-range NOE restraints with an average upper-bound distance of 5.32 ± 0.15 Å. Additionally, 54 medium-range restraints contribute to defining this region, with an average upper-bound distance of 4.07 ± 0.68 Å, again approaching the long-range regime. Collectively, these restraints provide a consistent and convergent definition of Region II across the ensemble.

Notably, all restraints defining Regions I and II are satisfied in the final structural ensemble, with no evidence of systematic distance and dihedral-angle violations. Representative long-range NOESY supporting these regions are shown in **Figures S1-S5**, and the corresponding atom assignments are provided in **Table S1**.

In contrast, the previously reported 1SCY structure displays greater backbone and side-chain variability, likely reflecting the limited availability of experimental restraints. For example, the Cys3–Cys21 disulfide bridge in 1SCY is poorly defined, probably due to the absence of NOE constraints between residues Phe2–Cys3–Asn4 and Lys20–Cys21–Ile22 (Martins et al. 1995).

Collectively, these results underscore the reliability and precision of the structure determination at the level of experimental restraints and demonstrate that the present approach yields a model with significantly improved backbone and side-chain packing as well as internal consistency, thereby establishing 9R5M as a precisely determined and well-defined NMR structure of Leiurotoxin I.

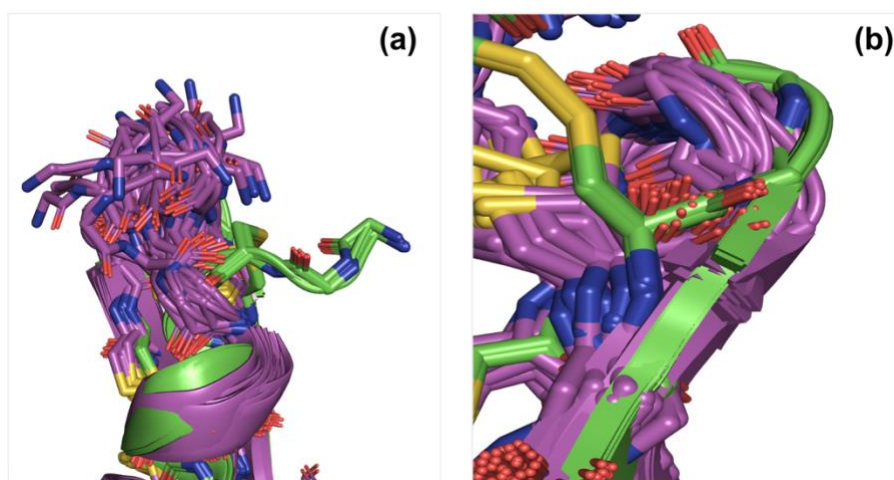


Figure 3. Regions of deviation between the 9R5M and 1SCY structures. (a-b) Regions I–II, respectively. Color code: 9R5M (green), 1SCY (magenta). Disulfide bonds are shown in yellow.

The NMR chemical shifts of Lei-Dab7 and the resulting three-dimensional structure were also deposited in the BMRB and PDB databases under accession codes 35019 and 9SWD, respectively. From the 643 distance restraints used in the structure calculation, 166 correspond to long-range NOEs, yielding an average of 5.3 long-range restraints per residue. As for Leiurotoxin I, the distribution of these long-range restraints is narrow, with an average upper distance limit of 5.33 ± 0.17 Å and minimum and maximum values of 5.0 Å and 5.5 Å, respectively. These demonstrate a high level of internal consistency and reliability of the Lei-Dab7 structure (**Figure 4**).

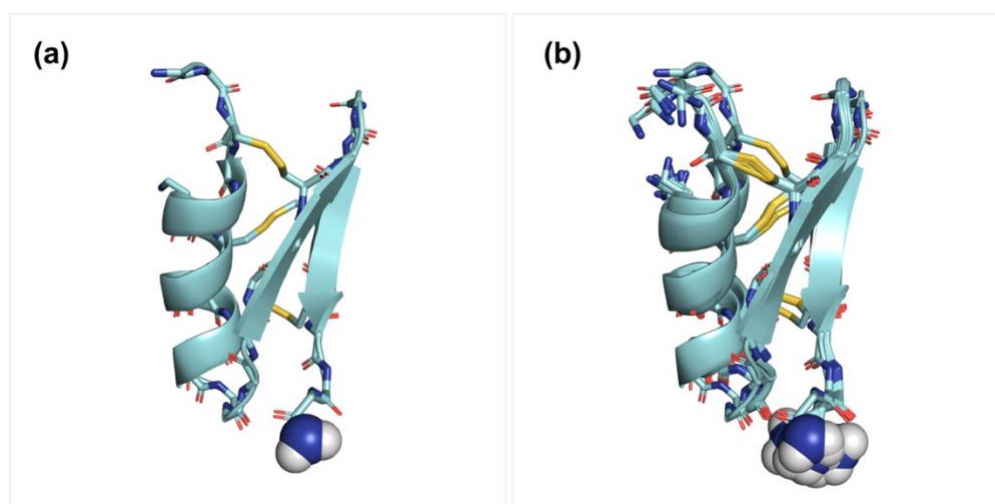


Figure 4. NMR solution structure of Lei-Dab7 (PDB ID: 9SWD). (a) Lowest-energy conformer. (b) Superposition of the ten lowest-energy conformers illustrating the well-converged structural ensemble. Disulfide bonds are shown in yellow, and C-terminal amide (–NH₂) groups are represented as spheres.

Structural statistics for the ten lowest-energy conformers are summarized in **Table 2** and demonstrate a high level of internal consistency and reliability of the Lei-Dab7 structure.

Table 2. Summary of structural statistics for Lei-Dab7 NMR structures.

	9SWD structure ^a
Distance restraints	
Total	643
Intra-residue ($ i - j = 0$)	149
Sequential ($ i - j = 1$)	200
Medium range ($ i - j > 1$ and $ i - j < 5$)	125
Long range ($ i - j \geq 5$)	166
Total dihedral-angle restraints	48
Number of restraints per residue	21.6
Number of long-range restraints per residue	5.3
RMSD from average coordinates ($\text{\AA}$) ^b	
Backbone atoms	0.23 ± 0.13
Heavy atoms	0.64 ± 0.06
Ramachandran statistics ^c	
Most favored regions (%)	100.0
Additional allowed regions (%)	0.0
Generously allowed (%)	0.0
Disallowed regions (%)	0.0
PSVS Overall quality Scores ^b	
Verify 3D	-0.12/-9.31
ProsaII	-0.41/-4.38
Procheck (phi-psi)	-0.85/-3.03
Procheck (all dihedrals)	-1.23/-7.27
Molprobity clash score	2.76/1.05

Statistical analyses were performed for residues 1–31

^a Lei-Dab7 NMR structure (PDB ID 9SWD)

^b Generated using PSVS 1.5 program (Tang et al. 2010).

^c From wwPDB validation report

Comparison with the Leurotoxin I structure reveals two major deviations. The first deviation occurs in the vicinity of the unnatural amino acid diaminobutanoic acid (Dab), which corresponds to the site of the synthetic mutation (**Figure 5a**). In Leurotoxin I, residue Met7 is involved in an extensive and well-defined network of 27 medium- and long-range NOE restraints with surrounding residues. Specifically, this network includes: two long-range restraints between Met7 and Asn4, with an average upper-bound distance of 4.07 ± 0.68 Å; one medium-range and five long-range restraints with Leu5, yielding average upper-bound distances of 4.08 Å and 5.40 ± 0.20 Å, respectively; four medium-range restraints with Cys8 (average 4.26 ± 0.75 Å); three medium-range and six long-range restraints with Gln9, with average upper-bound distances of 4.38 ± 0.22 Å and 5.24 ± 0.21 Å, respectively; five medium-range restraints with Leu10 (average 4.62 ± 0.35 Å); and one long-range restraint with Cys11. In contrast, no comparable NOE restraints were detected between Dab7 and its surrounding residues in the Lei-Dab7 structure. This marked difference in the density and distribution of experimental restraints likely accounts for the observed structural deviation in Region I, highlighting the critical role of the native Met7 side chain in stabilizing the local fold through extensive tertiary contacts.

In Region II (**Figure 5b**), the β -strand undergoes a reorientation, which is stabilized by a β -hairpin and constrained by the surrounding disulfide bond network. This conformation is consistently observed across the NMR ensemble and is supported by multiple long-range NOE restraints, indicating that it represents a well-defined structural feature rather than an artefact of structure calculation.

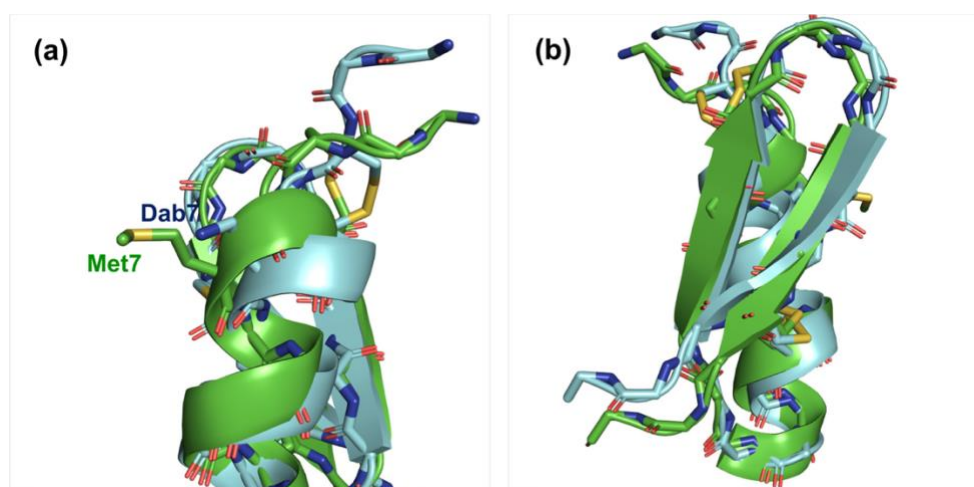


Figure 5. Regions of deviation between the Leurotoxin I (PDB ID: 9R5M) and Lei-Dab7 structures (PDB ID: 9SWD). (a) Region around Met7. (b) β -strand reorientation. Color code: 9R5M (green), 9SWD (cyan). Disulfide bonds are shown in yellow, and C-terminal amide ($-\text{NH}_2$) groups are represented as spheres.

Moreover, despite the β -strand reorientation and the presence of minor distance-restraint violations with mean values below 0.18 Å involving atom pairs Cys21 H–Cys26 H α , Cys21 H α –Cys26 H, Cys8 C β –Cys26 S γ , and Cys3 C β –Cys21 S γ , the disulfide bond network remains fully consistent across the Lei-Dab7 conformers (**Figure 6a**). Crucially, no significant differences are observed when these disulfide geometries are compared with their homologous counterparts in Leiurotoxin I, for which no distance-restraint violations were detected.

In addition, although the Met7Dab substitution lies within Region I, which deviates from the Leiurotoxin I structure, comparison of Met7 in Leiurotoxin I and Dab7 in Lei-Dab7 reveals increased conformational variability at position 7 in the latter (**Figure 6b**). This variability can be attributed to the absence of experimentally derived NMR restraints involving Dab7, limiting the definition of its side-chain conformation.

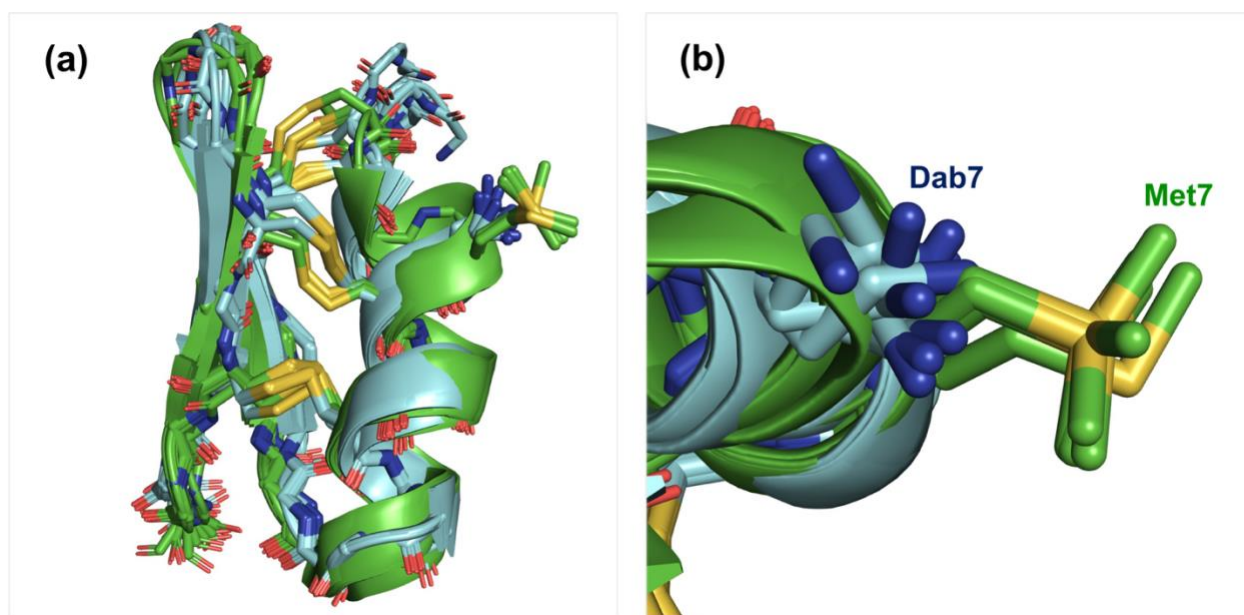


Figure 6. Structural comparison of Leiurotoxin I (PDB ID: 9R5M) and Lei-Dab7 (PDB ID: 9SWD), highlighting disulfide bond geometry and the site of the Met7Dab substitution. (a) Disulfide bonds. **(b)** Met7Dab substitution. Color code: 9R5M (green), 9SWD (cyan). Disulfide bonds are shown in yellow, and C-terminal amide ($-\text{NH}_2$) groups are represented as spheres.

Taken together, despite the localized deviations observed in Regions I and II, Lei-Dab7 exhibits a well-defined structure, supported by a dense and well-distributed network of distance restraints and consistent side-chain packing throughout the fold.

Based on the present refined three-dimensional structures of Leiurotoxin I and Lei-Dab7, the observed structural differences are localized and do not substantially alter the overall

fold or the main features of the putative binding interface (RXCQ motif (Auguste et al. 1990; Abia et al. 1986)). As such, these differences alone are unlikely to account for the selective affinity of Lei-Dab7 toward the SK2 channel, suggesting that additional factors such as molecular dynamic or channel-specific conformational selection may contribute to the observed selectivity.

Electronic Supplementary Material

A key feature of this work is the presentation of the well-defined NMR structure of Leurotoxin I reported to date, providing substantially improved accuracy compared with the previously published model (PDB ID: 1SCY). Notably, two localized regions that deviate beyond the intrinsic ensemble variability were identified. These regions are more precisely defined in the 9R5M ensemble, reflecting the higher density and improved distribution of long-range NMR restraints. For each region, we provide direct experimental validation in the Supplementary Information, including representative long-range NOESY strip plots, corresponding peak assignments, and upper-bound distance classifications. These data demonstrate that the observed deviations are supported by extensive long-range NOE restraints and therefore reflect experimentally defined conformations rather than modeling artifacts.

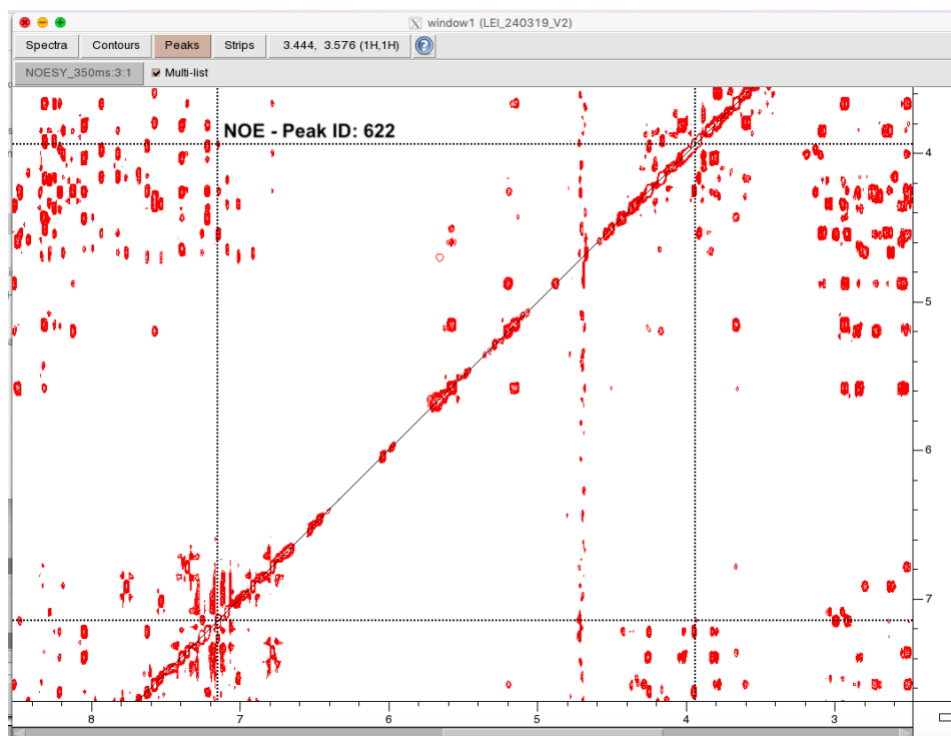


Figure S1. 2D-NOESY strip plots of Region I corresponding to peak ID 622. UPL = 5.5 Å

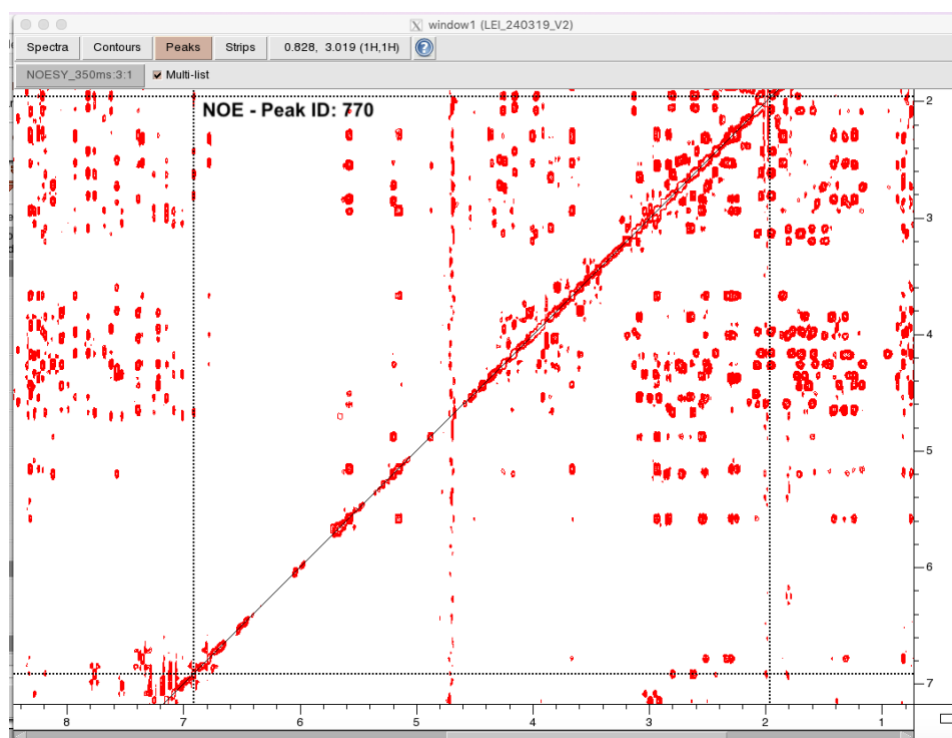


Figure S2. 2D-NOESY strip plots of Region I corresponding to peak ID 770. UPL = 5.5 Å

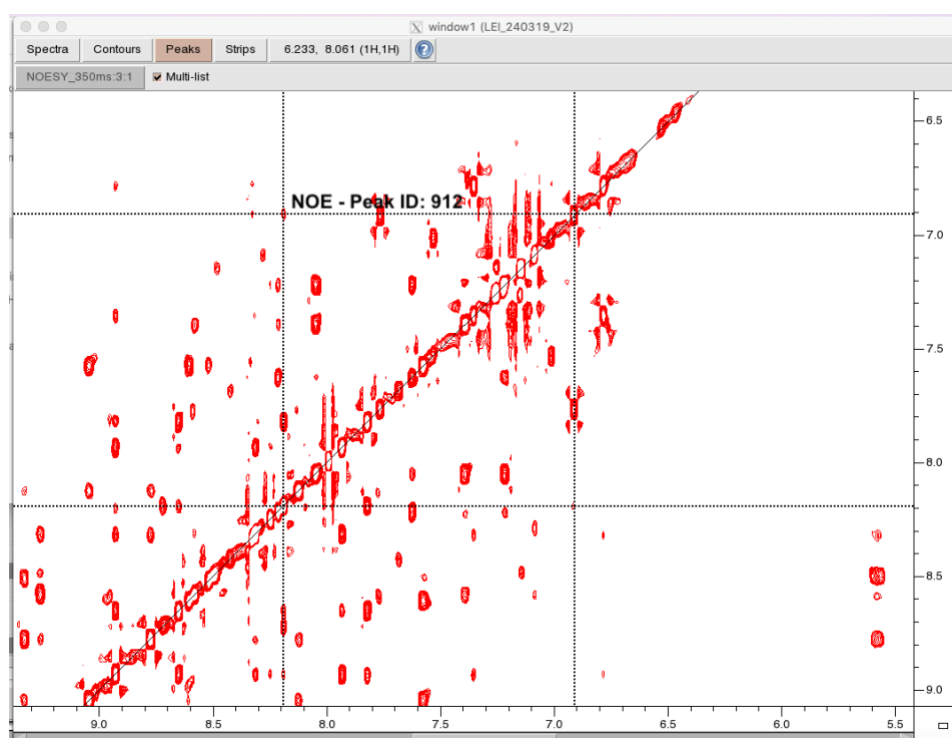


Figure S3. 2D-NOESY strip plots of Region I corresponding to peak ID 912. UPL = 5.5 Å

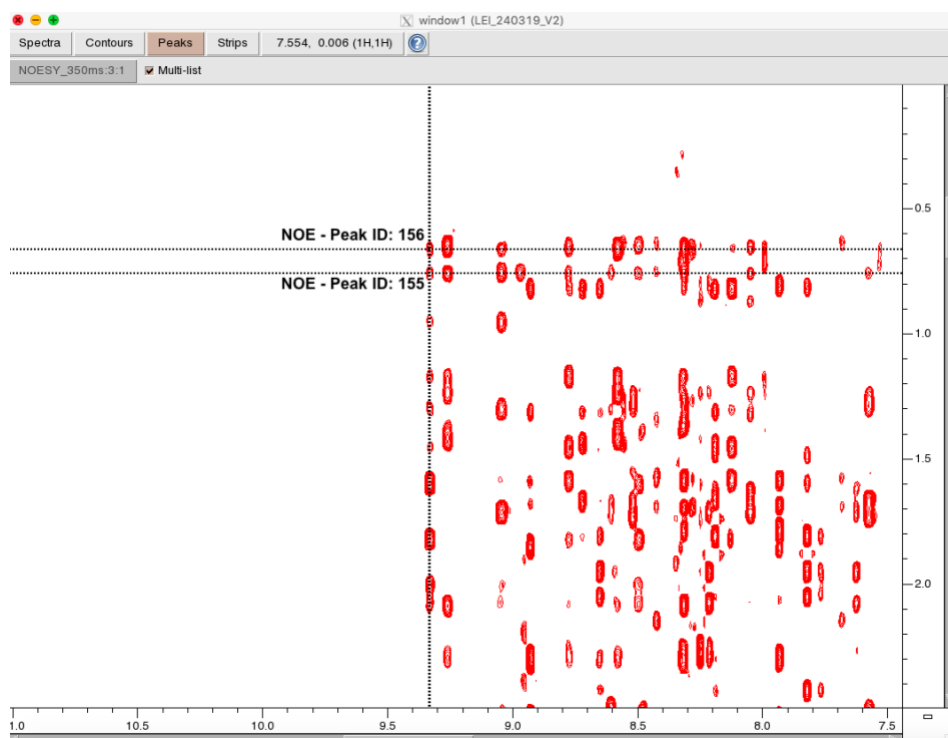


Figure S4. 2D-NOESY strip plots of Region II corresponding to peaks ID 155 and 156. UPL = 5.5 Å

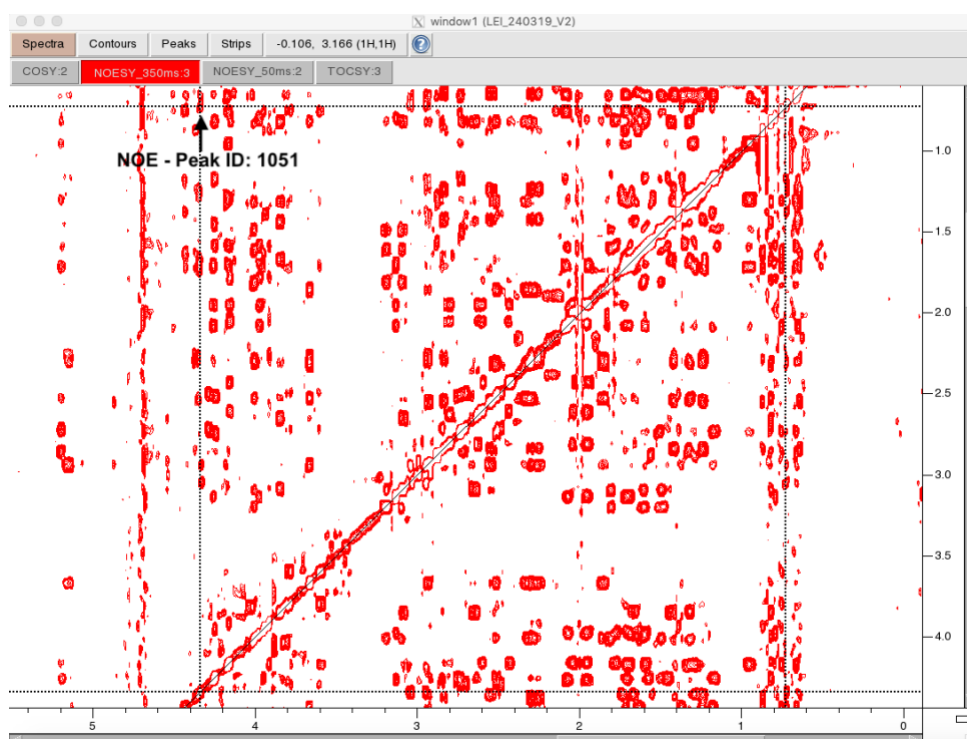


Figure S5. 2D-NOESY strip plots of Region II corresponding to peaks ID 1051. UPL = 5.5 Å

Table S1. List of NOEs identified from 2D NOESY for regions more precisely defined in the 9R5M ensemble.

	Residue 1		Atom 1	Residue 2		Atom 2	UPL	Peak ID
Region I	2	PHE	HB2	3	CYS	H	5.04	631
	2	PHE	HA	2	PHE	QE	4.41	746
	2	PHE	HA	2	PHE	QD	4.73	748
	2	PHE	QE	9	GLN	HG3	5.12	759
	2	PHE	QD	9	GLN	HG3	5.47	762
	2	PHE	QE	9	GLN	HB2	5.3	763
	2	PHE	QE	9	GLN	HB3	4.86	764
	2	PHE	QD	9	GLN	HB3	5.5	770
	2	PHE	QD	6	ARG	H	5.5	912
	2	PHE	HA	6	ARG	H	4.77	913
	2	PHE	HA	5	LEU	H	4.02	968
	2	PHE	HB3	6	ARG	H	5.29	970
	2	PHE	H	2	PHE	QE	5.18	1074
	2	PHE	H	2	PHE	HB2	4.07	1076
	2	PHE	QE	9	GLN	HG2	3.9	1089
	2	PHE	QD	6	ARG	QB	4.61	771
	1	ALA	QB	2	PHE	H	3.89	462
	3	CYS	H	3	CYS	HB2	3.75	626
	3	CYS	H	3	CYS	HB3	3.76	627
	3	CYS	HB3	4	ASN	H	5.12	1046
	1	ALA	HA	3	CYS	H	5.5	622
	1	ALA	QB	3	CYS	H	5.21	658
	4	ASN	HA	26	CYS	HB2	4.9	175
	4	ASN	H	26	CYS	HB2	4.98	871
	4	ASN	H	4	ASN	QD2	4.95	1001

	4	ASN	QB	7	MET	H	5.23	841
	4	ASN	QB	7	MET	HB2	5.34	1146
	4	ASN	QB	8	CYS	H	4.94	806
	4	ASN	QD2	22	ILE	HA	5.02	1004
	4	ASN	QD2	22	ILE	QG2	5.24	88
	4	ASN	QD2	24	ASP	H	5.34	1000
	4	ASN	QD2	25	LYS	HA	5.34	1003
	4	ASN	QD2	25	LYS	QB	4.76	1014
Region II	20	LYS	H	21	CYS	H	4.74	344
	20	LYS	HA	21	CYS	H	2.72	348
	20	LYS	HA	21	CYS	HA	5.13	362
	20	LYS	H	28	CYS	HA	3.98	378
	20	LYS	H	26	CYS	HA	4.58	380
	20	LYS	H	20	LYS	HB3	4.03	386
	20	LYS	H	20	LYS	HB2	4.03	387
	20	LYS	H	29	VAL	QG2	4.79	391
	20	LYS	H	20	LYS	QB	3.43	387
	20	LYS	H	20	LYS	QG	3.8	388
	20	LYS	H	27	GLU	QB	4.81	385
	20	LYS	HA	20	LYS	QG	3.72	432
	20	LYS	HA	21	CYS	QB	4.42	132
	20	LYS	QB	21	CYS	H	3.57	353
	20	LYS	QB	22	ILE	QG2	3.31	66
	20	LYS	QG	20	LYS	QE	3.26	415
	20	LYS	QG	21	CYS	H	4.78	356
	20	LYS	QG	27	GLU	H	5.33	153
	20	LYS	QD	22	ILE	QG2	5.27	419
	20	LYS	QD	29	VAL	QG2	5.34	419
	20	LYS	QE	29	VAL	QG1	4.91	417

	20	LYS	QE	29	VAL	QG2	4.39	73
	19	GLY	H	20	LYS	H	4.78	377
	19	GLY	HA2	20	LYS	H	2.84	379
	19	GLY	HA3	20	LYS	H	3.17	383
	5	LEU	QD2	20	LYS	H	4.99	389
	18	LEU	QD1	20	LYS	H	4.93	390
	5	LEU	QD2	20	LYS	HA	3.12	726
	19	GLY	HA3	20	LYS	HA	4.95	1149
	18	LEU	QD1	20	LYS	QE	5.34	72
	19	GLY	HA3	20	LYS	QG	5.06	1143
	21	CYS	H	22	ILE	H	4.57	345
	21	CYS	H	26	CYS	HA	5.38	347
	21	CYS	H	21	CYS	HB2	3.34	349
	21	CYS	HA	26	CYS	HA	3.89	361
	21	CYS	HA	27	GLU	H	4.53	365
	21	CYS	HB2	22	ILE	H	4.49	366
	5	LEU	QD2	21	CYS	HB3	4.32	137
	5	LEU	HG	21	CYS	H	5.28	355
	5	LEU	QD2	21	CYS	H	3.26	357
	5	LEU	QD2	21	CYS	HB2	4.32	374
	5	LEU	QD2	21	CYS	HA	5.45	727
	22	ILE	HA	24	ASP	H	5	24
	22	ILE	QG2	22	ILE	HG13	3.51	63
	22	ILE	HB	22	ILE	QD1	2.77	64
	22	ILE	HA	22	ILE	QD1	3.83	75
	22	ILE	QD1	23	GLY	HA3	4.58	76
	22	ILE	HA	22	ILE	QG2	2.94	78
	22	ILE	QD1	23	GLY	HA2	4.58	79
	22	ILE	H	22	ILE	QD1	3.89	96

	22	ILE	HG13	27	GLU	H	5.41	152
	22	ILE	HG12	27	GLU	H	5.41	154
	22	ILE	QD1	27	GLU	H	5.5	155
	22	ILE	QG2	27	GLU	H	5.5	156
	22	ILE	QD1	23	GLY	H	4.51	281
	22	ILE	H	27	GLU	H	5.23	296
	22	ILE	H	25	LYS	H	3.75	298
	22	ILE	H	26	CYS	HA	4.21	299
	22	ILE	H	22	ILE	HB	3.98	304
	22	ILE	H	22	ILE	HG13	4.14	305
	22	ILE	H	22	ILE	HG12	4.14	306
	22	ILE	H	22	ILE	QG2	4.64	308
	22	ILE	QD1	25	LYS	H	4.92	323
	22	ILE	QG2	22	ILE	HG12	3.51	338
	22	ILE	QD1	24	ASP	H	5.5	470
	22	ILE	QD1	25	LYS	HA	5.5	1051
	22	ILE	H	22	ILE	QG1	3.58	305
	22	ILE	HA	22	ILE	QG1	3.62	310
	22	ILE	QG2	22	ILE	QG1	3	63
	22	ILE	QG1	27	GLU	H	4.77	154
	22	ILE	QD1	23	GLY	QA	3.99	76
	22	ILE	QD1	25	LYS	QE	5.24	74
	4	ASN	QD2	22	ILE	HA	5.02	1004
	4	ASN	QD2	22	ILE	QG2	5.24	88

Part IV. NMR Relaxation

« ... the microsecond-to-millisecond (μs -ms) timescale dynamics are particularly significant, offering detailed insights into the kinetic, thermodynamic, and structural aspects of biological function. »

Yangzhuoyue Jin, Yingxian Cui and Tairan Yuwen. (2025), Magnetic Resonance Letters.

Chapter VI.

Introduction

NMR spectroscopy is an informative tool for probing molecular structure, dynamics, and interactions at atomic resolution. Since its development in the mid-20th century, it has become a central technique in chemistry, biology, and materials science, largely because it is non-destructive and can characterize molecules in their native environments. A particularly valuable feature of NMR lies in its sensitivity to dynamic processes, allowing researchers to access information that bridges molecular structure with function.

A key concept in NMR is relaxation, which refers to the process by which the net nuclear magnetization returns to equilibrium after perturbation by a radiofrequency pulse. This fundamental description was first introduced by Felix Bloch in his pioneering work on nuclear induction, where the Bloch equations were formulated to describe relaxation processes in terms of characteristic time constants (Bloch 1946). Importantly, relaxation has a microscopic physical origin: it is intimately governed by molecular motions that modulate local magnetic interactions. Thus, by characterizing NMR relaxation, one can indirectly extract quantitative information about molecular dynamics.

The microscopic origins of relaxation were clarified thereafter in the seminal work of Bloembergen, Purcell, and Pound, who developed a theory linking nuclear relaxation rates to molecular motion through spectral density functions (Bloembergen et al. 1948). The spectral density function describes how the molecular motion is distributed across different frequencies. In this framework, the longitudinal relaxation rate ($\{^{15}\text{N}\}\text{-}R_1$) reflects how nuclear spin populations exchange energy with their surroundings (the lattice), while the transverse relaxation rate ($\{^{15}\text{N}\}\text{-}R_2$) describes the dephasing of nuclear spin coherences due to stochastic fluctuations in local fields. Together, these relaxation parameters provide complementary and quantitative insights into the amplitudes and timescales of molecular motion.

Among the wide array of experimental techniques available to probe molecular dynamics, NMR relaxation is unique in its ability to quantify motions at atomic resolution across multiple orders of magnitude in timescale. Unlike spectroscopic approaches that are typically restricted to narrower temporal windows, NMR simultaneously accesses fast internal motions (picosecond–nanosecond) and slower conformational changes (microsecond–millisecond), thereby providing a comprehensive view of molecular behavior (Tiwari et al. 2024; Vallurupalli et al. 2012; Khandave et al. 2023). This capability is illustrated in **Figure 6.1**, where the dynamic range of NMR relaxation measurements is compared with that of biomolecular motions.

By exploiting this sensitivity, NMR relaxation has become an indispensable tool for investigating biomolecular function. In proteins, for example, side-chain and backbone motions are closely linked to enzyme catalysis, allosteric regulation, and molecular recognition (Walinda et al. 2018; Kleckner and Foster 2011; Lu and Yang 2021; Tiwari et al. 2024; Y. Jin et al. 2025; Palmer 2004). More broadly, relaxation-based analyses

provide mechanistic insights into dynamic processes that cannot be obtained from structural data alone.

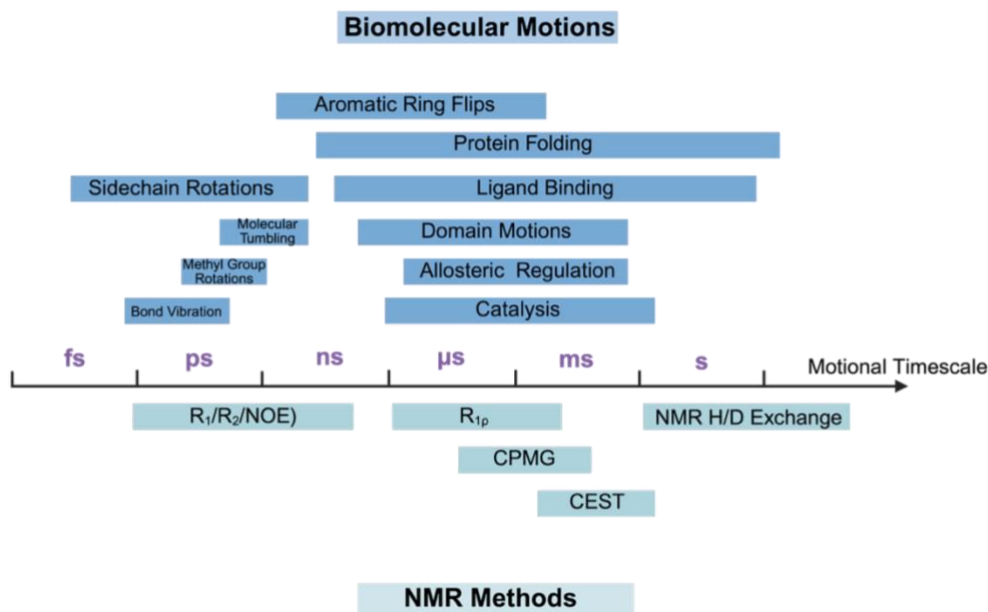


Figure 6.1. Timescales of biomolecular motions and NMR methods for probing molecular dynamics across different timescales. Adapted from (Y. Jin et al. 2025; Warecki et al. 2025)

For these reasons, NMR relaxation has emerged as a cornerstone technique for the study of molecular dynamics. The aim of the following chapters is to explore how NMR relaxation can be used as a quantitative probe of molecular dynamics at the atomic level. The specific objectives of this part of my research are threefold:

1. To review the theoretical foundations of NMR relaxation.

We will examine the principles that link stochastic molecular motion to relaxation processes, with particular emphasis on spectral density functions and their role in determining longitudinal ($\{^{15}\text{N}\}$ - R_1) and transverse ($\{^{15}\text{N}\}$ - R_2) relaxation rates, as well as the ^1H - $\{^{15}\text{N}\}$ heteronuclear Overhauser effect (^1H - $\{^{15}\text{N}\}$ NOE). The focus will be on motions in the picosecond–nanosecond timescale, which encompass fast internal fluctuations such as bond vibrations and side-chain reorientations. Furthermore, we will highlight how model-free analysis provides a framework to condense information from $\{^{15}\text{N}\}$ - R_1 , $\{^{15}\text{N}\}$ - R_2 and ^1H - $\{^{15}\text{N}\}$ NOE measurements into a single parameter, the generalized order parameter S^2 which quantitatively describes the amplitude of internal motion.

2. To survey experimental methods for quantifying relaxation at the microsecond–millisecond timescale.

We will present a range of NMR techniques, including spin-echo, Carr–Purcell–Meiboom–Gill (CPMG) dispersion, Chemical exchange saturation transfer (CEST), and, briefly, rotating-frame relaxation ($R_{1\rho}$). These approaches provide complementary windows into the microsecond–millisecond regime of molecular motion and, when combined, form a robust toolkit for probing dynamic processes with atomic resolution. Such processes include conformational exchange, enzyme catalysis, and protein folding, where understanding motions on these timescales is essential for linking structure to function.

3. To illustrate the functional relevance of relaxation measurements.

Through selected examples, we will demonstrate how quantitative analyses of relaxation provide mechanistic insights into biomolecular systems.

Taken together, these objectives aim to establish a comprehensive framework for understanding how relaxation measurements contribute to our knowledge of molecular behavior. By integrating theoretical, methodological, and applied perspectives, this dissertation seeks to demonstrate how NMR relaxation provides a bridge between structure, dynamics, and function across a wide range of biological systems.

Chapter VII.

Theoretical foundations of NMR relaxation on the picosecond–nanosecond (ps–ns) timescale

Molecular motions on the ps–ns timescale strongly influence NMR relaxation phenomena. These rapid fluctuations encompass bond vibrations, side-chain reorientations, and local backbone motions, all of which contribute to the modulation of nuclear spin interactions. By probing relaxation in this regime, NMR provides atomic level insights into fast internal dynamics that are otherwise inaccessible by most experimental methods.

The fundamental principle of NMR is that a nuclear spin placed in a static magnetic field B_0 , will precess around that field at the Larmor frequency $\omega_0 = \gamma B_0$, where γ is the gyromagnetic ratio of nuclei of interest. At thermal equilibrium, the populations of the nuclear spin states (α, β) follow the Boltzmann distribution, resulting in a net magnetization aligned along the direction of B_0 (along z). In this state, no net transverse magnetization is present. The evolution of the net magnetization vector $\mathbf{M}_0$ in a system of spin-1/2 nuclei placed in a static magnetic field can be conveniently described both by the classical vector model (Figure 7.1) and by the mathematical formalism introduced by Bloch (Abragam 1983; Bloch 1946).

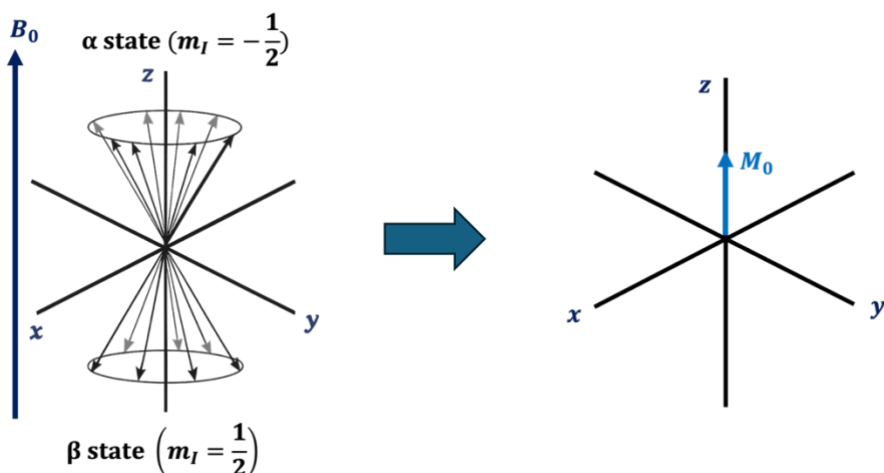


Figure 7.1. Net Magnetization at thermal equilibrium for a spin-1/2 nucleus with a positive gyromagnetic ratio ($\gamma > 0$).

To manipulate (perturb) these spins, a weak radiofrequency (rf) field oscillating at Larmor frequency ω_0 is applied in the transverse (xy) plane, enabling excitation and detection of nuclear magnetization (Ernst et al. 1990; Levitt 2008). In the laboratory frame, the rf-field is explicitly time-dependent (Redfield 1957; Wangsness and Bloch 1953; Bloch and Siegert 1940; Sacolick et al. 2010), which makes the description of spin evolution more complex.

To overcome the complexity introduced by the explicitly time-dependent rf-field in the laboratory frame, and to conveniently describe the precession of the magnetization in the xy -plane, it is useful to adopt a rotating frame of reference. The rotating frame rotates

about the z -axis at the Larmor frequency, such that the rapid precession of the bulk (or net) magnetization is effectively removed. As a result, only the residual rotations of the magnetization due to the applied rf-field B_1 , chemical shifts, and nuclear spin–spin couplings are observed (Abragam 1983; Levitt 2008; Silverstein Robert M et al. 2005).

In the rotating reference frame (rotating at $\omega_{rf} = \omega_0$ about the z -axis), the applied rf-field (rf-pulse) becomes time-independent, i.e. static in this frame, thereby simplifying the description of spin evolution.

Spins are typically manipulated (or perturbed) by applying an oscillating rf-field B_1 oriented in the transverse (xy) plane, for a well-defined duration τ_p and power. Such an rf-pulse rotates the net magnetization vector by a flip angle θ , thereby tipping it away from the z -axis into the transverse plane. This process is referred to as excitation (**Figure 7.2**).

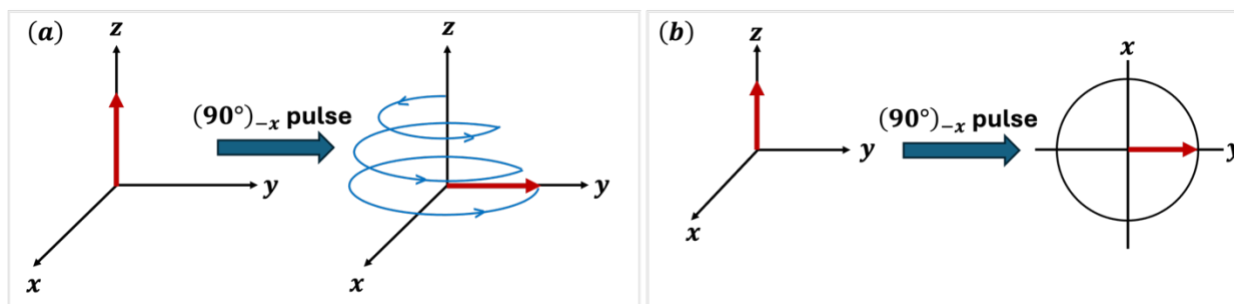


Figure 7.2. Excitation of the net magnetization by an rf pulse applied along the $-x$ -axis. The pulse produces a 90° rotation, tipping the equilibrium magnetization from the z -axis to y -axis. (a) In the laboratory frame. (b) In the rotating frame.

Once the rf excitation is removed, the transverse magnetization does not remain in its perturbed state but gradually returns towards thermal equilibrium, a process referred to as relaxation (Bloembergen et al. 1948; Levitt 2008).

For relaxation to occur, an additional static magnetic field or dynamics are needed. This field arises naturally from stochastic molecular motions, which produce oscillating local magnetic fields that modulate the relaxation rates. On the ps–ns timescale, two dominant mechanisms govern relaxation (Kleckner and Foster 2011):

1. Dipole–dipole (DD) interactions

DD interactions arise from the direct magnetic coupling between nearby nuclear spins (dipoles) as we can see in **Figure 7.3 (a)**. The interaction strength depends both on the internuclear distance and on the orientation of the internuclear vector relative to the external magnetic field B_0 . Because molecules undergo rotational diffusion (tumbling) in solution, the magnitude and orientation of these couplings are continuously modulated (**Figure 7.3 (b)**). While local magnetic fields arise from DD interactions, stochastic motion causes these fields to fluctuate in time with frequency components near the Larmor frequency, thereby modulating relaxation (Bloembergen et al. 1948; Levitt 2008; Abragam 1983).

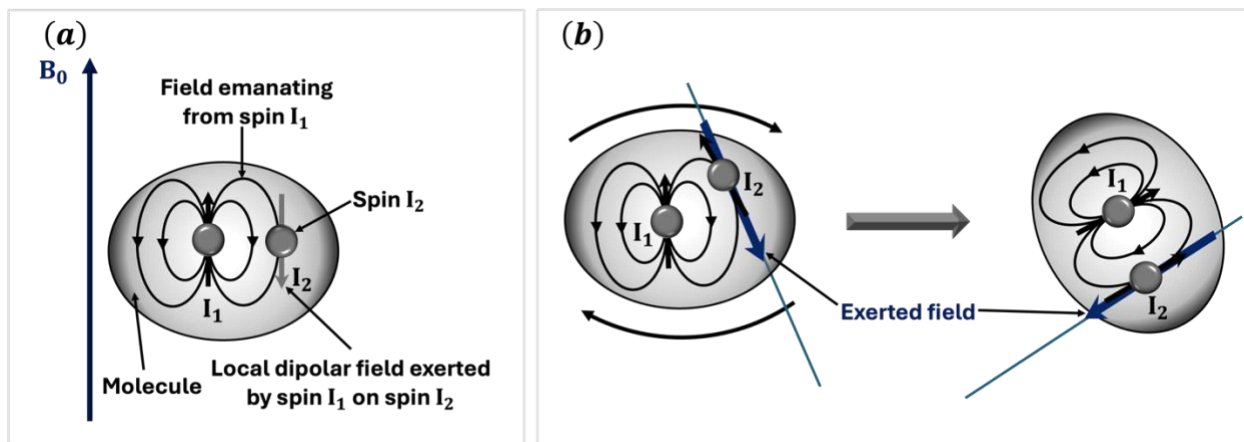


Figure 7.3. Illustration of dipolar coupling between two nuclear spins I_1 and I_2 . (a) Local dipolar fields exerted by spin I_1 on spin I_2 . (b) Modulation of the local dipolar field by molecular rotational diffusion. Figure adapted from (Levitt 2008)

2. Chemical shift anisotropy (CSA)

CSA arises from the anisotropic distribution of electrons around a nucleus, which leads to a shielding effect that depends on the orientation of the molecule relative to the external magnetic field B_0 (**Figure 7.4**). In solution, molecular reorientation causes the CSA interaction to fluctuate over time, causing local magnetic field variations that can modulate relaxation. Thus, CSA provides a second major mechanism alongside dipole–dipole interaction for nuclear spin relaxation on the ps–ns timescale (Cavanagh, J et al. 2006; Abragam 1983; Levitt 2008; Slichter 2010).

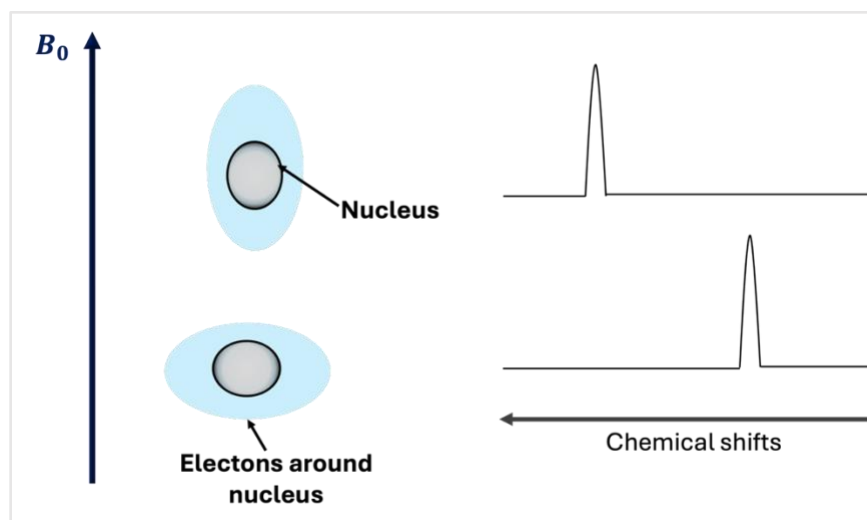


Figure 7.4. Illustration of CSA. Adapted from (Levitt 2008)

These two mechanisms provide the qualitative link between ps–ns timescale protein motions and the fluctuating local magnetic fields that modulate nuclear spin relaxation (Kleckner and Foster 2011). To establish a quantitative relationship between these stochastic fields and measurable NMR relaxation parameters, it is necessary to characterize the underlying molecular motions. This is achieved through the use of the spectral density function, which describes how molecular motions provide fluctuating magnetic fields at frequencies relevant to nuclear spin transitions. The key parameter governing these fluctuations is the rotational correlation time (τ_c), that defined the characteristic timescale over which a molecule reorients, corresponding approximately to the time needed for the molecule to rotate by ~ 1 radian due to Brownian motion (Abragam 1983; Lipari and Szabo 1982a, 1982b). When τ_c falls in the range of $\sim 10^{-12}$ to 10^{-9} s, the associated fluctuations efficiently sample frequencies near the nuclear Larmor frequency, making ps–ns motions highly effective in affecting relaxation (Chen et al. 2004; Farrow et al. 1995; Jarymowycz and Stone 2006; Palmer 2004; Renner et al. 2001). This framework provides a quantitative link between measurable NMR relaxation parameters $\{^{15}\text{N}\}\text{-R}_1$, $\{^{15}\text{N}\}\text{-R}_2$, $^1\text{H}\text{-}\{^{15}\text{N}\}$ NOE and the underlying molecular dynamics. By analyzing these observables, one can extract information about the amplitudes and timescales of internal motions at atomic resolution.

VII.1. Longitudinal and transverse relaxation rates and heteronuclear Nuclear Overhauser Effect

VII.1.1. Theory

Longitudinal and transverse ^{15}N relaxation arise from fluctuations of local magnetic fields generated by ^1H – $\{^{15}\text{N}\}$ dipole–dipole interactions and ^{15}N chemical shift anisotropy.

There are two distinct first-order relaxation processes: one along the z -axis (**Figure 7.5 a**) and one in the transverse xy -plane (**Figure 7.6 a**).

The former is referred to as longitudinal relaxation or spin–lattice relaxation ($\{^{15}\text{N}\}$ – R_1), and is characterized by the rate $R_1 = T_1^{-1}$, where T_1 is the time constant that characterizes the recovery of the longitudinal magnetization (M_z) toward its equilibrium value M_0 along the direction of the static magnetic field. Longitudinal relaxation time is quantified by the exponential recovery of the longitudinal magnetization observed as a time-dependent loss of signal intensity (**Figure 7.5 b**), as described by **Equation 7.1** (Kleckner and Foster 2011).

$$M_z(t) = M_z(0) \cdot (1 - e^{-R_1 t}) \quad \text{Equation 7.1}$$

where $M_z(0)$ is the magnetization at $t = 0$ i.e. the maximum intensity immediately after excitation, before relaxation has had time to occur.

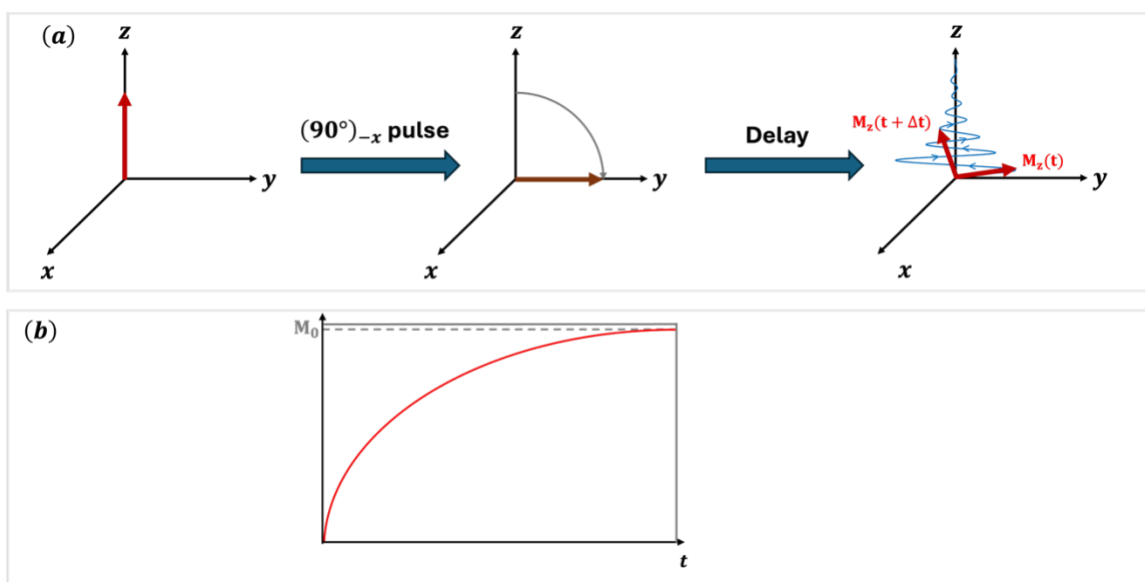


Figure 7.5. ^{15}N Longitudinal relaxation. (a) Magnetization at thermal equilibrium M_0 along the z -axis, rotation of the magnetization into the transverse plane by a 90° excitation pulse. (b) recovery of the longitudinal component M_z back to equilibrium, as described by the Bloch equations.

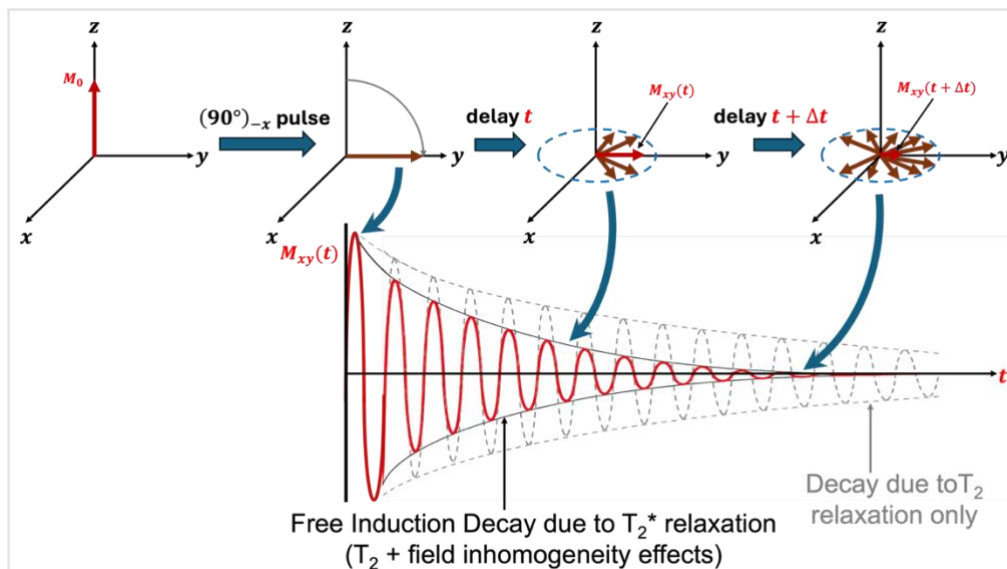


Figure 7.6. ^{15}N Transverse relaxation. At thermal equilibrium, the net magnetization M_0 lies along the z -axis. Following a 90° excitation pulse, it is rotated into the transverse xy -plane, where the transverse magnetization M_{xy} decays over time, producing the free induction decay (FID). The observed decay is governed by T_2^* , which combines intrinsic T_2 relaxation and dephasing caused by magnetic field inhomogeneities. Figure adapted from (Ridgway 2010)

The second process, called transverse relaxation or spin-spin relaxation ($\{^{15}\text{N}\}\text{-}R_2$) is defined by the rate $R_2 = T_2^{-1}$, where T_2 is the time constant that characterizes the decay of the net transverse magnetization (M_{xy}) in the xy -plane. It reflects the loss of phase coherence among spins due to local magnetic field inhomogeneities and fluctuating interactions. Transverse relaxation is quantified by the exponential decay of the transverse magnetization observed as a time-dependent decay of signal intensity (Figure 7.6 b)), as described by Equation 7.2 (Kleckner and Foster 2011).

$$M_{x,y}(t) = M_{x,y}(0) \cdot e^{-R_2 \cdot t} \quad \text{Equation 7.2}$$

where $M_{x,y}(0)$ is the magnetization at $t = 0$ i.e. the maximum intensity immediately after excitation, before relaxation has had time to occur.

The two relaxation rates exhibit distinct dependencies on the correlation time, as illustrated in Figure 7.7. As the correlation time (τ_c), increases, $\{^{15}\text{N}\}\text{-}R_1$ initially rises to a maximum before subsequently decreasing. At very short τ_c , $\{^{15}\text{N}\}\text{-}R_1$ is small because stochastic molecular motions provide local magnetic fields that oscillate too rapidly to efficiently modulate relaxation. As τ_c increase and the oscillation frequency approaches Larmor frequency, the local magnetic fields become resonant, and longitudinal relaxation rate reaches its maximum. When τ_c becomes longer, the stochastic molecular motions

are too slow to provide oscillations at the Larmor frequency, and $\{^{15}\text{N}\}\text{-}R_1$ therefore decreases (Keeler, 2002).

In contrast, while τ_c increases, $\{^{15}\text{N}\}\text{-}R_2$ continues to increase with correlation time and does not display a maximum. This is because slow molecular tumbling does not efficiently modulate DD interactions and CSA, allowing these mechanisms to contribute more strongly to transverse relaxation at long correlation times (Keeler, 2002). As a result, large molecules in the slow-motion limit are characterized by very rapid $\{^{15}\text{N}\}\text{-}R_2$.

In addition, to $\{^{15}\text{N}\}\text{-}R_1$ and $\{^{15}\text{N}\}\text{-}R_2$, an important relaxation phenomenon in NMR is the Overhauser Effect (NOE). The NOE arises from cross-relaxation between two coupled spins. Cross-relaxation refers to the transfer of the population difference between nuclear spin states, also referred to as polarization, between two coupled spins through relaxation pathways. It arises when stochastic molecular motions modulate the DD interaction between the spins, creating fluctuating local magnetic fields that simultaneously influence both nuclei. This effect provides valuable sensitivity enhancement for low gamma nuclei and depends strongly on internuclear distances. The relaxation pathways underlying the NOE can be formally described by the Solomon diagram (Solomon 1955), which depicts the transitions between the energy levels of a two coupled spins.

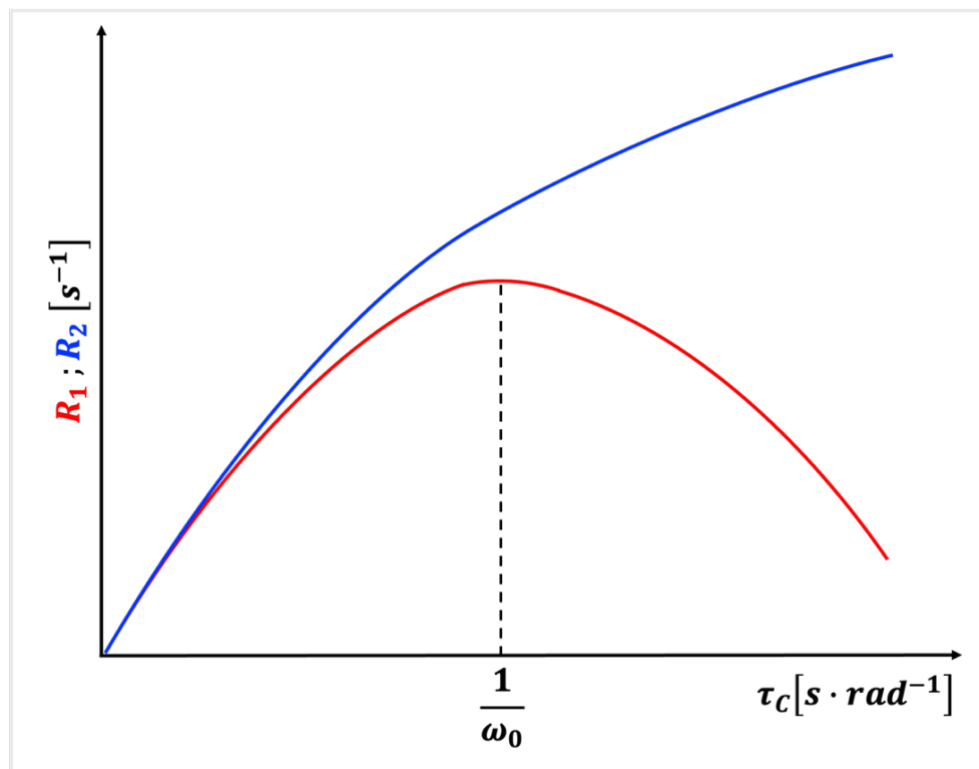


Figure 7.7. Comparison of the longitudinal and transverse relaxation rate constants as a function of the correlation time for the fixed Larmor frequency. Adapted from (Keeler 2002).

The Solomon diagram describe the relaxation between two coupled spin-1/2 nuclei, I and S . Each spin has two states: low energy α state and high energy β state and we can then come up with a 4 spin states and 4 energy levels for these two spins. This is shown in the **Figure 7.8**.

The rate constants for transitions between energy levels are classified according to which spins undergo a change of spin state during the transition. For two coupled spins, I and S they are defined as follows:

- W_I and W_S denote the relaxation rate constants associated with transitions between the α and β spin states of spin I and S respectively.
- W_0 is the relaxation rate constant associated with transitions in which both spins flip in opposite directions. This process is referred to as a zero-quantum transition.
- W_2 is the relaxation rate constant associated with transitions in which both spins flip in the direction. This process is referred to as a double-quantum transition.

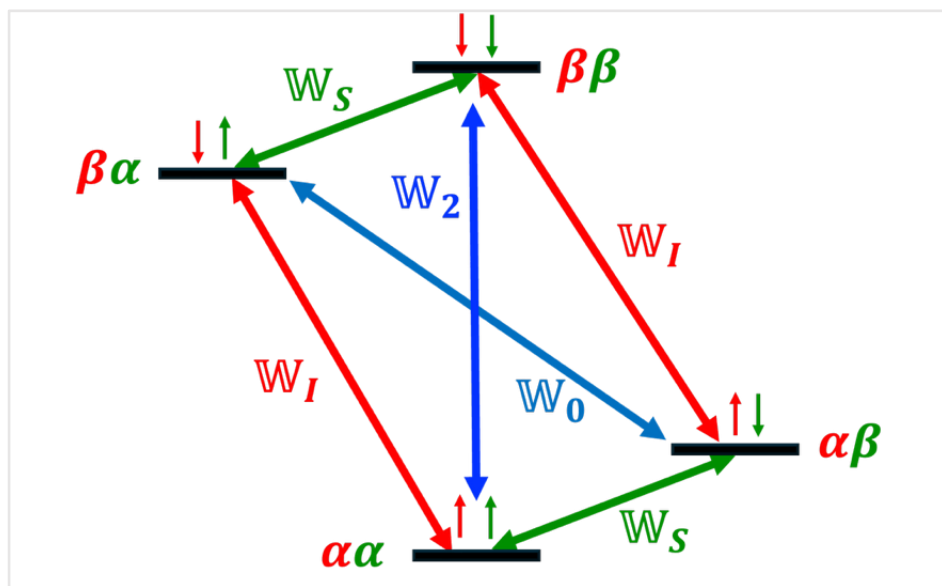


Figure 7.8. Relaxation induced transitions between energy levels and associated rate constants for two coupled spin-1/2. Adapted from (Solomon 1955)

Within the framework of relaxation theory, Solomon demonstrated that the cross-relaxation rate σ_{IS} , which underlies the NOE, can be expressed by the **Equation 7.3**:

$$\sigma_{IS} = W_2 - W_0 \quad \text{Equation 7.3}$$

As will be discussed in the next section, the cross-relaxation rate describes the transfer of longitudinal magnetization between coupled spins through by DD interactions. In the case of heteronuclear spin pairs such as ^1H – ^{15}N , this phenomenon is referred to as the $\{^{15}\text{N}\}$ heteronuclear Nuclear Overhauser Effect (^1H – $\{^{15}\text{N}\}$ NOE).

VII.1.2. NMR measurement of $\{^{15}\text{N}\}$ – R_1 , $\{^{15}\text{N}\}$ – R_2 and ^1H – $\{^{15}\text{N}\}$ NOE

A. Measurement of Longitudinal Relaxation Rate, $\{^{15}\text{N}\}$ – R_1

The measurement of the longitudinal relaxation rate $R_1 = T_1^{-1}$ is a fundamental aspect of NMR spectroscopy. Since the historical introduction of the Bloch equations to describe the time evolution of perturbed magnetization, knowledge of the spin–lattice relaxation time (T_1) has played a crucial role in understanding the dynamics of spin systems (Bhattacharyya and Kumar 2004; Bloch 1946). Among the various experimental approaches, the inversion-recovery (IR) sequence remains the most widely used method for determining T_1 (Bhattacharyya and Kumar 2004). This two-pulse experiment follows the pulse sequence $d_1 - 180^\circ - \tau - 90^\circ - \text{acquisition}$ (**Figure 7.9 (a)**), which is repeated for different values of the delay τ . In this sequence, d_1 represents the recovery delay, the period during which the spins return to thermal equilibrium, resulting in a bulk magnetization aligned along the z-axis. The 180° rf-pulse inverts the spin populations, such that β -spins become α -spins and α -spins become β -spins. The τ delay allows partial recovery of the longitudinal magnetization towards equilibrium, after which a 90° rf-pulse rotates the magnetization into the xy -plane for detection. By varying τ , the recovery of magnetization from the inverted to the equilibrium state can be monitored and fitted exponential to extract T_1 (**Figure 7.9 (b)**).

The most time-consuming step in this experiment is the recovery period d_1 , during which the magnetization must return to equilibrium before the next acquisition. To ensure complete recovery, d_1 is typically set to at least five times T_1 ($d_1 \geq 5T_1$) (Bhattacharyya and Kumar 2004). Consequently, T_1 measurements can require significant experimental time. To address this limitation, several accelerated methods have been proposed, such as the saturation-recovery (SR) (Bhattacharyya and Kumar 2004; Fukushima 1981; Kellman et al. 2002), saturation-inversion-recovery (SIR) (Fukushima 1981; Wang et al.

2017) and single-scan or slice selected inversion recovery (SSIR) techniques (Bhattacharyya and Kumar 2004), which reduce the overall measurement time while maintaining acceptable accuracy.

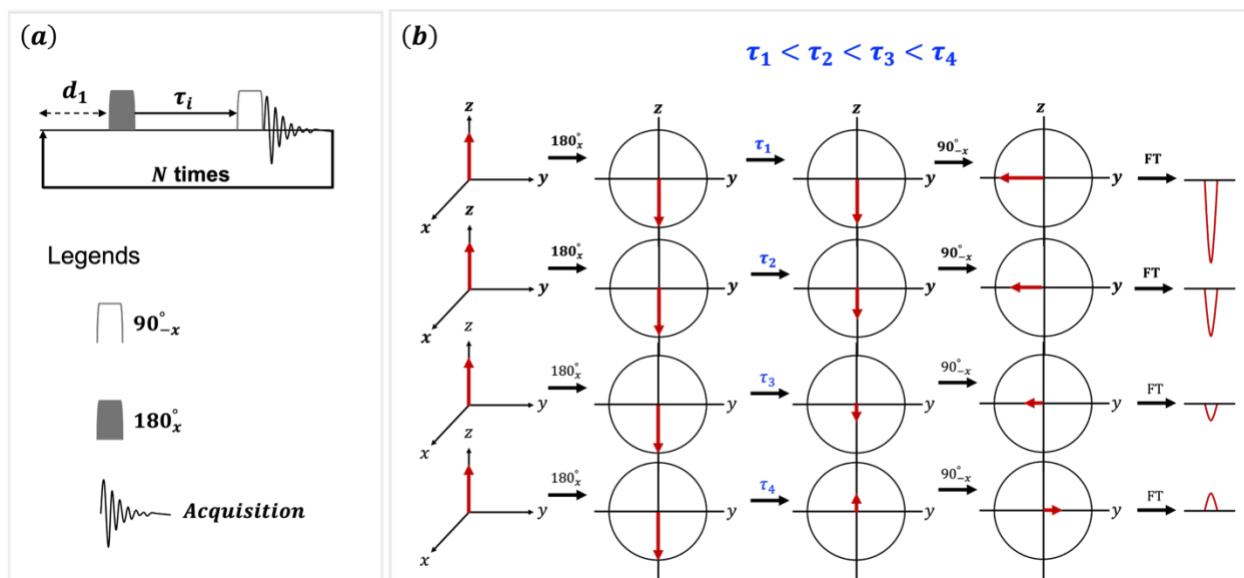


Figure 7.9. Longitudinal relaxation experiment. (a) Standard inversion recovery experiment, adapted from (Bhattacharyya and Kumar 2004). (b) Magnetization Evolution during the IR experiment.

B. Measurement of Transverse Relaxation Rate, $\{^{15}\text{N}\}\text{--}R_2$

The transverse relaxation rate $R_2 = T_2^{-1}$ characterizes the decay of transverse magnetization resulting from the loss of phase coherence among precessing spins in the xy -plane. Unlike longitudinal relaxation, which involves energy exchange between the spin system and the lattice, transverse relaxation originates primarily from spin–spin interactions and local magnetic field inhomogeneities (Levitt 2008; Hahn 1950; Kay and Torchia 1998). In the absence of magnetic field inhomogeneities, the width at half-height of an NMR spectral peak (in hertz) is inversely proportional to the intrinsic transverse relaxation time T_2 , according to $\Delta\nu_{1/2} = 1/(\pi T_2)$. In practice, however, experimentally observed linewidths generally reflect the apparent transverse relaxation time T_2^* , which includes additional contributions from magnetic field inhomogeneities.

The fundamental principle underlying the measurement of T_2 was introduced by *Erwin L. Hahn* in 1950, known as the spin-echo experiment (Hahn 1950). The basic pulse sequence consists of a $90^\circ - \tau - 180^\circ - \tau - \text{acquisition}$ block (Figure 7.10 (a)). The

purpose of this sequence is to refocus transverse magnetization dephasing caused by static magnetic field B_0 inhomogeneities (**Figure 7.10 (b)**).

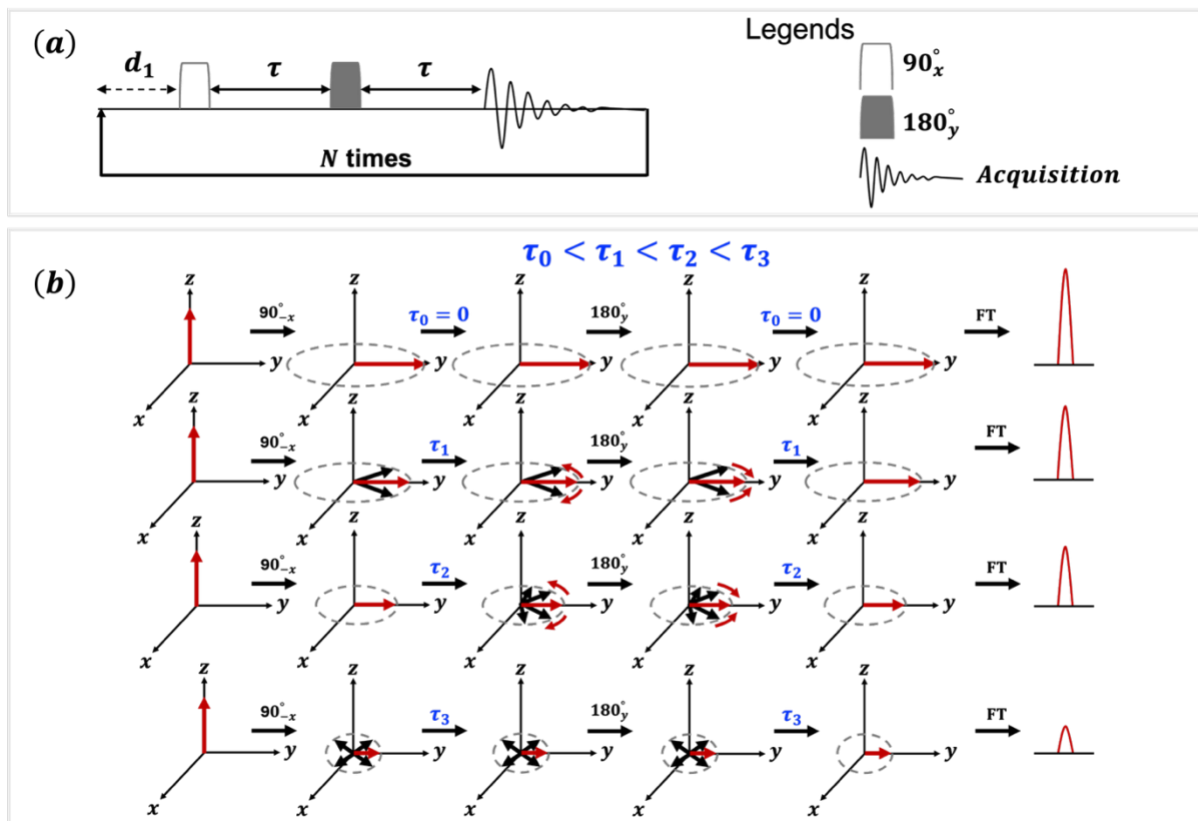


Figure 7.10. Transversal relaxation experiment. (a) Spin echo experiment. (b) Magnetization Evolution during the spin echo experiment. Magnetic field inhomogeneity prevents complete refocusing of all spins.

Following the initial 90° rf-pulse, transverse magnetization vectors begin to dephase during the first delay period τ due to variations in local magnetic fields. The subsequent 180° pulse inverts the accumulated spin phases, causing them to rephase during the second τ interval, resulting in an echo at time 2τ .

Perfect echo refocusing assumes that the homogeneous decay of transverse magnetization remains constant during the pulse sequence, so that the precession during the second τ interval mirrors that of the first. If the spins move into regions with different local fields or if the fields themselves fluctuate over time, the refocusing becomes imperfect and the echo intensity is reduced (Levitt 2008).

To overcome this limitation, *H. Y. Carr and E. M. Purcell (1954)* extended Hahn's concept by introducing a train of refocusing pulses at regular intervals, thereby generating a series of spin echoes (Carr and Purcell 1954). The corresponding pulse sequence is $90^\circ - [\tau - 180^\circ - \tau]_n - \text{acquisition}$. This Carr–Purcell sequence enhances the precision of T_2

measurements and reduces the influence of molecular diffusion in inhomogeneous fields by averaging over multiple echoes. However, small imperfections in the 180° refocusing pulses (e.g. slight deviations from the exact flip angle) accumulate over successive echoes, leading to systematic decay distortions and inaccurate T_2 estimations.

To correct for these cumulative pulse errors, *S. Meiboom and D. Gill (1958)* introduced a modification to the Carr–Purcell sequence, now widely known as the Carr–Purcell–Meiboom–Gill (CPMG) sequence (Meiboom and Gill 1958). In this version, the phase of the initial 90° excitation pulse is shifted by 90° relative to the 180° pulses. Typically, the 90° pulse is applied along the y -axis, while the 180° pulses are applied along the x -axis.

This simple phase adjustment compensates for systematic pulse imperfections and prevents the accumulation of phase errors over successive echoes. As a result, the CPMG sequence provides stable echo amplitudes and has become the standard method for T_2 measurement in modern NMR applications (Carver and Richards 1972; Myint et al. 2012).

Compared to the Hahn echo experiment, the CPMG technique offers several distinct advantages:

1. The frequency of the refocusing can be varied to probe chemical exchange processes and relaxation dispersion phenomena (Bain 2003; Long et al. 2008; Vallurupalli et al. 2009; Yip and Zuiderweg 2004).
2. When the delay between refocusing pulses is sufficiently short, the effects of scalar-coupling on R_2 measurements are effectively suppressed (Stables et al. 1999), allowing more accurate determination of relaxation rates in coupled spin systems (S. R. Williams et al. 1985).
3. The CPMG sequence minimizes errors from imperfect rf-pulses (Carr and Purcell 1954; Gullion et al. 1990; Maudsley 1986; Meiboom and Gill 1958; Shaka et al. 1988) and mitigates the effects of inhomogeneous rf-fields (Ziegler et al. 1992).
4. Phase cycling within the CPMG pulse train suppresses intensity oscillations caused by off-resonance effects (Long et al. 2008; Myint et al. 2012; Yip and Zuiderweg 2004), leading to smoother relaxation decay curves.

Despite its robustness, the CPMG experiment remains more complex than the Hahn echo method. Systematic errors can still accumulate, particularly when long echo trains are used. These cumulative errors are not easily eliminated, even when employing hard pulses (Abdi et al. 2021, 2021; Hughes 1977; Hürlimann 2001; Ishima and Torchia 2005; Vold et al. 1973) or soft pulses (Czisch et al. 1997; Korzhnev et al. 2000; Pawley et al. 2006; Ross et al. 1997).

To address these issues, *A. D. Bain et al.* (Bain et al. 2011) demonstrated that combining CPMG with an {0013}-phase cycling scheme significantly improves the robustness and

accuracy of relaxation measurements. This modification ensures consistent echo amplitudes, suppresses pulse-related artifacts, and extends the applicability of the CPMG method to more demanding experimental conditions.

C. $\{^{15}\text{N}\}$ heteronuclear Nuclear Overhauser Effect, $^1\text{H}\text{--}\{^{15}\text{N}\}$ NOE

The $\{^{15}\text{N}\}$ heteronuclear Nuclear Overhauser enhancement, also referred to as the heteronuclear Nuclear Overhauser effect ($^1\text{H}\text{--}\{^{15}\text{N}\}$ NOE), describes the net change in signal intensity of a nucleus I that arises from DD interactions with a coupled spin S (Ferrage et al. 2009, 2008; Kharchenko et al. 2020; Sedaghat Doost et al. 2019). In other words, cross-relaxation of spin I , arising from DD interactions with a nearby spin S , whether directly bonded or simply located within approximately 5 Å, leads to changes in the population of the spin states of I (Neuhaus 2011; Vögeli 2014; “Solving Problems with NMR Spectroscopy - Edition 2 - By Atta-ur Rahman, Muhammad Iqbal Choudhary and Atia-tul- Wahab Elsevier Health Inspection Copies” 2015). Thus, the $^1\text{H}\text{--}\{^{15}\text{N}\}$ NOE reflects how fluctuations of local magnetic fields, modulate the transfer of longitudinal magnetization (or polarization) between the coupled spins (Kuhlmann and Grant 1971; Kuhlmann et al. 1970).

Typically, nuclei in a molecule may interact magnetically via two main mechanisms: DD interaction (direct), which arises through space, and scalar-coupling interaction (indirect), also known as J-coupling, which occurs through chemical bonds. In the case of the heteronuclear NOE, only DD interactions (through-space) are relevant (Claridge 2016). These interactions depend strongly on the inter-nuclear distance and the spectral density at high frequencies (ω_{H}), making $^1\text{H}\text{--}\{^{15}\text{N}\}$ NOE sensitive to probe fast local dynamics (Ferrage et al. 2009).

Theoretically, the $^1\text{H}\text{--}\{^{15}\text{N}\}$ NOE is defined as the change in signal intensity of one nucleus when the equilibrium population of another coupled nucleus is perturbed. This variation is expressed as a relative intensity ratio, according to the **Equation 7.4** (Gil and Navarro-Vázquez, n.d.) :

$$\eta = \frac{I_{\text{sat}} - I_0}{I_0} \quad \text{Equation 7.4}$$

where I_{sat} is the signal intensity of the heteronucleus in the presence of proton saturation, and I_0 is the reference intensity obtained without saturation. Saturation refers to the condition in which continuous or repeated rf-pulse equalizes the spin-state populations, eliminating net longitudinal magnetization.

The enhancement factor η can be either positive or negative, depending on the molecular tumbling rate, the sign of the gyromagnetic ratios of the interacting spins, and the magnetic field strength of the spectrometer (Sedaghat Doost et al. 2019). Generally, small molecules (fast tumbling) exhibit positive NOE enhancements, whereas large molecules such as large proteins (slowly tumbling) can display reduced or even negative NOE values.

In practice, $^1\text{H}\{-^{15}\text{N}\}$ NOE measurements are performed using two interleaved experiments:

- Reference spectrum I_0 , acquired without proton saturation.
- Saturated spectrum I_{sat} acquired after continuous wave or proton pulsed saturation, typically applied for several seconds to achieve a steady-state condition.

Both spectra are recorded under identical experimental parameters, except for the presence or absence of proton saturation before the heteronuclear detection period. The $^1\text{H}\{-^{15}\text{N}\}$ NOE value (η) for each resonance is then determined using **Equation 7.5**:

$$\eta = \frac{I_{sat}}{I_0} \quad \text{Equation 7.5}$$

The difference in signal intensity directly reflects the extent of cross-relaxation between the nitrogen and the attached proton.

Although the three NSR parameters $\{^{15}\text{N}\}\text{--}R_1$, $\{^{15}\text{N}\}\text{--}R_2$ and $^1\text{H}\{-^{15}\text{N}\}$ NOE provide valuable insights into local molecular motion, their direct interpretation often leads to ambiguities and inconsistencies (Farrow et al. 1994; Kay et al. 1989). These three parameters are influenced by different aspects of molecular dynamics occurring on distinct timescales. For instance, $\{^{15}\text{N}\}\text{--}R_1$ primarily reflects fast motions and overall tumbling, $\{^{15}\text{N}\}\text{--}R_2$ is sensitive to both fast and slower processes including conformational exchange, and $^1\text{H}\{-^{15}\text{N}\}$ NOE predominantly responds to very fast motions (Lipari and Szabo 1982a, 1982b). Because these parameters probe overlapping but not identical motional regimes, attempts to extract dynamic information from them independently may yield contradictory interpretations.

For example, a nucleus may display a relatively high $\{^{15}\text{N}\}\text{--}R_2$ value suggesting slow motion while simultaneously exhibiting a low $^1\text{H}\{-^{15}\text{N}\}$ NOE value, which indicates fast local motion. Similarly, residues that show comparable $\{^{15}\text{N}\}\text{--}R_1$ relaxation rates may differ markedly in $\{^{15}\text{N}\}\text{--}R_2$ or $^1\text{H}\{-^{15}\text{N}\}$ NOE, even though they are located within the same structural region (Kay et al. 1989). Such discrepancies arise because the three relaxation parameters depend on different combinations of motional amplitudes and timescales, and their sensitivities to local versus global motions are not the same.

Moreover, experimental relaxation rates represent ensemble averaged quantities, integrating the effects of both global molecular tumbling and internal motions. Even when the global rotational correlation time of a molecule is known, the contribution from local flexibility remains obscured, making it difficult to separate the two effects (Farrow et al. 1994). The problem is further complicated by chemical exchange processes that influence $\{^{15}\text{N}\}\text{--R}_2$ but not $\{^{15}\text{N}\}\text{--R}_1$ or $^1\text{H}\text{--}\{^{15}\text{N}\}$ NOE, introducing additional complexity into the interpretation (Palmer 2004). As a result, relying solely on $\{^{15}\text{N}\}\text{--R}_1$, $\{^{15}\text{N}\}\text{--R}_2$ or $^1\text{H}\text{--}\{^{15}\text{N}\}$ NOE data does not provide a consistent or quantitative picture of molecular motion.

To overcome these inconsistencies, *Lipari and Szabo* (Lipari and Szabo 1982a, 1982b) introduced the model-free formalism, which offers a unified framework for describing internal dynamics without assuming any specific motional model or geometry.

VII.2. Model Free Analysis (MFA)

The model-free analysis was introduced to provide a unified framework for characterizing internal molecular dynamics. In this approach, the complex dependence of relaxation rates on molecular motion is reduced to a few physically meaningful parameters. The method assumes that internal motions are independent of, and occur on a much faster timescale than overall molecular tumbling, an assumption that is not always valid (Chen and Tjandra 2008; Kleckner and Foster 2011). The term “model-free” reflects the fact that this approach does not rely on an explicit structural model to describe the nature of the motion (Kleckner and Foster 2011). Nevertheless, it remains a theoretical model that interprets NSR parameters ($\{^{15}\text{N}\}\text{--R}_1$, $\{^{15}\text{N}\}\text{--R}_2$ and $^1\text{H}\text{--}\{^{15}\text{N}\}$ NOE) through four distinct types of parameters:

1. The rotational diffusion tensor. It is a mathematical formalism that describes how a molecule undergoes rotational motion in solution as a result of Brownian motion (Ghose et al. 2001; Ollila et al. 2018). In the most general case, the rotational diffusion tensor is defined by six parameters: D_{xx} , D_{yy} , D_{zz} , θ , φ , ψ , where D_{xx} , D_{yy} , and D_{zz} are the principal components of the diffusion tensor, and the Euler angles (θ , φ , ψ) specify the orientation of the diffusion tensor frame relative to a chosen molecular coordinate system (e.g., the coordinate frame of a PDB structure) (Blackledge et al. 1998; Walker et al. 2004). In the simpler axially symmetric diffusion, only four parameters are optimized: $D_{\perp}$, $D_{\parallel}$, θ , and φ , where $D_{\perp}$ and $D_{\parallel}$ represent the diffusion coefficients perpendicular and parallel to the principal rotational axis, respectively (Blackledge et al. 1998), and we have $D_{zz} = D_{\parallel}$ and $D_{xx} = D_{yy} = D_{\perp}$ (Ghose et al. 2001). The diffusion parameters are

optimized by nonlinear least squares using the Levenberg–Marquardt algorithm to minimize the objective function E , defined as:

$$E = \sum_{i=1}^{N_{res}} \frac{(\rho_i^{exp} - \rho_i^{cal})^2}{\sigma_i^2} \quad \text{Equation 7.6}$$

where N_{res} is the total number of N–H vectors included in the analysis, parameter ρ is defined as a ratio of the transverse and longitudinal relaxation rates, and σ_i denotes the experimental error in ρ_i for vector number i .

$$\rho = \left(\frac{2 R'_2}{R'_1} - 1 \right)^{-1} \quad \text{Equation 7.7}$$

The value of ρ^{exp} is directly derived from the measured relaxation parameters, while ρ^{cal} is calculated using expressions for the spectral densities $J(\omega)$, depending on the rotational diffusion model (Ferrage et al. 2009; Ghose et al. 2001). In **Equation 7.7**, primes denote relaxation rates corrected to remove high-frequency contributions to the spectral density (Walker et al. 2004).

2. The site-specific internal effective correlation time τ_e . It quantifies the timescale of reorientation of the N–H bond vector at each residue. It can be decomposed into two distinct internal motional components: a fast timescale τ_f ($\tau_f < 20$ ps (Jarymowycz and Stone 2006; Palmer 2004; Sakhrani et al. 2021)), typically associated with local bond vibrations or side-chain fluctuations, and a slow timescale τ_s ($\tau_f < \tau_s < \tau_c$ (Palmer 2004)), which reflects larger amplitude motions such as loop.
3. The site-specific generalized squared order parameter S^2 . It describes the spatial restriction or amplitude of internal motion that governs the reorientation of the N–H bond vector. It reflects how confined or flexible a N–H bond vector is within its local motional environment. For instance, if the N–H bond vector undergoes isotropic diffusion within a cone, as illustrated in **Figure 7.11**, the value of S^2 decreases markedly as the cone semi-angle θ increases from 0° to approximately 75° , and remains low for $75^\circ < \theta < 180^\circ$ (Kay and Torchia 1998). In general, $S^2 = 1$ indicates a completely rigid bond (no internal motion), whereas $S^2 = 0$

corresponds to unrestricted internal motion. Furthermore, S^2 can be partitioned into two distinct motional components: a fast component S_f^2 , associated with higher internal motions and a slow component S_s^2 , which represents slower internal motions. Therefore, $S^2 = S_f^2 S_s^2$ (Clare et al. 1990).

4. The site-specific exchange broadening R_{ex} . It represents the contribution to R_2 arising from chemical exchange processes occurring on the μs – ms timescale. These processes involve conformational exchange that modulate the local magnetic environment, leading to additional dephasing of transverse magnetization and consequently, an increase in R_2 .

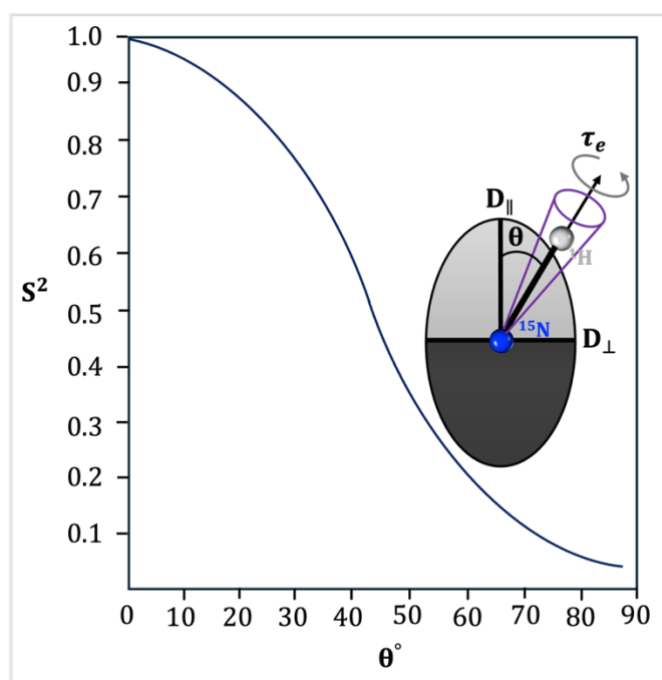


Figure 7.11. Relationship between internal motion and model-free parameters for axially symmetric diffusion tensor. Adapted from (Kay and Torchia 1998; Kleckner and Foster 2011; Palmer 2004).

The extraction of the four model-free parameters for each N–H bond vector is achieved through analysis of NSR relaxation data using the spectral density function $J(\omega)$. This function describes how molecular motions modulate local magnetic fields at different angular frequencies ω . In practice, five spectral density terms are commonly evaluated, each corresponding to a specific frequency relevant to NMR relaxation:

- $J(0)$: the spectral density at zero frequency, reflecting contributions from very slow motions, such as conformational exchange (Andrec et al. 1999, 1999; Palmer 2004; Pengt and Wagner 1992).

- $J(\omega_N)$: the spectral density at the nitrogen Larmor frequency ω_N , which primarily influences R_1 (Andrec et al. 1999, 1999; Palmer 2004; Pengt and Wagner 1992).
- $J(\omega_H)$: the spectral density at the proton Larmor frequency ω_H , which contributes to the heteronuclear NOE enhancement (Andrec et al. 1999, 1999; Palmer 2004; Pengt and Wagner 1992).
- $J(\omega_H \pm \omega_N)$: the spectral density at the sum or difference frequency, representing simultaneous modulation of both nuclei and contributing to cross-relaxation effects (Andrec et al. 1999, 1999; Palmer 2004; Pengt and Wagner 1992).

The experimentally measured NSR relaxation parameters $\{^{15}\text{N}\}\text{--}R_1$, $\{^{15}\text{N}\}\text{--}R_2$ and $^1\text{H}\text{--}\{^{15}\text{N}\}$ NOE are quantitatively linked to the five spectral density functions through the following equations (Andrec et al. 1999; Farrow et al. 1995; Palmer 2004; Pengt and Wagner 1992):

$$R_1 = \frac{d^2}{4} [J(\omega_H - \omega_N) + 3J(\omega_N) + 6J(\omega_H + \omega_N)] + c^2 J(\omega_N) \quad \text{Equation 7.8}$$

$$R_2 = \frac{d^2}{8} [4J(0) + J(\omega_H - \omega_N) + 3J(\omega_N) + J(\omega_H) + J(\omega_H + \omega_N)] + \frac{c^2}{6} [4J(0) + 3J(\omega_N)] \quad \text{Equation 7.9}$$

$$h\text{NOE} = 1 + \left(\frac{\gamma_H}{\gamma_N}\right) \frac{d^2}{4R_1} [6J(\omega_H + \omega_N) - J(\omega_H - \omega_N)] \quad \text{Equation 7.10}$$

$$d = \frac{\gamma_H \gamma_N \hbar \mu_0}{8\pi^2 r_{NH}^2} \quad c = \frac{\Delta\sigma \omega_N}{\sqrt{3}}$$

where μ_0 is the permeability of free space; $\hbar$ is Planck's constant; γ_H and γ_N are the gyromagnetic ratios of the **H** and **N** spins, respectively; r_{NH} is the N-H bond length; $\Delta\sigma$ is the ^{15}N CSA.

However, it has been shown that, because $\omega_H \gg \omega_N$, the spectral density function values at the three high frequencies ($\omega_H - \omega_N$, ω_H , and $\omega_H + \omega_N$) can be combined into a single effective term $J(0.87\omega_H)$, which represents molecular motions occurring near the proton Larmor frequency (Farrow et al. 1995; Jarymowycz and Stone 2006; Kay et al. 1989; Renner et al. 2001). This approximation forms the basis of the reduced spectral density mapping formalism, in which all relaxation parameters are expressed in terms of only three effective spectral densities: $J(0)$, $J(\omega_N)$ and $J(0.87\omega_H)$.

Using these simplifications, the full relaxation equations can be rewritten in terms of only three spectral density functions:

$$R_1 = \frac{d^2}{4} [3J(\omega_N) + 7J(0.87\omega_H)] + c^2 J(\omega_N) \quad \text{Equation 7.11}$$

$$R_2 = \frac{d^2}{8} [4J(0) + 3J(\omega_N) + 13J(0.87\omega_H)] + \frac{c^2}{6} [4J(0) + 3J(\omega_N)] \quad \text{Equation 7.12}$$

$$hNOE = 1 + \left(\frac{\gamma_H}{\gamma_N} \right) \frac{d^2}{4R_1} [5J(0.87\omega_H)] \quad \text{Equation 7.13}$$

Assuming that, each N–H bond undergoes axially symmetric rotational diffusion, two distinct modeling approaches can be applied within the MFA formalism: isotropic and anisotropic modeling.

VII.2.1. Isotropic Modeling

In the isotropic model, the molecule is assumed to tumble uniformly in all directions, characterized by a single rotational diffusion coefficient (D_{iso}) or, equivalently, the single overall correlation time (τ_c) and $\tau_c^{-1} = 6D_{iso}$. Isotropic modeling is characterized by a ratio of the principal components of the diffusion tensor $D_{\parallel}/D_{\perp} < 1.2$ (Hall and Fushman 2003; Tjandra, Feller, et al. 1995).

The MFA of NSR parameters is typically carried out using five independent isotropic models, all derived from the original formulation proposed by *Lipari and Szabo* (Lipari and Szabo 1982a, 1982b) and later extended by *Clore et al.* (Clore et al. 1990) to account for two distinct timescales of internal motion, fast (τ_f) and slow (τ_s) motions. In this approach, the spectral density function is expressed as:

$$J(\omega) = \frac{2}{5} \left[\frac{S^2 \tau_c}{1 + (\omega \tau_c)^2} + \frac{(1 - S_f^2) \tau'_f}{1 + (\omega \tau'_f)^2} + \frac{(S_f^2 - S^2) \tau'_s}{1 + (\omega \tau'_s)^2} \right] \quad \text{Equation 7.14}$$

where the square of the overall order parameter is the product of the order parameters for the first and second internal motions, $S^2 = S_f^2 S_s^2$ and τ_c is defined as the global correlation time and closely related to the isotropic component of diffusion D_{iso} by: $\tau_c^{-1} = 6D_{iso}$. $(\tau_i')^{-1} = \tau_c^{-1} + \tau_i^{-1}$ with $i = s$ or $i = f$, is an internal correlation time describing the internal motions. All five models of the spectral density consider the isotropic rotational diffusion tensor by using the following external correlation function:

$$C_0^{axial}(t) = S^2 + (1 - S_f^2)e^{-t/\tau_f} + (S_f^2 - S^2)e^{-t/\tau_s} \quad \text{Equation 7.15}$$

The five model-free formulations, their parameters, and corresponding spectral density functions are summarized in **Table 7.1**.

Table 7.1. Isotropic spectral density functions and corresponding fitted parameter subsets

Models	Subset of fitted parameters	Spectral density function
1	S^2	$J(\omega) = \frac{2}{5} \cdot \tau_c \cdot \left[\frac{S^2}{1 + (\omega \cdot \tau_c)^2} \right]$
2	$S^2, \tau_e(ps - ns)$	$J(\omega) = \frac{2}{5} \cdot \tau_c \cdot \left[\frac{S^2}{1 + (\omega \cdot \tau_c)^2} + \frac{(1 - S^2)(\tau_c + \tau_e) \cdot \tau_e}{(\tau_c + \tau_e)^2 + (\omega \cdot \tau_c \cdot \tau_e)^2} \right]$
3	S^2, R_{ex}	$J(\omega) = \frac{2}{5} \cdot \tau_c \cdot \left[\frac{S^2}{1 + (\omega \cdot \tau_c)^2} \right]; R_{2eff} = R_2 + R_{ex}$
4	$S^2, \tau_e(ps - ns), R_{ex}$	$J(\omega) = \frac{2}{5} \cdot \tau_c \cdot \left[\frac{S^2}{1 + (\omega \cdot \tau_c)^2} + \frac{(1 - S^2)(\tau_c + \tau_e) \cdot \tau_e}{(\tau_c + \tau_e)^2 + (\omega \cdot \tau_c \cdot \tau_e)^2} \right]; R_{2eff} = R_2 + R_{ex}$
5	$S_f^2, S^2 = S_f^2 S_s^2, \tau_e(ns)$	$J(\omega) = \frac{2}{5} \cdot \tau_c \cdot \left[\frac{S^2}{1 + (\omega \cdot \tau_c)^2} + \frac{(1 - S_f^2)(\tau_c + \tau_f) \cdot \tau_f}{(\tau_c + \tau_f)^2 + (\omega \cdot \tau_c \cdot \tau_f)^2} \cdot \frac{(S_f^2 - S^2)(\tau_c + \tau_s) \cdot \tau_s}{(\tau_c + \tau_s)^2 + (\omega \cdot \tau_c \cdot \tau_s)^2} \right]$

VII.2.2. Anisotropic Modeling

The anisotropic modeling accounts for the directional dependence of rotational motion, where the rates of molecular tumbling differ along the principal axes of the diffusion tensor. This anisotropy reflects deviations from spherical symmetry and is typically described by the parameters $D_{\parallel}$, $D_{\perp}$ and the orientation of the tensor relative to the molecular frame. It is characterized by a ratio of the principal components of the diffusion tensor $D_{\parallel}/D_{\perp} > 1.2$ (Hall and Fushman 2003; Tjandra, Feller, et al. 1995).

The spectral density function for anisotropic modeling is expressed as follows:

$$J(\omega) = \frac{2}{5} \sum_{i=0}^2 A_i \left[\frac{S^2 \tau_i}{1 + (\omega \tau_i)^2} + \frac{(1 - S_f^2) \tau'_{f,i}}{1 + (\omega \tau'_{f,i})^2} + \frac{(S_f^2 - S^2) \tau'_{s,i}}{1 + (\omega \tau'_{s,i})^2} \right] \quad \text{Equation 7.16}$$

where the square of the overall generalized order parameter is the product of the order parameters for the first and second internal motions, $S^2 = S_f^2 S_s^2$, $(\tau'_i)^{-1} = \tau_e^{-1} + \tau_i^{-1}$ with $\tau_0 = (6D_{\perp})^{-1}$, $\tau_1 = (D_{\parallel} + 5D_{\perp})^{-1}$ and $\tau_2 = (4D_{\parallel} + 2D_{\perp})^{-1}$.

All five models of the spectral density consider the axially symmetric anisotropic rotational diffusion tensor by using the following external correlation function:

$$C_0^{axial}(t) = \frac{1}{5} (A_0 e^{-t/\tau_0} + A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2}) \quad \text{Equation 7.17}$$

where $A_0 = (1.5 \cos^2 \theta - 0.5)^2$; $A_1 = 3 \sin^2 \theta \cos^2 \theta$ and $A_2 = 0.75 \sin^4 \theta$ with θ being the angle between the N–H bond vector and the unique axis of the rotational diffusion tensor (Barbato et al. 1992; Jarymowycz and Stone 2006; Sakhrani et al. 2021). The five model-free formulations, their parameters, and corresponding spectral density functions are summarized in **Table 7.2**.

Table 7.2. Anisotropic spectral density functions and corresponding fitted parameter subsets

Models	Subset of fitted parameters	Spectral density function
1	S^2	$J(\omega) = \frac{2}{5} \cdot \sum_{i=0}^2 A_i \cdot \left[\frac{S^2 \tau_i}{1 + (\omega \cdot \tau_i)^2} \right]$
2	$S^2, \tau_e(ps - ns)$	$J(\omega) = \frac{2}{5} \cdot \sum_{i=0}^1 A_i \cdot \left[\frac{S^2 \tau_i}{1 + (\omega \cdot \tau_i)^2} + \frac{(1 - S^2) \cdot \tau'_i}{1 + (\omega \cdot \tau'_i)^2} \right]$
3	S^2, R_{ex}	$J(\omega) = \frac{2}{5} \cdot \sum_{i=0}^2 A_i \cdot \left[\frac{S^2 \tau_i}{1 + (\omega \cdot \tau_i)^2} \right]; R_2^{obs} = R_2 + R_{ex}$
4	$S^2, \tau_e(ps - ns), R_{ex}$	$J(\omega) = \frac{2}{5} \cdot \sum_{i=0}^2 A_i \cdot \left[\frac{S^2 \tau_i}{1 + (\omega \cdot \tau_i)^2} + \frac{(1 - S^2) \cdot \tau'_i}{1 + (\omega \cdot \tau'_i)^2} \right]; R_2^{obs} = R_2 + R_{ex}$
5	$S_f^2, S^2 = S_f^2 S_s^2, \tau_e(ns)$	$J(\omega) = \frac{2}{5} \cdot \sum_{i=0}^2 A_i \cdot \left[\frac{S^2 \tau_i}{1 + (\omega \cdot \tau_i)^2} + \frac{(S_f^2 - S^2) \tau'_i}{1 + (\omega \cdot \tau'_i)^2} \right]$

VII.2.3. Model Selection in Model-Free Analysis

Model selection is a critical step in the MFA. The goal is to determine, for each N–H bond vector, the simplest model that adequately explains the experimental relaxation parameters ($\{^{15}\text{N}\}$ -R₁, $\{^{15}\text{N}\}$ -R₂ and ^1H - $\{^{15}\text{N}\}$ NOE) without introducing unnecessary complexity. Inappropriate model selection may lead to either underfitting, where essential dynamical features are ignored, or overfitting, where the model captures noise rather than true molecular motion.

Underfitting occurs when an overly simple model (e.g., Model 1 or 2) fails to capture slower internal motions or exchange contributions, leading to systematic deviations in the back calculated $\{^{15}\text{N}\}$ -R₁, $\{^{15}\text{N}\}$ -R₂ and ^1H - $\{^{15}\text{N}\}$ NOE values.

Overfitting occurs when additional parameters improve the numerical fit but lack physical justification. This can lead to unstable or nonphysical parameter values such as a perfect back calculated $\{^{15}\text{N}\}$ -R₁, $\{^{15}\text{N}\}$ -R₂ and ^1H - $\{^{15}\text{N}\}$ NOE values.

Model selection can be performed using Akaike's Information Criterion (AIC), defined as:

$$AIC = \chi^2 + 2k \quad \text{Equation 7.18}$$

where

$$\chi^2 = \sum_{i=1}^n \frac{(R_i - R'_i)^2}{\sigma_i^2} \quad \text{Equation 7.19}$$

is the chi-square statistic measuring the goodness of fit between the experimental relaxation data (R) and the back-calculated values (R') (Abragam 1983). Here, σ denotes the experimental error, i is the residue index, k is the number of parameters in a given model, and n is the size of the data set for each residue. The AIC provides an optimal balance between underfitting (selection of overly simple models) and overfitting (selection of unnecessarily complex models), and has been shown to outperform traditional chi-square or F-test criteria in model selection (d'Auvergne and Gooley 2003).

Therefore, for accurate and reliable estimation of MFA parameters, it is recommended to measure NSR experiments at two or more static magnetic field strengths (Morin 2011). Performing measurements at multiple fields helps to separate contributions from DD and CSA relaxation mechanisms, thereby improving the precision and robustness of parameters fitting (Farrow et al. 1994; Kay et al. 1989; Mandel et al. 1995).

Chapter VIII.

**Theoretical Foundations of NMR Relaxation on the
Microsecond–Millisecond (μs –ms) Timescale**

VIII.1. Introduction

Biomolecular dynamics span across an extensive range of timescales, governed by the interdependent intramolecular and intermolecular interactions within complex free-energy landscapes. Such dynamic processes are essential for biological function (Alderson and Kay 2021; Boehr et al. 2006; Henzler-Wildman and Kern 2007; M. Karplus and Kuriyan 2005) and are frequently implicated in pathological dysfunction (Fawzi et al. 2011; Neudecker et al. 2012; Sekhar et al. 2015), motivating their investigation through a wide array of experimental and computational approaches. Among the diverse classes of biomolecular motions, microsecond-to-millisecond (μs – ms) dynamics are critically involved in fundamental biological processes, including enzyme catalysis, protein folding, ligand binding, and allosteric regulation (Palmer 2014). Characterization of these motions yields detailed information on the kinetic, thermodynamic, and structural bases of biological function (Kleckner and Foster 2011).

Molecular dynamics occurring on the μs – ms timescale often give rise to excited states that possess higher free energy than the predominantly populated ground state (Figure 8.1 (a)).

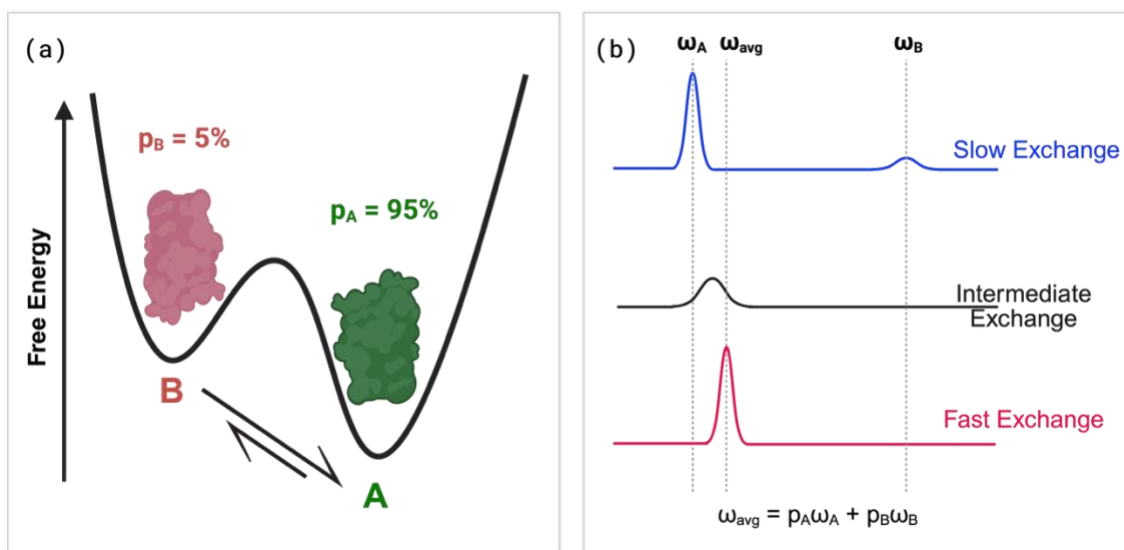


Figure 8.1. Conformational exchange between two states. (a) Schematic free-energy landscape illustrating a two-state exchange process, highlighting the low-population “invisible” excited state with a population $p_B = 5\%$. (b) Illustration of two-state exchange regimes adapted from (Y. Jin et al. 2025). Simulated NMR spectra corresponding to slow, intermediate, and fast exchange conditions, generated previously (Y. Jin et al. 2025), are shown to demonstrate the spectral signatures associated with each exchange regime.

These excited states are commonly referred to as “invisible states” due to their low populations (typically accounting for at most ~5% of the total population), their short lifetimes and the experimental challenges associated with their direct observation (Baldwin and Kay 2009; Alderson and Kay 2020). Interconversion between distinct conformational states on the μs – ms timescale is referred to as conformational exchange (Palmer et al. 2001). Because the interconverting conformers frequently adopt different structures, the magnetic environments experienced by nuclear spins in each state can differ substantially. Consequently, the exchange process gives rise to a stochastic modulation of nuclear chemical shifts, commonly termed chemical exchange. This modulation of chemical shifts can be exploited to probe the kinetics and thermodynamics of the exchange process and to extract valuable structural information about the transiently populated states (excited states) sampled during the exchange phenomenon.

Exchange processes can be described using a variety of exchange models, the simplest of which assumes a two-state exchange mechanism. For simplicity, we consider here an isolated ^{15}N - ^1H spin in a protein that undergoes exchange between two conformational states: A (the ground state) and B (the excited state).



Equation 8.1

The exchange process is characterized by several key parameters, including the exchange rate between the two states ($k_{\text{ex}} = k_{\text{AB}} + k_{\text{BA}}$, where k_{AB} and k_{BA} represent rate constant for transition from ground state to excited state and vice versa, respectively), the populations of the ground and excited states (p_{A} and p_{B} , respectively), the chemical shift difference between the two states ($\Delta\varpi_{\text{AB}} = \varpi_{\text{B}} - \varpi_{\text{A}}$ in ppm or $\Delta\omega_{\text{AB}} = \omega_{\text{B}} - \omega_{\text{A}}$ in rad/s), as well as the longitudinal relaxation rates ($R_{1\text{A}}$, $R_{1\text{B}}$) and transverse relaxation rates ($R_{2\text{A}}$, $R_{2\text{B}}$) associated with each state.

In two-state exchange model, the exchange regime is determined by the relative magnitude between k_{ex} expressed in s^{-1} and $|\Delta\omega_{\text{AB}}|$, which can be categorized as:

- **Slow Exchange** ($k_{\text{ex}} \ll |\Delta\omega_{\text{AB}}|$). In the slow-exchange regime, the NMR signals corresponding to the ground and excited states are observed separately, reflecting their distinct chemical shifts, intensities, and linewidths (**Figure 8.1 (b)**). This behavior arises because there is negligible interconversion between states A and B during the frequency-detection period of the NMR experiment (Kleckner and Foster, 2011). In this limit, the intensity of each resonance directly reports on the population of the corresponding conformational state (Kleckner and Foster, 2011).

- Intermediate Exchange ($k_{ex} \approx |\Delta\omega_{AB}|$). In the intermediate-exchange regime, a single resonance is observed at a chemical shift located between ϖ_A and ϖ_B (**Figure 8.1 (b)**). Importantly, exchange-induced line broadening predominantly affects the excited state ($k_{BA} = p_A \cdot k_{ex}$) rather than the ground state ($k_{AB} = p_B \cdot k_{ex}$), owing to its lower population and increased sensitivity to exchange processes (Jin et al. 2025; Kleckner and Foster 2011).
- Fast Exchange ($k_{ex} \gg |\Delta\omega_{AB}|$). In the fast-exchange regime, the ground and excited states give rise to a single resonance whose frequency corresponds to the population-weighted average of the individual state chemical shifts ($\omega_{avg} = p_A\omega_A + p_B\omega_B$). This behavior arises from rapid interconversion between the states, leading to effective averaging during the frequency-detection period of the NMR experiment (Jin et al., 2025; Kleckner and Foster, 2011).

To investigate biomolecular dynamics on the μ s–ms timescale, a variety of NMR techniques have been developed. Commonly used approaches include Carr–Purcell–Meiboom–Gill (CPMG) relaxation dispersion (Korzhnev et al. 2004; Palmer et al. 2001), chemical exchange saturation transfer (CEST) (Yuwen, Kay, et al. 2018; Vallurupalli et al. 2012, 2017), and rotating-frame longitudinal relaxation dispersion ($R_{1\rho}$) (Rangadurai et al. 2019; Palmer and Massi 2006) (**Figure 8.2**). This chapter focuses on the first two methods, which are employed throughout this thesis.

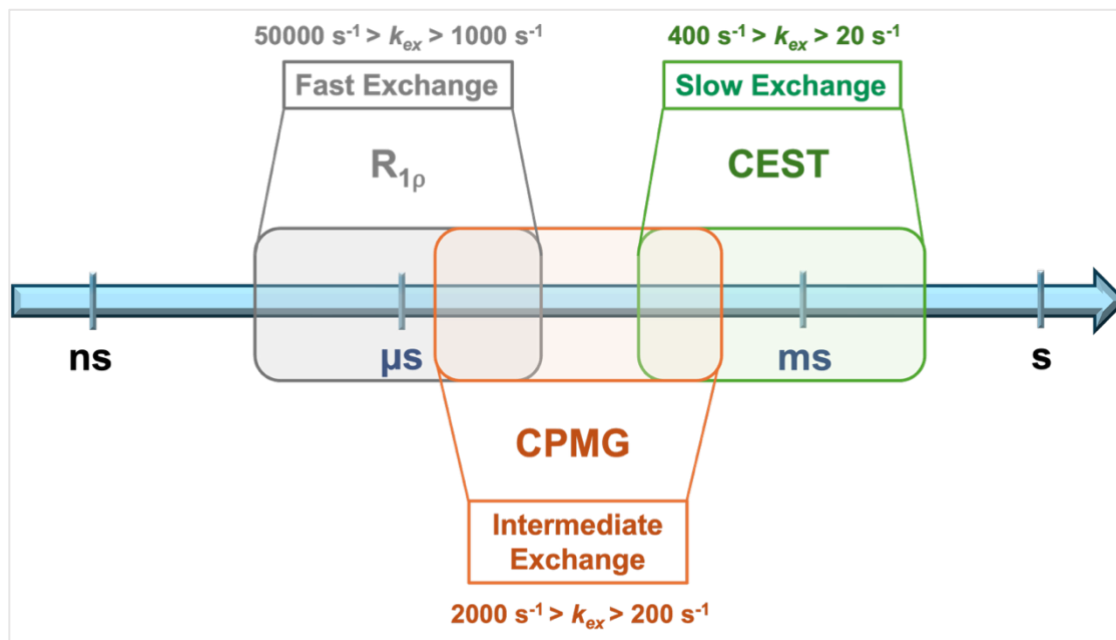


Figure 8.2. Timescales of biomolecular motions and NMR methods for probing molecular dynamics across different timescales.

VIII.2. CPMG relaxation dispersion experiment

VIII.2.1. Overview of CPMG relaxation dispersion

Carr–Purcell–Meiboom–Gill (CPMG) relaxation dispersion, named after its developers Herman Carr, Edward Purcell, Solomon Meiboom, and David Gill (Meiboom and Gill 1958; Carr and Purcell 1954), is one of the most widely used NMR techniques for characterizing low-population, “invisible” excited states (**Figure 8.3 (a)**) in biomolecules (Sekhar and Kay 2019; Y. Jin et al. 2025). It is a spin-echo–based method, as described in **Chapter VII, Section VII.1.2.B**.

In a CPMG experiment, transverse magnetization is maintained in the xy -plane during the relaxation period (T_{CPMG}), while a train of 180° refocusing pulses (commonly referred to as CPMG pulses or refocusing frequency/rate) is applied at defined time intervals (**Figure 8.3 (b)**). The evolution of magnetization during the pulse sequence is illustrated in **Figure 8.3 (c)**, where, without loss of generality, the carrier frequency is assumed to be placed on resonance with the ground state (ϖ_A) (Vallurupalli et al. 2012). Because biomolecules typically exhibit relatively large intrinsic transverse relaxation rates (R_2), the relaxation period T_{CPMG} is usually chosen to be on the order of several tens of milliseconds (Jin et al. 2025).

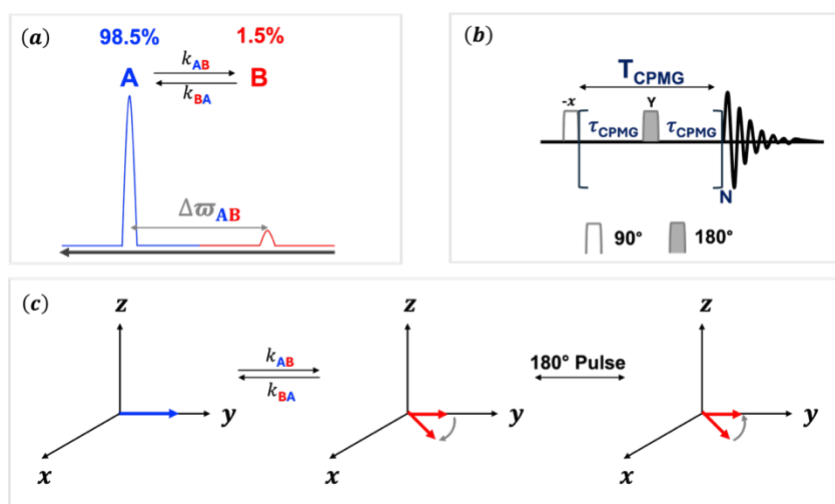


Figure 8.3. Standard CPMG relaxation dispersion experiment. (a) ^{15}N spectrum of an isolated spin exchanging between two states with chemical shifts ϖ_A and ϖ_B . The minor state is shown in the spectrum for purposes of illustration but cannot be observed in the systems of interest. (b) Basic CPMG experiment. A variable number (N) of 180° refocusing pulses is applied during a constant-time relaxation element of duration. (c) Illustration of the mechanism underlying the CPMG experiment, with the ground-state (blue magnetization) assumed to be on resonance. The 180° pulses invert the sense in which “excited state” spins (red magnetization) precess around the z -axis. Adapted from (Vallurupalli et al. 2012)

The CPMG experiment measures an effective transverse relaxation rate,

$$R_{2,eff} = R_2 + R_{ex} \quad \text{Equation 8.2}$$

where R_2 is the intrinsic transverse relaxation rate and R_{ex} is the chemical exchange contribution arising from conformational exchange. For systems undergoing exchange on μs -ms timescale, $R_{2,eff}$ is modulated by the application of refocusing frequency at a frequency,

$$\nu_{CPMG} = 1/4\tau_{CPMG} \quad \text{Equation 8.3}$$

where $2\tau_{CPMG}$ denotes the delay between successive 180° pulses in the refocusing pulse train.

Each 180° refocusing pulse reverses the sense of spin precession about the z -axis, thereby refocusing chemical shift evolution and attenuating the effects of chemical exchange. At low refocusing frequencies, exchange effects are insufficiently refocused, resulting in a large R_{ex} contribution and significant signal attenuation (**Figure 8.4**). In contrast, at high refocusing frequencies, exchange-induced dephasing is efficiently quenched, leading to a reduction in R_{ex} and a corresponding increase in signal intensity (**Figure 8.4**).

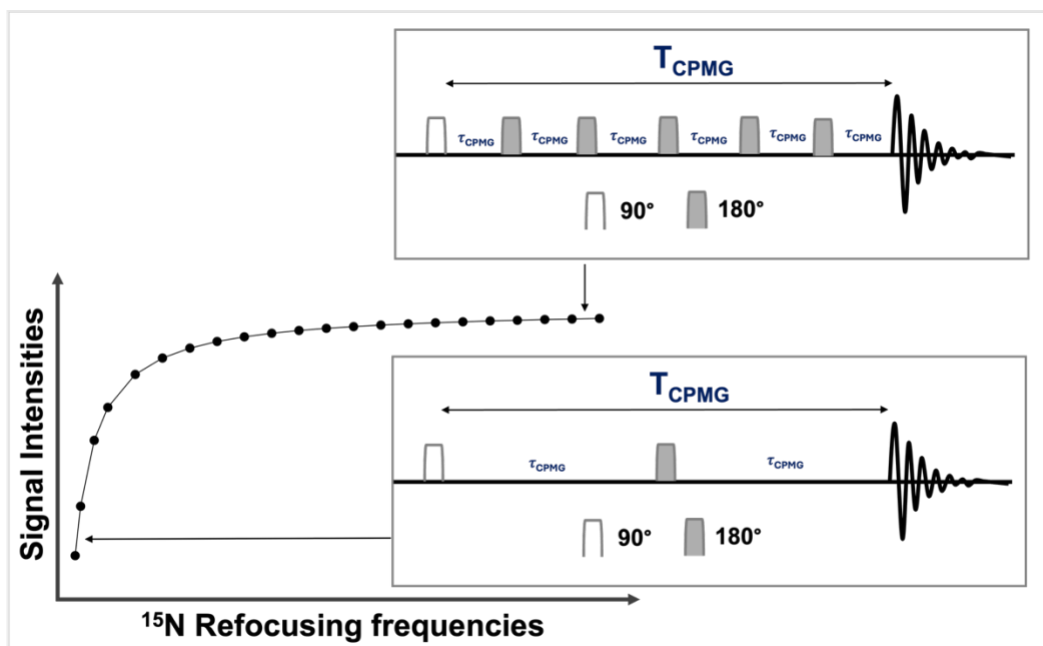


Figure 8.4. Evolution of ^{15}N signal intensities as a function of CPMG refocusing frequency.

This dependence of $R_{2,\text{eff}}$ on the refocusing frequency; known as CPMG relaxation dispersion; arises because the chemical shift differences between exchanging states ($\Delta\omega_{AB}$) are effectively averaged out by rapid refocusing (Y. Jin et al. 2025). Consequently, the exchange contribution R_{ex} is progressively suppressed at higher refocusing rates ($R_{\text{ex}} \rightarrow 0$ as $\nu_{\text{CPMG}} \rightarrow \infty$), enabling quantitative characterization of exchange kinetics, populations, and chemical shift differences.

$R_{2,\text{eff}}$ can be calculated from the measured signal intensities and T_{CPMG} according to the following relationship (Mulder et al. 2001):

$$R_{2,\text{eff}}(\nu_{\text{CPMG}}) = -\frac{1}{T_{\text{CPMG}}} \cdot \ln\left(\frac{I(\nu_{\text{CPMG}})}{I_0}\right) \quad \text{Equation 8.4}$$

where I_0 is the signal intensity in the reference spectrum recorded without the T_{CPMG} relaxation delay and $I(\nu_{\text{CPMG}})$ is the corresponding signal intensity in the spectrum measured at a frequency of ν_{CPMG} .

Plotting $R_{2,\text{eff}}$ as a function of ν_{CPMG} yields two distinct types of profiles: (i) relaxation dispersion profiles observed for residues undergoing conformational exchange, and (ii) flat profiles for residues that do not participate in exchange (Figure 8.5).

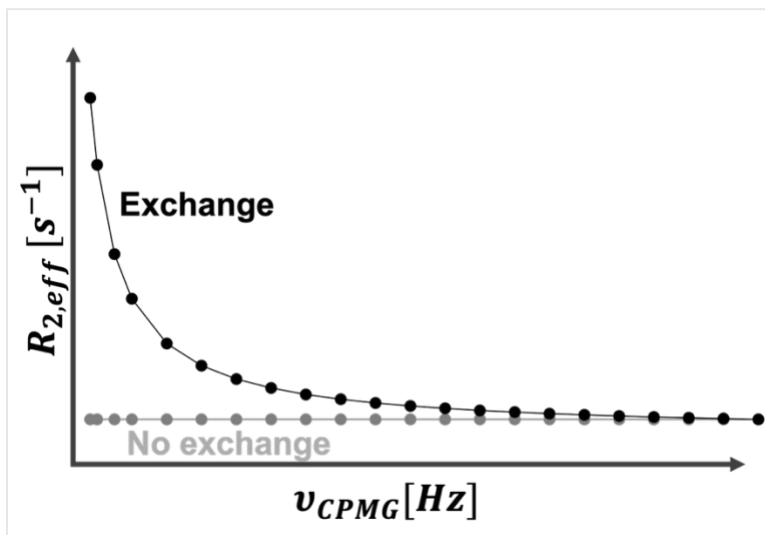


Figure 8.5. ^{15}N CPMG profiles.

VIII.2.2. Experimental considerations in CPMG relaxation dispersion measurements

CPMG relaxation dispersion experiments in biomolecular NMR are most performed using ^{15}N (Kleckner and Foster 2011; Jin et al. 2025; Kay et al. 1992) or ^{13}C (Kleckner and Foster 2011; Y. Jin et al. 2025; Vallurupalli et al. 2009; Lundström et al. 2009) spin probes, owing to the reduced influence of homonuclear couplings ($^1J_{\text{H}-\text{H}}$) and cross-relaxation effects (e.g. ^{13}C - ^{13}C cross-relaxation), which simplifies both experimental implementation and data analysis. In addition, TROSY-based CPMG experiments have been developed for ^{15}N spin probes, making them particularly well suited for investigations of high molecular-weight biomolecules (Jin et al. 2025; Loria et al. 1999).

In the traditional CPMG experiment, relaxation dispersion profiles are constructed by measuring signal intensities at multiple CPMG refocusing frequencies ν_{CPMG} , a procedure that is experimentally time-consuming. This approach has therefore been largely superseded by constant-time CPMG schemes, in which relaxation dispersion profiles are obtained by varying the number of refocusing pulses applied within a fixed relaxation period T_{CPMG} .

Although CPMG experiments can also be conducted using ^1H spin probes, their experimental implementation is considerably more challenging. This increased complexity arises from artifacts associated with ^1H - ^1H homonuclear couplings and cross-relaxation effects (Y. Jin et al. 2025; Ishima and Torchia 2003; Ishima et al. 1999). Consequently, such experiments often require sample deuteration (^2H) or the use of specialized pulse schemes designed for studies of fully protonated samples (Y. Jin et al. 2025; Yuwen and Kay 2019).

Focusing on ^{15}N CPMG relaxation dispersion experiments, which are employed throughout this research, it is important to note that ^{15}N nuclei are directly bonded to NMR-active ^1H spins. The associated heteronuclear scalar-couplings $^1J_{\text{H}-^{15}\text{N}}$ can introduce potential artifacts during experiments and must therefore be carefully suppressed. To understand the origin of these artifacts, it is necessary to introduce two key concepts: in-phase and anti-phase magnetization.

A. In-phase magnetization

To illustrate the concept of in-phase magnetization, we consider an isolated ^1H - ^{15}N spin pair subjected to a simple one-pulse experiment (**Figure 8.6 (a)**). At equilibrium, the magnetization of both ^1H and ^{15}N nuclei lies along the z -axis and carries a positive sign (**Figure 8.6 (b)**).

Application of a 90° pulse on the ^{15}N channel rotates the ^{15}N magnetization from the z -axis into the transverse plane (xy -plane), for example along the y -axis, while retaining its positive sign. Because the ^1H magnetization remains aligned along the z -axis, both ^{15}N transverse magnetizations are in-phase at $t = 0$, immediately after the 90° pulse on the ^{15}N channel.

Consequently, detectable transverse magnetization is generated on the ^{15}N nucleus. The observed ^{15}N signal is split into a doublet due to the $^1J_{\text{H}-^{15}\text{N}}$ scalar-coupling. Each component of the doublet corresponds to a distinct spin state of the coupled proton: one resonance arises from ^{15}N nuclei coupled to a ^1H in the α -state, while the other corresponds to coupling with a ^1H in the β -state.

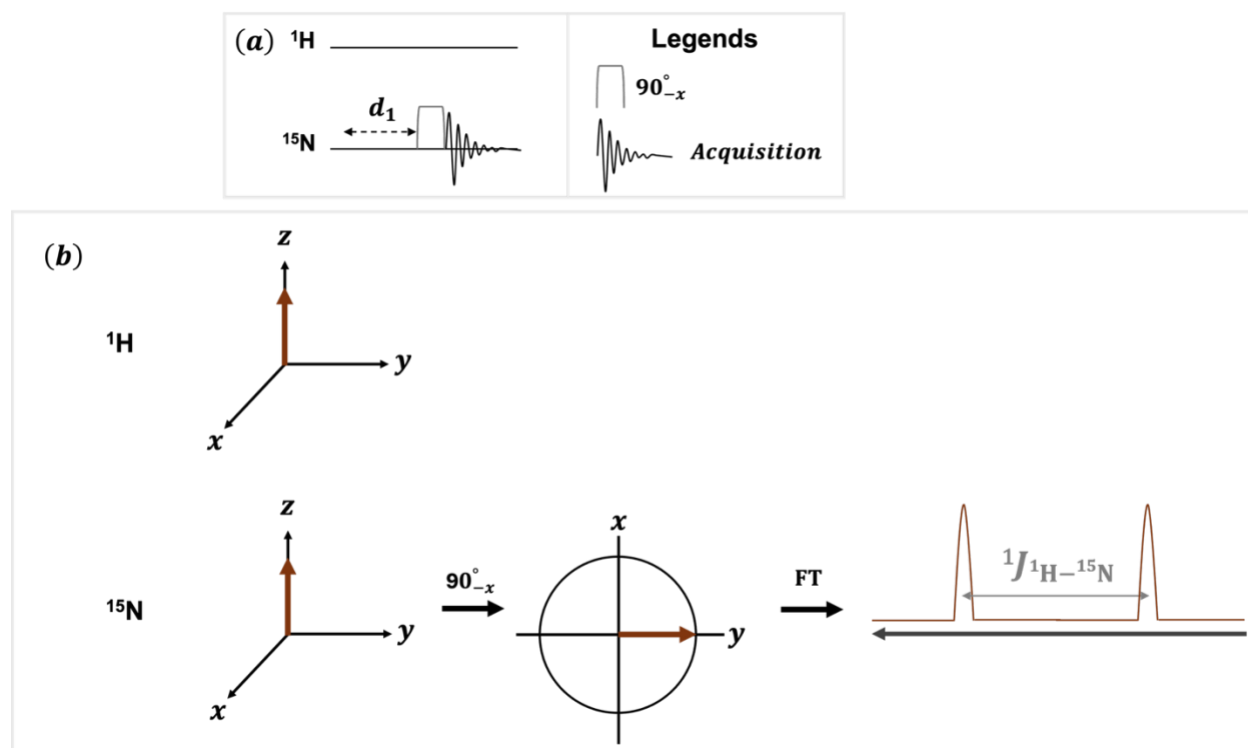


Figure 8.6. In-phase magnetization. (a) One-pulse sequence. (b) ^{15}N Magnetization Evolution and detected signal.

B. Anti-phase magnetization

In NMR spectroscopy, anti-phase magnetization arises when the evolution of one nuclear spin depends on the spin state of a coupled partner, most commonly through scalar-coupling. Although anti-phase magnetization carries valuable information about spin–spin interactions and dynamics, it is less directly observable and more susceptible to relaxation, making its control a critical aspect of pulse-sequence design.

To illustrate the concept of anti-phase magnetization, we consider an isolated ^1H – ^{15}N spin pair undergoing an Insensitive Nuclei Enhancement by Polarization Transfer (INEPT) experiment (**Figure 8.7 (a)**). At equilibrium, the magnetization of both the ^1H and ^{15}N nuclei is aligned along the z -axis and carries a positive sign.

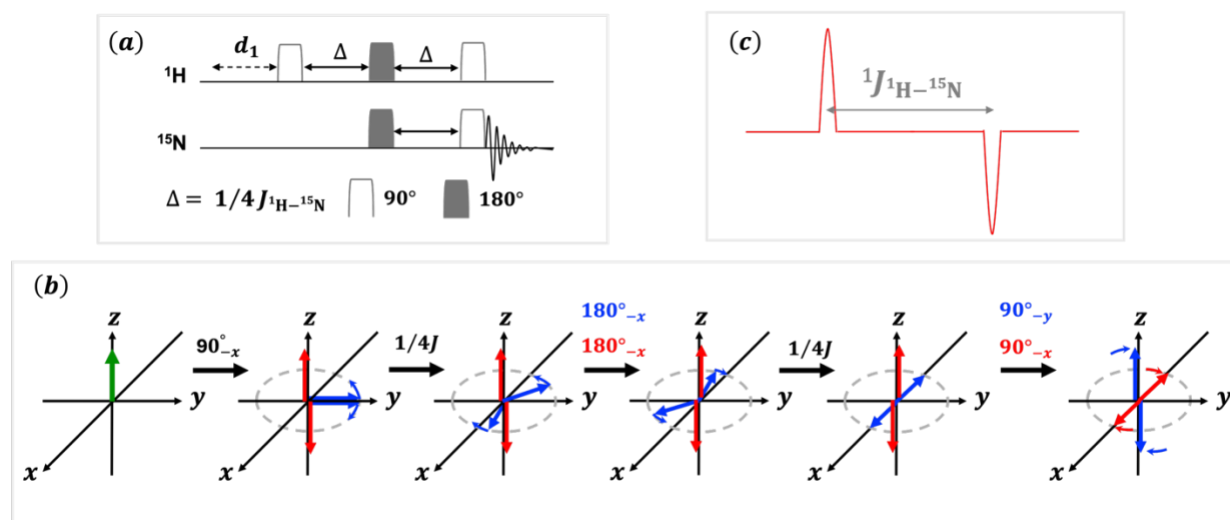


Figure 8.7. Anti-phase magnetization. (a) INEPT pulse sequence. (b) Evolution of ^1H (blue) and ^{15}N (red) magnetizations. (c) Detected signal

Application of a 90° pulse on the ^1H channel rotates the proton magnetization from the z -axis into the xy -plane, where it evolves under the influence of the $^1J_{^1\text{H}-^{15}\text{N}}$ scalar-coupling (**Figure 8.7 (b)**). During this evolution, ^1H magnetization coupled to a ^{15}N spin in the α -state precesses in counterclockwise direction, whereas ^1H magnetization coupled to a ^{15}N spin in the β -state precesses in the opposite direction (clockwise).

After a delay of $1/4J_{^1\text{H}-^{15}\text{N}}$, 180° pulses are applied simultaneously on both the ^1H and ^{15}N channels. The 180° pulse applied on the ^1H channel changes the phase of the proton coherence in the xy -plane, while the 180° pulse inverts the ^{15}N spin populations. As a result, after the second $1/4J_{^1\text{H}-^{15}\text{N}}$ evolution period, the two ^1H magnetization components become aligned along the x -axis but with opposite signs. This configuration corresponds to anti-phase magnetization.

Subsequent application of a 90° pulse simultaneously on both the ^1H and ^{15}N channels transfers the ^1H population difference to the ^{15}N spin, generating anti-phase ^{15}N

magnetization in the xy -plane. Consequently, detectable anti-phase magnetization is produced on the ^{15}N spin (Figure 8.7 (b-c))

Returning to the ^{15}N CPMG relaxation dispersion experiment, the conventional pulse sequence can be divided into several key elements, as illustrated in the simplified scheme shown in Figure 8.8. These elements include an initial INEPT, a CPMG relaxation period, an incremental evolution time, a reverse INEPT, and signal acquisition. Each step plays a specific role in preparing, modulating, and detecting the ^{15}N magnetization.

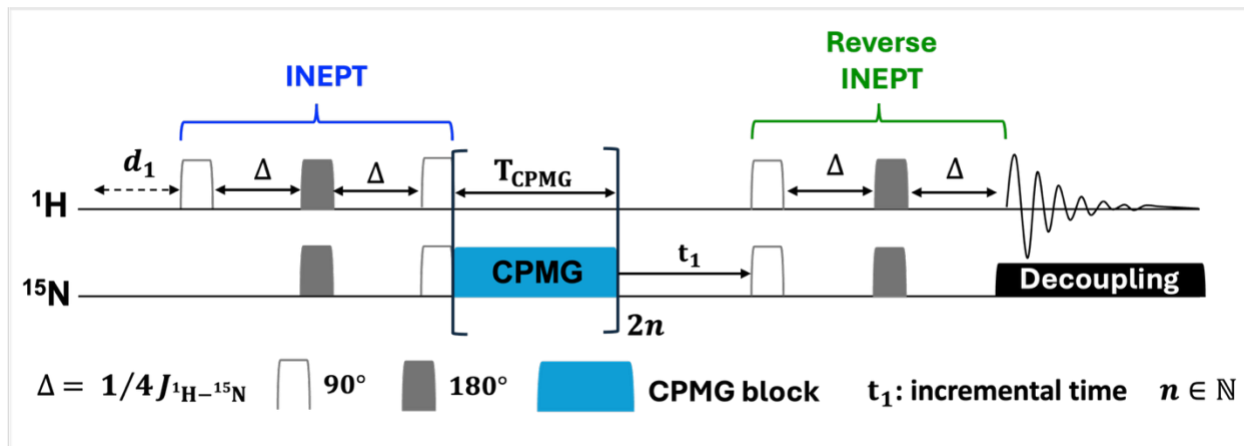


Figure 8.8. Simplified conventional ^{15}N relaxation dispersion pulse sequence. Adapted from (Palmer et al. 1992).

The experiment begins with an INEPT block which transfers polarization (population difference) from the highly sensitive ^1H spins to the less sensitive ^{15}N spins via the $^1J_{^{1}\text{H}-^{15}\text{N}}$ scalar-coupling. As described above, this step generates anti-phase ^{15}N magnetization in the xy -plane.

Following INEPT, the anti-phase ^{15}N magnetization is subjected to the conventional CPMG pulse train constituting the CPMG relaxation period. During this period, chemical shift evolution is modulated by the application of defined refocusing frequency, thereby probing exchange contributions to the transverse relaxation.

After the CPMG relaxation period, an incremental evolution period is introduced to encode ^{15}N chemical shift information. This period allows frequency labeling of the ^{15}N spin prior to detection and is incremented systematically to generate the indirect ^{15}N dimension of the experiment. Finally, the NMR signal is recorded during the acquisition period, typically on the ^1H channel, with the observed signal intensity reflecting the cumulative relaxation experienced by the magnetization during the CPMG periods.

Although the INEPT transfer initially generates anti-phase ^{15}N magnetization, this magnetization does not remain purely anti-phase during the subsequent CPMG relaxation period due to the presence of the $^1J_{^{1}\text{H}-^{15}\text{N}}$ scalar-coupling. During the delays between

successive CPMG refocusing pulses (τ_{CP}), evolution under the $^1J_{^1H-^{15}N}$ transiently interconverts anti-phase and in-phase ^{15}N coherences. Because anti-phase magnetization relaxes more rapidly than in-phase magnetization (Hansen, Vallurupalli, and Kay 2008), magnetization converted into anti-phase coherence decays more rapidly. Since the extent of this interconversion depends on the τ_{CP} , the measured effective transverse relaxation rate may acquire a τ_{CP} –dependent contribution that is unrelated to conformational exchange. As result, apparent relaxation dispersion may be observed even in the absence of genuine conformational exchange, giving rise to artificial dispersion profiles. The frequently used strategy to suppress such artifacts is the relaxation-compensated CPMG (rc-CPMG) approach (Loria et al. 1999). The rc-CPMG scheme was designed to average the relaxation of in-phase and anti-phase coherences in a controlled manner, so that this contribution becomes independent of τ_{CP} .

In the rc-CPMG experiment, the pulse sequence consists of two CPMG relaxation periods of equal and constant duration, each of duration $T_{CPMG}/2$. These two periods are separated by a P-element, as illustrated in the simplified ^{15}N rc-CPMG pulse sequence shown in **Figure 8.9**.

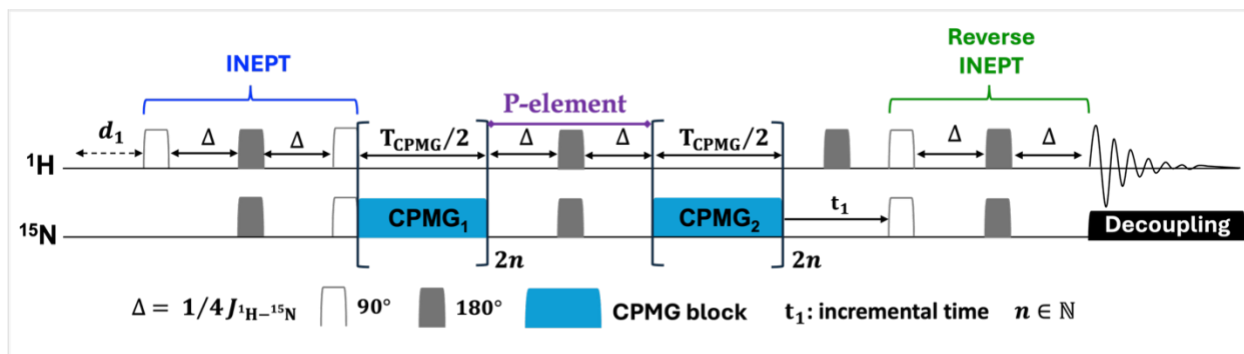


Figure 8.9. Simplified ^{15}N rc-CPMG relaxation dispersion pulse sequence. Adapted from (Loria et al. 1999)

Following INEPT, the anti-phase ^{15}N magnetization is subjected to a CPMG pulse train constituting the first CPMG relaxation period (CPMG₁). During this period, chemical shift evolution is modulated by the application of defined refocusing frequency, thereby probing exchange contributions to the transverse relaxation of anti-phase magnetization. After the first CPMG relaxation period, the P-element convert the anti-phase ^{15}N magnetization into in-phase ^{15}N magnetization. A second CPMG block (CPMG₂), identical or symmetric to the first, is then applied. This symmetric arrangement about the P-element is designed to ensure that equal amounts of in-phase and anti-phase ^{15}N magnetization are sampled during the total T_{CPMG} period, independent of the CPMG refocusing frequency ν_{CPMG} . The incremental evolution time, reverse INEPT block, and signal acquisition are implemented in the same manner as in the conventional ^{15}N CPMG experiment discussed above.

To improve robustness against pulse imperfections and resonance offsets, the P-element is usually implemented as a composite pulse (Hansen, Vallurupalli, and Kay 2008; Hansen, Vallurupalli, Lundström, et al. 2008), i.e., a sequence of pulses with defined phases and durations that together achieve an accurate effective rotation while minimizing experimental errors.

Another widely used strategy for suppressing $^1J_{\text{H}-^{15}\text{N}}$ scalar-coupling induced artifacts involves the application of ^1H decoupling during the T_{CPMG} relaxation period, as implemented in the CW-CPMG (Hansen, Vallurupalli, and Kay 2008) and ST-CW-CPMG (Jiang et al. 2015) experiments. Continuous ^1H decoupling during T_{CPMG} maintains the ^{15}N magnetization in an in-phase state, thereby preventing interconversion with anti-phase magnetization. Because in-phase magnetization exhibits more favorable relaxation properties and contributes less background to the effective transverse relaxation rate $R_{2,\text{eff}}$, as discussed above, CW-CPMG and ST-CW-CPMG enable sampling of a larger number of CPMG refocusing frequencies ν_{CPMG} within a fixed relaxation period T_{CPMG} (Hansen, Vallurupalli, and Kay 2008).

However, the use of continuous-wave (CW) ^1H decoupling requires very high ^1H decoupling power (typically ~ 15 kHz), which makes it challenging to study high-salt samples, particularly when using cryogenic probes (Jin et al. 2025). Furthermore, CW ^1H decoupling can introduce slow-pulsing artifacts, primarily due to off-resonance effects and radiofrequency field inhomogeneities (pulse imperfections). To mitigate these artifacts, composite-pulse decoupling (CPD) schemes, such as $90_x^\circ 240_y^\circ 90_x^\circ$ (Chatterjee et al. 2018), are commonly employed. These schemes improve decoupling efficiency over a broader frequency range and enhance robustness against offset and pulse imperfections, thereby reducing decoupling-related artifacts in CW-based CPMG experiments.

Artifacts in ^{15}N CPMG relaxation dispersion experiments can also arise from the presence of D_2O in the sample (Jin et al. 2025), which is commonly used for spectrometer frequency locking. Solvent exchange leads to interconversion between ^{15}N – ^1H and ^{15}N – ^2H groups at backbone amide and other labile sites, resulting in artificial relaxation dispersion effects (Kumari et al. 2018; Kim et al. 2013). To minimize such artifacts, it is recommended to use the lowest practical fraction of D_2O in the sample (e.g., 5% v/v instead of the typical 10%).

At this stage, we have shown that artificial ^{15}N relaxation dispersion profiles arising from $^1J_{\text{H}-^{15}\text{N}}$ scalar-coupling in the absence of conformational exchange can be suppressed using rc-CPMG, CW-CPMG, and ST-CW-CPMG experiments. In all of these approaches, a large number of CPMG refocusing pulses are applied during the constant-time relaxation period T_{CPMG} . Therefore, small errors arising from off-resonance effects and pulse imperfections can accumulate over the pulse train and lead to experimental artifacts, thereby reducing the accuracy of the extracted dynamical parameters.

Although careful calibration of the CPMG pulses can mitigate these effects, a commonly employed and highly effective strategy is the use of specialized phase-cycling schemes within the CPMG pulse train. Phase cycling involves systematically varying the phases of rf-pulses, such that desired magnetization pathways add constructively while undesired pulse-induced artifacts cancel upon signal averaging. In this way, phase cycling improves robustness against pulse imperfections and enhances the reliability of relaxation measurements.

Among the various phase-cycling schemes that have been developed, the {0013}-phase cycle (Bain et al. 2011; Yip and Zuiderweg 2004) is most widely used and is generally preferred for ^{15}N CPMG relaxation dispersion experiments (Jin et al. 2025). This phase cycle has been successfully implemented in several pulse-sequence variants, including rc-CPMG (Long et al. 2008) and ST-CW-CPMG (Jiang et al. 2015), where it significantly reduces artifacts arising from pulse imperfections and off-resonance effects. Furthermore, to ensure reliable and accurate estimation of exchange parameters ($k_{ex}, p_B, \Delta\varpi_{AB}$) in biomolecular systems using ^{15}N CPMG relaxation dispersion experiments, it is commonly recommended to perform measurements at multiple static magnetic field strengths (Millet et al. 2000) and/or at different temperatures (Korzhnev et al. 2004).

VIII.3. CEST experiment

VIII.3.1. Overview of CEST experiment

Chemical exchange saturation transfer (CEST) techniques were originally developed over six decades ago to investigate slow chemical exchange between "visible" states in small molecules (Forsén and Hoffman 1963). The underlying concept was to selectively perturb the magnetization at one site (site A) by applying a weak radiofrequency field and to observe the resulting effect on the magnetization at a second site (site B), arising from the reversible transfer of protons between the two molecular sites (sites A and B). Conversely, and in a completely symmetric manner, perturbation of the magnetization at site B produces a corresponding effect on the magnetization at site A. Since then, CEST has emerged as a widely used methodology in magnetic resonance imaging (MRI) and related fields (Ward et al. 2000; Van Zijl and Yadav 2011). More recently, its routine application to the study of biomolecular conformational exchange between "visible" and "invisible" states has gained substantial popularity, particularly following its adaptation for protein NMR spectroscopy (Vallurupalli et al. 2012; Y. Jin et al. 2025).

To illustrate the principle of the CEST experiment, we consider, without loss of generality, a simplified CEST pulse scheme (**Figure 8.10 (b)**). We assume a two-state exchange

model operating in the slow-exchange limit ($k_{ex} \ll \Delta\omega_{AB}$) where $\Delta\omega_{AB}$ denotes the difference in resonance frequencies between the visible ground state (state-A) and the invisible excited state (state-B) (Figure 8.10 (a)).

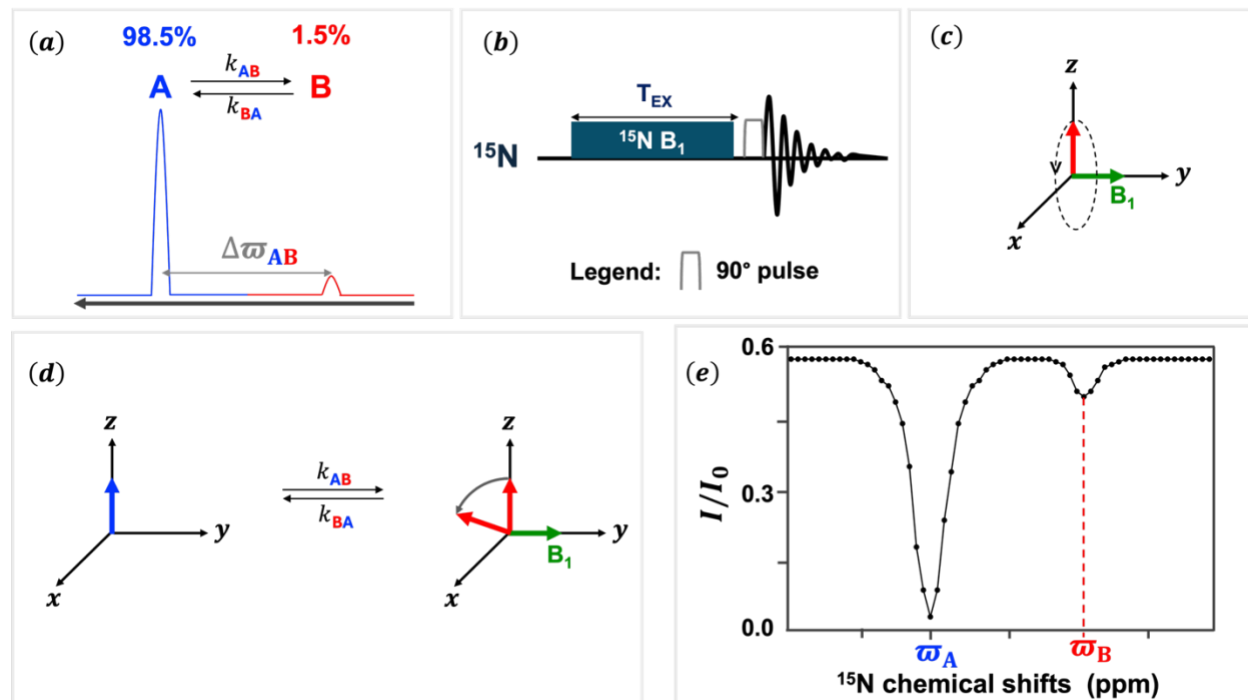


Figure 8.10. Standard CEST experiment. (a) ^{15}N spectrum of an isolated spin exchanging between two states with chemical shifts ω_A and ω_B . The minor state is shown in the spectrum for purposes of illustration but cannot be observed in the systems of interest. (b) Schematic CEST experiment. A weak B_1 field is applied along the y -axis (green) for a time T_{EX} before acquisition of the spectrum. (c) When the B_1 field is on resonance with ω_B , precession occurs around the y -axis. (d) Phase accumulation in the minor state and chemical-exchange modulation reduce the observed major-state magnetization intensity. (e) CEST profile obtained by quantifying the intensity of the visible-state peak as a function of position of the weak B_1 irradiation field. Adapted from (Vallurupalli et al. 2012).

When the frequency of the weak perturbing rf-field B_1 is far from both ω_A and ω_B , spins in neither state are significantly affected, and the intensity of the state-A resonance remains unchanged relative to the case where no B_1 field is applied.

When the B_1 field is applied at ω_A , the observed state-A magnetization is directly perturbed, leading to the major dip in the CEST profile (Figure 8.10 (e)). This contribution corresponds primarily to direct saturation of the ground-state resonance.

In contrast, when the B_1 field is applied at ω_B , it selectively perturbs spins transiently populating state-B. This perturbation induces Rabi oscillations (transfer of population between two states induced by B_1 field) for the nuclei transiently populating the state-B,

leading to precession around the y -axis in the xz -plane (**Figure 8.10 (c)**). This behavior is analogous to the transverse precession about the z -axis observed during CPMG experiments. As a result, the longitudinal magnetization of state-B is perturbed. Through chemical exchange, this perturbation is transferred back to the observable ground-state population. This exchange-modulated contribution gives rise to an additional, usually weaker, dip centered at ω_B in the CEST profile (**Figure 8.10 (e)**).

It is important to emphasize that attributing this signal decrease solely to a classical saturation transfer mechanism upon irradiation at ω_B is misleading in many cases (Vallurupalli et al. 2017). In typical biomolecular systems, the lifetime of the state-B $\tau_B = 1/k_{BA}$ is significantly shorter than the timescale of $1/R_{B,avg}$ required to establish steady-state saturation. $R_{B,avg} = (R_{1,B} + R_{2,B})/2$ (Vallurupalli et al. 2012). Consequently, spins exchange out of state-B before full saturation can be achieved, or spins leave state-B before full saturation. Instead, the applied B_1 field partially dephases the magnetization while spins transiently reside in the minor state (**Figure 8.10 (d)**).

A CEST profile is generated by plotting the observed magnetization intensity as a function of the B_1 irradiation offset. The intensity is typically normalized and expressed as a ratio relative to a reference experiment acquired without the CEST irradiation period (I/I_0). The major and minor dips observed in the CEST profile correspond to the ground state (state-A) and the excited state (state-B), respectively (**Figure 8.10 (e)**).

In most applications, CEST experiments are performed under slow-exchange conditions. However, it has been demonstrated more recently that CEST methodologies can also be used to probe systems undergoing exchange in the intermediate-to-fast timescale (Tiwari et al. 2024; Khandave et al. 2023; Rangadurai et al. 2020).

In a CEST experiment, the ground-state magnetization is initially aligned along the longitudinal z -axis and remains longitudinal during the exchange period T_{EX} . Consequently, relaxation during the exchange period T_{EX} is dominated by longitudinal relaxation (R_1). Owing to the relatively slow intrinsic R_1 of biomolecules, T_{EX} is typically set to several hundreds of milliseconds.

VIII.3.2. Experimental considerations in CEST measurements

In the previous section, we described how CEST profiles are generated through the application of a weak B_1 field. An important practical question then arises regarding how to select an optimal B_1 field strength for a given set of exchange parameters (k_{ex} and p_B). By analogy with CPMG relaxation dispersion experiments, it is commonly recommended to acquire a pair of CEST datasets using two different B_1 fields: one in the regime where $\omega_1 < k_{ex}$ and another where $\omega_1 > k_{ex}$; $\omega_1 = 2\pi B_1$ with B_1 in Hz.

However, in the analysis of CEST profiles, it is often implicitly assumed that the transverse relaxation rate of the minor state, $R_{2,B}$, is much smaller than the exchange rate k_{ex} ($R_{2,B} \ll k_{ex}$), an assumption that is not always valid in practice.

Recently, it has been demonstrated that when $R_{2,B} \gg k_{ex}$, precise site-specific exchange parameters cannot be reliably extracted from the analysis of two CEST datasets (Khandave et al. 2024). In this regime, the B_1 fields required to accurately characterize chemical exchange depend not only on k_{ex} but also on $R_{2,B}$. Accordingly, it has been proposed that the choice of B_1 fields should be governed by an effective exchange parameter K (Khandave et al. 2024),

$$K = \sqrt{k_{ex}(k_{ex} + R_{2,B})} \quad \text{Equation 8.5}$$

which incorporates contributions from both k_{ex} and $R_{2,B}$. When $R_{2,B} \ll k_{ex}$, $K \approx k_{ex}$, and the selection of B_1 fields is primarily determined by k_{ex} . In contrast, when $R_{2,B} \gg k_{ex}$, CEST datasets recorded using higher B_1 fields dictated by K , rather than by k_{ex} alone (Khandave et al. 2024), are required to obtain accurate and reliable exchange parameters.

As described above, in case of exchange, CEST profile consists of a major dip at ω_A , and smaller dip centered at ω_B . Recent studies have established that CEST profile does not often contain well-separated dips but a single asymmetric dip that can be analyzed to study biomolecular dynamics occurring on the intermediate-to-fast timescale (Tiwari et al. 2024; Khandave et al. 2023; Rangadurai et al. 2020). Therefore, one should take care when analyzing CEST profiles. In the case of suspicion of a single asymmetric dip, recording CPMG relaxation dispersion experiment using CW-CPMG or ST-CW-CPMG experiments may help to distinguish specific site in exchange from those who are not in exchange as shown in this research (manuscript under review at *Journal of Magnetic Resonance*). Accordingly, a combined analysis of CPMG relaxation dispersion and CEST datasets may provide a more robust framework for the extraction of exchange parameters.

In what follows, we focus on ^{15}N CEST experiments, which are employed throughout this research.

For biomolecular studies, ^{15}N CEST experiments targeting backbone amide groups are most commonly employed (Vallurupalli et al. 2012). Because backbone ^{15}N nuclei are directly bonded to ^1H , the application of ^1H decoupling is essential to suppress $^1J_{^1\text{H}-^{15}\text{N}}$ scalar-coupling induced splitting of both the major and minor dips in the CEST profile. Such splitting can significantly hinder accurate extraction of exchange parameters, particularly at lower B_1 field (Y. Jin et al. 2025; Zhou and Yang 2014). For the range of B_1 fields most commonly used in ^{15}N CEST experiments (typically 10–50 Hz), it is generally recommended to employ the $90_x^\circ 240_y^\circ 90_x^\circ$ CPD scheme (Chatterjee et al. 2018; Vallurupalli et al. 2012). Using ^1H decoupling field strength of 2.35 kHz at a static magnetic field of 11.7 T, this CPD scheme produces decoupling sidebands at $\pm 1/(2p_{\text{w}_{\text{INV}}})$ Hz relative to ω_{A} , corresponding to approximately ~ 20.0 ppm (Vallurupalli et al. 2012). These sidebands typically give rise to very weak spurious peaks in a small subset of CEST profiles (Vallurupalli et al. 2012). Here, $p_{\text{w}_{\text{INV}}} = 1.17/(^1\text{H field})$ denotes the duration of the inversion pulse, where the factor 1.17 accounts for the total nutation (rotation) time of the CPD.

As in ^{15}N CPMG relaxation-dispersion experiments, artifacts in ^{15}N CEST experiments can also arise from solvent-exchange effects involving labile amide protons (Tiwari et al. 2019). To minimize such artifacts, it is recommended to use the lowest practical fraction of D_2O in the sample.

In ^{15}N CEST experiment, the irradiation frequency spacing is typically chosen to be comparable to the weak B_1 field (usually 10–50 Hz) (Y. Jin et al. 2025). Given that, the ^{15}N spectral window commonly spans approximately ~ 35 ppm, acquiring a full ^{15}N CEST profile often requires collecting a large number of individual datasets, rendering the experiment time-consuming. To address this limitation, accelerated CEST methodologies such as DANTE-CEST (Yuwen, Kay, et al. 2018) and COS-CEST (Yuwen, Bouvignies, et al. 2018) have been developed.

A. DANTE-CEST

The DANTE-CEST (D-CEST) approach (Yuwen, Kay, et al. 2018) uses a train of short, high-power B_1 pulses, known as a DANTE sequence (Gareth A. Morris and Freeman 2011; Bodenhausen, Freeman, et al. 1976), to achieve multi-site excitation during the saturation period T_{EX} . In this scheme, the interval between successive DANTE pulses determines the spacing between adjacent excitation regions, commonly referred to as D-CEST spectral window (SW_{CEST}). As a result, D-CEST profiles exhibit periodicity with a period equal to SW_{CEST} . To remove ambiguities in the extracted ^{15}N chemical shifts of the major- and excited-state, it is recommended to acquire data using at least two different SW_{CEST} .

By simultaneously probing multiple frequency offsets within a single experiment, D-CEST significantly reduces the total measurement time, typically providing an approximately fourfold decrease compared to conventional CEST experiments (Figure 8.11). As a consequence, D-CEST is particularly advantageous for studies of large proteins, such as the TEM-1 enzyme, where it enables the acquisition of D-CEST profiles with high signal-to-noise ratios (Figure 8.12). In contrast to conventional CEST, this improved efficiency and facilitates more reliable extraction of exchange parameters in systems where experimental time and sensitivity are limiting factors.

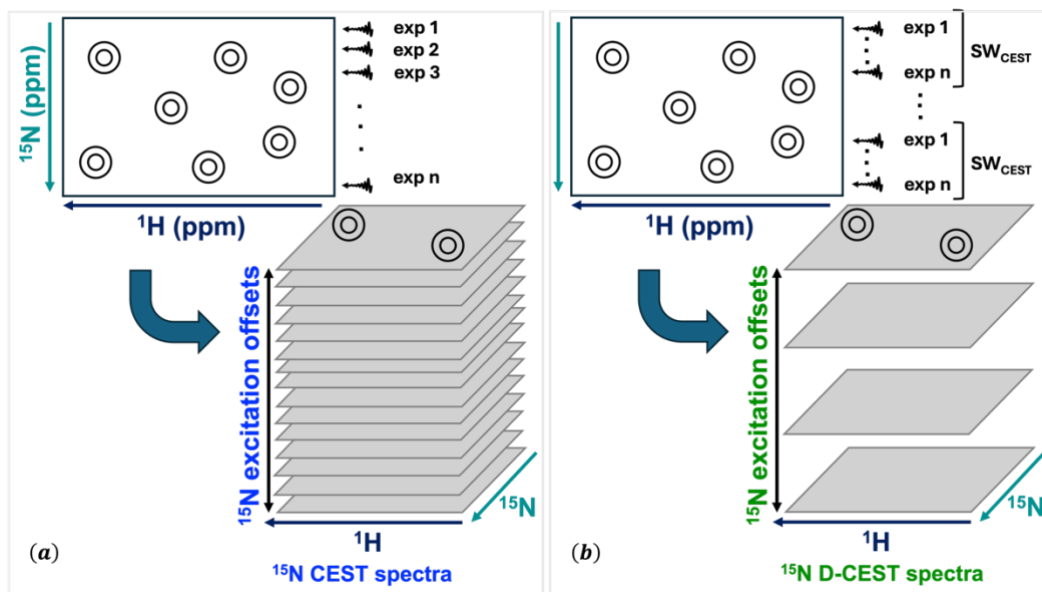


Figure 8.11. Comparison of standard ^{15}N CEST and ^{15}N D-CEST acquisition strategies. (a) Conventional CEST samples offsets sequentially. (b) D-CEST enables simultaneous excitation of multiple offsets, reducing experimental time.

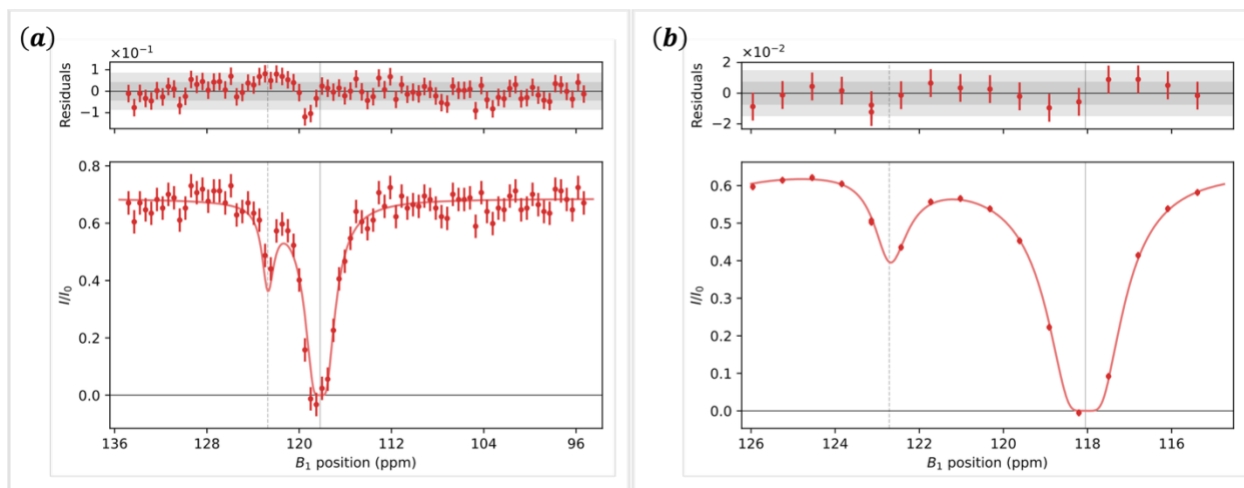


Figure 8.12. Comparison of signal-to-noise ratios obtained from standard ^{15}N CEST and ^{15}N D-CEST experiments for TEM-1 residue A172. The CEST profile was acquired over a total experimental time of 5 days and 5 hours using $B_1 = 25$ Hz. The D-CEST profile was acquired over a total experimental time of 2 days and 5 hours using $B_1 = 20$ Hz and $SW_{\text{CEST}} = 800$ Hz

B. COS-CEST

The COS-CEST experiment employs cosine-modulated shaped pulses to achieve multi-site excitation, in contrast to the rectangular pulses used in D-CEST. As a result, COS-CEST deposits less radiofrequency power than D-CEST and is therefore better suited for studies of fully protonated proteins using ^1H CEST. In such applications, higher sensitivity can be achieved by selectively avoiding excitation of the water resonance and aliphatic proton regions (Yuwen, Bouvignies, et al. 2018). Because COS-CEST was not employed in the present research, this approach will not be discussed further.

VIII.4. CPMG and CEST Data Analysis

The CEST and CPMG intensity profiles can be analyzed using *ChemEx* (<https://github.com/gbouvignies/chemex>) as illustrated in **Figure 8.13**.

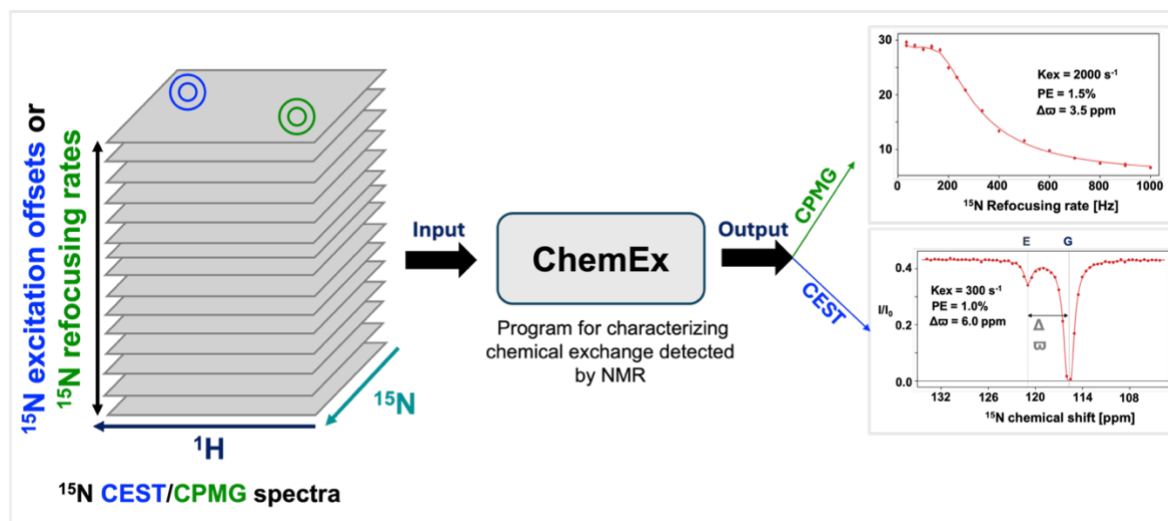


Figure 8.13. Schematic illustration of the analysis of ^{15}N CEST and ^{15}N CPMG profiles using *ChemEx*.

Best-fit exchange parameters were determined by minimizing the following target function through non-linear least-squares optimization:

$$\chi^2(\xi) = \sum_i^n \frac{(I_i^{\text{Exp}} - I_i^{\text{Calc}}(\xi))^2}{\sigma_i^2} \quad \text{Equation 8.6}$$

where I_i^{Exp} is the experimental intensity, I_i^{Calc} is the corresponding calculated intensity, $\xi = \{x_1, x_2, \dots, x_n\}$ refers to the different fitting parameters, σ is the uncertainty in the experimental measurement, and the summation extends over all experimental data points (i) included in the fitting process.

For the ^{15}N CEST experiments, signal intensities were calculated by numerically integrates (Korzhnev et al. 2004) the Bloch–McConnell equations (McConnell 1958) over the T_{EX} period, describing the evolution of an isolated scalar coupled ^1H - ^{15}N spin system exchanging between two states A and B with rate constant k_{AB} and k_{BA} :

$$\frac{d}{dt} \begin{pmatrix} E/2 \\ I_{x,A} \\ I_{y,A} \\ I_{z,A} \\ I_{x,B} \\ I_{y,B} \\ I_{z,B} \end{pmatrix} = \begin{pmatrix} 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & -R_{2,A} - k_{AB} & -\omega_A & \omega_1 & k_{BA} & 0 & 0 \\ 0 & \omega_A & -R_{2,A} - k_{AB} & 0 & 0 & k_{BA} & 0 \\ 2R_{1,A}I_{A,eq} & -\omega_1 & 0 & -R_{2,A} - k_{AB} & 0 & 0 & k_{BA} \\ 0 & k_{AB} & 0 & 0 & -R_{2,B} - k_{BA} & -\omega_B & 0 \\ 0 & 0 & k_{AB} & 0 & \omega_B & -R_{2,B} - k_{BA} & 0 \\ 2R_{1,B}I_{B,eq} & 0 & 0 & k_{AB} & -\omega_1 & 0 & -R_{2,B} - k_{BA} \end{pmatrix} \begin{pmatrix} E/2 \\ I_{x,A} \\ I_{y,A} \\ p_A \\ I_{x,B} \\ I_{y,B} \\ p_B \end{pmatrix}$$

Equation 8.7 adapted from (Vallurupalli et al., 2012)

where E is the identity operator, $I_{j,k}$ is component of the angular momentum for state A or B ; $j \in \{x, y, z\}$ and $k \in \{A, B\}$, $R_{1,k}$ is the longitudinal rates, $R_{2,k}$ is the transverse relaxation rates, ω_A and ω_B are the ground- and minor-state frequencies respectively, $I_{A,eq}$ and $I_{B,eq}$ are the equilibrium populations of states A and B respectively.

The global fitting parameters, common to all residues, are $k_{ex} = k_{AB} + k_{BA}$ and p_B ; with $k_{AB} = p_B k_{ex}$ and $k_{BA} = p_A k_{ex}$. Residue-specific fitting parameters included ϖ_A and ϖ_B , as well as $R_{1,A}$, $R_{1,B}$, $R_{2,A}$ and $R_{2,B}$ of ^{15}N spins. Usually, relaxation rates for the A and B states are constrained to be equal ($R_{2,A} = R_{2,B}$ and $R_{1,A} = R_{1,B}$).

The data were fit using two sets of initial conditions:

- $I_{x,A/B} = I_{y,A/B} = 0$; $I_{z,A} = p_A$ and $I_{z,B} = p_B$.
- $I_{x,A/B} = I_{y,A/B} = 0$; $I_{z,A} = -p_A$ and $I_{z,B} = -p_B$.

These two conditions are used in all of the calculations, and the difference in $I_{z,A}$ is retained.

Chapter IX.

Research Contributions

IX.1. Structural and Functional Insights into CspA (Under Review at *Journal of Magnetic Resonance*)

Structural and Functional Insights into a Mesophilic Cold Shock Protein CspA with Enhanced Precision

Marius Wanko Nembot¹, Georges Feller², Alexander N. Volkov^{3,4}, Ulric B. le Paige⁵, Guillaume Bouvignies⁵ and Christian Damblon¹.

¹Structural Biological Chemistry Laboratory, MolSys Research Unity, University of Liege, Liège, Belgium.

²Department of Life Sciences, Laboratory of Biochemistry, Center for Protein Engineering-InBioS, University of Liège, Liège, Belgium

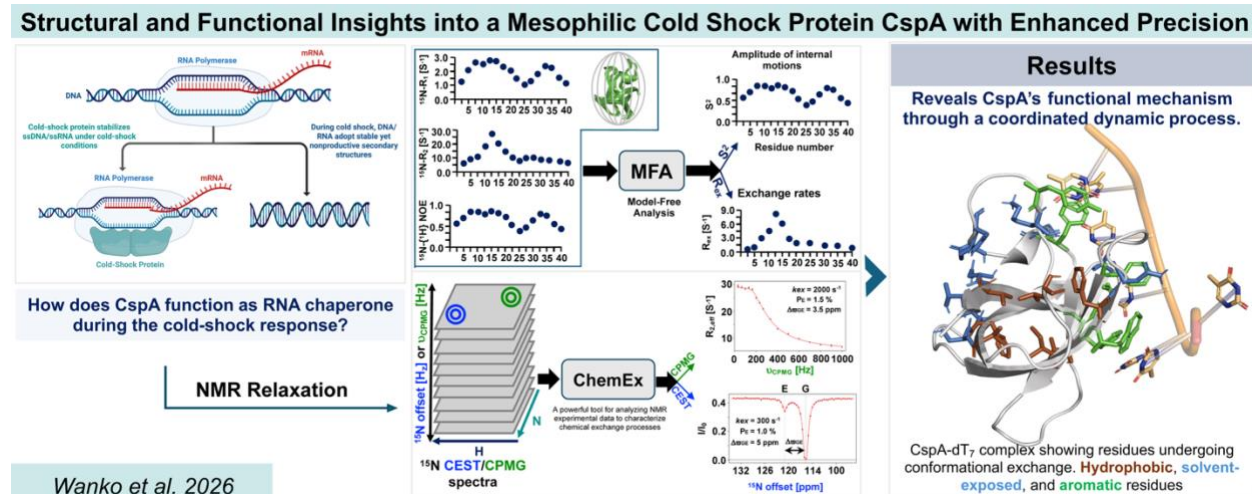
³VIB-VUB Center for Structural Biology, Vlaams Instituut voor Biotechnologie (VIB), Brussels, Belgium.

⁴Jean Jeener NMR Center, Vrije Universiteit Brussel (VIB), Pleinlaan 2, Brussels, Belgium.

⁵Chimie Physique et Chimie du Vivant (CPCV), Département de Chimie, École normale supérieure, PSL University, Sorbonne Université, CNRS, Paris, France

To whom correspondence should be addressed: Prof. Christian Damblon, Laboratory of Structural Biological Chemistry Laboratory, MolSys Research Unity, University of Liege, Belgium, Building B6c, Quartier Agora, Allée du 6 Août, 13, 4000 Liège (Sart-Tilman), Belgium, Telephone : +32 (0) 43663788 ; Email : c.damblon@uliege.be ; amwanko@uliege.be

Graphical Abstract



ABSTRACT

Cold-shock proteins (CSPs) are highly conserved nucleic acid-binding proteins that act as chaperones during cellular adaptation to low temperatures. Here, we present a comprehensive structural and dynamic characterization of *Escherichia coli* CspA using high-resolution NMR spectroscopy. The solution structure of CspA (PDB ID: 30IB) is supported by extensive NMR experimental restraints, minimal violations, and favorable stereochemistry, establishing it as a well-converged NMR structure. Crucially, we investigated backbone dynamics across multiple timescales, with a particular focus on the microsecond–millisecond regime using a combination of ^{15}N Carr–Purcell–Meiboom–Gill (CPMG) relaxation dispersion and ^{15}N chemical exchange saturation transfer (CEST) experiments, together with visible peak-position constraints. To our knowledge, this represents the first application of such a combined ^{15}N CEST, ^{15}N CPMG and visible peak-position constraints to probe conformational exchange in CSPs. Our results show that, in addition to the conserved aromatic residues of RNP1 and RNP2 motifs that mediate π -stacking interactions with nucleic acids, an unexpectedly broad network of hydrophobic core and solvent-exposed polar residues undergoes conformational exchange. Notably, residues in the $\beta 3$ – $\beta 4$ and $\beta 4$ – $\beta 5$ loops display complex dynamics not fully captured by model-free analysis formalism, highlighting their role in binding site flexibility. Complementary AF3/YASARA modelling of the CspA bound to heptathymidine (dT7) further supported that aromatic and polar residues form π -stacking and ionic interactions with ssDNA bases, corroborating the functional relevance of these dynamic regions. Therefore, our findings demonstrate that CspA relies on a dynamic network extending from conserved motifs through the hydrophobic core and flexible loops, conferring the structural adaptability required for efficient nucleic acid recognition and chaperone activity.

INTRODUCTION

Extremophilic microorganisms can thrive across a remarkable temperature range from approximately -20°C , as in psychrophiles inhabiting permafrost soil and sea ice (Collins and Feller 2023), to temperatures near or above the boiling point of water, characteristic of hyperthermophiles found in deep-sea hydrothermal vents (Prieur 1997). These organisms have evolved exquisite cellular strategies to cope with extreme thermal stress. Psychrophiles generally grow within a narrow range of low-temperatures ranging from -1 to 10°C (Y. Lee et al. 2018), whereas thermophiles and hyperthermophiles typically require temperatures above 60°C (Prieur 1997) for proliferation. Proteins play a central role in thermal adaptation, as their structural stability and catalytic function are directly affected by environmental temperature. The exceptional thermal stability of extremophilic proteins has been well documented (Champdoré et al. 2007) and is widely attributed to an adaptive increase in resistance to thermal denaturation.

Among the molecular strategies employed by microorganisms to withstand sudden changes in temperature, the synthesis of CSPs is one of the most rapid and effective responses. They are found in a wide variety of Gram-positive and Gram-negative bacteria and are among the most evolutionarily conserved protein families. Upon abrupt temperature downshifts, CSPs are rapidly upregulated to preserve cellular homeostasis, stabilize nucleic acids, and facilitate the continued translation of essential proteins (Y. Lee et al. 2018; J. Lee et al. 2013; B. Jin et al. 2014; G. Horn et al. 2007). CSPs constitute a highly conserved protein family (Sinhaikul et al. 2002; Zeeb et al. 2006), ubiquitously distributed across all Archaea and Eubacteria, with the number of paralogous CSP genes per genome ranging from two in *Geacillus stearothermophilus* (CspB and CspD) to nine in the mesophilic bacterium *Escherichia coli* (Roy and Ray 2024) (CspA, CspB, CspC, CspD, CspE, CspF, CspG, CspH, and CspI).

CSPs contain one or more cold-shock domains (CSDs), which possess nucleic acid-binding activity. CSDs preferentially associate with single-stranded RNA and DNA (ssRNA/ssDNA) in a cooperative manner and with low sequence specificity (Schindler et al. 1999; Behl et al. 2020), but they have little to no affinity for double-stranded DNA (Behl et al. 2020; G. Horn et al. 2007). The protein-nucleic acid interaction is mediated by well-conserved cold-shock domain motifs, RNP1 (K/R-G-F/Y-G/A-F-V/I-X-F/Y) and RNP2 (L/I-F/Y-V/I-G/K-N/G-L), which occur with minor variations in all CSPs (G. Horn et al. 2007; Ermolenko and Makhatadze 2002; Schröder et al. 1995; Burd and Dreyfuss 1994; Landsman 1992). These motifs enable CSPs to recognize and bind nucleic acids, a property central to their role as RNA chaperones. By binding nucleic acids, CSPs modulate translation. Under low-temperature stress, RNA molecules often adopt stable yet nonproductive secondary structures that impede transcription and translation. CSPs counteract this effect by binding to these structured RNAs and destabilizing them, thereby promoting single-stranded conformations conducive to efficient gene expression (Yan Zhang and Gross 2021; Yonghong Zhang et al. 2022).

Previous studies have investigated the structure of the mesophilic CspA using both conventional and automated nuclear magnetic resonance (NMR) spectroscopy (Newkirk et al. 1994; Tang et al. 2010), as well as X-ray crystallography (Heinemann 1994). Conventional NMR refers to manual spectral assignment and structure determination based on experimentally derived restraints, whereas the automated NMR structure determination workflow (Shen et al. 2008), which incorporates CS-Rosetta (Shen et al.

2008; Tang et al. 2010), combines sparse NMR chemical shifts ($^1\text{H}^{\text{N}}$, $^1\text{H}^{\alpha}$, ^{15}N , $^{13}\text{C}_{\alpha}$, $^{13}\text{C}_{\beta}$ and $^{13}\text{C}'$) with *de novo* protein structure prediction (Bonneau et al. 2001; Bradley et al. 2003; Rohl et al. 2004).

While automated NMR structure determination can rival the conventional X-ray crystallography approach (Tang et al. 2010), the overall quality of both NMR-derived and crystallographic structures could be further improved, although the types of improvements required differ between the two techniques. In contrast, the molecular dynamics of CspA and CspB have been examined by NMR (Zeeb et al. 2006; Zeeb and Balbach 2005; Feng et al. 1998). However, these studies relied primarily on nuclear spin relaxation (NSR) experiments combined with model-free analysis (MFA), which characterizes fast internal motions on the picosecond-to-nanosecond (ps–ns) timescale. Because the MFA assumes that slower motions (microsecond-to-millisecond timescale), such as conformational exchange, contribute only through an additional relaxation term R_{ex} , it may not provide the most comprehensive insights into protein function. To date, neither a well-defined NMR structure in agreement with experimental restraints nor detailed molecular dynamics on the microsecond–millisecond timescale of CspA have been reported. This study therefore aims to determine a well-defined NMR structure of CspA and to characterize its dynamics using advanced NMR experiments, with the goal of providing deeper insight into its functional mechanisms.

MATERIALS AND METHODS

Protein Expression, Isotopic Enrichment, and Purification. The native gene sequence for CspA was synthesized and inserted into the expression vector pET22b at the NdeI site. The protocol for isotope labelling was adapted as described previously (Marley et al. 2001). Colonies of the *E. coli* BL21 (DE3) cells transformed with the vector were used to inoculate a 50 ml preculture of Terrific broth TB (12 g/l Bactotryptone (Difco), 24 g/l yeast extract (Difco), 4 ml/l glycerol, 12.54 g/l K_2HPO_4 , 2.31 g/l KH_2PO_4 , pH 7.2) containing 100 mg/l ampicillin, which was run overnight at 37°C. Five-milliliter aliquots were used to inoculate 250 ml of TB in 2-liter Erlenmeyer flasks and the cultures were incubated at 37°C and 250 rpm. Upon reaching an OD_{600} of ~ 2.0 , the cells were harvested by a 5 min centrifugation at $3000 \times g$. The cell pellet was resuspended in the isotopically labelled M9 medium (14.6 g/l Na_2HPO_4 , 5.5 g/l KH_2PO_4 , 1 g/l NaCl, 0.24 g/l $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.02 g/l $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$) with 100 mg/l ampicillin, 1g/l $^{15}\text{NH}_4\text{Cl}$ and 5 g/l D-glucose- $^{13}\text{C}_6$ (Cambridge Isotope Laboratories) or only 1 g/l $^{15}\text{NH}_4\text{Cl}$ for relaxation studies, and then incubated at 37 °C and 250 rpm to allow the recovery of growth and the clearance of unlabeled metabolites. After 1h30, the Erlenmeyer flasks were transferred at 18°C for 30 min, after which protein expression was induced by 1 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG), followed by overnight culture at 18°C and 250 rpm. The cells were harvested by centrifugation at $4000 \times g$ for 10 min at 4°C. The cell pellets were resuspended in 10 mM Tris-HCl, pH 7.2 containing Benzonase, RNase and a protease inhibitor cocktail (Complete, Roche), then disrupted in a prechilled high-pressure cell disrupter (Constant Systems) at 28 kpsi, and centrifuged at $20000 \times g$ for 30 min at 4 °C. The lysis supernatant was then brought to 90% saturation in $(\text{NH}_4)_2\text{SO}_4$ and the precipitated proteins collected by centrifugation were suspended in 10 mM Tris-HCl, pH

7.2. This fraction was diluted to 30% saturation $(\text{NH}_4)_2\text{SO}_4$ and the soluble fraction was recovered after centrifugation at $45000 \times g$.

The protein mixture was loaded on a Phenyl-Sepharose CL-4B column (2.5×40 cm) equilibrated in 10 mM HEPES and saturated with 30% $(\text{NH}_4)_2\text{SO}_4$, pH 7.2. Proteins were eluted with a decreasing gradient from 30% to 0% saturation in $(\text{NH}_4)_2\text{SO}_4$. Fractions containing CspA were then loaded on a Q-Sepharose HP column (2.5×40 cm) equilibrated in 10 mM HEPES, pH 7.2 and eluted with a linear gradient from 0 to 0.4 M NaCl. Fractions containing CspA were concentrated by ultrafiltration on membrane with a 3 kDa cutoff and loaded on a Superdex 75 column (2.5×100 cm) and eluted with 50 mM KH_2PO_4 , 100 mM KCl and 0.1 mM EDTA, pH 6.5. The purified protein samples (~ 5 mg/ml) were stored at -70°C . Before the NMR experiments, the defrosted sample was filtered through a $0.22 \mu\text{m}$ membrane. The purification process was followed by electrophoresis on Tricine-SDS-PAGE (Schägger 2006). The protein concentration was determined by the BCA Protein Assay (Pierce).

NMR Experiments and Assignments. NMR experiments were performed on Bruker Avance III HD 500 MHz and 700 MHz spectrometers equipped with helium-cooled cryogenic probes (CryoProbe™). 2,2-Dimethyl-2-silapentane-5-sulfonate (DSS) was used as the internal chemical shift reference. CspA samples were prepared at concentrations of 0.5–1.0 mM in 50 mM potassium phosphate buffer (pH 6.5) containing 100 mM KCl and 0.1 mM EDTA. All NMR experiments for structure determination were conducted at 25°C .

Backbone resonance assignments were obtained from HNCACB and HN(CO)CACB triple-resonance experiments. Aliphatic side-chain assignments were derived from HBHA(CO)NH, H(C)CH-TOCSY, and (H)CCH-TOCSY experiments. Aromatic side-chain resonances were assigned using 2D (HB)CB(CGCD)HD and 2D (HB)CB(CGCDCE)HE experiments. The intense water resonance was suppressed using the WATERGATE water suppression scheme.

Nuclear Overhauser effect (NOE) distance restraints were obtained from three-dimensional ^1H – ^{15}N , ^1H – ^{13}C aliphatic, and ^1H – ^{13}C aromatic NOESY–HSQC spectra recorded at 700 MHz with a triple-resonance ^1H –(^{13}C , ^{15}N) cryogenic probe, using a mixing time of 120 ms (Sattler 1999; Yamazaki et al. 1994; Zhu et al. 1994).

All NMR spectra were processed with Bruker TopSpin version 3.6 (Bruker BioSpin) and NMRPipe (Delaglio et al. 1995) and analyzed using CcpNmr Analysis v2 (Vranken et al. 2005). Chemical shifts and NMR-derived restraints have been deposited in the Biological Magnetic Resonance Data Bank (Hoch et al. 2023) (BMRB ID: 34897).

NMR Structure Determination. NOE assignments and structure calculations were performed using a combination of automated and manual methods in CYANA version 3.98.15 (Peter Güntert 2004; Koradi et al. 1998; Peter Güntert et al. 1991), incorporating dihedral angle (φ and ψ) constraints derived from chemical shifts using TALOS+ (Shen et al. 2009; Cornilescu et al. 1999). After seven iterative cycles of structure calculation, a total of 100 conformers were calculated using a torsion-angle dynamics protocol, and the 20 conformers with the lowest final target function values were selected for further analysis. The selected CYANA conformers were subsequently refined using an explicit water refinement protocol in the Crystallography and NMR System (CNS) (Brünger et al. 1998) with the toppallhdg5.3.pro/parallhdg5.3.pro force field (Linge, Williams, et al. 2003).

The 10 lowest-energy water-refined structures were validated using the Protein Structure Validation Software suite (PSVS) (Bhattacharya et al. 2007). Structural figures were generated using PyMOL (<http://www.pymol.org>).

In Silico Structure Prediction and Refinement. The three-dimensional structure of the heptathymidine-binding protein CspA, referred to as CspA-dT7 was predicted using AlphaFold 3 (Abramson et al. 2024) following the protocol. Predictions were generated via the official AlphaFold Server (<https://alphafoldserver.com>) using the default multimer modeling pipeline to account for potential nucleic acids. Multiple sequence alignments (MSAs) were automatically constructed using UniRef90 and MGnify, and structural templates were identified from PDB70. Five independent models were generated, each accompanied by the predicted template modelling (pTM) score and the interface predicted template modeling (ipTM) score. The five models were selected for refinement. These models were subjected to energy minimization and refinement using YASARA (Land and Humble 2018). The refinement protocol employed the AMBER14 force field with explicit solvent (TIP3P water model), followed by a 500 ps molecular dynamics simulation under constant pressure (1 bar) and temperature (25° C) to relieve steric clashes and optimize hydrogen-bonding networks.

NMR Investigation of Backbone Dynamics. ^{15}N spin relaxation experiments (^{15}N - R_1 , ^{15}N - R_2 , and ^{15}N - $\{^1\text{H}\}$ heteronuclear NOE) were recorded on Bruker Avance III 500 MHz (11.7 T) and 700 MHz (16.4 T) spectrometers equipped with cryogenically cooled triple-resonance probes at 25 °C. Longitudinal relaxation rates (R_1) were measured using relaxation delays of 10.0, 50.0, 100.0 ($\times 2$), 200.0, 300.0, 400.0, 500.0 ($\times 2$), 700.0, 1000.0, and 1500.0 ms, with a recycle delay of 2.0 s. Transverse relaxation rates (R_2) were obtained using relaxation delays of 8.48, 16.96, 33.92 ($\times 2$), 50.88, 67.84, 84.80 ($\times 2$), 101.76, 118.72, 152.64, and 203.52 ms, with the same recycle delay.

^{15}N - $\{^1\text{H}\}$ NOE values were determined from experiments with interleaved pulse sequences recorded with and without proton saturation. The recycle delay and proton saturation time for the ^{15}N - $\{^1\text{H}\}$ NOE measurements were both set to 2.5 s.

For all ^{15}N spin relaxation experiments, the ^{15}N carrier frequency was set to 117.037 ppm, and the ^1H carrier frequency was positioned at the water resonance. At 700 MHz, the spectral widths were 11,160.714 Hz (F_2 , ^1H) and 3,263.708 Hz (F_1 , ^{15}N); at 500 MHz, they were 7,812.500 Hz (F_2 , ^1H) and 2,280.772 Hz (F_1 , ^{15}N). Data were acquired with 1024 (F_2) and 64 (F_1) complex points.

^{15}N CPMG relaxation dispersion experiments were performed as previously described (Hansen et al. 2007). The spacing between successive ^{15}N 180° refocusing pulses was adjusted to generate effective CPMG field frequencies (ν_{CPMG}) of 0 ($\times 2$), 33, 67, 100, 133, 167 ($\times 2$), 200, 267, 333, 400, 467, 533 ($\times 2$), 667, 833, and 1000 Hz, during a constant-time relaxation period of 30 ms. The ^{15}N carrier frequency was set to 120 ppm, and spectra were recorded in an interleaved manner with a recycle delay of 3.0 s.

As with the ^{15}N CPMG, ^{15}N CEST profiles were recorded on the 700 MHz (16.4 T) Bruker Avance III HD spectrometer equipped with a cryogenically cooled triple resonance probe. To accelerate data acquisition, ^{15}N CEST experiments employed the DANTE-CEST (D-CEST) approach (Yuwen, Kay, et al. 2018), which uses a train of short, high-power radiofrequency pulses [DANTE sequence (Gareth A. Morris and Freeman 2011;

Bodenhausen, Freeman, et al. 1976)] to achieve multi-site excitation during the saturation period ($T_{EX} = 455$ ms), with a recycle delay of 2.0 s. During the T_{EX} period, ^1H decoupling was achieved using 90x240y90x composite pulses (Levitt 1982). ^{15}N D-CEST profiles were sampled every 25 Hz. To remove degeneracies in the extracted chemical shifts of excited-state nuclei, two different D-CEST spectral windows were used ($SW_{CEST} = 700$ and 800 Hz). Two B_1^{D-CEST} field strengths were employed: $B_1^{D-CEST} = 20$ Hz and $B_1^{D-CEST} = 60$ Hz (Vallurupalli et al. 2017; Khandave et al. 2024, 2023). B_1^{D-CEST} fields were calibrated using the nutation method (Guenneugues et al. 1999). The ^1H – ^{15}N correlation spectra were recorded with 64 complex points in the indirect (^{15}N) dimension (45 ppm sweep width) using 16 scans per increment.

Visible peak positions (ground-state ^{15}N chemical shifts) were determined from the D-CEST spectra ($SW_{CEST} = 700$ and 800 Hz) using the ground-state peak positions obtained in the absence of the two B_1^{D-CEST} (20 Hz and 60 Hz) irradiation period. Uncertainties in the ground-state ^{15}N chemical shifts were estimated from these four spectra.

Data Processing and Analysis. ^{15}N relaxation spectra were processed and visualized using Bruker TopSpin version 3.6. ^{15}N – $\{^1H\}$ NOE values were calculated as the ratio of peak heights in spectra recorded with and without proton saturation. ^{15}N – R_1 and ^{15}N – R_2 relaxation rates values were obtained by fitting peak intensities using Dynamics Center (Bruker BioSpin).

^{15}N CPMG relaxation dispersion and ^{15}N D-CEST data sets were processed using the NMRPipe software package (Delaglio et al. 1995). Visualization and peak assignment in the ^1H – ^{15}N correlation spectra were performed with NMRFAM-Sparky (W. Lee et al. 2015). Peak intensities were extracted using *PeakFit* (<https://github.com/gbouvignies/PeakFit>). Effective transverse relaxation rates, $R_{2,eff}$ were then calculated from the ratio of peak intensities according to:

$$R_{2,eff}(\nu_{CPMG}) = -\frac{1}{T_{relax}} \ln \left(\frac{I(\nu_{CPMG})}{I_0} \right)$$

where I_0 is the intensity of a peak in the reference spectrum recorded without the CPMG relaxation period, $I(\nu_{CPMG})$ is the corresponding peak intensity in the spectrum measured at a CPMG field strength of ν_{CPMG} , and T_{relax} is the constant-time CPMG relaxation delay. Exchange parameters were obtained by simultaneously fitting the ^{15}N CPMG relaxation dispersion data and the ^{15}N D-CEST profiles, including the visible peak positions, as previously described (Khandave et al. 2023; Vallurupalli et al. 2011), using *ChemEx* (<https://github.com/gbouvignies/chemex>), which numerically integrates (Korzhnev et al. 2004) the Bloch–McConnell equations (McConnell 1958) for various exchange models. Best-fit exchange parameters were determined by minimizing the following target function through non-linear least-squares optimization:

$$\chi^2 = \sum_i^n \frac{(m_i^{Exp} - m_i^{Calc})^2}{\sigma_i^2}$$

where m_i^{Exp} is the experimental measure (i.e. peaks intensities in the case of CPMG/D-CEST datasets or visible peak positions), m_i^{Calc} is the corresponding calculated peak intensity or visible peak position using the Bloch–McConnell equations over the duration of the T_{EX} period – both calculated values were determined from the Bloch–McConnell equations as described previously (Khandave et al. 2023), σ is the uncertainty in the experimental measurement, and the summation extends over all experimental data points (i) included in the fitting process.

For each residue, the experimental uncertainty (σ) was estimated from the signal-to-noise ratio of the recorded ^{15}N CPMG relaxation dispersion and ^{15}N D-CEST profiles. Uncertainties in the best-fit exchange parameters were derived from the covariance matrix obtained during the Levenberg–Marquardt optimization.

A two-state exchange model, $A \rightleftharpoons B$, was used to analyze the ^{15}N CPMG relaxation dispersion, ^{15}N D-CEST profiles and visible peak-position constraints simultaneously. The global fitting parameters, common to all residues, were the exchange rate ($k_{ex} = k_{AB} + k_{BA}$) and the minor B-state population (p_B). Residue-specific fitting parameters included the ^{15}N chemical shifts of the major A state and minor B state, as well as the longitudinal (R_1) and transverse (R_2) relaxation rates of ^{15}N spins. Relaxation rates for the A and B states were constrained to be equal ($R_{2,A} = R_{2,B}$ and $R_{1,A} = R_{1,B}$).

Model-free analysis and model selection. ^{15}N relaxation data collected at 500 and 700 MHz were analyzed using model-free analysis (Hall and Fushman 2003; Tjandra, Feller, et al. 1995; Lipari and Szabo 1982b, 1982a), following recommended best practices (Morin 2011). The workflow comprised: (i) calculation of the molecular moment of inertia tensor from structural coordinates (PDB 301B), (ii) alignment and fitting of an effective, axially symmetric rotational diffusion tensor, and (iii) model-free analysis of ^{15}N – R_1 , ^{15}N – R_2 , and ^{15}N – $\{^1\text{H}\}$ NOE data with statistical model selection.

For low-amplitude, fast motions, the ratio R_2'/R_1' is essentially independent of internal dynamics and primarily reflects overall tumbling through the spectral density function. Here, primes denote relaxation rates corrected to remove high-frequency contributions to the spectral density (Walker et al. 2004).

The anisotropic rotational diffusion tensor was modelled as axially symmetric with four parameters ($D_{\parallel}, D_{\perp}, \theta, \varphi$), where the Euler angles (θ, φ) define the orientation of the diffusion frame relative to the molecular frame. Parameter estimation used per-residue ^{15}N R_2'/R_1' values together with atomic coordinates (Tjandra, Kuboniwa, et al. 1995). Residues exhibiting pronounced internal dynamics were excluded from the fit (i.e., ^{15}N – $\{^1\text{H}\}$ NOE < 0.61 and markedly increased ^{15}N R_2 values).

The four diffusion parameters were optimized by nonlinear least squares using the Levenberg–Marquardt algorithm to minimize the objective function E (Dosset et al. 2000; Fushman et al. 1999; Tjandra, Feller, et al. 1995; Walker et al. 2004), which is defined as the squared deviation between experimental and predicted R_2/R_1 ratios for the selected rigid-limit N–H bond vectors:

$$E = \sum_{i=1}^{N_{res}} \frac{(\rho_i^{exp} - \rho_i^{cal})^2}{\sigma_i^2}$$

where N_{res} is the total number of N–H bond vectors included in the analysis, the parameter ρ is defined as a ratio of the transverse and longitudinal relaxation rates,

$$\rho = \left(\frac{2 R'_2}{R'_1} - 1 \right)^{-1}$$

and σ_i denotes the experimental error in ρ_i for bond vector number i . The value of ρ^{exp} is directly derived from the measured relaxation parameters, whereas ρ^{cal} is calculated using expressions for the spectral densities $J(\omega)$ that depend on the rotational diffusion model (Fushman and Cowburn 2001; Ghose et al. 2001).

For CspA protein, the optimized axial tensor shows an anisotropy of $D_{\parallel}/D_{\perp} = 1.2$, indicating nearly isotropic overall rotation. The corresponding rotational correlation time was $\tau_c = 3.9$ ns, computed from the isotropic average $D_{iso} = (D_{xx} + D_{yy} + D_{zz})/3$ via $\tau_c = 1/(6D_{iso})$.

Since small anisotropy, characterized by a ratio of the principal components of the diffusion tensor $D_{\parallel}/D_{\perp} \approx 1.2$ (Hall and Fushman 2003; Tjandra, Feller, et al. 1995), results in model-free parameters that are not significantly different when assuming anisotropic versus isotropic overall tumbling, the model-free analysis (Lipari and Szabo 1982b, 1982a) of ^{15}N – R_1 , ^{15}N – R_2 , and ^{15}N – $\{^1\text{H}\}$ NOE data (Kay et al. 1989; Morin 2011) was performed using five isotropic models derived from the original formulation of Lipari and Szabo as extended by Clore et al. (1990) (Clore et al. 1990) to include two separated timescales of internal motion, fast (τ_f) and slow (τ_s). In this approach, the spectral density is modeled as:

$$J(\omega) = \frac{2}{5} \left[\frac{S^2 \tau_c}{1 + (\omega \tau_c)^2} + \frac{(1 - S_f^2) \tau'_f}{1 + (\omega \tau'_f)^2} + \frac{(S_f^2 - S^2) \tau'_s}{1 + (\omega \tau'_s)^2} \right]$$

where the square of the overall generalized order parameter is the product of the order parameters for the first and second internal motions, $S^2 = S_f^2 S_s^2$ and τ_c is defined as the global correlation time and closely related to the isotropic component of diffusion D_{iso} by (Bloembergen et al. 1948): $(\tau_c)^{-1} = 6D_{iso}$. $(\tau'_i)^{-1} = \tau_c^{-1} + \tau_i^{-1}$ with $i = s$ or $i = f$, is an internal correlation time that describes the internal motions.

All five models of the spectral density employ an isotropic rotational diffusion tensor using the following external correlation function:

$$C_0^{axial}(t) = S^2 + (1 - S_f^2) e^{-t/\tau_f} + (S_f^2 - S^2) e^{-t/\tau_s}$$

The five model-free formulations, their parameters, and corresponding spectral density functions are summarized in **Table 1**. Models 1–4 assume a single effective timescale for internal motion ($S_s^2 = 1$), where S^2 reflects motions occurring on the pico- to nanosecond timescale. Models 1 and 3 assume the internal correlation time approaches zero ($\tau_f \rightarrow 0$), describing very fast motions ($\tau_f < 20$ ps). Models 2 and 4 incorporate internal motions on a finite pico- to nanosecond timescale ($\tau_f = \tau_e$) (Mandel et al. 1995). Models 3 and 4 are

extensions of Models 1 and 2, respectively, that include an additional exchange term R_{ex} to account for conformational exchange and pseudo-first-order processes occurring on the micro- to millisecond timescale, as captured here by using CPMG (Bloom et al. 1965; Allerhand and Gutowsky 1965) and D-CEST (Yuwen, Kay, et al. 2018) at 700 MHz. Model 5 incorporates two distinct internal timescales: (i) ultrafast motions ($\tau_f \rightarrow 0$) with order parameter S_f^2 and (ii) slower nanosecond motions ($\tau_s = \tau_e$) with order parameter S_s^2 (Clore et al. 1990).

Model selection was performed using Akaike's Information Criterion (AIC), defined as:

$$AIC = \chi^2 + 2k$$

where

$$\chi^2 = \sum_{i=1}^n \frac{(R_i - R'_i)^2}{\sigma_i^2}$$

is the chi-square statistic measuring the goodness of fit between the experimental relaxation data (R) and the back-calculated values (R') (Abragam 1983). Here, σ denotes the experimental error, i is the residue index, k is the number of adjustable parameters in a given model, and n is the size of the data set for each residue. The AIC provides an optimal balance between underfitting (selection of overly simple models) and overfitting (selection of unnecessarily complex models) and has been shown to outperform traditional chi-square or F-test criteria in model selection (d'Auvergne and Gooley 2003). With six experimentally derived relaxation parameters per residue at two fields ($n = 6$), models 4 and 5 each involve three adjustable parameters ($k = 3$), yielding three degrees of freedom. Therefore, to prevent overfitting, models 1–3 were first evaluated, and models 4 and 5 were only considered when the model with the lowest AIC did not reproduce the experimental R_1 and R_2 values within 99% agreement.

Table 1. Spectral density functions and corresponding fitted parameter subsets.

Models	Subset of fitted parameters	Spectral density function
1	S^2	$J(\omega) = \frac{2}{5} \cdot \tau_c \cdot \left[\frac{S^2}{1 + (\omega \cdot \tau_c)^2} \right]$
2	$S^2, \tau_e(ps - ns)$	$J(\omega) = \frac{2}{5} \cdot \tau_c \cdot \left[\frac{S^2}{1 + (\omega \cdot \tau_c)^2} + \frac{(1 - S^2)(\tau_c + \tau_e) \cdot \tau_e}{(\tau_c + \tau_e)^2 + (\omega \cdot \tau_c \cdot \tau_e)^2} \right]$
3	S^2, R_{ex}	$J(\omega) = \frac{2}{5} \cdot \tau_c \cdot \left[\frac{S^2}{1 + (\omega \cdot \tau_c)^2} \right]; R_{2eff} = R_2 + R_{ex}$
4	$S^2, \tau_e(ps - ns), R_{ex}$	$J(\omega) = \frac{2}{5} \cdot \tau_c \cdot \left[\frac{S^2}{1 + (\omega \cdot \tau_c)^2} + \frac{(1 - S^2)(\tau_c + \tau_e) \cdot \tau_e}{(\tau_c + \tau_e)^2 + (\omega \cdot \tau_c \cdot \tau_e)^2} \right]; R_{2eff} = R_2 + R_{ex}$
5	$S_f^2, S^2 = S_f^2 S_s^2, \tau_e(ns)$	$J(\omega) = \frac{2}{5} \cdot \tau_c \cdot \left[\frac{S^2}{1 + (\omega \cdot \tau_c)^2} + \frac{(1 - S_f^2)(\tau_c + \tau_f) \cdot \tau_f}{(\tau_c + \tau_f)^2 + (\omega \cdot \tau_c \cdot \tau_f)^2} \cdot \frac{(S_f^2 - S^2)(\tau_c + \tau_s) \cdot \tau_s}{(\tau_c + \tau_s)^2 + (\omega \cdot \tau_c \cdot \tau_s)^2} \right]$

RESULTS

Solution NMR structure of CspA. NMR chemical shifts and the resulting three-dimensional structure were deposited in the BMRB and PDB databases under accession codes 35048 and 30IB, respectively. Nearly complete assignments were obtained for the well-defined regions (K4–L70), with 94% backbone and 87% side-chain completeness, corresponding to an overall assignment level of 87% (733/844 atoms). Consistent with other cold-shock proteins, the ensemble of structures revealed that CspA adopts a conserved fold characterized by five antiparallel β -strands (β 1– β 5) forming a β -barrel with an oligonucleotide/oligosaccharide-binding (OB) fold topology (Y. Lee et al. 2018). The five antiparallel β -strands comprise: β 1 (M5–N13), β 2 (G17–T22), β 3 (V30–F33), β 4 (G48–I55) and β 5 (A63–T68). The side chains of V9, F12, I21, V30, V32, I37, L45, V51, F53, A64, and V67 are oriented toward the interior of the β -barrel, where they pack together to form a well-defined hydrophobic core. For clarity, we refer to the conventionally determined NMR structure reported here as 30IB (PDB ID; **Fig. 1**), the previously determined conventional NMR structure as 3MEF (Feng et al. 1998) (PDB ID), and the sparse-constraint CS-Rosetta structure as the CS-Rosetta model (PDB ID : 2L15). Comparative structural statistics, including heavy-atom RMSD and Ramachandran analysis, for 30IB, 3MEF, and the CS-Rosetta model are summarized in **Table 2**. Comparative analysis of the NMR restraint sets highlights the substantially higher experimental definition of the 30IB structure relative to the 3MEF and CS-Rosetta models. The 30IB ensemble is based on 867 distance restraints, including 347 long-range restraints (5.0 per residue), compared to 131 total restraints and 61 long-range restraints (1.2 per residue) for the sparse datasets (CS-Rosetta). In addition, comparative structural

statistics indicate that the 30IB ensemble is in good agreement with the experimental restraints. The precision of the ensemble is reflected in low RMSD values (0.53 ± 0.09 Å for backbone atoms and 0.92 ± 0.10 Å for heavy atoms), which are comparable to or lower than those of previously reported structures. In terms of stereochemical quality, 30IB exhibits a high proportion of residues in favored Ramachandran regions (94.3%) and no residues in disallowed regions, together with low percentages of Ramachandran (5.7%) in the additional allowed regions. By comparison, the other structures show differing levels of agreement with the experimental restraints. 3MEF structure displays elevated proportions of Ramachandran outliers (14.8% vs 1.6%) and side-chain outliers (43.4% vs 2.3%). 2L15 structure shows low levels of geometric outliers (0.2% Ramachandran and 0.4% side-chain) and no dihedral angle violations exceeding 10° but exhibits residues in favored Ramachandran regions (93.7%) compared to 30IB. PSVS (Bhattacharya et al. 2007) validation scores are consistent with these observations. The 30IB structure exhibits favorable ProsaII (Sippl 1993) and PROCHECK Z-scores (Laskowski et al. 1996), which are improved relative to 3MEF and comparable to those of the CS-Rosetta model. The MolProbity (Lovell et al. 2003) clash score is also substantially lower than that of 3MEF (18.11 vs 64.74), although higher than that of the CS-Rosetta model (5.58).

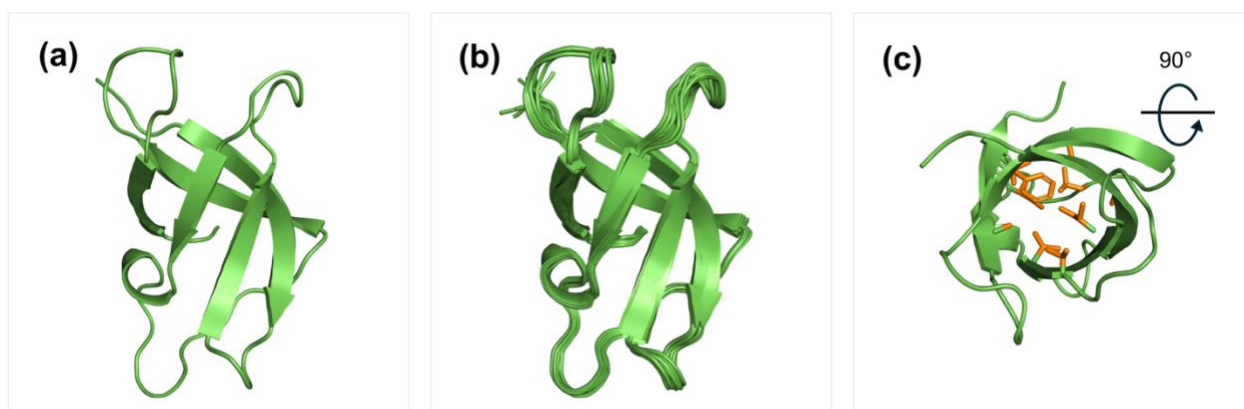


Fig. 1. 30IB NMR structure. (a) Cartoon representations of the lowest energy model of the 30IB ensemble. (b) Cartoon representations of the 10 conformers of the 30IB ensemble. (c) Packing of hydrophobic residues (V9, I21, V30, V32, I37, L45, V51, F53, A64, and V67) in the lowest energy 30IB conformer.

Table 2 Summary of structural statistics for *E. coli* CspA NMR structures. In this table, the results from 3MEF structure and CS-Rosetta structure are obtained from Y. Tang et al. (2010) (Tang et al. 2010)

	30IB structure ^a	3MEF structure ^b	CS-Rosetta structure ^c
Distance restraints			
Total	867		131
Intra-residue ($ i - j = 0$)	210		17
Sequential ($ i - j = 1$)	235		45
Medium range ($ i - j > 1$ and $ i - j < 5$)	75		8
Long range ($ i - j \geq 5$)	347		61
Total dihedral-angle restraints	118		68
Number of restraints per residue	14.3		3.3
Number of long-range restraints per residue	5.0		1.2
Average number of dihedral angle violations per residue ($^{\circ}$) ^{d,e}			
1.0 – 10.0 $^{\circ}$	7.1		3.0
> 10 $^{\circ}$	2.53		0.8
Maximum dihedral angle violation	5.96		16.70
RMSD from average coordinates ($\text{\AA}$) ^{e,f}			
Backbone atoms	0.53 $\pm$ 0.09	0.5 $\pm$ 0.1	0.8 $\pm$ 0.2
Heavy atoms	0.92 $\pm$ 0.10	1.1 $\pm$ 0.1	1.2 $\pm$ 0.2
Ramachandran statistics (%) ^{e,f}			
Most favored regions	94.3	78.3	93.7
Additional allowed regions	5.7	21.7	6.3
Generously allowed	0.0	0.0	0.0
Disallowed regions	0.0	0.0	0.0
Overall quality at a glance ^{e,g}			
	Value	Value	Value
Clash score	5	32	2
Ramachandran outliers (%)	1.6	14.8	0.2
Sidechain outliers (%)	2.3	43.4	0.4
PSVS Overall quality Score ^{e,f,h}			
	Raw/Z-score	Raw/Z-score	Raw/Z-score
Verify 3D	0.28/-2.89	0.43/-0.48	0.45/-0.16
ProsaII	0.61/-0.17	0.77/0.50	0.85/0.83
Procheck (phi-psi)	-0.24/-0.63	-1.37/-5.07	-0.28/-0.79
Procheck (all dihedrals)	-0.07/-0.41	-1.47/-8.69	0.00/0.00
Molprobtity clash score	18.11/-1.58	64.74/-9.58	5.58/0.57

Analysis statistics for residues 2–70

^a NMR structure determined in this work using conventional methods.

^b Previously reported NMR structure determined by conventional methods

^c Structure obtained from CS-Rosetta structure generation, compared with constraints; note that these distance constraints were not used in generating the CS-Rosetta structure

^d Statistics from the wwPDB validation report for 30IB.

^e Statistics from Y. Tang *et al.* (2010) (Tang *et al.* 2010) for 2L15.

^g Statistics from the wwPDB validation report.

^h Order residue ranges [$S(\phi) > S(\psi)$ 1.8]. 30IB generated structure: 4–27, 29–37, 40–60, 62–66. Generated using PSVS 1.5

At the level of NOE restraints, the distribution of long-range distances is narrow, with an average upper bound of 5.29 ± 0.17 Å and values ranging from 5.00 to 5.50 Å, indicating a high degree of internal consistency in the restraint set. In line with this, comparison of the 30IB structural ensemble with previously reported models (1MJC, 3MEF, and 2L15) identifies three localized regions that deviate beyond the ensemble variability (**Fig. 2**): (i) Region I, encompassing the N-terminus and β -strand β 1 (residues S2–M6); (ii) Region II, comprising the β 1– β 2 loop and β -strand β 2 (residues N13–I21); and (iii) Region III, including the C-terminal end of β -strand β 4 and the β 4– β 5 loop (residues I55–A64). In each case, the observed differences are supported by multiple long-range NOE restraints in the 30IB dataset, consistent with an experimental origin.

In Region I (**Fig. 2a**), the conformation observed in the 30IB ensemble adopts an intermediate orientation relative to those in the 1MJC, 3MEF, and 2L15 structures. This positioning is supported by 7 long-range NOE restraints, with an average upper-bound distance of 5.36 ± 0.76 Å. In addition, 76 medium-range NOE restraints further define this region, with an average upper-bound distance of 4.10 ± 0.64 Å, approaching the long-range NOE restraints. Together, these restraints form a dense and internally consistent network, supporting the observed conformation of Region I.

In Region II (**Fig. 2b**), the 30IB ensemble deviates from the corresponding regions in the 1MJC, 3MEF, and 2L15 structures. This conformation is supported by 12 long-range NOE restraints, with an average upper-bound distance of 5.33 ± 0.71 Å. A substantial number of medium-range restraints (82 restraints) further define this region, with an average upper-bound distance of 4.12 ± 0.56 Å, approaching the long-range NOEs. Collectively, these restraints define a dense and coherent network that supports the observed conformation of Region II.

In Region III (**Fig. 2c**), the conformation in the 30IB ensemble deviates from those observed in the 1MJC, 3MEF, and 2L15 structures, while remaining well defined. This region is supported by 17 long-range NOE restraints, with an average upper-bound distance of 5.33 ± 0.65 Å. A large set of medium-range restraints (132 restraints) further defines this region, with an average upper-bound distance of 4.09 ± 0.67 Å. Taken together, these data establish a dense and self-consistent restraint network that strongly supports the observed conformation of Region III. Representative strip plots of long-range NOEs for these regions are shown in the **Supplementary Information (Fig. S1)**.

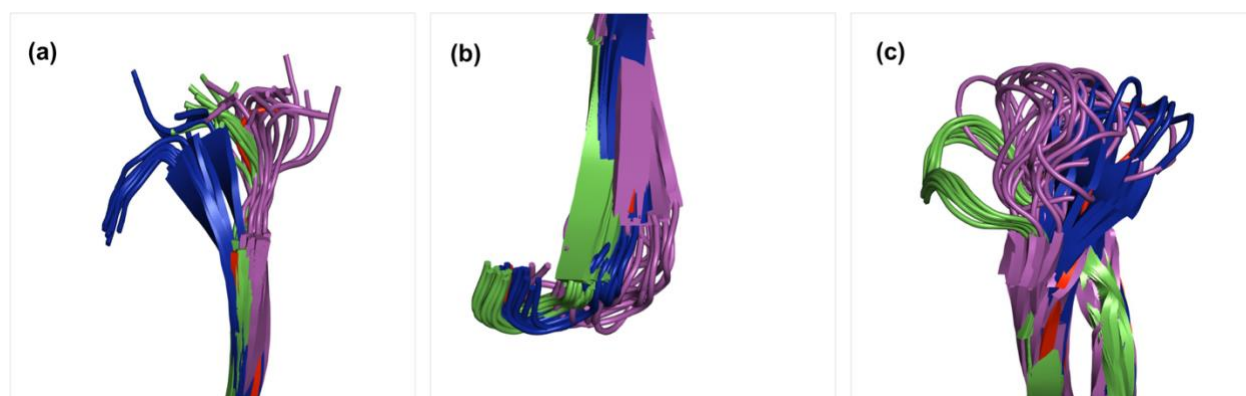


Fig. 2. Regions of deviation between the 30IB, 1MJC, 3MEF, and 2L15 structures. (a–c) Regions I–III, respectively. Color code: 30IB (green), 1MJC (red), 3MEF (magenta), and 2L15 (blue).

Because 1MJC is an X-ray crystal structure, a direct restraint-level comparison with the solution NMR ensemble 30IB is not possible. Instead, the quality and limitations of 1MJC were assessed independently based on its wwPDB validation report and established crystallographic criteria. The structure was refined at 2.0 Å resolution with an R value of 0.187, consistent with a physically reasonable model according to classical crystallographic standards (Cruickshank 1999; A T Brünger 1992). Independent validation using Xtriage indicates no evidence for twinning and only moderate anisotropy (0.252), supporting the absence of major crystallographic artifacts (Padilla and Yeates 2003). The Wilson B-factor (24.6 Å²) and the average refined atomic B-factor (30.0 Å²) are relatively elevated for a 2.0 Å structure, reflecting increased atomic displacement and/or conformational heterogeneity around the mean atomic positions rather than refinement instability (Cruickshank 1999; Parthasarathy and Murthy 1997). However, the structure does not satisfy current high-quality crystallographic standards (Evans and Murshudov 2013), primarily due to incomplete data completeness (89.1%) and the absence of an R_{free} test set, which precludes rigorous cross-validation against overfitting (Kleywegt and Alwyn Jones 1997). This distinction is particularly relevant when interpreting structural differences in the context of NMR relaxation measurements, as quantitative comparisons between crystallographic B-factors and NMR-derived order parameters (S^2) requires highly complete, cross-validated crystal structures (Clore and Schwieters 2006). Consequently, while 1MJC provides a reliable reference for the global fold and topology, it is not treated here as a reference for assessing localized conformational differences relative to the NMR ensemble. Accordingly, structural deviations observed between 30IB and 1MJC are interpreted in light of the extensive long-range NOE restraint network defining the NMR ensemble.

In Silico Structure Prediction and Refinement. An AlphaFold3 model of the CspA–dT7 complex was generated (**Fig. 3 (a–b)**) as described in the Materials and Methods section. The predicted structure adopts the canonical OB-barrel fold with five antiparallel β -strands. For the best-ranked model, the confidence scores for the prediction were pTM = 0.86 and ipTM = 0.69. Since ipTM values between 0.6 and 0.8 indicate intermediate confidence in the predicted protein–nucleic acid interface, the model was further refined using molecular dynamics-based energy minimization in YASARA (see Materials and

Methods). The final refined model was deposited in ModelArchive (<https://www.modelarchive.org/doi/10.5452/ma-7fflj>).

Because cold-shock proteins adopt a highly conserved β -barrel fold, we compared the CspA structure in our AF3/YASARA-derived complex with previously experimentally determined Bs-CspB complexes (Zeeb et al. 2006; Max et al. 2006). As shown in **Fig. 3 (c-d)**, the CspA structure in the model closely agrees with both the NMR Bs-CspB–dT7 complex (Zeeb et al. 2006), in which the dT7 moiety is unresolved and the X-ray Bs-CspB–dT6 complex (Max et al. 2006). The only notable deviation is observed in the I55–P62 region, which lies outside the nucleic-acid interaction interface and therefore does not affect ligand binding.

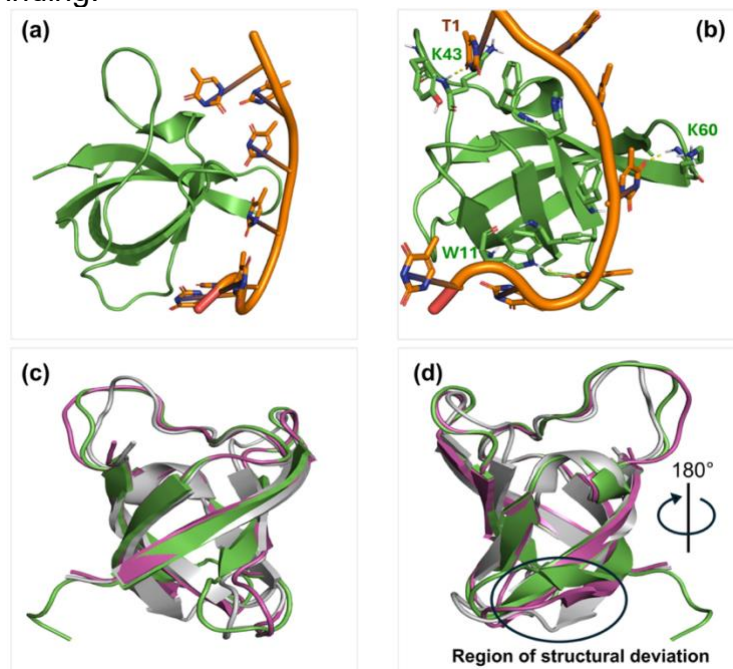


Fig. 3. In silico structure of CspA–dT7. (a) cartoon representation of the CspA–dT7. (b) CspA side chains residues involving π -stacking and ionic interactions with the dT7 bases. (c-d) Superposition of CspA from the CspA–dT7 model (green) with Bs-CspB from the Bs-CspB–dT7 NMR structure (gray) and the Bs-CspB–dT6 X-ray structure (magenta).

To further assess the consistency of the model with experimental data, we compared the predicted interaction interface with residues previously identified by NMR ssDNA titration as exhibiting significant chemical-shift perturbations and subsequently mapped as nucleic-acid-interacting residues in resolved NMR or X-ray structures. In the Bs-CspB–dT7 NMR complex (corresponding AF3/YASARA CspA–dT7 residues in parentheses), residues W8 (W11), F15, F17 (F20), F27 (F30), H29 (H33), F30 (F34), and F38 (Y42) were identified as key interaction sites in the NMR structure and shown to engage the ssDNA primarily through aromatic π -stacking interactions. Similarly, in the Bs-CspB–dT6 X-ray complex, residues F17 (F20), F27 (F30), H29 (H33), F30 (F34), and F38 (Y42) were identified as major interaction residues as they form π -stacking contacts with the ssDNA. Notably, these aromatic residues form the core of the predicted ssDNA-binding interface

in the AF3/YASARA CspA–dT7 model. The consistent involvement of the same interaction across two CSP homologs (CspA and CspB), two related ligands (dT6 and dT7), two independent experimental techniques (NMR and X-ray crystallography) and one computational approach (AF3/YASARA), indicates that our model reproduces the canonical CSP nucleic-acid binding surface. Together, the agreement in overall folding and the convergence of experimentally identified interaction residues indicate that our CspA–dT7 model provides a reasonable structural framework for interpreting the experimentally observed results.

NMR Investigation of Backbone Dynamics

Backbone Dynamics on the ps-ns timescale. Site-specific ^{15}N - R_1 , ^{15}N - R_2 , and ^{15}N - $\{^1\text{H}\}$ NOE values were obtained for 65 residues at 16.4 T (700 MHz) and 11.7 T (500 MHz). The results are shown in **Fig. 4** and indicate uniform relaxation behavior across the structured core.

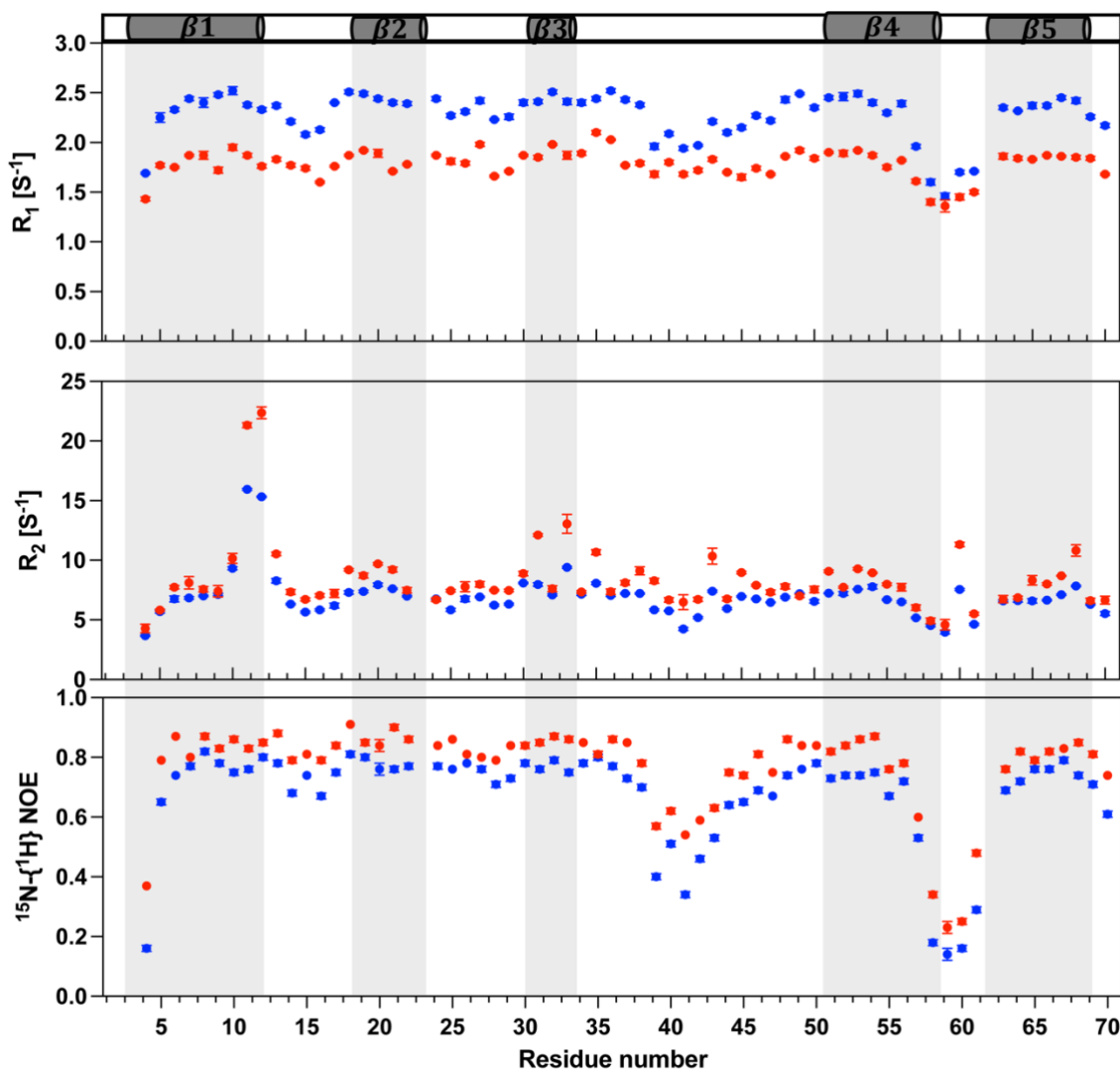


Fig. 4. Experimental ^{15}N - R_1 and ^{15}N - R_2 relaxation rates and ^{15}N - $\{^1\text{H}\}$ NOE for CspA residues at 700 MHz (Red) and 500 MHz (blue), shown with associated errors.

As seen often for this fold, the loop regions and the N- and C-termini were found to be more dynamic on the pico- to nanosecond timescale than the rest of the protein was, as indicated by decreased $^{15}\text{N}\text{--}\{^1\text{H}\}$ NOE values. Concurrently, increased dynamics on the micro- to millisecond timescale could be identified for residues at the C-termini of the $\beta 1$ strand based on elevated R_2 relaxation rates.

To quantify the ps-ns dynamics, model-free analysis was performed using relaxation data acquired at both 700 and 500 MHz. The best-fit model for each residue was selected using Akaike's Information Criterion (AIC)(d'Auvergne and Gooley 2003) from five models of increasing complexity (see Materials and Methods and **Table 1**).

For CspA, 12 residues were best fit by Model 1, which is consistent with fast ps-ns internal motions reflected by reduced order parameters ($S^2 < 1$), whereas no residues required Model 3. Six residues were fit to Model 2 and 26 to Model 4, indicating that single-timescale internal motions (τ_e) on the ps-ns timescale contributed significantly to their relaxation. Eighteen residues were fit to Model 5, indicating that internal motions on two distinct timescales contributed to their relaxation. Finally, three residues were not adequately fit by any of the five models, suggesting that a more complex spectral density function is required to capture their dynamics. Per-residue model-free parameters, together with the corresponding AIC values and selected models, are provided in **Table S1** of the Supplementary Information.

As summarized in **Fig. 5**, model-free analysis indicates that CspA is overall well ordered, with an average generalized order parameter S^2 of 0.81 ± 0.01 . Several residues implicated in nucleic acid binding exhibit S^2 values above the protein average, including G17 (0.91), F18 (0.93), F20 (0.84), I21 (0.89), V30 (0.87), F31 (0.91), H33 (0.88), and F34 (0.85), consistent with relatively restricted backbone dynamics in these regions. It should be noted that S^2 values depend on the chosen model-free description, and differences between residues should therefore be interpreted cautiously. Interestingly, residues in the $\beta 3\text{--}\beta 4$ loop (F33-Q49) and $\beta 4\text{--}\beta 5$ loop (S57-G61) presented reduced $^{15}\text{N}\text{--}\{^1\text{H}\}$ NOE values while still exhibiting high generalized order parameters ($S^2 \approx 0.8$). This apparent discrepancy likely reflects differences in the timescale sensitivity of the two measurements: the $^{15}\text{N}\text{--}\{^1\text{H}\}$ NOE is particularly sensitive to very fast local motions (<100 ps), whereas the order parameter describes the overall restriction of the N-H bond vector averaged over ps-ns motions (Ferrage et al. 2009; Gong and Ishima 2007; Kharchenko et al. 2020; Idiyatullin et al. 2001). As a result, small-amplitude fast fluctuations can reduce the NOE while having little impact on S^2 , as described previously (Kay et al. 1989). Alternatively, the discrepancy may indicate the presence of more complex motional behavior, potentially spanning multiple timescales, that is not fully captured by the simple model-free analysis formalism employed here.

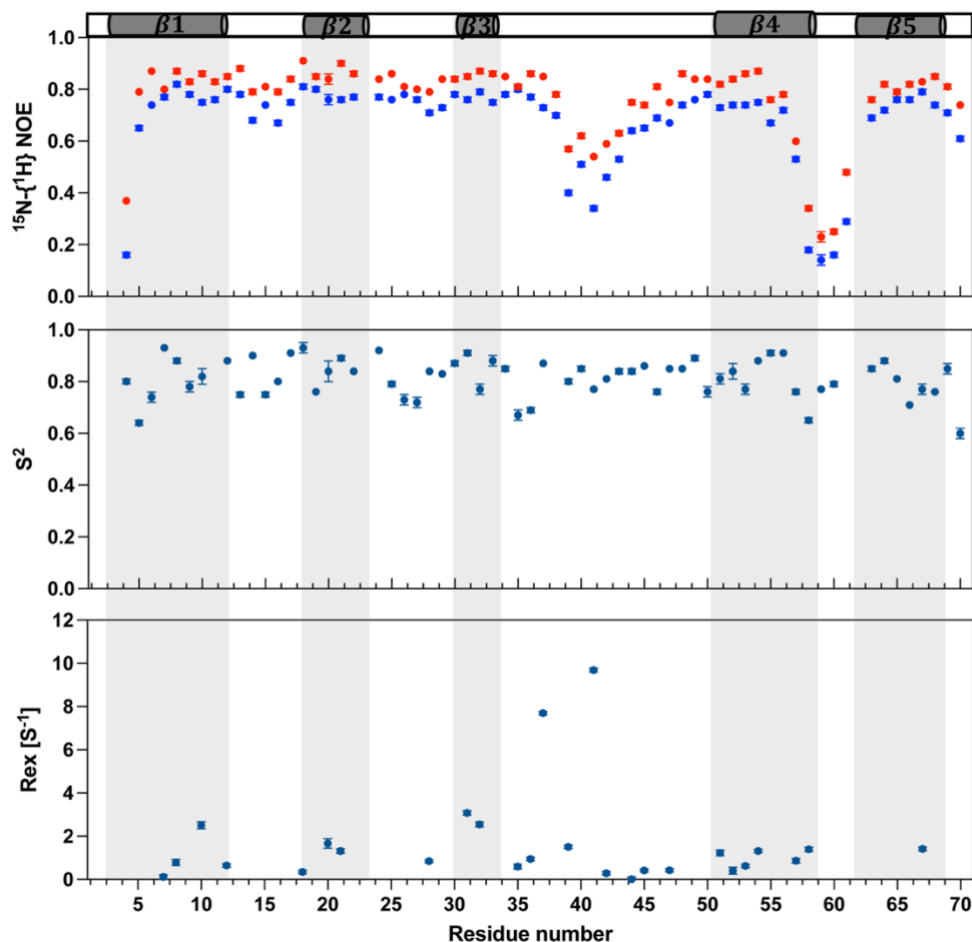


Fig. 5. Residue-wise MFA derived S^2 and conformational exchange parameters, shown with associated errors.

Backbone Dynamics Reveal Structural Features of the Invisible CspA Population.

To investigate potential conformational exchange on the microsecond–millisecond timescale, we employed a comprehensive set of chemical exchange-based NMR methods, combining ^{15}N CPMG relaxation dispersion (Hansen et al. 2007), ^{15}N D-CEST (Gareth A. Morris and Freeman 2011; Bodenhausen, Freeman, et al. 1976), and ^{15}N visible peak-position constraints (Khandave et al. 2023; Skrynnikov et al. 2002).

All datasets were analyzed jointly using a global two-state exchange model ($A \rightleftharpoons B$) implemented in ChemEx, combining CPMG, D-CEST and peak-position constraints. In this framework, k_{ex} and p_B were shared across residues, while residue-specific parameters included $\Delta\omega_{AB}$ and relaxation rates, as detailed in the Materials and Methods. The analysis revealed that a significant number of residues undergo conformational exchange in the intermediate-to-fast exchange regime, where ^{15}N D-CEST profiles no longer display well-separated dips corresponding to distinct ground and excited state resonances (Khandave et al. 2024; Rangadurai et al. 2020; Tiwari et al. 2024). Instead, a single asymmetric dip is observed (**Fig. 6 a–d**) that can still be analyzed to extract exchange parameters and minor-state chemical shifts.

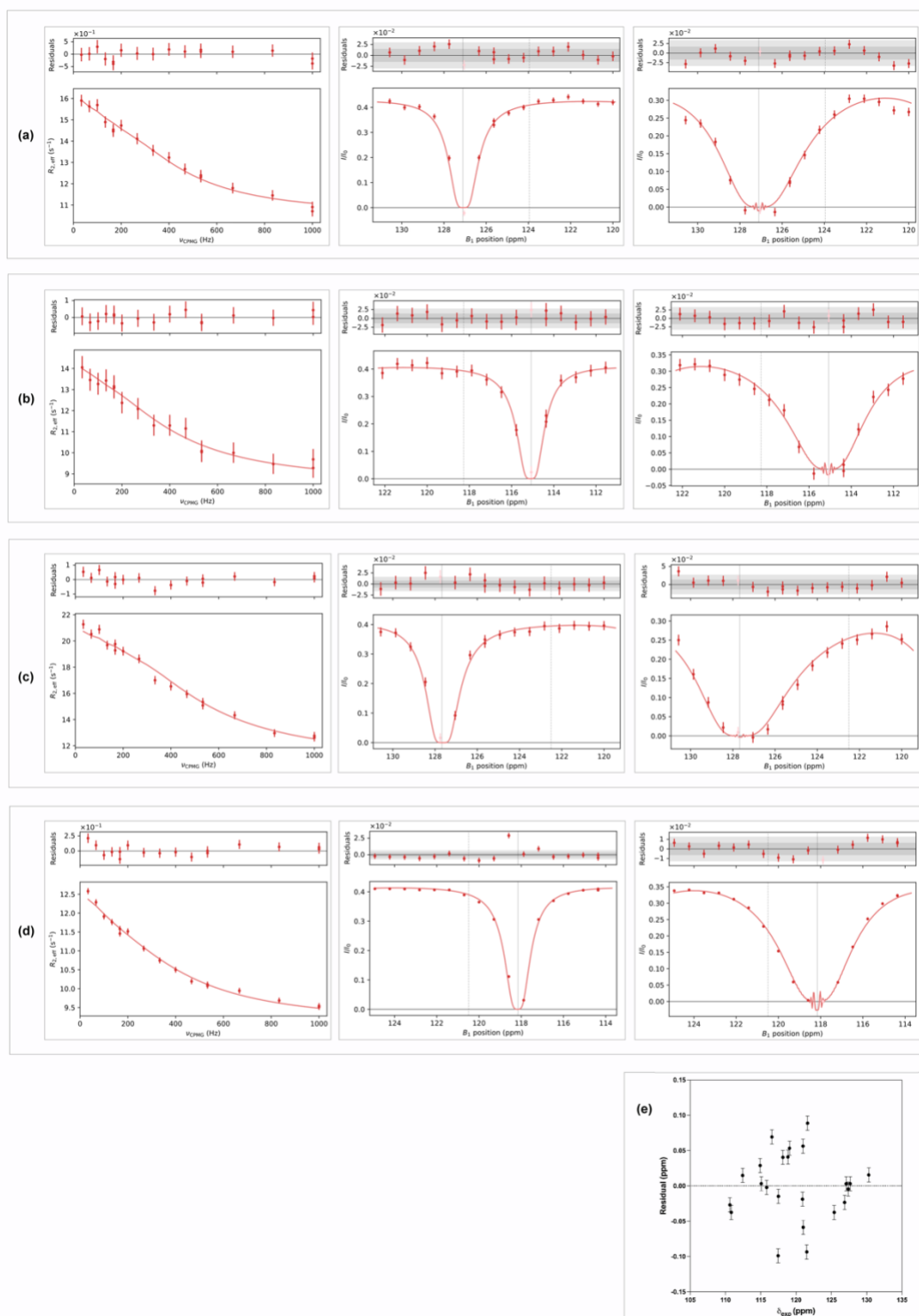


Fig. 6. ^{15}N CPMG and ^{15}N D-CEST profiles for residues N13, F20, H33 and V76 (panels a–d respectively) of the CspA recorded at 25 °C, $B_0 = 16.4$ T, with SW_{CEST} of 800 Hz and $B_1^{D\text{-CEST}}$ field strengths of 20 Hz and 60 Hz. (e) Residuals from the linear regression between experimental and predicted ground-state chemical shifts.

The fitted ground-state chemical shifts agree closely with the observed peak positions (**Fig. 6 e**). Consistent with the ^{15}N D-CEST results, the ^{15}N CPMG relaxation dispersion profiles also indicate that exchange processes occur in the intermediate-to-fast regime (Y. Jin et al. 2025) (**Fig. 6**). Together, these data provide direct evidence for microsecond–millisecond conformational exchange in CspA using a combined ^{15}N CPMG and D-CEST approach.

Global analysis identified 25 residues underwent two-state exchange with $k_{\text{ex}} = 2216 \pm 60 \text{ s}^{-1}$ and $p_{\text{B}} = 0.90 \pm 0.05\%$. The agreement between the experimental data and this two-state model establishes the presence of a sparsely populated excited state ($p_{\text{B}} < 1\%$)—the “invisible” population—in dynamic equilibrium with the ground state. These exchanging residues comprise three functionally relevant groups (**Fig. 7**): (i) **Aromatic residues at the binding interface**—solvent-exposed aromatics (W11, F18, F20, F31, H33, F34 and Y42) that cluster on the nucleic acid-binding interface and may undergo transient conformational adjustments to facilitate π -stacking interactions with single-stranded nucleic acids; (ii) **Hydrophobic core residues**—buried residues within the β -barrel core (F12, I21, V30, L45, V51, F53, and V67) whose exchange may reflect subtle internal fluctuations, providing structural flexibility for ligand accommodation without necessarily implying global unfolding; and (iii) **Polar residues in flexible loops**—solvent-exposed polar side chains (N13, T22, S35, Q38, N39, K43, S52, T54, K60, N66, and T68) whose dynamics are consistent with loop flexibility and solvent-mediated rearrangements that may regulate binding surface accessibility and contribute to conformational selection during nucleic acid recognition. Complete residue-specific parameters, including minor-state ^{15}N chemical shifts, are provided in **Table S2**.

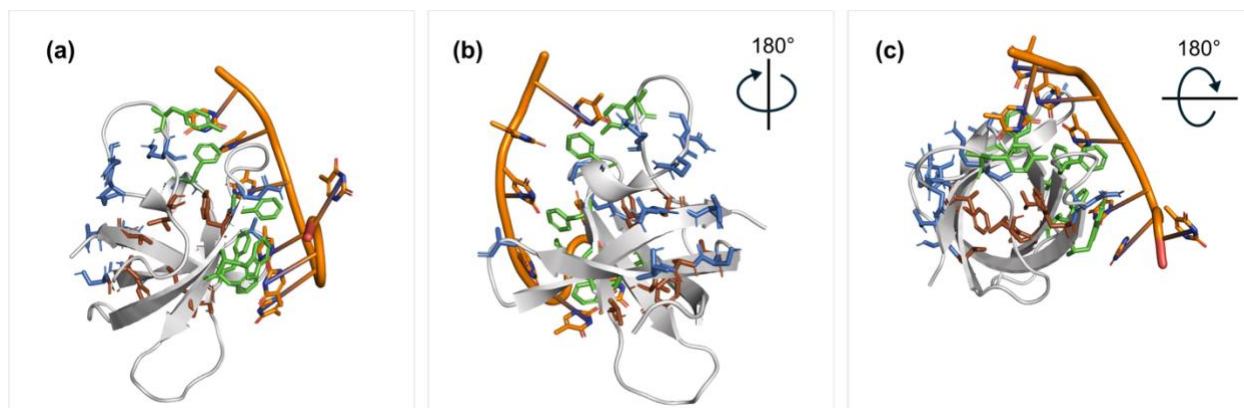


Fig. 7. Structural representation of CspA-dT₇ complex showing residues undergoing conformational exchange (a-c). Hydrophobic, solvent-exposed, and aromatic residues are color-coded in brown, marine, and green, respectively.

DISCUSSION

The comparative analysis of restraints, deviations, and violations highlights the role of experimental restraints in structure definition. The 30IB ensemble is defined by a larger number of experimental restraints than the 3MEF and 2L15 structures, particularly long-range distance restraints and dihedral angle constraints, which are important for defining the global fold and side-chain packing. This is further supported by favorable stereochemical quality, as reflected in Ramachandran statistics and minimal side-chain outliers. In comparison, 3MEF exhibits higher levels of residual violations and a larger proportion of geometric outliers, whereas the 2L15 structure shows low outlier percentages and similar RMSD values. These differences may reflect variations in restraint density rather than fundamental differences in the overall fold. Together, these data support the 30IB ensemble as a well-defined, experimentally supported structural representation.

To gain insight into the structural basis of nucleic acid recognition, CspA binding to the ssDNA dT7 was investigated using an *in-silico* approach. In the predicted CspA–dT7 complex, the overall fold of CspA closely matches the NMR structure of Bs-CspB bound to dT7 (Zeeb et al. 2006) and the X-ray Bs-CspB–dT6 complex (Max et al. 2006). Furthermore, residues W11, F20, F31, H33, F34 and Y42 form a distinct hydrophobic patch at the nucleic acid-binding site of CspA (**Fig. 3 (a-b)**). This arrangement is in excellent agreement with the binding interface observed in the Bs-CspB/dT7 and Bs-CspB/dT6 complexes, as determined by both structural analysis and NMR titration experiments. The predicted CspA–dT7 complex also revealed ionic interactions between the ssDNA and residues W11, K43, and K60 (**Fig. 3b**). These interactions may stabilize nucleic acid binding and may contribute to the ability of CspA to recognize and destabilize secondary structures in single-stranded regions during the cold-shock response. Therefore, the observed ionic contacts and π -stacking interactions provide a structural basis for interpreting the dynamic and functional properties of CspA, particularly its role in facilitating transcriptional and translational adaptation under stress conditions.

We investigated the backbone dynamics of CspA on two distinct timescales: picosecond–nanosecond (ps–ns) using ^{15}N spin relaxation and model-free analysis, and microsecond–millisecond (μs – ms) using ^{15}N CPMG and CEST experiments, together with ^{15}N visible peak-position constraints. Nuclear spin relaxation studies have previously been reported for several cold-shock proteins, including CspA (Feng et al. 1998), Bs-CspB (Zeeb et al. 2006), Lm-Csp (J. Lee et al. 2013), Ta-Csp (B. Jin et al. 2014), and Cp-Csp (Y. Lee et al. 2018), revealing consistent patterns of flexibility in loop regions. In all cases, residues in the $\beta 3$ – $\beta 4$ and $\beta 4$ – $\beta 5$ loops displayed reduced ^{15}N – $\{^1\text{H}\}$ NOE values, whereas residues proximal to the $\beta 1$ and $\beta 2$ strands, as well as those in the connecting loop, presented elevated R_2 values. However, these earlier studies were performed at a single magnetic field. Our multi-field analysis with model-free fitting reveals that the $\beta 3$ – $\beta 4$ and $\beta 4$ – $\beta 5$ loop regions undergo complex motions: residues in these loops exhibit reduced ^{15}N – $\{^1\text{H}\}$ NOE values while maintaining high generalized order parameters ($S^2 \approx 0.8$). Since these loops regions form part of the nucleic acid-binding interface, their dynamic behavior may reflect the functional plasticity required to accommodate and stabilize diverse ssDNA or ssRNA conformations. Our results therefore suggest that loop

flexibility may represent an intrinsic feature of CspA that could contribute to its chaperone-like activity during cold-shock adaptation.

While the microsecond–millisecond dynamics of CspA have been characterized previously (Feng et al. 1998), the structural features and functional role of transiently populated excited states remained unknown. Using a comprehensive approach combining ^{15}N CPMG relaxation dispersion, ^{15}N D-CEST, and visible peak positions in the ^{15}N D-CEST spectra, we identified 25 residues undergoing conformational exchange on the microsecond–millisecond timescale ($k_{\text{ex}} \approx 2200 \text{ s}^{-1}$, $p_B \approx 0.9\%$). The exchange rate places this process in the intermediate-to-fast regime on the NMR timescale, enabling characterization of both kinetics and structural features of the excited state. The detection of exchange across residues distributed throughout the protein suggests a process that is not confined to a single local site and is consistent with a coordinated (or coupled) dynamic event. Notably, residues undergoing exchange belong to three functionally distinct groups: (i) aromatic residues in the nucleic acid-binding interface, (ii) buried hydrophobic residues in the β -barrel core, and (iii) polar residues in flexible surface loops. To test whether residues undergoing exchange in spatially distinct regions are compatible with a single underlying exchange process (rather than unrelated local events), we performed cluster-specific joint fits using representative residues from each of the three regions. Parameter uncertainties for the cluster-specific fits, as well as for the global joint fit (combining ^{15}N CPMG, ^{15}N D-CEST, and visible peak positions), were estimated using 1000 Monte Carlo trials (Choy et al. 2005; Press 2007).

Aromatic residues F18, F20, and F31 were selected to represent the binding interface; L45, V53, and N66 to represent the hydrophobic core; and N13, K43, and T68 to represent polar residues located in flexible loop regions. Residues were chosen based on the strength and clarity of their exchange signatures, ensuring a balanced sampling of the three regions. Exchange rate parameters and excited-state populations obtained from the cluster-specific joint fits, together with uncertainties, are reported in **Table 3**, alongside the results from the global joint fit.

Table 3. Exchange parameters and associated uncertainties derived from 1000 Monte Carlo trials.

	$k_{\text{ex}} (\text{s}^{-1})$	$p_B (\%)$	χ_{red}^2 ¹
Global Fit (CPMG + D-CEST + Visible Peak Positions)	2216 ± 26	0.89 ± 0.02	0.94
Cluster 1 (Aromatic residues)	2146 ± 47	1.09 ± 0.09	0.93
Cluster 2 (Hydrophobic core residues)	2203 ± 14	0.63 ± 0.04	0.93
Cluster 3 (Polar residues in flexible loops)	2164 ± 91	1.04 ± 0.09	0.94
^{15}N CPMG + Visible Peak Positions	2312 ± 45	1.21 ± 0.10	0.95

¹ $\chi_{\text{red}}^2 = \chi^2 / \text{dof}$ where *dof* is the degrees of freedom and corresponds to the difference between the number of experimental data points per residue and the number of fitted parameters for that residue.

The cluster-specific fits yield exchange parameters of similar order of magnitude across regions, consistent with a shared exchange regime. In particular, the extracted exchange rates are broadly similar ($k_{ex} \sim 2.1\text{--}2.2 \times 10^3 \text{ s}^{-1}$), whereas the estimated minor-state populations show modest cluster-to-cluster variation. The hydrophobic-core subset returns a lower p_B than the global joint fit (**Table 3**). We interpret this deviation cautiously, as it may reflect reduced parameter identifiability for this subset in the intermediate-to-fast exchange regime such as smaller $\Delta\omega_{AB}$ values and stronger $p_B\text{--}\Delta\omega_{AB}$ trade-offs (**Fig S2**) rather than compelling evidence for a distinct exchange process.

Because the extracted exchange parameters place the process in the intermediate-to-fast exchange regime, parameter degeneracies can occur in single-field CPMG analyses, and CEST parameters identifiability can be limited when ω_1 is below k_{ex} (Vallurupalli et al. 2017; Khandave et al. 2024). To evaluate the extent to which the additional observables provide orthogonal constraints, we repeated the analysis using only ^{15}N CPMG data in combination with visible peak positions, estimating uncertainties by 1000 Monte Carlo trials. This reduced analysis yielded $k_{ex} = 2312 \pm 45 \text{ s}^{-1}$ and $p_B = 1.21 \pm 0.10 \%$, with broader uncertainties and reduced parameter coupling than the full joint fit (**Table 3**; **Fig. 8 (a)**). The improved precision obtained in the global joint analysis indicates that incorporating ^{15}N D-CEST data and peak-position information provides orthogonal constraints that enable more reliable determination of k_{ex} and p_B than the CPMG alone does, despite the absence of a second magnetic field.

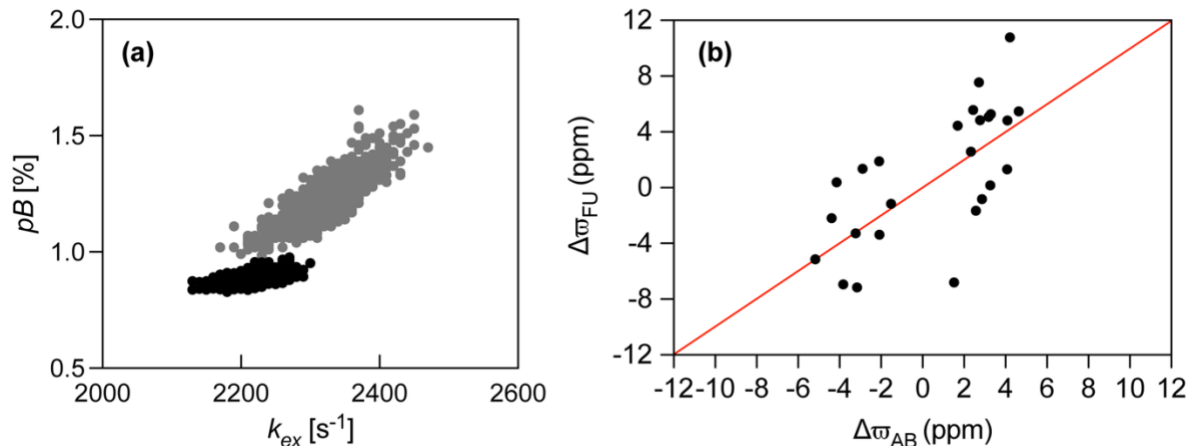


Fig. 8. Consistency of exchange parameters across ^{15}N CPMG and combined ^{15}N CPMG and D-CEST analyses. (a) Superimposition of the k_{ex} - p_B correlations from the two analyses highlighting differences in parameters. Color code: black, correlation from ^{15}N CPMG and D-CEST fits, in combination with visible peak positions; steel, correlation from ^{15}N CPMG fits, in combination with visible peak positions. (b) Comparison of residue-specific chemical shift differences derived from ^{15}N CEST/CPMG-derived chemical-shift differences ($\Delta\omega_{AB}$) and residue-specific $\Delta\omega_{FU}$ values.

Overall, the spatial distribution of residues exhibiting exchange—spanning the binding surface, core, and loop regions—is consistent with a coordinated conformational process that propagates across the protein scaffold. Given the limitations of the present dataset, we present the two-state model as a parsimonious working description that is compatible with the combined observables, without claiming that it is uniquely established.

Finally, we compared the $\Delta\omega_{AB}$ values obtained from the global joint fit with the $\Delta\omega_{FU}$ values to assess whether the extracted chemical shift differences were indicative of local unfolding. The $\Delta\omega_{FU}$ values were calculated using POTENCI (Nielsen and Mulder 2018), which provides improved accuracy for random-coil chemical shift predictions. As shown in **Fig. 8 (b)**, the relationship is weak, indicating that the extracted chemical-shift differences are not well described by a simple folded-to-random-coil transition, nor does it support a local unfolding mechanism as the dominant explanation of the observed exchange.

Taken together, the robustness analyses show that a parsimonious two-state model provides a consistent description of the exchange signatures observed across spatially distinct regions, and that combining ^{15}N CPMG with ^{15}N D-CEST and peak-position constraints improves parameter identifiability despite the use of a single magnetic field. The spatial distribution of residues undergoing exchange—spanning the binding surface, hydrophobic core, and flexible loops—is therefore consistent with coordinated conformational fluctuations that can propagate through the protein scaffold. Such coupled motions may contribute to the structural plasticity required for CspA to act as a nucleic acid chaperone.

To assess whether such dynamics represent a conserved feature of cold-shock proteins, we compared these results with those of dynamic studies of related CSPs from organisms adapted to different thermal environments. The aromatic residues undergoing conformational exchange in CspA (W11, F18, F20, F31, H33, F34 and Y42) are located within the conserved RNA-binding motifs RNP1 and RNP2, where they mediate π -stacking interactions with nucleic acid bases (T1, T3, T4, T5 and T6) in the modeled CspA–dT7 complex. This observation is consistent with a direct link between microsecond–millisecond dynamics and the nucleic acid-binding interface, suggesting that conformational exchange may facilitate adaptive recognition of diverse ssRNA and ssDNA sequences. Notably, the involvement of these aromatic residues in nucleic acid binding appears to be a conserved feature across the cold-shock protein family. Chemical shift perturbation studies have identified analogous aromatic residues as critical for nucleic acid binding in CSPs from organisms adapted to different thermal environments: in psychrophilic CspA, residues W8, F15, F17, F28, and H30 undergo significant chemical shift changes upon dT7 binding (J. Lee et al. 2013; Y. Lee et al. 2018); in the mesophilic protein Bs-CspB, residues W8, F9, F15, F17, F29, and F30 display marked perturbations upon ssRNA and ssDNA binding (Max et al. 2007; Sachs et al. 2012; Zeeb et al. 2006); and in thermophilic and hyperthermophilic CspA, the corresponding conserved aromatic residues (W8, F17, F30 (B. Jin et al. 2014) and Y14, F16, H28, F37 (Jin et al. 2014; Von König et al. 2020), respectively) are directly involved in nucleic acid recognition. The strong conservation of these aromatic residues within the RNP motifs, despite sequence variation and point mutations across organisms, underscores their functional importance. The combination of π -stacking interactions and conformational flexibility at these aromatic residues suggests a structural and dynamic basis for CSP chaperone activity. We

propose that transient sampling of multiple conformations enables CSPs to recognize diverse nucleic acid sequences, while π -stacking interactions stabilize binding and may disrupt secondary structures. This mechanism would promote the single-stranded conformations necessary for efficient translation during the cold-shock response.

Chemical shift perturbation studies typically highlight residues at or near the binding interface, where local electronic environments are most strongly affected by ligand binding (Hobbs et al. 2022; Kleckner and Foster 2011). In contrast, residues within the hydrophobic core are generally less sensitive to chemical shift perturbation, as their local environments remain largely unchanged upon complex formation (Mike P. Williamson 2013). Our ^{15}N CPMG and D-CEST analyses revealed seven buried core residues (F12, I21, V30, L45, V51, F53, and V67) that underwent microsecond–millisecond exchange—a substantially larger set than previously detected in related CSPs. Earlier relaxation studies identified only four core residues through elevated R_2 values (V29, L43, V53, and V66) (Y. Lee et al. 2018) and three through direct measurement of exchange rates (T48, F49, and V53) (J. Lee et al. 2013). The detection of exchange in buried regions suggests that CspA dynamics extends well beyond the binding interface, involving coupled fluctuations that propagate through the hydrophobic core. This observation highlights an important aspect of CspA functional mechanism: internal core dynamics may modulate the flexibility of surface loops, thereby tuning the conformational plasticity required for promiscuous nucleic acid recognition. The coupling between core and loop dynamics is consistent with a coordinated mechanism in which binding-competent conformations are sampled through cooperative motions spanning the entire protein structure. Such widespread conformational exchange could not be detected by chemical shift perturbation alone, highlighting the complementary insights provided by relaxation-based approaches for characterizing functionally relevant protein dynamics.

Among the eleven solvent-exposed polar residues identified as undergoing exchange (N13, T22, S35, Q38, N39, K43, S52, T54, K60, N66, and T68), K43 and K60 form ionic interactions with T1 and T4, respectively, in the modeled CspA–dT7 complex. Since solvent-exposed polar residues have previously been shown to interact with dT7 bases (B. Jin et al. 2014; J. Lee et al. 2013; Y. Lee et al. 2018; Zeeb et al. 2006), our findings further support the hypothesis that such interactions contribute to stabilizing nucleic acid binding. Moreover, the dynamic behavior of these residues may enhance the ability of CspA to recognize diverse nucleic acid sequences and to destabilize secondary structures in single-stranded regions, a property that is central to its role in the cold-shock response. In addition, several residues of the $\beta 3$ – $\beta 4$ loop (F33, Y42, K43, and L45) and the $\beta 4$ – $\beta 5$ loop (K60) also undergo conformational exchange. While we previously noted a discrepancy between the ^{15}N – $\{^1\text{H}\}$ NOE and S^2 values for residues in these loops, their involvement in conformational exchange further reinforces the view that the $\beta 3$ – $\beta 4$ and $\beta 4$ – $\beta 5$ loop regions experience complex motions that extend beyond the description provided by model-free analysis.

CONCLUSIONS

Our study provides a comprehensive characterization of the CspA structure and dynamics, combining extensive NMR restraints with multiple relaxation methods to probe motions across ps–ns and μ s–ms timescales. We show that conformational exchange involves not only the conserved aromatic residues of the RNP1 and RNP2 motifs, which mediate π -stacking interactions with nucleic acids, but also an unexpectedly large set of hydrophobic core and solvent-exposed polar residues. Importantly, several residues of the β 3– β 4 loop (F33, Y42, K43, and L45) and β 4– β 5 loop (K60) undergo exchange, reinforcing our conclusion that these regions experience complex motions not fully captured by model-free analysis. Consistent with this, the modeled CspA–dT7 complex suggests that aromatic and polar residues at the binding interface (e.g., W11, F20, F31, H33, F34, Y42, K43, and K60) can form π -stacking and ionic interactions with ssDNA, highlighting how loop flexibility and dynamic side chains may contribute to nucleic acid recognition. Together, these findings indicate that CspA relies on coordinated motions extending from its binding motifs through the hydrophobic core and flexible loops, providing structural adaptability that is likely important for nucleic acid recognition, destabilization of secondary structures, and chaperone activity during the cold-shock response. Our results therefore support 3OIB as a well-converged NMR ensemble of CspA and offer new mechanistic insights into how molecular motions may underpin its biological function.

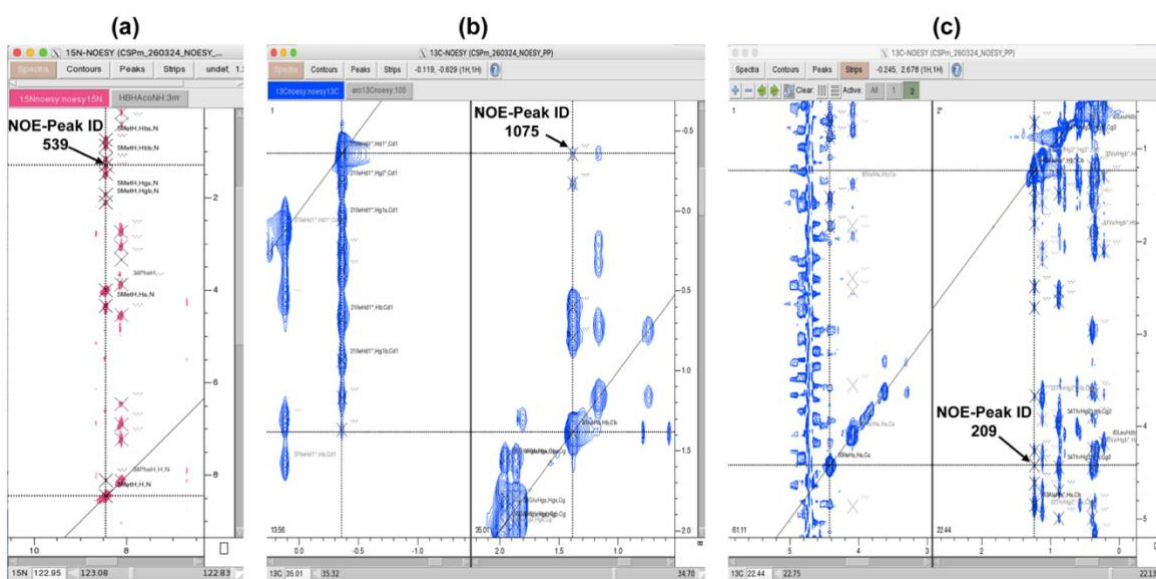
ACKNOWLEDGMENTS

This study made use of NMRbox: National Center for Biomolecular NMR Data Processing and Analysis, a Biomedical Technology Research Resource (BTRR), which is supported by NIH grant P41GM111135 (NIGMS)

Electronic Supplementary Material

Solution NMR structure of CspA.

One key feature of this work is the presentation of the well-defined NMR structure of CspA to date, offering improved accuracy relative to previously reported CspA structures. Specifically, we identify four well-defined regions in which the 3OIB structure deviates from the 1MJC, 3MEF, and 2L15 models. For each region, we provide direct experimental validation in the Supplementary Information, including long-range NOESY strip plots, corresponding peak assignments, and upper-bound distance classifications. These data demonstrate that the observed deviations are supported by extensive long-range NOE restraints and reflect experimentally defined conformations rather than modeling artifacts.



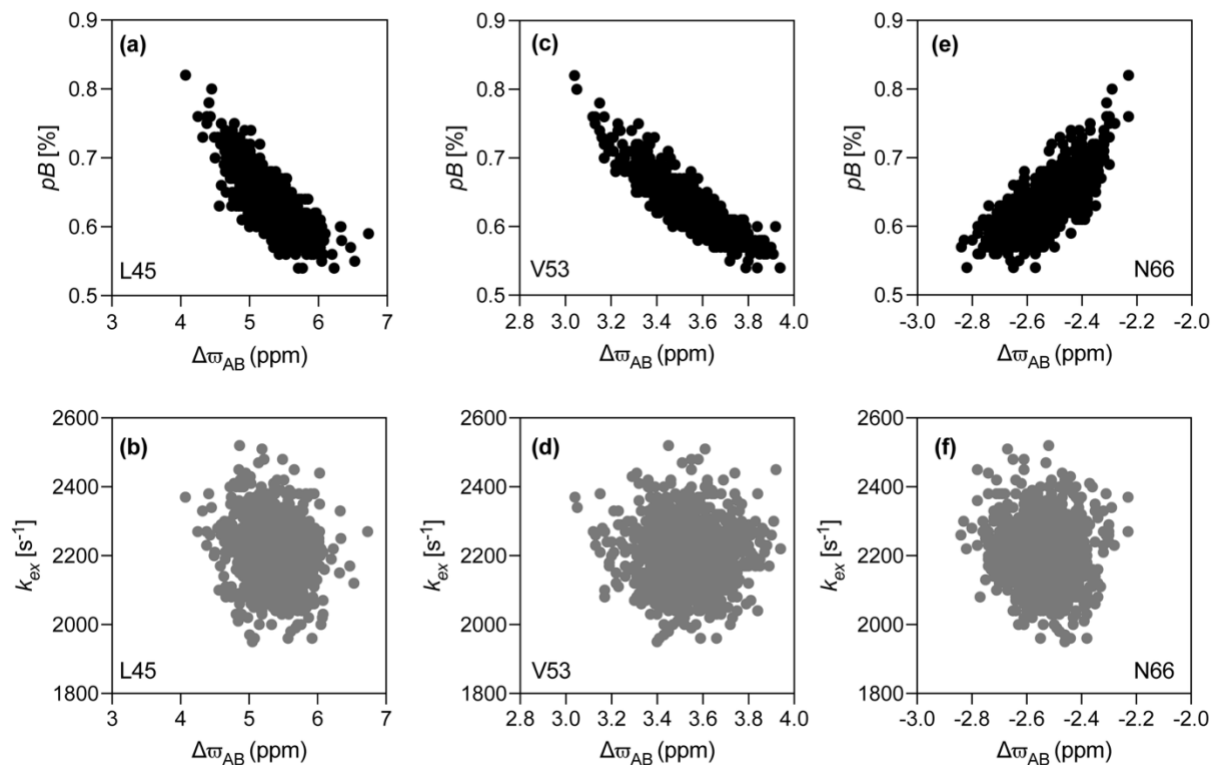


Figure S2. Correlation analysis of fitted exchange parameters. (a, c, e) Correlation between p_B and $\Delta\omega_{AB}$ for residues L45, V53, and N66, showing parameter trade-offs. (b, d, f) Corresponding correlations between k_{ex} and $\Delta\omega_{AB}$, showing weaker dependence and a narrower distribution of k_{ex} values.

Model-free Analysis**Table S2.** Per-residue model-free analysis parameters, including residue-specific generalized order parameter (S^2), local correlation time (τ_e), and conformational exchange rate (R_{ex}).

Residue	Chosen Model	AIC	S^2	$\pm\Delta S^2$	τ_e (ps)	$\pm\Delta\tau_e$	R_{ex} (s^{-1})	$\pm\Delta R_{ex}$
LYS[4]	M5	2390.00	0.80	0.006	242.00	1660.00		
MET[5]	M5	98.60	0.64	0.014	1570.00	91.70		
THR[6]	M5	137.00	0.74	0.018	1890.00	175.00		
GLY[7]	M4	76.90	0.93	0.003	170.00	27.70	0.117	0.102
ILE[8]	M4	51.90	0.88	0.006	19.00	3.85	0.782	0.143
VAL[9]	M5	55.60	0.78	0.017	823.00	129.00		
LYS[10]	M4	26.20	0.82	0.028	1370.00	179.00	2.5	0.166
TRP[11]	-	-	-	-	-	-	-	-
PHE[12]	M4	929.00	0.88	0.005	77.50	6.37	0.643	0.0779
ASN[13]	M5	819.00	0.75	0.008	820.00	54.50		
ALA[14]	M2	7.32	0.90	0.004	94.30	8.72		
ASP[15]	M5	255.00	0.75	0.008	820.00	54.50		
LYS[16]	M1	440.00	0.80	0.003	0.00	0.00		
GLY[17]	M2	11.50	0.91	0.004	52.00	4.37		
PHE[18]	M4	330.00	0.93	0.017	1010.00	227.00	0.348	0.0865
GLY[19]	M1	11700.00	0.76	0.003	0.00	0.00		
PHE[20]	M4	107.00	0.84	0.043	928.00	293.00	1.66	0.222
ILE[21]	M4	36.30	0.89	0.005	16.70	5.11	1.31	0.102
THR[22]	M1	1760.00	0.84	0.004	0.00	0.00		
ASP[24]	M1	1600.00	0.92	0.003	0.00	0.00		
ASP[25]	M5	322.00	0.79	0.012	983.00	107.00		
GLY[26]	M5	34.70	0.73	0.022	1160.00	121.00		
SER[27]	M5	161.00	0.72	0.019	1180.00	89.30		
LYS[28]	M4	154.00	0.84	0.004	49.70	2.52	0.841	0.0598
ASP[29]	M1	5230.00	0.83	0.004	0.00	0.00		
VAL[30]	M1	2820.00	0.87	0.005	0.00	0.00		
PHE[31]	M4	465.00	0.91	0.006	65.80	8.18	3.07	0.103
VAL[32]	M4	172.00	0.77	0.018	1540.00	78.10	2.54	0.118
HIS[33]	M5	75.70	0.88	0.016	571.00	270.00		
PHE[34]	M5	84.20	0.85	0.011	937.00	123.00		
SER[35]	M4	506.00	0.67	0.024	1460.00	88.80	0.597	0.115

ALA[36]	M4	195.00	0.69	0.011	1700.00	37.50	0.941	0.0866
ILE[37]	M4	1390.00	0.87	0.005	30.50	2.74	7.69	0.0775
GLN[38]	-	-	-	-	-	-	-	-
ASN[39]	M4	161.00	0.80	0.007	113.00	9.61	1.5	0.0868
ASP[40]	M5	407.00	0.85	0.008	395.00	47400.00		
GLY[41]	M4	874.00	0.77	0.004	127.00	5.97	9.68	0.0907
TYR[42]	M4	575.00	0.81	0.004	115.00	5.91	0.283	0.0777
LYS[43]	M2	120.00	0.84	0.005	146.00	14.60		
SER[44]	M4	46.60	0.84	0.006	146.00	15.50	0.00531	0.113
LEU[45]	M4	190.00	0.86	0.003	90.50	4.51	0.414	0.0595
ASP[46]	M1	2400.00	0.76	0.006	0.00	0.00		
GLU[47]	M4	43.80	0.85	0.004	70.60	3.02	0.428	0.0738
GLY[48]	M1	10500.00	0.85	0.004	0.00	0.00		
GLN[49]	M5	199.00	0.89	0.011	680.00	138.00		
LYS[50]	M5	37.40	0.76	0.022	1390.00	166.00		
VAL[51]	M4	168.00	0.81	0.019	1040.00	89.80	1.22	0.139
SER[52]	M4	64.40	0.84	0.029	1090.00	190.00	0.408	0.162
PHE[53]	M4	523.00	0.77	0.016	1200.00	68.90	0.621	0.0874
THR[54]	M4	292.00	0.88	0.004	37.10	3.93	1.31	0.075
ILE[55]	M5	105.00	0.91	0.007	287.00	165.00		
GLU[56]	M2	25.50	0.91	0.003	118.00	11.00		
SER[57]	M4	400.00	0.76	0.008	85.40	5.86	0.86	0.102
GLY[58]	M4	1510.00	0.65	0.007	94.10	4.78	1.39	0.1
ALA[59]	M2	387.00	0.77	0.004	79.30	3.36		
LYS[60]	M2	2690.00	0.79	0.005	114.00	6.34		
GLY[61]	-	-	-	-	-	-	-	-
ALA[63]	M1	2090.00	0.85	0.005	0.00	0.00		
ALA[64]	M5	87.70	0.88	0.007	446.00	106.00		
GLY[65]	M1	14600.00	0.81	0.005	0.00	0.00		
ASN[66]	M1	20100.00	0.71	0.004	0.00	0.00		
VAL[67]	M4	482.00	0.77	0.018	1080.00	77.20	1.41	0.0963
THR[68]	M1	6840.00	0.76	0.004	0.00	0.00		
SER[69]	M5	62.70	0.85	0.015	709.00	132.00		
LEU[70]	M5	20.20	0.60	0.015	990.00	44.20		

Backbone Dynamics on the μs -ms timescale**Table S3** Complete set of residue-specific parameters obtained from two-state fits of ^{15}N CPMG relaxation-dispersion and ^{15}N D-CEST data, including ground-state chemical shifts δ_A , chemical-shift differences between ground and excited states ($\Delta\omega_{AB}$), and longitudinal and transverse relaxation rates R_1 and R_2 respectively.

Residue	δ_A (ppm)			$\Delta\omega_{AB}$ (ppm)			R_1 (s^{-1})			R_2 (s^{-1})		
W11	110.78	$\pm$	0.02	4.22	$\pm$	0.21	1.99	$\pm$	0.02	21.01	$\pm$	0.24
F12	121.05	$\pm$	0.02	-4.14	$\pm$	0.22	1.93	$\pm$	0.03	20.07	$\pm$	0.30
N13	127.11	$\pm$	0.02	-3.15	$\pm$	0.19	1.86	$\pm$	0.04	10.75	$\pm$	0.37
F18	112.44	$\pm$	0.01	2.72	$\pm$	0.08	2.00	$\pm$	0.01	10.35	$\pm$	0.05
F20	115.07	$\pm$	0.01	3.20	$\pm$	0.10	1.93	$\pm$	0.02	8.68	$\pm$	0.23
I21	121.72	$\pm$	0.02	-2.89	$\pm$	0.14	1.78	$\pm$	0.03	8.64	$\pm$	0.24
T22	126.81	$\pm$	0.01	1.53	$\pm$	0.09	1.86	$\pm$	0.02	8.94	$\pm$	0.14
V30	120.92	$\pm$	0.02	2.88	$\pm$	0.13	1.81	$\pm$	0.02	10.71	$\pm$	0.22
F31	130.30	$\pm$	0.02	-3.82	$\pm$	0.17	1.95	$\pm$	0.02	8.69	$\pm$	0.22
H33	127.68	$\pm$	0.03	-5.17	$\pm$	0.39	1.92	$\pm$	0.06	11.06	$\pm$	0.57
F34	123.27	$\pm$	0.01	-1.51	$\pm$	0.08	1.79	$\pm$	0.02	10.31	$\pm$	0.13
S35	117.34	$\pm$	0.02	3.27	$\pm$	0.15	1.78	$\pm$	0.04	15.60	$\pm$	0.23
Q38	127.38	$\pm$	0.01	-2.07	$\pm$	0.11	1.85	$\pm$	0.02	10.12	$\pm$	0.21
N39	118.86	$\pm$	0.02	4.09	$\pm$	0.22	1.42	$\pm$	0.04	13.71	$\pm$	0.32
Y42	121.39	$\pm$	0.01	2.58	$\pm$	0.09	1.48	$\pm$	0.02	12.29	$\pm$	0.12
K43	125.37	$\pm$	0.02	-4.38	$\pm$	0.23	1.65	$\pm$	0.03	14.48	$\pm$	0.30
L45	119.10	$\pm$	0.02	4.10	$\pm$	0.28	1.63	$\pm$	0.04	8.35	$\pm$	0.55
V51	115.81	$\pm$	0.01	2.45	$\pm$	0.09	1.95	$\pm$	0.02	8.07	$\pm$	0.14
S52	114.93	$\pm$	0.01	1.70	$\pm$	0.07	1.87	$\pm$	0.02	9.25	$\pm$	0.10
F53	117.45	$\pm$	0.01	2.78	$\pm$	0.10	1.94	$\pm$	0.02	9.52	$\pm$	0.13
T54	110.58	$\pm$	0.02	3.30	$\pm$	0.14	1.91	$\pm$	0.03	9.79	$\pm$	0.21
K60	115.06	$\pm$	0.02	4.64	$\pm$	0.29	1.20	$\pm$	0.05	17.80	$\pm$	0.41
N66	116.66	$\pm$	0.01	-2.09	$\pm$	0.10	1.78	$\pm$	0.02	9.49	$\pm$	0.17
V67	118.16	$\pm$	0.01	2.34	$\pm$	0.08	1.86	$\pm$	0.01	9.07	$\pm$	0.11
T68	120.91	$\pm$	0.02	-3.22	$\pm$	0.13	1.85	$\pm$	0.03	10.40	$\pm$	0.19

IX.2. Antibody Modulation of TEM-1 Conformational Dynamics (manuscript in preparation)

In this paper, the X-ray crystallographic analyses and kinetic studies were performed by Dr. Frédéric Cawez and are included for contextual purposes only; they do not form part of my original contributions.

Antibody Binding Modulates the Conformational Energy Landscape of TEM-1 β -Lactamase

Marius Wanko Nembot[†], Frédéric Cawez[‡], Maximiliano Figueroa[§], Alain Brans^{||}, Ulric B. le Paige[#], Guillaume Bouvignies[#], Frédéric Kerff^{†*}, Christian Damblon^{†*} and Moreno Galleni^{†*}.

[†]Structural Biological Chemistry Laboratory, MolSys Research Unity, University of Liege, Liège, Belgium.

[‡]InBioS, Center for Protein Engineering, Biological Macromolecules, Department of Life Sciences, University of Liège, Liège, Belgium

[§]Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Biológicas, Universidad de Concepción, Concepción, Chile

^{||}Center for Protein Engineering, Protein Factory, Department of Life Sciences, University of Liège, Liège, Belgium

[#]Chimie Physique et Chimie du Vivant (CPCV), Département de Chimie, École normale supérieure, PSL University, Sorbonne Université, CNRS, Paris, France

Corresponding Authors

Moreno Galleni: mgalleni@uliege.be

Christian Damblon: C.Damblon@uliege.be

Marius Wanko Nembot: amwanko@uliege.be

Keywords: Enzyme, TEM-1, Nanobody, Conformational exchange, NMR, CPMG, CEST

INTRODUCTION

The production of β -lactamases by nosocomial strains represents the most widespread mechanism of resistance developed by bacteria to escape the lethal action of β -lactam antibiotics (Bush 2010). β -Lactamases catalyse the irreversible hydrolysis of these compounds. The product of this reaction cannot inhibit their natural targets [the DD-transpeptidases, also known as penicillin-binding proteins (Frère 1995)]. Up to date, more than 500 different enzymes have been identified. Based on their sequences, three-dimensional structure and catalytic properties, they are divided into four groups (Bush 2010). β -Lactamases which display an essential serine residue can be categorized into three classes, A, C, and D, while a smaller number of enzymes, referred to as class B β -lactamases, are Zn (II)-dependent enzymes. The emergence of resistant strains has been a recurrent problem from the very beginning of the clinical use of penicillin. This has resulted in the development of new beta-lactam compounds whose inactivate or escape the activity of the most common enzymes. Bacteria, however, have responded by developing improved resistance mechanisms. In particular, the appearance of new or modified β -lactamases exhibiting broadened specificity spectra. The situation is worsened by the facts that an increasing number of clinical isolate's strains produce several β -lactamases and that the corresponding genetic materials can easily spread in the bacterial population by horizontal gene transfer (Davies 1994). Moreover, some bacterial strains have acquired resistance factors who make them resistant to all known antibiotics. Since new β -lactamases appear as a response to the introduction of new β -lactams, the use of non- β -lactam inhibitors or inactivators of the β -lactamases and DD-transpeptidases might be a solution. Today, compounds based on the diazabicyclo[3.2.1]octanone (DBO) scaffold are used in chemotherapy. Another strategy is the selection of protein/peptide derived from proteinaceous inhibitors that can bind the β -lactamases with high affinities. For example, camelid single-domain antibodies (VHH) that inhibits TEM-1 β -lactamase were isolated and hereafter referred to as cAb_{TEM13} or VHH. The VHHs are minimally sized, highly soluble entities that bind the antigen with nanomolar affinity (K. E. Conrath et al. 2001). Compared to monoclonal antibody fragments, VHHs are made of one polypeptide chain. They can easily be produced in recombinant organisms such as *Escherichia coli* (*E. coli*) and *Pichia pastoris*. They retain full activity compared to the HcAb from which they originate. On the opposite, Fabs, scFvs and other mAb fragments are made of two polypeptide chains, and their folding without the supporting scaffold of the original mAb may lead to specificity or sensitivity issues. Producing dimeric fragments is also more challenging.

The intrinsic biophysical robustness and monomeric architecture of VHHs therefore provide distinct advantages for both mechanistic studies and therapeutic development. Their small size allows access to recessed or conformationally dynamic epitopes, including active-site-adjacent or allosteric regions that may be less accessible to larger antibody formats. Accordingly, VHHs constitute attractive molecular tools for the detection, structural characterization, and inhibition of class A β -lactamases, including members of the TEM family. Beyond simple steric blockade of the catalytic site, high-affinity VHH binding may modulate enzyme function through conformational selection or dynamic allostery (Galdadas et al. 2021; Huang et al. 2020), thereby offering a complementary strategy to classical small-molecule inhibition.

In this work, we characterized the camelid antibody fragment cAb_{TEM13} targeting TEM-1 beta-lactamase, a widely disseminated class A β -lactamase impacting both human and veterinary health. Our objective was not only to evaluate its inhibitory properties but also to gain mechanistic insight into how VHH binding modulates enzyme function. To this end, we employed a complementary and integrative biophysical strategy. Steady-state and pre-steady-state kinetic analyses defined the inhibition profile and quantified binding and catalytic parameters. High-resolution X-ray crystallography of the TEM-1/cAb_{TEM13} complex provided a structural framework, revealing a binding epitope located adjacent to, but not directly occluding, the active site and highlighting local conformational rearrangements upon complex formation. Molecular dynamics (MD) simulations extended these static structural observations by characterizing the time-resolved behavior of the complex, validating observed conformational shifts and probing their propagation through the enzyme scaffold. Finally, NMR relaxation measurements enabled residue-specific characterization of conformational dynamics across multiple timescales, providing direct experimental evidence of dynamic redistribution upon VHH binding. Importantly, these approaches are mutually reinforcing. Crystallography defines the structural architecture of the complex; MD simulations test its stability and explore dynamic trajectories inaccessible to static structures; kinetic measurements link structural changes to functional consequences; and NMR relaxation directly reports on changes in internal motions in solution. Together, this multiscale analysis bridges structure, dynamics, and function.

Collectively, these complementary studies suggest functional coupling within TEM-1 upon VHH binding. The redistribution of conformational dynamics extends from the distal VHH interface to regions proximal to the catalytic site, indicating long-range communication between structurally separated regions of the enzyme. Such coordinated dynamic changes are consistent with a mechanism in which inhibition arises not solely from steric interference but from modulation of the enzyme's conformational landscape.

MATERIALS AND METHODS

X-ray Crystallography.

Determination of the holot_{TEM-1} (TEM-1/cAb_{TEM13}) and holot_{TEM-1} (TEM-121/cAb_{TEM13}) complexes. Prior to the crystallization, the TEM-1 β -lactamase solution was combined with the cAb_{TEM13} solution. The complex was concentrated and purified by a size-exclusion chromatography using a Superdex 75 10/300GL (Cytiva, United-States) and a 20 mM hepes pH 7,8 buffer. The holot_{TEM-1} complex was concentrated to 14 mg·mL⁻¹. The crystals were grown at 20°C using the sitting drop vapor diffusion method with drops made of 0.2 μ L of protein sample and 0.2 μ L of precipitant solution. The precipitant solution contained 0.1 M Bis-Tris propane pH 7.0, 1 M tri-ammonium citrate. Crystals were transferred in a cryo-protectant solution containing 1.8 M ammonium sulfate and 45 % (v/v) glycerol and 4.5 M Na formate and 50 % (v/v) glycerol before being frozen in liquid nitrogen.

Data collection, phasing, model construction and refinement for both structures.

Diffraction data were collected at the Proxima 2a and Proxima 1 beamlines of the Soleil synchrotron (Saint Aubin, France) for the holot_{TEM-1} and holot_{TEM-121} complexes

respectively. Indexing, integration and scaling of the data were carried out with the XDS Program Package (PMID: 20124692). The structure of the TEM-1 β -lactamase alone (PDB 1M40) and the structure of the cAb-H7S VHH specific of the BlaP β -lactamase from *Bacillus licheniformis* (PDB 4M3J) were used to obtain the initial phases of the holot_{TEM-1} complex by molecular replacement using the Phaser Crystallographic program (PMCID: [PMC2483472](#)). The same program was used to obtain the initial phases of holot_{TEM-121} complex by molecular replacement using the structure of the previously solved holot_{TEM-1} complex. The structures were built using the Coot program (Crystallographic Object-Oriented Toolkit) (PMCID: PMC2852313) and refined with Phenix refine tool of the Phenix suite (PMID: 22505256). The amino acid numbering scheme of the β -lactamases were based on the TEM-1 structure 1M40. The figures were prepared using PyMOL software (The PyMOL Molecular Graphics System, Version 3.1.3 Enhanced for Mac OS X, Schrödinger, LLC.). The PDB code for the structure of the holot_{TEM-1} complex is 9U0R.

NMR Sample Preparation. Uniformly [U-¹⁵N, ²H]-labeled and [U-¹⁵N, U-¹³C, ²H]-labeled Apot_{TEM-1} (free enzyme) and VHH were expressed and purified as described previously (Tomanicek et al. 2010; Meilleur et al. 2009; Tomanicek et al. 2011; Cawez et al. 2018) with some modifications (see Supporting Information for details) using 1 g/L of ¹⁵NH₄Cl and 4 g/L of {¹³C, ¹H}-glucose as the sole nitrogen and carbon sources in 100% D₂O. Uniformly [U-¹⁵N, ²H]- or [U-¹⁵N, U-¹³C, ²H]-labeled NMR samples of Apot_{TEM-1} and Holot_{TEM-1} (TEM-1/cAb_{TEM13} complex) were prepared at ~ 500 μ M in 50 mM potassium phosphate buffer (pH 6.6) containing 95% H₂O/5% D₂O and 40 μ M 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal chemical shift reference. Apot_{TEM-1} and nanobodies were mixed at a 1:1.1 molar ratio to form Holot_{TEM-1}.

NMR Spectroscopy.

¹⁵N Nuclear Spin Relaxation (NSR). ¹⁵N spin relaxation experiments ({¹⁵N}-R₁, {¹⁵N}-R₂, and ¹H-{¹⁵N} heteronuclear NOE) were acquired on Bruker Avance III spectrometers operating at 11.7 and 16.4 T, each equipped with a cryogenically cooled triple-resonance probe, at 30 °C. Longitudinal relaxation rates (R₁) (Zhu et al. 2000) were measured using 12 relaxation delays ranging from 10 to 165 ms, including two repetitions for error estimation. Transverse relaxation rates (R₂) (Zhu et al. 2000) were measured using 12 relaxation delays ranging from 8.48 to 152.64 ms, including two repetitions for error estimation. ¹H-{¹⁵N} heteronuclear NOE (¹H-{¹⁵N} NOE) (Zhu et al. 2000) values were determined from interleaved experiments recorded with and without proton saturation.

¹⁵N Relaxation Dispersion. ¹⁵N CPMG relaxation dispersion experiments were acquired at a static magnetic field strength of 16.4 T equipped with a cryogenically cooled triple-resonance probe, using the pulse sequence as described previously (Vallurupalli et al. 2007; Tollinger et al. 2001). Data were collected at 30 °C with 21 ν_{CPMG} values (T_{CPMG} = 50 ms) ranging from 40 to 1000 Hz, including three ν_{CPMG} repetitions for error estimation. Each 2D spectrum was acquired with 16 transients and (64, 1024) complex points in (t₁, t₂), corresponding to acquisition times of 20.04 and 91.75 ms, respectively, with a 3.0 s delay between scans.

¹⁵N CEST Experiments. NMR CEST experiments on Apot_{TEM-1} were performed as described previously (Yuwen, Kay, et al. 2018). A 2 s delay was alternated with DANTE (Bodenhausen, Morris, et al. 1976) trains of square pulses to enable simultaneous multi-

site excitation during the saturation period ($T_{EX} = 500$ ms). Two D-CEST spectral windows ($SW_{CEST} = 700$ and 800 Hz) were used, and CEST profiles were sampled every 25 Hz with two B_1^{D-CEST} field strengths (20 and 42 Hz), as recommended in prior studies (Khandave et al. 2024, 2023; Vallurupalli et al. 2017). B_1^{D-CEST} fields were calibrated using the nutation method (Guenneugues et al. 1999).

Visible peaks-positions. Visible peak positions were extracted from the ^{15}N D-CEST spectra ($SW_{CEST} = 700$ and 800 Hz) by referencing the peak positions obtained in control experiments recorded without the two B_1^{D-CEST} irradiation periods. Uncertainties in the visible peak positions were estimated from these spectra.

Data Analysis.

Model-free analysis, model selection and per-residue parameters. All NSR spectra were processed using Bruker TopSpin 3.6 (Bruker BioSpin) and analyzed using model-free analysis in Dynamics Center (Bruker BioSpin). For reliability, ^{15}N relaxation data collected at 11.7 and 16.4 T were analysed using model-free analysis (Hall and Fushman 2003; Tjandra, Feller, et al. 1995; Lipari and Szabo 1982b), following recommended best practices (Morin 2011). The workflow comprised: (i) calculation of the molecular moment of inertia tensor from structural coordinates (PDB 1M40 and 9U0R for Apo_{TEM-1} and Holo_{TEM-1} respectively), (ii) alignment and fitting of an effective, axially symmetric rotational diffusion tensor, and (iii) model-free analysis of $\{^{15}\text{N}\}\text{--R}_1$, $\{^{15}\text{N}\}\text{--R}_2$, and $^1\text{H}\text{--}\{^{15}\text{N}\}$ heteronuclear NOE data with statistical model selection.

For low-amplitude, fast motions, the ratio R'_2/R'_1 is essentially independent of internal dynamics and primarily reflects overall tumbling through the spectral density function. Here, primes denote relaxation rates corrected to remove high-frequency contributions to the spectral density (Walker et al. 2004).

The anisotropic rotational diffusion tensor was modelled as axially symmetric with six parameters (D_{xx} , D_{yy} , D_{zz} , θ , φ , ψ), where the Euler angles (θ , φ , ψ) define the orientation of the diffusion frame relative to the molecular frame. Parameter estimation used per-residue ^{15}N R'_2/R'_1 values together with atomic coordinates (Tjandra, Kuboniwa, et al. 1995). Residues exhibiting pronounced internal dynamics were excluded from the fit.

The six diffusion parameters were optimized by nonlinear least squares using the Levenberg–Marquardt algorithm to minimize the objective function E (Dosset et al. 2000; Fushman et al. 1999; Tjandra, Feller, et al. 1995; Walker et al. 2004), defined as the squared deviation between experimental and predicted R_2/R_1 ratios for the selected rigid-limit N–H bond vectors:

$$E = \sum_{i=1}^{N_{res}} \frac{(\rho_i^{exp} - \rho_i^{cal})^2}{\sigma_i^2}$$

where N_{res} is the total number of N–H bond vectors included in the analysis, the parameter ρ is defined as a ratio of the transverse and longitudinal relaxation rates,

$$\rho = \left(\frac{2 R'_2}{R'_1} - 1 \right)^{-1}$$

and σ_i denotes the experimental error in ρ_i for bond vector number i . The value of ρ^{exp} is directly derived from the measured relaxation parameters, while ρ^{cal} is calculated using expressions for the spectral densities $J(\omega)$ that depend on the rotational diffusion model (Fushman and Cowburn 2001; Ghose et al. 2001).

The anisotropic rotational diffusion tensor was modeled as axially symmetric, and its principal components and orientation were obtained by nonlinear numerical optimization using per-residue backbone relaxation parameters. The nonlinearly optimized principal components of the rotational diffusion tensor for Apo_{TEM-1} were $D_{xx} = 1.44 \times 10^7 \pm 1.16 \times 10^5 \text{ s}^{-1}$, $D_{yy} = 1.23 \times 10^7 \pm 9.37 \times 10^4 \text{ s}^{-1}$, and $D_{zz} = 1.31 \times 10^7 \pm 1.13 \times 10^5 \text{ s}^{-1}$ corresponding to an effective rotational correlation time ($\tau_c = 1/(6D_{iso})$) of 12.60 ns and an anisotropy $D_{\parallel}/D_{\perp}$ (Woessner 1962) of 1.16. For Holo_{TEM-1}, the optimized diffusion components were $D_{xx} = 6.50 \times 10^6 \pm 6.78 \times 10^4 \text{ s}^{-1}$, $D_{yy} = 8.35 \times 10^6 \pm 7.61 \times 10^4 \text{ s}^{-1}$, and $D_{zz} = 7.82 \times 10^6 \pm 8.00 \times 10^4 \text{ s}^{-1}$, giving an anisotropy of 1.56 and an effective rotational correlation time of 22.10 ns.

The principal components and orientation of the rotational diffusion tensor were used for performing standard local model-free analysis (Lipari and Szabo 1982b, 1982a) of $\{^{15}\text{N}\}$ -R₁, $\{^{15}\text{N}\}$ -R₂, and $^1\text{H}\{-^{15}\text{N}\}$ heteronuclear NOE (Kay et al. 1989; Morin 2011) was performed using five isotropic models derived from the original formulation of Lipari and Szabo as extended by Clore et al. (1990) (Clore et al. 1990) to include two separated timescales of internal motion, fast (τ_f) and slow (τ_s). In this approach, the spectral density is modeled as:

$$J(\omega) = \frac{2}{5} \sum_{i=0}^2 A_i \left[\frac{S^2 \tau_i}{1 + (\omega \tau_i)^2} + \frac{(1 - S_f^2) \tau'_{f,i}}{1 + (\omega \tau'_{f,i})^2} + \frac{(S_f^2 - S^2) \tau'_{s,i}}{1 + (\omega \tau'_{s,i})^2} \right]$$

where the square of the overall generalized order parameter is the product of the order parameters for the first and second internal motions, $S^2 = S_f^2 S_s^2$, $(\tau'_i)^{-1} = \tau_e^{-1} + \tau_i^{-1}$ with $\tau_0 = (6D_{\perp})^{-1}$, $\tau_1 = (D_{\parallel} + 5D_{\perp})^{-1}$ and $\tau_2 = (4D_{\parallel} + 2D_{\perp})^{-1}$.

All five models of the spectral density consider the axially symmetric anisotropic rotational diffusion tensor by using the following external correlation function:

$$C_0^{axial}(t) = \frac{1}{5} (A_0 e^{-t/\tau_0} + A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2})$$

where $A_0 = (1.5 \cos^2 \theta - 0.5)^2$; $A_1 = 3 \sin^2 \theta \cos^2 \theta$ and $A_2 = 0.75 \sin^4 \theta$ with θ being the angle between the N-H bond vector and the unique axis of the rotational diffusion tensor (Barbato et al. 1992; Jarymowycz and Stone 2006; Sakhrani et al. 2021).

The five model-free formulations, their parameters, and corresponding spectral density functions are summarized in **Table 1**. Models 1–4 assume a single effective timescale for internal motion ($S_s^2 = 1$), where S^2 reflects motions occurring on the pico- to nanosecond timescale. Models 1 and 3 assume the internal correlation time approaches zero ($\tau_f \rightarrow 0$), describing very fast motions ($\tau_f < 20 \text{ ps}$). Models 2 and 4 incorporate internal motions on a finite pico- to nanosecond timescale ($\tau_f = \tau_e$) (Mandel et al. 1995). Models 3 and 4 are

extensions of Models 1 and 2, respectively, that include an additional exchange term R_{ex} to account for conformational exchange and pseudo-first-order processes occurring on the micro- to millisecond timescale, as captured here by using CPMG (Bloom et al. 1965; Allerhand and Gutowsky 1965) and D-CEST (Yuwen, Kay, et al. 2018) at 16.4 T. Model 5 incorporates two distinct internal timescales: (i) ultrafast motions ($\tau_f \rightarrow 0$) with order parameter S_f^2 and (ii) slower nanosecond motions ($\tau_s = \tau_e$) with order parameter S_s^2 (Clore et al. 1990).

Model selection was performed using Akaike's Information Criterion (AIC), defined as:

$$AIC = \chi^2 + 2k$$

where

$$\chi^2 = \sum_{i=1}^n \frac{(R_i - R'_i)^2}{\sigma_i^2}$$

is the chi-square statistic measuring the goodness of fit between the experimental relaxation data (R) and the back-calculated values (R') (Abragam 1983). Here, σ denotes the experimental error, i is the residue index, k is the number of adjustable parameters in a given model, and n is the size of the data set for each residue. The AIC provides an optimal balance between underfitting (selection of overly simple models) and overfitting (selection of unnecessarily complex models) and has been shown to outperform traditional chi-square or F-test criteria in model selection (d'Auvergne and Gooley 2003). With six experimentally derived relaxation parameters per residue at two fields ($n = 6$), models 4 and 5 each involve three adjustable parameters ($k = 3$), yielding three degrees of freedom. Therefore, to prevent overfitting, models 1–3 were first evaluated, and models 4 and 5 were only considered when the model with the lowest AIC did not reproduce the experimental R_1 and R_2 values within 99% agreement.

Table 1. Spectral density functions and corresponding fitted parameter subsets.

Models	Subset of fitted parameters	Spectral density function
1	S^2	$J(\omega) = \frac{2}{5} \cdot \sum_{i=0}^2 A_i \cdot \left[\frac{S^2 \tau_i}{1 + (\omega \cdot \tau_i)^2} \right]$
2	$S^2, \tau_e(ps - ns)$	$J(\omega) = \frac{2}{5} \cdot \sum_{i=0}^2 A_i \cdot \left[\frac{S^2 \tau_i}{1 + (\omega \cdot \tau_i)^2} + \frac{(1 - S^2) \cdot \tau'_i}{1 + (\omega \cdot \tau'_i)^2} \right]$
3	S^2, R_{ex}	$J(\omega) = \frac{2}{5} \cdot \sum_{i=0}^2 A_i \cdot \left[\frac{S^2 \tau_i}{1 + (\omega \cdot \tau_i)^2} \right]; R_2^{obs} = R_2 + R_{ex}$
4	$S^2, \tau_e(ps - ns), R_{ex}$	$J(\omega) = \frac{2}{5} \cdot \sum_{i=0}^2 A_i \cdot \left[\frac{S^2 \tau_i}{1 + (\omega \cdot \tau_i)^2} + \frac{(1 - S^2) \cdot \tau'_i}{1 + (\omega \cdot \tau'_i)^2} \right]; R_2^{obs} = R_2 + R_{ex}$
5	$S_f^2, S^2 = S_f^2 S_s^2, \tau_e(ns)$	$J(\omega) = \frac{2}{5} \cdot \sum_{i=0}^2 A_i \cdot \left[\frac{S^2 \tau_i}{1 + (\omega \cdot \tau_i)^2} + \frac{(S_f^2 - S^2) \tau'_i}{1 + (\omega \cdot \tau'_i)^2} \right]$

¹⁵N CPMG Relaxation Dispersion and ¹⁵N D-CEST. ¹⁵N CPMG and ¹⁵N D-CEST spectra were processed and analyzed using the NMRPipe suite (Delaglio et al. 1995). Peak assignments were visualized and labeled in the ¹H–¹⁵N maps with Sparky(W. Lee et al. 2015) and peak intensities were extracted using PeakFit (<https://github.com/gbouvignies/PeakFit>). Effective transverse relaxation rates, $R_{2,eff}$ were calculated from the ratio of peak intensities according to

$$R_{2,eff} = -\frac{1}{T_{relax}} \ln \left(\frac{I(v_{CPMG})}{I_0} \right)$$

where T_{relax} is the constant CPMG relaxation period, $I(v_{CPMG})$ is the peak intensity at a given v_{CPMG} , and I_0 is the reference intensity.

Ap_{TEM-1} exchange parameters were obtained by independently fitting ¹⁵N CPMG relaxation dispersion data (Tollinger et al. 2001; Vallurupalli et al. 2007) and ¹⁵N D-CEST (Yuwen, Kay, et al. 2018), together with visible peak-position constraints (Khandave et al. 2023), whereas Hol_{TEM-1} parameters were determined by fitting simultaneously the ¹⁵N CPMG relaxation data and peak-position constraints. All ¹⁵N CPMG and D-CEST profiles were analysed using a two-state exchange process, $A \rightleftharpoons B$ where A and B refer to the ground and excited states, respectively, with populations p_A and p_B ($= 1 - p_A$), and $k_{ex} = k_{AB} + k_{BA}$, using ChemEx (<https://github.com/gbouvignies/chemex>). ChemEx numerically integrates (Korzhnev et al. 2004) the Bloch–McConnell equations (McConnell

1958) for various exchange models. Best-fit exchange parameters were obtained by minimizing the target function:

$$\chi^2 = \sum_i^n \frac{(m_i^{Exp} - m_i^{Calc})^2}{\sigma_i^2}$$

where m_i^{Exp} is the experimental measure (i.e. peaks intensities in the case of CPMG/D-CEST datasets or visible peak positions), m_i^{Calc} is the corresponding calculated peaks intensities or visible peak positions using the Bloch–McConnell equations over the duration of the T_{EX} period - both calculated values were determined from the Bloch–McConnell equations as described previously (Khandave et al. 2023), σ is the uncertainty in the experimental measurement, and the summation extends over all experimental data points (i) included in the fitting process.

Uncertainties in the best-fit parameters were determined using 1000 Monte-Carlo trials (Choy et al. 2005; Press 2007). In the Monte Carlo simulations, Gaussian noise was added to profiles generated from an initial fit, and the fitting was repeated. After N iterations, the distribution of fitted parameters provided the uncertainty estimates.

Global fitting parameters, shared across all residues, were the exchange rate, $k_{ex} = k_{AB} + k_{BA}$, and the minor B-state population p_B . Residue-specific parameters included the ^{15}N chemical shifts of the A and B states, the longitudinal (R_1) and transverse (R_2) relaxation rates. Relaxation rates for the two states were constrained to be equal ($R_{2,A} = R_{2,B}$ and $R_{1,A} = R_{1,B}$)

RESULTS

Structural Insights into cAb_{TEM-13} Binding to TEM-1 and Kinetic Characterization.

The crystal structure of holot_{TEM-1} (TEM-1/cAb_{TEM13}) was determined by X-ray crystallography. Holot_{TEM-1} crystallized in space group P4₃2₁2 and was refined to 1.64 Å resolution. The datasets exhibited good data quality, with high completeness (99.6%) and acceptable signal-to-noise ratios ($\langle I \rangle / \langle \sigma I \rangle$ of 19.7). Data collection and refinement statistics are summarized in **Table 2**. The cAb_{TEM13} binds to the hinge region of TEM-1, located in the loop connecting the $\alpha 10$ helix and $\beta 3$ strand, primarily through interactions involving CDR2 and CDR3. Specifically, residue D214 of TEM-1 forms two hydrogen bonds with Y52 and S103, located in CDR2 and CDR3 of the VHH, respectively (**Figure 1(a-c)**). In addition, D273, positioned on the $\alpha 12$ helix, establishes two hydrogen bonds with W100 from CDR3 of the VHH (**Figure 1 (c)**). The interaction interface is further stabilized by a hydrophobic cluster formed between Y52 and Y56 of CDR2 and the side chains of P219, R222, L225, and I231 located in the hinge region of TEM-1 (**Figure 1 (d)**).

Table 2. Data Collection and Refinement Statistics

	TEM-1/cAb _{TEM13}
Unit cell parameters (Å)	$a = 94.72, b = 94.72, c = 139.68$
Resolution (Å)	48.4 – 1.64 (1.74–1.64) ^a
R _{work} /R _{free}	14.7/18.2
R _{merge} (%)	13.2 (330) ^a
Completeness (%)	99.6 (97.4) ^a
$\langle I \rangle / \langle \sigma(I) \rangle$	19.7 (0.96) ^a
Number of protein residues	263
Number of atoms	3030
number of water molecules	429
rmsd bond lengths (Å)	0.015
rmsd angles (deg)	1.15
Ramachandran favored/allowed regions (%)	98.4/1.6

^a Values in parentheses are for the highest resolution shell.

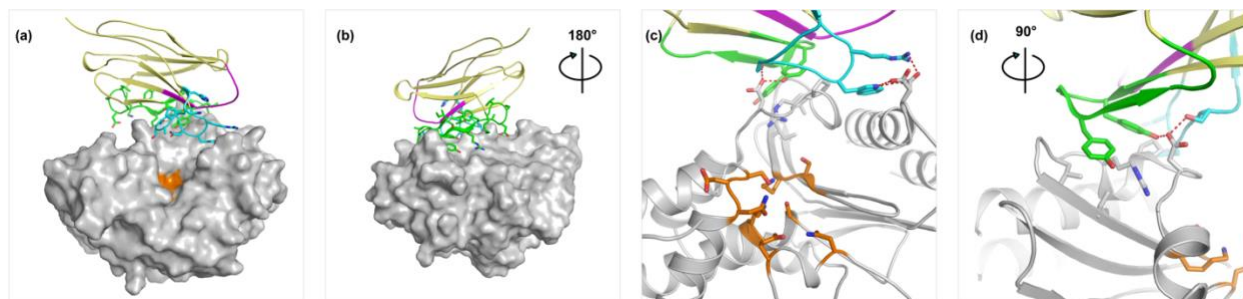


Figure 1. Crystal structure of the holotEM-1 complex. (a) Surface representation of TEM-1 with the VHH shown as a cartoon. Key side chains at the interaction interface are represented as sticks. The CDRs are colored as follows: CDR1 (magenta), CDR2 (green), and CDR3 (cyan). (b) Same representation as in (a) after a 180° rotation, highlighting the VHH binding interface. (c) Hydrogen-bonding interactions between cAb_{TEM13} and TEM-1. TEM-1 is shown in gray, with catalytic motifs S70XXK73 (motif 1), S130D131N132 (motif 2), and K234S235G236 (motif 3) highlighted in orange, together with residues E166 and N170 from the Ω-loop. Red dashed lines indicate hydrogen bonds between the VHH and TEM-1. (d) Hydrophobic interactions between CDR2 of the VHH and residues located in the hinge region of TEM-1.

Quantitative binding measurements using biolayer interferometry (BLI) to assess the specificity of cAb_{TEM13} toward representative β -lactamases from the four molecular classes A, B, C, and D were performed. These interaction assays demonstrated that the cAb_{TEM13} specifically binds to TEM-1 and TEM-121, a mutant of TEM-1 (**Figure 2 (a)**).

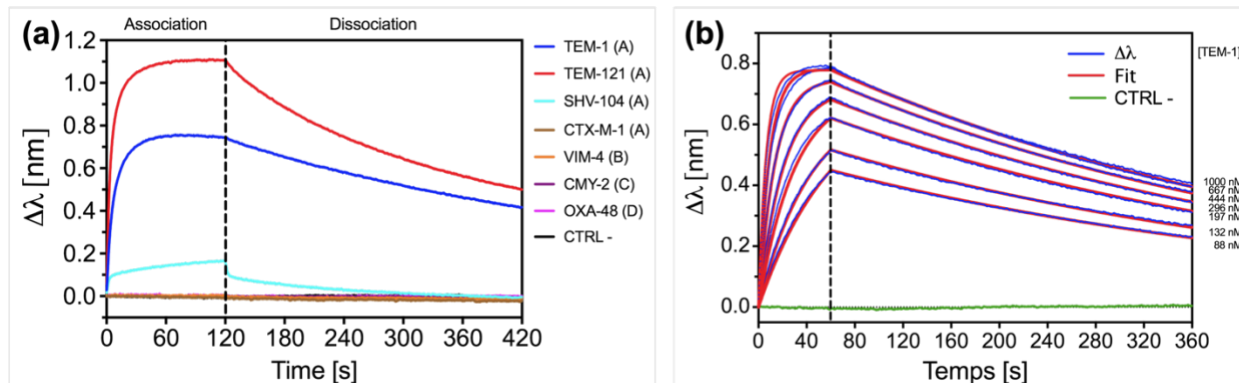


Figure 2. Binding characterization of cAb_{TEM13}. (a) Specificity of binding between the cAb_{TEM13} and representative β -lactamases from different molecular classes, as assessed by BLI. The names and corresponding classes (in brackets) of the β -lactamases are indicated. The negative control (CTRL-) corresponds to the loading of TEM-1 directly onto the sensor. All experiments were performed independently in duplicate. A: association phase; D: dissociation phase. (b) Quantitative analysis of the interaction between cAb_{TEM13} and TEM-1.

Subsequently, the association (k_{on}) and dissociation (k_{off}) rate constants, as well as the equilibrium dissociation constant (K_D), for the interaction between cAb_{TEM13} and TEM-1 (**Figure 2 (b)**) were determined. The resulting kinetic parameters are summarized in **Table 3**.

Table 3. Association (k_{on}), dissociation (k_{off}), and equilibrium dissociation (K_D) constants derived from global fitting of bio-layer interferometry data obtained at a minimum of seven TEM-1 concentrations, using a 1:1 binding model.

	k_{on} ($10^5 \text{ M}^{-1} \text{ s}^{-1}$)	k_{off} (10^{-3} s^{-1})	K_D (nM)
TEM-1	1.7 ± 0.1	2.2 ± 0.2	13 ± 2

Backbone Dynamics on the ps-ns timescale. Site-specific $\{^{15}\text{N}\}$ -R₁, $\{^{15}\text{N}\}$ -R₂ and ^1H - $\{^{15}\text{N}\}$ NOE values were obtained for 242 (ApO_{TEM-1}) and 218 (HolO_{TEM-1}) of the 251 non-proline backbone amide resonances at 16.4 and 11.7 T. These data, summarized respectively in **Figure 3** and **Figure 4**, provide residue-level insight into fast protein dynamics (Kay and Torchia 1998). For both enzyme forms, the $\{^{15}\text{N}\}$ -R₁ profiles exhibit the same residue-specific pattern at 16.4 and 11.7 T, and, as expected, $\{^{15}\text{N}\}$ -R₁(11.7 T) > $\{^{15}\text{N}\}$ -R₁(16.4 T). The $\{^{15}\text{N}\}$ -R₂ values are also in very good agreement between the two fields, and consistent with the CSA contribution to transverse relaxation (which is

proportional to ω_N^2 , $\{^{15}\text{N}\}\text{-R}_2(16.4\text{ T}) > \{^{15}\text{N}\}\text{-R}_2(11.7\text{ T})$ (Gagné et al. 1998; Savard and Gagné 2006).

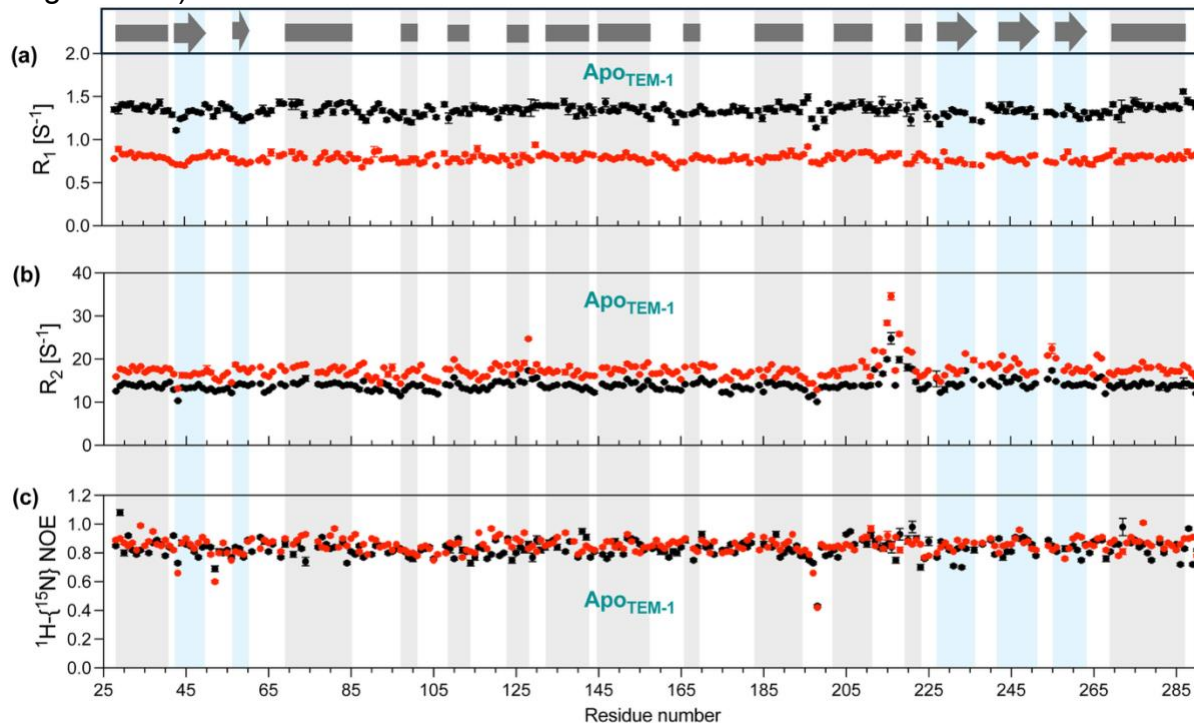


Figure 3. Experimental $\{^{15}\text{N}\}\text{-R}_1$, $\{^{15}\text{N}\}\text{-R}_2$ and $^1\text{H}\text{-}\{^{15}\text{N}\}$ NOE values for free TEM-1 β -lactamase at 16.4 T (color code: red) and 11.7 T (color code: black), recorded at 30° C. (a, b, c) Apo_{TEM-1} $\{^{15}\text{N}\}\text{-R}_1$, $\{^{15}\text{N}\}\text{-R}_2$ and $^1\text{H}\text{-}\{^{15}\text{N}\}$ NOE respectively.

Comparison of the relaxation parameters between the two functional states reveals the expected trend: $\{^{15}\text{N}\}\text{-R}_1(\text{Apo}_{\text{TEM-1}}) > \{^{15}\text{N}\}\text{-R}_1(\text{Holo}_{\text{TEM-1}})$, whereas $\{^{15}\text{N}\}\text{-R}_2(\text{Holo}_{\text{TEM-1}}) > \{^{15}\text{N}\}\text{-R}_2(\text{Apo}_{\text{TEM-1}})$, reflecting the increased overall tumbling time of the cAb_{TEM13}-bound complex. Notably, residues in the hinge region (212–224) display elevated $\{^{15}\text{N}\}\text{-R}_2$ values in Apo_{TEM-1}, indicative of conformational exchange on the microsecond-to-millisecond (μs -ms) timescale. These elevated $\{^{15}\text{N}\}\text{-R}_2$ values are absent in Holo_{TEM-1}, suggesting that VHH binding suppresses this local exchange process. The $^1\text{H}\text{-}\{^{15}\text{N}\}$ NOE values at 11.7 and 16.4 T show identical patterns for both enzymes. Strikingly high $^1\text{H}\text{-}\{^{15}\text{N}\}$ NOE values are observed across the entire sequence, including the N- and C-termini, indicating that both Apo_{TEM-1} and Holo_{TEM-1} are well ordered throughout, consistent with earlier observations (Savard and Gagné 2006; Doucet et al. 2007).

Model-free analysis (MFA) provided quantitative characterization of both global and local motions. The global rotational correlation time (τ_c) and the degree of anisotropy of overall molecular rotation were determined as detailed in the **Supporting Information**. As summarized in **Figure 5**, the MFA demonstrates that both Apo_{TEM-1} and Holo_{TEM-1} are highly ordered, with identical average generalized order parameter, $S^2 = 0.86 \pm 0.01$. These results are consistent with the uniformly high $^1\text{H}\text{-}\{^{15}\text{N}\}$ NOE values observed across the sequence and are in agreement with previously reported analyses of TEM-1 backbone dynamics (Savard and Gagné 2006; Doucet et al. 2007). These findings, taken together, indicate that the ps–ns backbone dynamics of TEM-1 are essentially unchanged

upon VHH binding. Per-residue model-free parameters, together with the corresponding AIC values and selected models, are provided in **Table S2** and **S3** of the **Supplementary Information**.

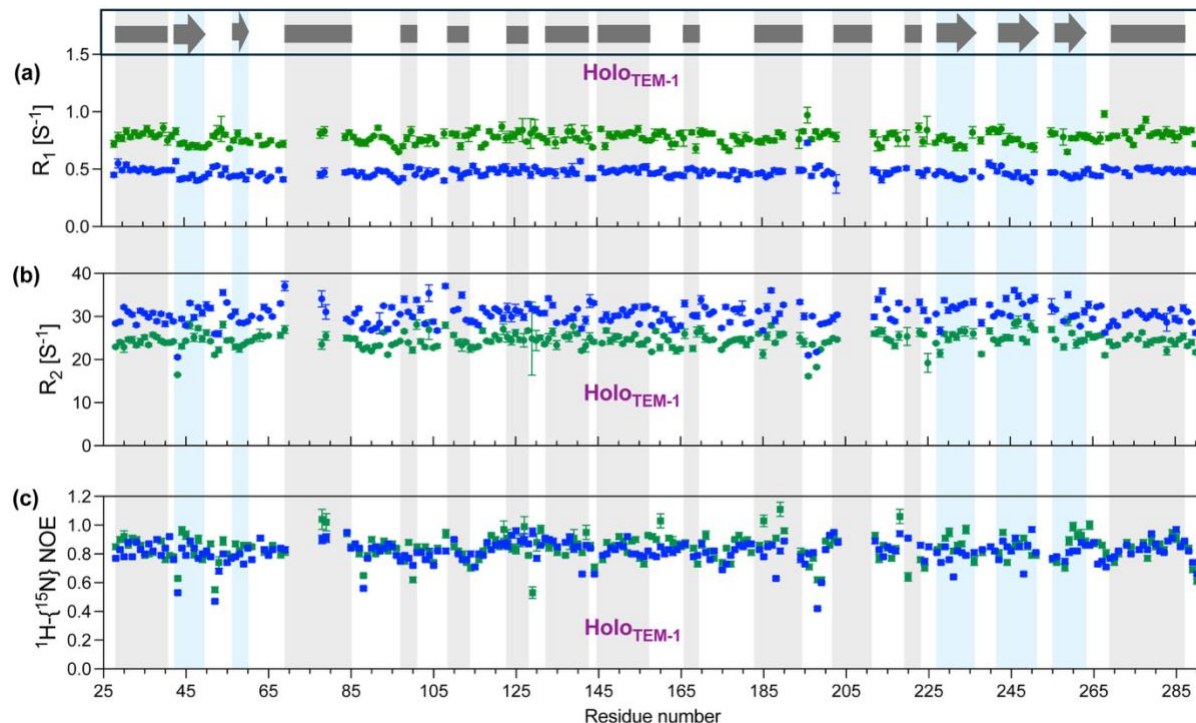


Figure 4. Experimental $\{^{15}N\}$ - R_1 , $\{^{15}N\}$ - R_2 and $^1H\{-^{15}N\}$ NOE values for TEM-1 β -lactamase at 16.4 T (color code: blue) and 11.7 T (color code: green) with cAb_{TEM13}, recorded at 30° C using 500 μ M protein and an [inhibitor]/[protein] ratio of 1.1. (a, b, c) Holo_{TEM-1} $\{^{15}N\}$ - R_1 , $\{^{15}N\}$ - R_2 and $^1H\{-^{15}N\}$ NOE respectively.

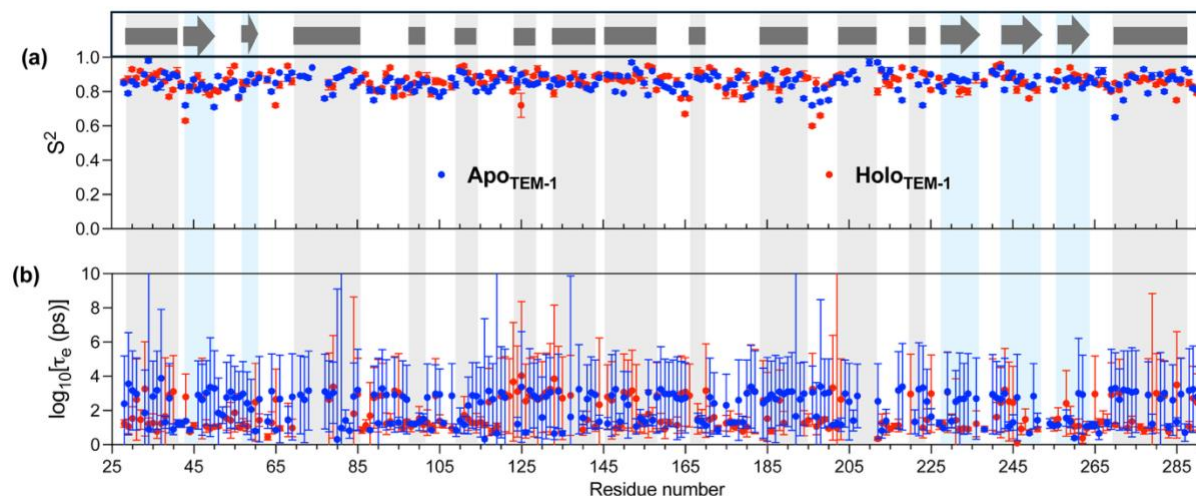


Figure 5. Residue-specific model-free analysis results for TEM-1 β -lactamase showing (a) generalized order parameters S^2 and (b) local correlation times (τ_e) on the ps–ns timescale, in the absence (blue) and presence (red) of cAb_{TEM13}.

Backbone NMR Dynamics Reveal a Minor, Structurally Distinct TEM-1 States. To gain insight into TEM-1 inhibition by the cAb_{TEM13}, we investigated potential conformational exchange on the microsecond–millisecond timescale in Apo_{TEM-1} and Holo_{TEM-1}.

For Apo_{TEM-1}, global and residue-specific fitted parameters reveal exchange processes occurring on two distinct timescales. One set of twenty-two residues (R43, V44, L169, N170, A172, I173, W210, E212, A213, D214, K215, V216, A217, G218, G236, E240, R241, G242, S243, G245, Y264 and G267) exhibits slower exchange rate (**Figure 6**), with $k_{ex} = 112 \pm 3 \text{ s}^{-1}$ and relative population, $p_B = 1.85 \pm 0.03 \%$. Residues undergoing chemical exchange cluster around the catalytic serine (S70) and along the interface between the α -helices and β -sheets that form the α/β domains, including the cAb_{TEM13}-binding site and Ω -loop (**Figure 6**).

A second set of sixteen residues (E37, E48, M69, T71, Y105, S106, T128, S130, D131, H153, H158, E171, S203, L225, I231 and I287) exhibits intermediate-exchange dynamics between two states, with $k_{ex} = 772 \pm 3 \times 10^{-12} \text{ s}^{-1}$ and $p_B = 2.32 \pm 1.04 \times 10^{-14} \%$. These parameters were highly reproducible across the 1000 Monte Carlo trials, with estimated uncertainties of $3 \times 10^{-12} \text{ s}^{-1}$ for k_{ex} and $1.04 \times 10^{-14} \%$ for p_B . The exceptionally small uncertainties suggest that the fits are strongly constrained by the single-field CPMG data or that Monte Carlo analysis alone may not reliably estimate parameter uncertainties under these conditions. To evaluate the accuracy of the k_{ex} and p_B values obtained from single-field CPMG analysis, several additional robustness checks were therefore performed.

First, a three-stage grid search over k_{ex} was performed, in which k_{ex} was systematically fixed after grid search and p_B was fitted. A logarithmically spaced grid was used to ensure coverage of the full sensitivity range. In stage 1, a coarse grid search was conducted with k_{ex} values ranging from 30 to 30000 s^{-1} using 61 points. In stage 2, a refined grid search was performed around the minimum, with k_{ex} spanning 200–3000 s^{-1} over 118 points. In stage 3, an optional ultra-refined grid search was carried out with k_{ex} values between 600 and 1000 s^{-1} using 80 points. Following the three-stage grid search, the estimated k_{ex} values were 754, 766, and 772 s^{-1} for stages 1–3, respectively (**Figure 7 (a)**), with corresponding p_B values of 2.07%, 2.05%, and 2.32%. As the grid search was progressively refined, both k_{ex} and p_B converged toward the globally fitted values obtained from the 1000 Monte Carlo trials.

Next, an analogous three-stage grid search was performed over p_B , in which p_B was fixed and k_{ex} was fitted. In stage 1, a logarithmically spaced grid spanning p_B values from 0.001 to 0.20 was used with 47 points. In stage 2, a refined grid search was conducted around the minimum, with p_B ranging from 0.012 to 0.035 over 23 points. In stage 3, an optional ultra-refined grid search was performed with p_B values between 0.005 and 0.05 using 51 points. Against, as the grid search was progressively refined, the fitted parameters converged toward the globally determined values obtained from the 1000 Monte Carlo trials. The resulting p_B estimates were 3.17%, 2.61%, and 2.29% for stages 1–3, respectively (**Figure 7 (b)**), with corresponding k_{ex} values of 846, 806, and 769 s^{-1} . The close agreement across grid resolutions shows the internal consistency of the fitting procedure and supports the accuracy of the exchange parameters derived from the Monte Carlo analysis.

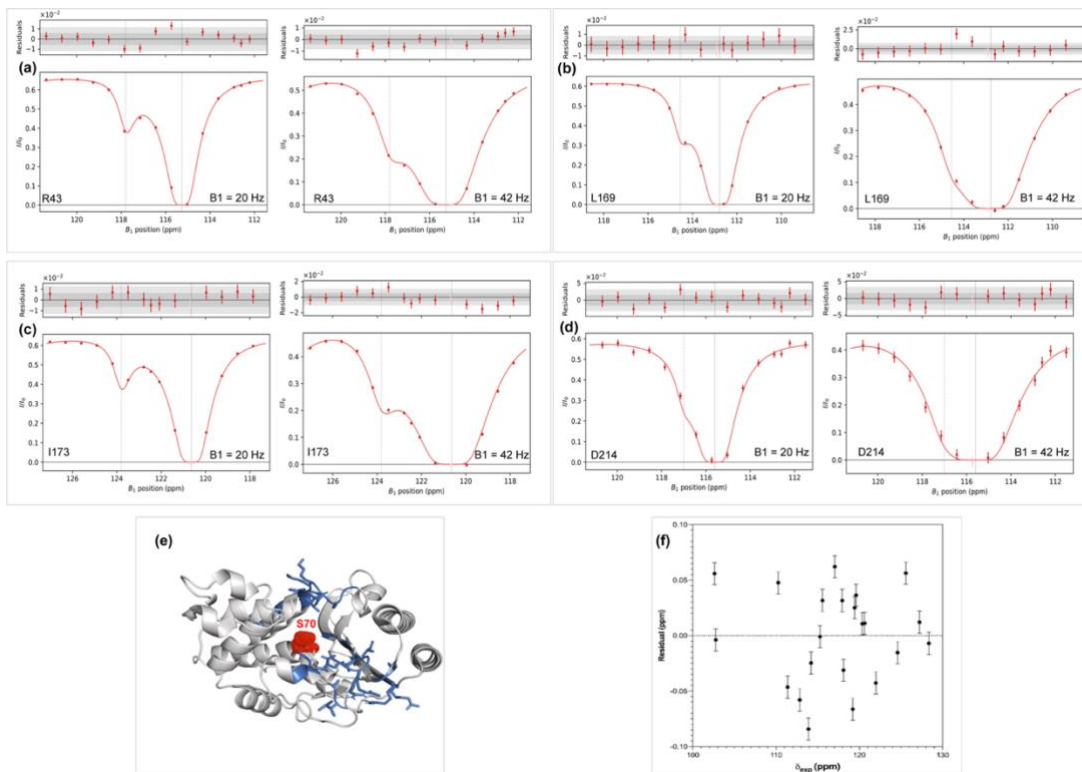


Figure 6: Conformational exchange in apoTEM-1 recorded at 30 °C and $B_0 = 16.4$ T using $\text{SW}_{\text{CEST}} = 700$ Hz and $B_1^{D\text{-CEST}}$ field strengths of 20 Hz and 42 Hz. (a–d) ^{15}N D-CEST profiles for residues R43, L169, I173, and D214, respectively. (e) Residues shown in blue exhibit ^{15}N D-CEST–detected conformational exchange in apoTEM-1, active-site S70 in red. (f) Residuals from the linear regression between experimental and predicted visible-state peak positions. Panel (e) was generated in PyMOL (Schrödinger, LLC) using PDB entry 1M40.

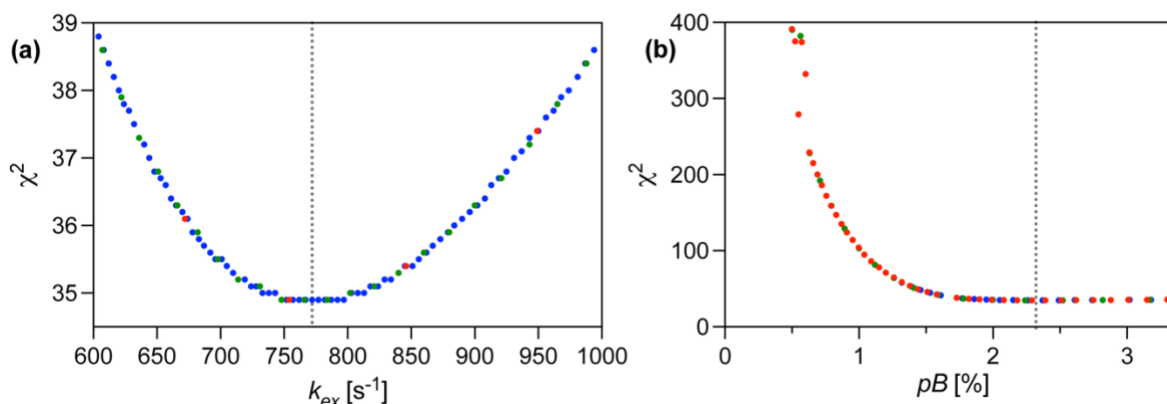


Figure 7: Logarithmically spaced grid search over k_{ex} and p_B . (a) k_{ex} grid-search results. (b) p_B grid-search results. Stage 1 is shown in red, stage 2 in green, and stage 3 in blue.

Residues exhibiting intermediate-exchange are located in the Ω -loop, the S130–D131–N132 motif and across β -sheet elements of the α/β domain (**Figure 8 (e)**). The S130–D131–N132 motif has been shown to contribute to local structural stability and may also influence enzymatic function (Jacob et al. 1990). In particular, S130 participates in the hydrogen-bonding network, while N132 contributes to transition state stabilization through a hydrogen bond with the O16 atom of benzylpenicillin (Jacob et al. 1990). The dynamic behavior observed in this region suggests that the S130–D131–N132 motif undergoes conformational exchange that may influence the organization of the hydrogen-bonding network. Such motions could facilitate subtle rearrangements required for efficient substrate binding and transition state stabilization.

^{15}N CEST and CPMG residues undergoing exchange are predominantly clustered around the catalytic serine, although not all participating residues are localized to this region. Taken together, the ^{15}N CEST- and CPMG-derived exchange profiles reveal at least two distinct conformational processes in Apo $_{\text{TEM-1}}$. Residues exhibiting exchange exclusively in the ^{15}N CEST data most likely report on a relatively slow, low-population conformational transition that is spatially localized (**Figure 6 (e)**). We propose that this motion reflects pre-organization or micro-adjustments within the active site or adjacent structural elements that modulate catalytic geometry. In contrast, residues detected only in the ^{15}N CPMG relaxation dispersion experiments are consistent with an intermediate-timescale process that reflects a more collective structural rearrangement.

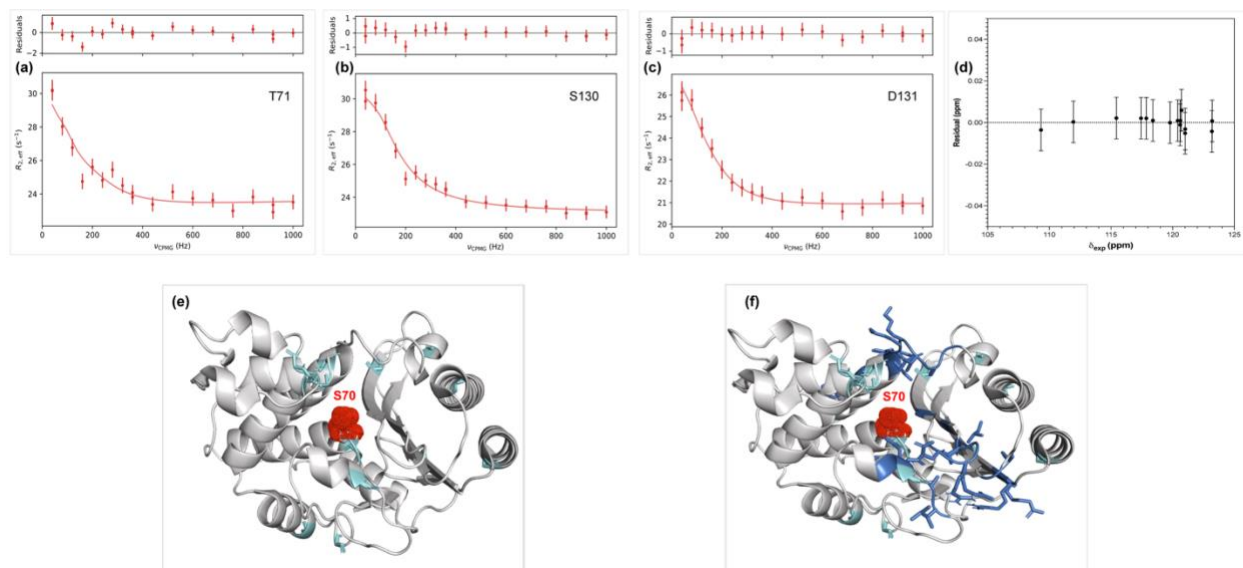


Figure 8: Conformational exchange in apo $_{\text{TEM-1}}$ recorded at 30 °C and $B_0 = 16.4$ T using ^{15}N CPMG with $T_{\text{EX}} = 50$ ms. (a–c) ^{15}N CPMG profiles for residues T71, S130 and D131, respectively. (d) Residuals from the linear regression between experimental and predicted visible-state peak positions. (e) Residues shown in cyan exhibit ^{15}N CPMG-detected conformational exchange in apo $_{\text{TEM-1}}$. (f) apo $_{\text{TEM-1}}$ overall residues (for both D-CEST & CPMG detected; blue and cyan respectively) in exchange. Panels (e–f) were generated in PyMOL (Schrödinger, LLC) using PDB entry 1M40

The combined analysis of ^{15}N CEST and CPMG data was initially approached using a two-state exchange model. However, satisfactory global fits could not be obtained, suggesting that the exchange behavior is more complex than a single $A \rightleftharpoons B$ process. Furthermore, residues displaying detectable excited-state signatures in the CEST profiles and those exhibiting CPMG relaxation dispersion were largely distinct, indicating that the two experiments probe different exchange processes.

Several three-state exchange schemes were therefore considered, including linear ($A \rightleftharpoons B \rightleftharpoons C$) and triangular ($A \rightleftharpoons B \rightleftharpoons C$ and $A \rightleftharpoons C$) models. Owing to the increased number of exchange parameters and the limited experimental constraints available, particularly the use of single-field CPMG data, these models resulted in overparameterization and poor parameter identifiability.

The observation that CEST and CPMG effects are detected for largely distinct sets of residues suggests that the two experiments may probe different exchange processes involving a common ground state. One possible interpretation of these observations is a forked three-state scheme in which the ground state A exchanges independently with two low-populated states, B and C ($B \rightleftharpoons A \rightleftharpoons C$). However, no explicit fit of such a model was performed, and it is therefore discussed only as a conceptual framework for interpreting the experimental observations rather than as a demonstrated kinetic mechanism.

Our data support a model in which apo_{TEM-1} samples two kinetically distinct, independent two-state exchange processes, consistent with previous reports on the conformational dynamics of TEM-1 (Knoverek et al. 2021). The detection of exchange across residues distributed throughout the protein, including the active site, further indicates that these processes reflect the selective sampling of distinct regions within a heterogeneous conformational landscape. Complete residue-specific parameters, including minor-state ^{15}N chemical shifts, are provided in **Tables S4 and S5**.

For Holot_{TEM-1}, the ^{15}N CPMG experiment performed on TEM-1 in complex with the inhibitory cAb_{TEM13}, reveal a marked increase in the molecular dynamics (**Figure 9**), characterized by $k_{ex} = 846 \pm 17 \text{ s}^{-1}$ and $p_B = 3.28 \pm 0.06 \%$.

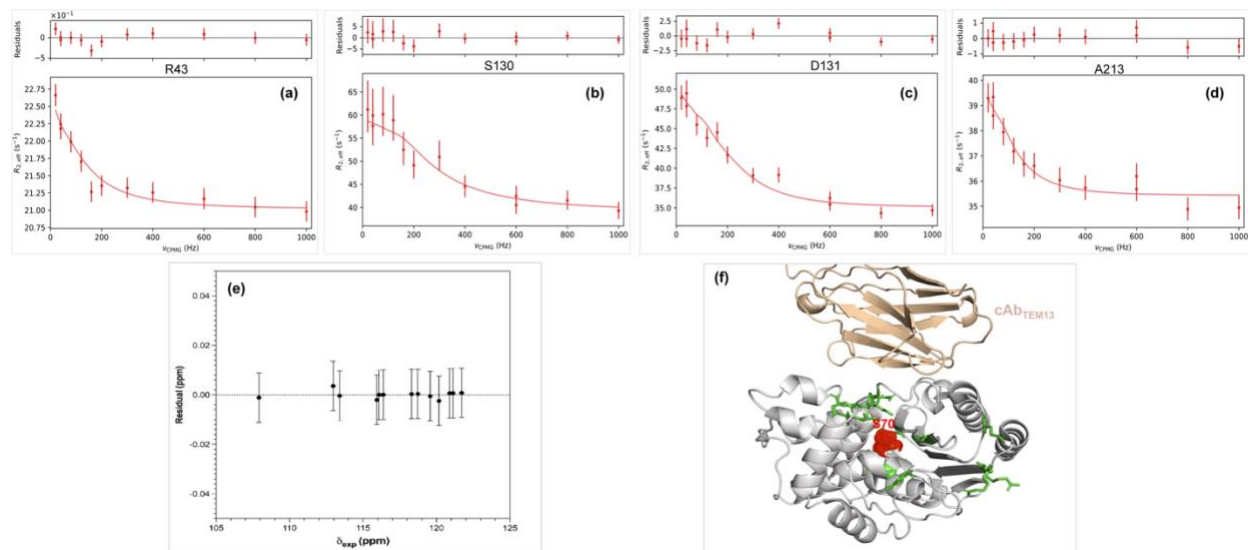


Figure 9: Conformational exchange in holotEM-1 recorded at 30 °C and $B_0 = 16.4$ T using ^{15}N CPMG with $T_{EX} = 50$ ms. (a–d) ^{15}N CPMG profiles for residues R43, S130, D131 and A213, respectively. (e) Residuals from the linear regression between experimental and predicted visible-state peak positions. (f) Residues shown in green exhibit ^{15}N CPMG–detected conformational exchange in HoloTEM-1. Panel (f) was generated in PyMOL (Schrödinger, LLC) using PDB entry 9U0R.

The thirteen residues exhibiting exchange can be grouped into two categories: (i) residues displaying an increase contribution from slow exchange relative to previous ApoTEM-1 ^{15}N D-CEST and ^{15}N CPMG measurements. Precisely, residues R43, M68, Y105, S106, S130, D131, L169, A213, D214, K234, G238, G245 and Q269 that exhibits molecular dynamics in holotEM-1 shows significantly enhanced dynamics relative to the apoTEM-1 state, consistent with an overall increase in conformational fluctuations (**Figure 10 (a–d)**). (ii) Additional residues M68, K234, G238, and Q269 exhibit exchange only in the cAb_{TEM13}-bound form, suggesting that VHH binding alters exchange kinetics and populations, making these processes experimentally detectable (**Figure 10 (e–f)**). Collectively, these results show that cAb_{TEM13} binding modulates the intrinsic conformational dynamics of TEM-1, including microsecond–millisecond motions at sites distant from the binding interface. The presence of correlated dynamic changes throughout the protein upon cAb_{TEM13} binding, including at sites distal to the binding interface, is consistent with long-range dynamic coupling. This is consistent with prior evidence that the hinge region; targeted by the VHH; acts as a key initiation site for allosteric communication within TEM-1 (Meneksedag et al. 2013; Avery et al. 2022; Galdadas et al. 2021). Complete residue-specific parameters for holotEM-1, including minor-state ^{15}N chemical shifts, are provided in **Table S6**.

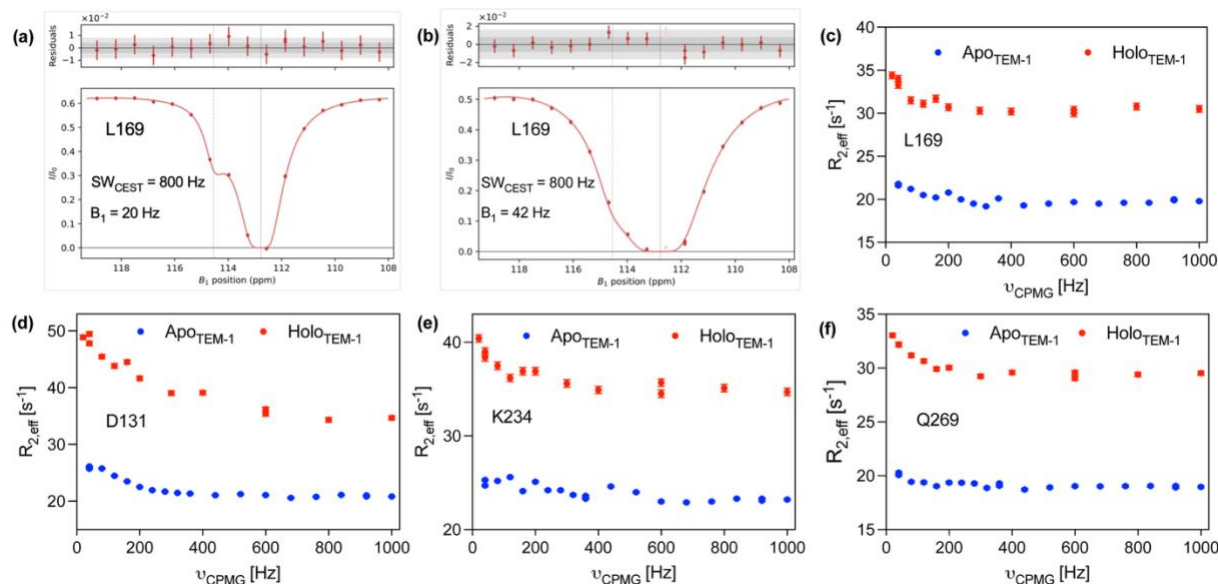


Figure 10. Representative ^{15}N CPMG relaxation dispersion and D-CEST profiles illustrating two classes of residues exhibiting chemical exchange in holo_{TEM-1}. Panels show: (a–c) ^{15}N D-CEST and CPMG profiles for L169; (d) ^{15}N CPMG profiles for D131 in apo_{TEM-1} and holo_{TEM-1}; (e) ^{15}N CPMG profiles for K234 in apo_{TEM-1} and holo_{TEM-1}; and (f) ^{15}N CPMG profiles for Q269 in apo_{TEM-1} and holo_{TEM-1}.

DISCUSSION AND CONCLUSIONS

Fast Backbone Dynamics Establish Highly Ordered Enzymes. Understanding how enzyme dynamics relate to function, and how inhibitors modulate these dynamics, is central to deciphering the mechanisms of enzymatic inhibition, particularly in systems where the three-dimensional structure alone does not provide sufficient insight, as is the case for class A β -lactamases TEM-1 bound to cAb_{TEM13}. To elucidate the mechanisms underlying VHH inhibition of TEM-1, we characterized fast and slow backbone dynamics in both the Apo and Holo enzyme states.

The Apo_{TEM-1} NSR data obtained in this study were compared with previously reported measurements (Savard and Gagné 2006) acquired at 500, 600 and 800 MHz. Because $^1\text{H}\{-^{15}\text{N}\}$ steady-state NOE data were not available at 800 MHz in the earlier study, our $^1\text{H}\{-^{15}\text{N}\}$ steady-state NOE values measured at 700 MHz were instead compared with published data recorded at 600 MHz. Overall, the NSR data obtained here reproduce the same residue-specific patterns observed in the reported measurements (**Figures 11**), thereby validating the robustness of the fast-timescale dynamic parameters derived for Apo_{TEM-1}.

Consequently, MFA, which characterizes global tumbling through the global correlation time (τ_c) and local backbone motions through generalized order parameter (S^2), led to results that are in excellent agreement with previously reported data (**Figure 11 (g)**). Specifically, we obtained a τ_c of 12.6 ns and an average generalized order parameter of $S^2 = 0.86 \pm 0.01$, values that closely match earlier estimates of τ_c of 12.3 ns (Fisette et

al. 2010) and an average order parameter of 0.85 (Savard and Gagné 2006). Taken together, these observations indicate that Apo_{TEM-1} is well-ordered on fast timescales, in agreement with previous reports, with limited backbone flexibility as reflected in its ps–ns dynamics.

The global tumbling time of Holo_{TEM-1} increased to $\tau_C \approx 22.1$ ns, in line with the higher molecular weight of the complex upon cAb_{TEM13} binding (from ~ 30 to ~ 45 kDa). Despite this increase in molecular weight, the average generalized order parameter remained unchanged relative to apo_{TEM-1}, indicating that cAb_{TEM13} binding does not alter fast-timescale backbone order.

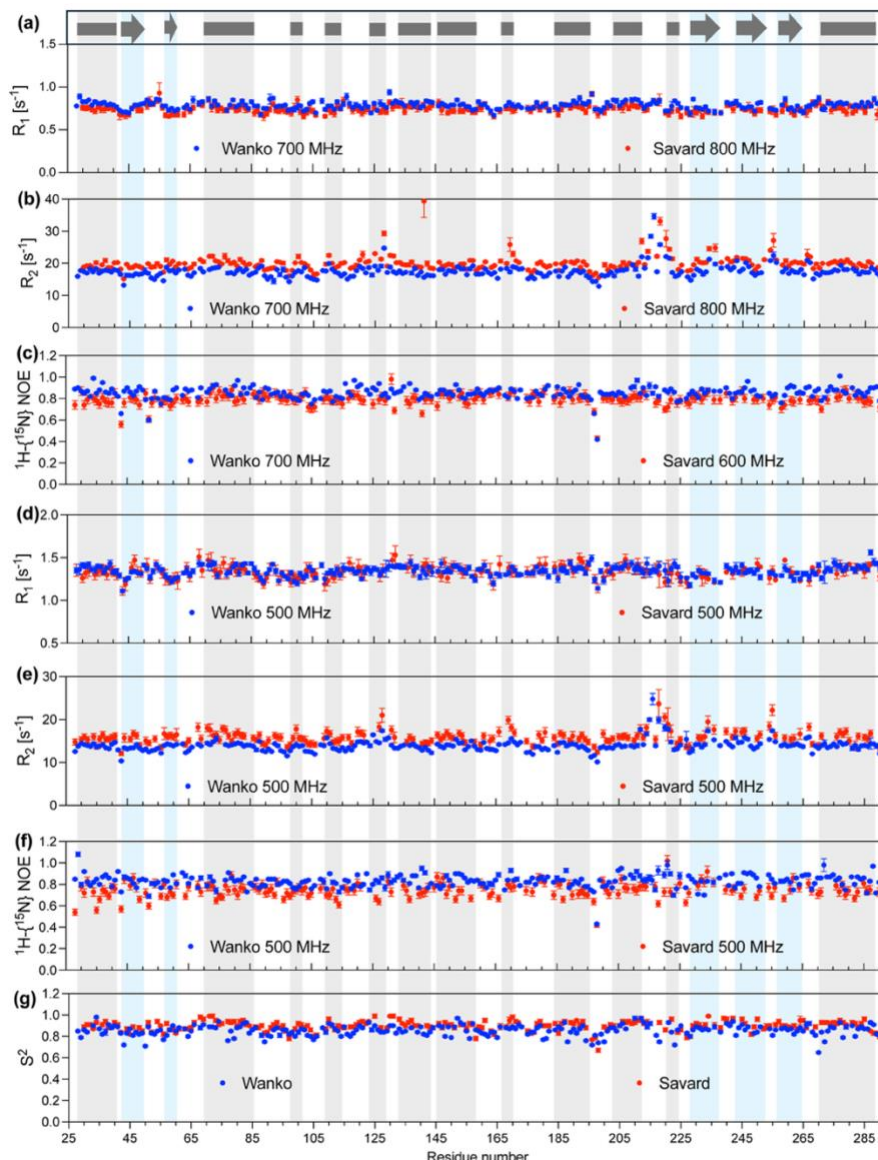


Figure 11. Comparison of NMR data for TEM-1 obtained in this study with previously reported measurements. Panels (a–c) show the comparison of our 700 MHz NMR data with the NMR data reported by Savard *et al.*; panels (d–f) show the corresponding comparison of our 500 MHz NMR data with the Savard *et al.* dataset. (g) Comparison of generalized order parameter S^2 .

Modulation of the Conformational Energy Landscape in cAb_{TEM13}-Bound TEM-1.

The global fitting parameters derived from our apo_{TEM-1} ¹⁵N CEST experiments were compared with those reported in a recent study (Knoverek et al. 2021; Doucet et al. 2007; Fisette et al. 2010; Savard and Gagné 2006), which focused specifically on residues within the Ω-loop. In that work, the exchange rate and excited-state population were reported as $k_{ex} = 98 \pm 6 \text{ s}^{-1}$ and $p_B = 1.05 \pm 0.03 \%$, respectively. These values are in close agreement with the CEST parameters obtained in the present study ($k_{ex} = 112 \pm 3 \text{ s}^{-1}$ and $p_B = 1.85 \pm 0.03 \%$). Residues undergoing slow exchange in the earlier report were localized around the Ω-loop pocket, including L169, N170, G238, E240, G242, and S243. Notably, all these residues also exhibit slow-timescale dynamics in our measurements, indicating that the same conformational process is detected despite differences in experimental scope. By contrast, global fitting parameters (k_{ex} and p_B) from ¹⁵N CPMG relaxation dispersion experiments were not reported previously. Nevertheless, several residues (M9, T71, E171, and G236) were identified as exhibiting intermediate-timescale dynamics. Except for G236, which displays slow exchange behavior in our dataset, the remaining residues similarly exhibit intermediate-timescale dynamics in the present study. Together, agreement between residue-specific dynamic regimes observed here and those reported previously, supports the robustness of the detected slow and intermediate motions and indicates that these processes extend beyond the Ω-loop to involve a broader network of residues throughout the enzyme.

NMR relaxation analysis of the Holo_{TEM-1} enzyme on the millisecond–microsecond timescale proved challenging, primarily due to its ~ 45 kDa molecular weight, which results in increased transverse relaxation rate and substantial line broadening. The loss of peak intensity reduced the sensitivity of the millisecond–microsecond relaxation measurements, thereby limiting the number of residues amenable to quantitative analysis. Although ¹⁵N D-CEST experiments were not performed and slower timescale processes were therefore not comprehensively examined, ¹⁵N CPMG relaxation dispersion experiments enabled characterization of intermediate-timescale dynamics. A modest subset of residues (thirteen) exhibited significant exchange contributions in this regime. Despite their limited number, these residues are distributed throughout the protein rather than confined to a single localized region. This spatial distribution indicates that VHH binding perturbs conformational fluctuations beyond the immediate binding interface and is consistent with long-range dynamic coupling between distal regions and the active site.

Direct comparison of the apo_{TEM-1} and holo_{TEM-1} states reveals a modulation of exchange parameters upon binding of the inhibitory cAb_{TEM13}. The Holo_{TEM-1} form displays increased exchange rates and minor-state populations relative to apo_{TEM-1}. Although excited states are observed in the apo and holo forms, the underlying exchanging states remain largely similar. These observations suggest that nanobody binding primarily modulates the populations and kinetics of pre-existing conformations, rather than indicating the emergence of new states or a substantial reshaping of the conformational energy landscape.

Therefore, VHH binding fine-tunes the dynamic equilibrium within the existing energy landscape. The redistribution of conformational dynamics across residues extending beyond the binding interface suggests functional coupling between the VHH interaction

site and catalytically relevant regions. Although the present data do not define a specific allosteric signaling pathway, they are consistent with a model in which inhibition involves dynamic allosteric modulation of the conformational landscape, whereby redistribution of low-populated conformational substates may alter the functional ensemble of the enzyme. Previous simulation studies of apoTEM-1 have identified the cAbTEM13-binding site, commonly referred to as the hinge region (residues 212–224), as the point of origin of the allosteric signal (Galdadas et al. 2021). Consistent with this model, our data show that the hinge region in apoTEM-1 undergoes slow-timescale dynamics, indicating conformational heterogeneity in this region. Notably, this region is also the primary site of perturbation in nonequilibrium simulations (Galdadas et al. 2021) and adopts two distinct conformations in high-resolution X-ray structures (PDB ID: 1M40). Located on the loop connecting the distal end of helix H11 to β -strand β 7, is the highly conserved residue W229, which is preserved across multiple classes of β -lactamases (Meneksedag et al. 2013). The indole side chain of W229 is sandwiched between two other conserved residues, P226 and P252, positioned on the H11– β 7 and β 8– β 9 loops, respectively. This P226–W229–P252 π -stacking interaction forms a rigid structural motif that may play a key role in modulating allosteric communication between the active site and the allosteric site (Meneksedag et al. 2013; Avci et al. 2016).

Disruption of this hydrophobic triad; either by the W229A mutation (Meneksedag et al. 2013) or by small-molecule allosteric inhibitors (J. R. Horn and Shoichet 2004); has been shown to alter internal motions and perturb the conformational dynamics required for efficient catalysis. More recently, computational studies have described signal transmission from this allosteric site through cooperative coupling of loop dynamics, including loops distal to the perturbation site (Galdadas et al. 2021). Upon interaction with cAbTEM13, Pro226 is displaced, disrupting the Pro226–Trp229–Pro252 π -stacking interaction (**Figure 12**) and based on previous studies, may be linked to the observed overall enhancement of molecular dynamics.

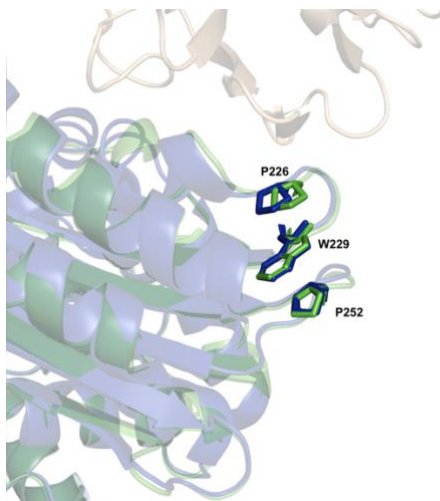


Figure 12. Perturbation of the P226–W229–P252 π -stacking interaction upon cAbTEM-1 binding. ApoTEM-1 is shown in blue, holotEM-1 in green, and the cAbTEM13 in tinted shades.

This dynamic perturbation propagates toward the active site, where subtle micro-adjustments and larger-amplitude motions of structural elements required to maintain catalytic geometry are altered. Specifically, three effects are observed: (i) intermediate-timescale motions are quenched for a subset of residues, (ii) the population of minor conformational state is substantially increased, and (iii) the underlying conformational landscape is modulated upon VHH binding. Although only thirteen residues exhibit detectable intermediate-regime exchange in Holo_{TEM-1}, the detection of these enhanced minor-state population across residues distributed throughout catalytically relevant regions, including Ω -loop and SDN motif, provides strong evidence for coupling between the distal allosteric cAb_{TEM13}-binding site and the enzyme's catalytic machinery. Interestingly, residues within the catalytically critical S130–D131–N132 loop display enhanced dynamics upon cAb_{TEM13} binding. To our knowledge, this study constitutes the first direct NMR-based characterization of the dynamics of this loop, which has been shown by site-directed mutagenesis to be critical for structural stability and function (Jacob et al. 1990).

These results indicate that the VHH may act as an allosteric inhibitor. Binding at a distal site induces a redistribution of the conformational landscape of TEM-1 β -lactamase, with dynamic effects propagating from the binding interface to distant regions, including catalytically relevant sites, thereby modulating enzymatic activity. Such long-range dynamic effects are consistent with allosteric communication between structurally separated regions of the enzyme, in agreement with previous studies (Galdadas et al. 2021; Huang et al. 2020; Meneksedag et al. 2013; Avci et al. 2016; J. R. Horn and Shoichet 2004). Although the present data do not directly demonstrate a defined allosteric signaling pathway, they are consistent with a model in which VHH binding modulates intrinsic dynamic networks within TEM-1, thereby contributing to inhibition through dynamic allosteric effects.

ACKNOWLEDGMENTS

This study made use of NMRbox: National Center for Biomolecular NMR Data Processing and Analysis, a Biomedical Technology Research Resource (BTRR), which is supported by NIH grant P41GM111135 (NIGMS)

This work was supported by the The Walloon Region and the Fond Européen de Développement Régional (FEDER) [Grant portfolio PHENIX n°244, project PHENIX_Analytique_ULiège n°397]

This work was supported by the Belgian Federal Public Service Health, Food Chain Safety and Environment (grant no. RF 17/6317 RU-BLA-ESBL-CPE) and the Fonds National de la Recherche Scientifique (CDR grant no. J0081.20).

Electronic Supplementary Material

Table S1. Per-residue model-free analysis parameters for Apo_{TEM-1}, including residue-specific generalized order parameter (S^2), local correlation time (τ_e), and conformational exchange rate (R_{ex}).

Residue	Chosen Model	AIC	S^2	$\pm\Delta S^2$	τ_e (ps)	$\pm\Delta\tau_e$	R_{ex} (s ⁻¹)	$\pm\Delta R_{ex}$
GLU[3]	TM5	34.20	0.85	0.005	2.43E-10	6.35E-10		
THR[4]	TM5	57.90	0.79	0.013	3.63E-09	9.72E-10		
LEU[5]	TM5	27.60	0.86	0.007	1.49E-09	2.02E-10		
VAL[6]	TM5	52.50	0.84	0.010	9.22E-10	1.29E-10		
LYS[7]	-	-	-	-	-	-	-	-
VAL[8]	TM5	26.50	0.90	0.009	7.20E-11	3.21E-10		
LYS[9]	TM5	17.80	0.98	0.008	1.25E-13	2.45E-03		
ASP[10]	TM5	24.50	0.87	0.012	6.66E-10	1.21E-10		
ALA[11]	TM5	121.00	0.82	0.007	1.50E-09	1.93E-10		
GLU[12]	TM5	165.00	0.84	0.032	7.71E-09	1.04E-08		
ASP[13]	TM2	34.40	0.91	0.006	2.05E-11	2.31E-12		
GLN[14]	TM5	12.40	0.88	0.005	5.11E-10	1.07E-10		
LEU[15]	TM4	106.00	0.90	0.009	7.96E-12	2.07E-12	0.56	0.19
GLY[16]	TM2	58.90	0.89	0.004	1.73E-11	1.62E-12		
ALA[17]	TM4	18.00	0.83	0.012	1.62E-11	1.58E-12	0.40	0.24
ARG[18]	TM2	149.00	0.72	0.006	2.38E-11	9.02E-13		
VAL[19]	TM2	13.00	0.83	0.010	7.37E-12	1.37E-12		
GLY[20]	TM1	33.70	0.87	0.006				
TYR[21]	TM5	57.90	0.81	0.005	7.91E-10	9.96E-11		
ILE[22]	TM5	24.80	0.84	0.011	4.20E-10	1.20E-10		
GLU[23]	TM5	122.00	0.82	0.009	1.07E-09	7.83E-11		
LEU[24]	TM5	48.90	0.83	0.011	2.27E-09	7.72E-10		
ASP[25]	TM5	38.50	0.71	0.008	1.94E-09	1.58E-10		
LEU[26]	TM5	45.90	0.89	0.009	7.30E-11	1.09E-10		
ASN[27]	TM2	269.00	0.83	0.006	5.29E-11	3.02E-12		
SER[28]	TM5	19.40	0.82	0.012	5.73E-10	1.14E-10		
GLY[29]	TM5	18.80	0.85	0.008	1.23E-09	1.36E-10		
LYS[30]	TM5	28.80	0.86	0.004	5.62E-10	6.73E-11		
ILE[31]	TM5	222.00	0.77	0.007	6.08E-10	3.08E-11		
LEU[32]	TM5	110.00	0.81	0.012	9.27E-10	7.58E-11		
GLU[33]	TM5	78.40	0.86	0.005	2.65E-10	6.82E-11		
SER[34]	TM5	77.70	0.90	0.006	1.16E-10	5.75E-11		

PHE[35]	TM4	66.40	0.85	0.012	6.29E-12	1.26E-12	0.77	0.25
ARG[36]	TM5	107.00	0.88	0.004	4.54E-10	3.15E-10		
GLU[38]	TM4	206.00	0.87	0.011	2.24E-11	2.60E-12	1.53	0.22
GLU[39]	TM5	76.70	0.80	0.006	1.35E-09	1.51E-10		
ARG[40]	TM2	21.30	0.85	0.004	7.15E-12	8.96E-13		
PHE[41]	TM5	70.60	0.91	0.004	4.68E-10	3.65E-10		
MET[43]	TM4	18.80	0.88	0.020	2.89E-11	6.03E-12	1.59	0.47
MET[44]	TM5	43.70	0.91	0.008	6.17E-10	3.31E-10		
THR[46]	TM5	19.60	0.89	0.008	8.27E-10	1.96E-10		
PHE[47]	TM5	106.00	0.89	0.007	4.15E-10	3.24E-10		
LYS[48]	TM5	226.00	0.88	0.007	1.45E-09	2.02E-10		
VAL[49]	TM1	112.00	0.94	0.005				
CYS[52]	TM5	49.80	0.76	0.007	1.29E-09	1.56E-10		
GLY[53]	TM5	38.70	0.85	0.012	6.83E-10	1.21E-10		
ALA[54]	TM5	151.00	0.78	0.005	1.09E-09	6.38E-11		
VAL[55]	TM5	68.30	0.88	0.006	4.86E-13	6.19E-04		
LEU[56]	TM5	44.30	0.89	0.010	1.07E-13	1.30E-02		
SER[57]	TM2	18.60	0.92	0.008	2.61E-11	4.07E-12		
ARG[58]	TM2	56.40	0.93	0.007	6.62E-12	2.79E-12		
VAL[59]	TM5	89.40	0.83	0.011	6.65E-10	9.28E-11		
ASP[60]	TM5	50.70	0.86	0.010	1.54E-09	2.48E-10		
ALA[61]	TM1	115.00	0.88	0.007				
GLY[62]	-	-	-	-	-	-	-	-
GLN[63]	TM4	73.20	0.81	0.018	7.12E-12	1.27E-12	1.97	0.38
GLU[64]	TM5	138.00	0.75	0.006	8.59E-10	4.55E-11		
GLN[65]	TM5	161.00	0.81	0.004	1.75E-11	6.40E-09		
LEU[66]	TM5	31.70	0.80	0.009	1.90E-09	1.89E-10		
GLY[67]	TM5	32.60	0.86	0.011	8.31E-10	1.14E-10		
ARG[68]	TM2	84.70	0.88	0.006	1.66E-11	1.38E-12		
ARG[69]	TM5	16.50	0.81	0.016	8.21E-10	1.54E-10		
ILE[70]	TM2	20.50	0.86	0.010	2.01E-11	2.00E-12		
HIS[71]	TM5	259.00	0.86	0.005	6.81E-10	1.21E-10		
TYR[72]	TM5	195.00	0.82	0.006	4.32E-10	1.03E-10		
SER[73]	TM2	48.70	0.84	0.005	1.68E-11	1.24E-12		
GLN[74]	TM2	376.00	0.80	0.005	1.59E-11	8.80E-13		
ASN[75]	TM4	45.40	0.84	0.018	2.12E-11	3.25E-12	1.37	0.34
ASP[76]	TM2	28.50	0.87	0.005	3.15E-11	1.98E-12		
LEU[77]	TM5	183.00	0.86	0.007	5.73E-10	9.02E-11		
VAL[78]	TM2	143.00	0.81	0.009	7.05E-12	9.97E-13		

GLU[79]	TM5	62.10	0.80	0.004	9.33E-10	4.89E-11		
TYR[80]	TM5	212.00	0.77	0.007	8.64E-10	3.18E-11		
SER[81]	TM2	129.00	0.80	0.006	1.76E-11	1.05E-12		
VAL[83]	TM5	95.30	0.88	0.006	7.17E-10	7.68E-11		
THR[84]	TM4	49.60	0.84	0.019	6.96E-12	2.34E-12	1.95	0.40
GLU[85]	TM4	228.00	0.92	0.015	2.56E-11	5.50E-12	1.47	0.31
LYS[86]	TM4	16.70	0.91	0.013	1.48E-11	3.07E-12	0.63	0.27
HIS[87]	TM5	15.50	0.87	0.008	1.95E-10	4.52E-11		
LEU[88]	TM5	27.00	0.85	0.004	7.63E-10	8.36E-11		
THR[89]	TM5	31.40	0.80	0.006	6.85E-10	9.10E-11		
ASP[90]	TM5	15.40	0.89	0.006	3.05E-10	1.09E-10		
GLY[91]	TM5	160.00	0.82	0.008	4.66E-13	1.08E-05		
MET[92]	TM5	15.30	0.80	0.006	1.37E-09	1.04E-10		
THR[93]	TM5	13.80	0.83	0.006	4.62E-10	1.24E-10		
VAL[94]	TM5	119.00	0.86	0.008	2.20E-13	2.26E-02		
ARG[95]	TM5	415.00	0.90	0.006	1.15E-09	4.58E-10		
GLU[96]	TM5	220.00	0.88	0.008	5.59E-10	6.85E-10		
LEU[97]	-	-	-	-	-	-	-	-
CYS[98]	TM1	15.60	0.93	0.007				
SER[99]	TM4	54.90	0.86	0.015	1.57E-11	2.51E-12	2.01	0.37
ALA[100]	TM5	95.90	0.90	0.008	2.36E-09	1.68E-09		
ALA[101]	TM2	135.00	0.86	0.006	5.20E-12	1.34E-12		
ILE[102]	TM5	84.00	0.84	0.009	1.53E-09	2.69E-10		
THR[103]	TM5	1260.00	0.84	0.008	7.49E-10	7.90E-11		
MET[104]	TM5	28.90	0.85	0.006	4.93E-10	1.93E-10		
SER[105]	TM5	74.90	0.90	0.008	3.88E-11	2.31E-10		
ASP[106]	TM5	23.80	0.90	0.011	6.38E-10	2.92E-10		
ASN[107]	TM5	49.40	0.83	0.008	1.14E-09	1.06E-10		
THR[108]	TM2	12.50	0.87	0.007	4.51E-12	2.30E-12		
ALA[109]	TM5	63.00	0.84	0.009	1.31E-09	1.51E-10		
ALA[110]	TM4	79.80	0.85	0.013	4.53E-12	1.42E-12	1.21	0.26
LEU[112]	TM5	143.00	0.88	0.006	2.39E-14	1.74E-04		
LEU[113]	-	-	-	-	-	-	-	-
LEU[114]	TM5	18.60	0.84	0.011	1.75E-09	4.10E-10		
THR[115]	TM2	35.10	0.89	0.005	3.73E-11	2.50E-12		
THR[116]	TM5	6.61	0.82	0.008	4.65E-10	1.01E-10		
ILE[117]	TM5	30.30	0.81	0.010	1.12E-09	1.22E-10		
GLY[118]	TM5	17.60	0.84	0.013	8.17E-10	1.06E-10		
GLY[119]	TM2	52.50	0.88	0.009	2.19E-11	2.39E-12		

LYS[121]	TM2	154.00	0.90	0.007	1.68E-11	2.30E-12		
GLU[122]	TM5	28.00	0.89	0.013	7.62E-10	2.48E-10		
LEU[123]	TM5	123.00	0.80	0.007	1.74E-09	1.77E-10		
THR[124]	TM2	18.10	0.89	0.010	2.20E-11	3.04E-12		
ALA[125]	TM5	2890.00	0.79	0.004	5.75E-10	3.28E-11		
PHE[126]	TM5	16.90	0.89	0.005	2.08E-10	8.70E-11		
LEU[127]	TM1	27.90	0.97	0.013				
HIS[128]	TM2	76.40	0.93	0.004	1.15E-11	2.82E-12		
ASN[129]	TM2	107.00	0.91	0.006	3.59E-11	3.20E-12		
MET[130]	TM4	23.10	0.85	0.009	2.29E-11	1.97E-12	0.31	0.18
GLY[131]	TM5	129.00	0.78	0.009	1.14E-09	8.16E-11		
ASP[132]	TM2	214.00	0.92	0.004	2.63E-11	2.16E-12		
HIS[133]	TM5	56.70	0.86	0.007	5.72E-10	1.20E-10		
VAL[134]	TM5	29.10	0.85	0.007	1.74E-09	3.07E-10		
THR[135]	TM5	27.00	0.83	0.009	9.29E-10	1.07E-10		
ARG[136]	TM5	97.90	0.82	0.006	9.19E-10	7.49E-11		
LEU[137]	TM5	158.00	0.80	0.007	9.87E-10	8.91E-11		
ASP[138]	TM5	48.40	0.84	0.008	5.17E-10	2.65E-10		
ARG[139]	TM5	79.80	0.84	0.008	4.51E-10	1.67E-10		
TRP[140]	TM5	138.00	0.79	0.005	5.55E-10	1.11E-10		
GLU[141]	TM1	183.00	0.89	0.005				
GLU[143]	TM4	99.30	0.88	0.008	2.66E-11	2.60E-12	0.41	0.22
LEU[144]	TM2	49.50	0.87	0.011	2.13E-11	2.34E-12		
ASN[145]	TM4	91.50	0.89	0.014	1.33E-11	2.70E-12	0.76	0.32
GLU[146]	TM5	96.60	0.87	0.007	5.95E-10	1.95E-10		
ALA[147]	TM5	101.00	0.91	0.006	2.59E-10	6.24E-11		
ILE[148]	TM2	105.00	0.89	0.006	6.69E-12	1.61E-12		
ASN[150]	TM5	42.00	0.82	0.006	1.98E-10	6.60E-11		
ASP[151]	TM2	241.00	0.84	0.004	1.92E-11	9.35E-13		
GLU[152]	TM2	24.00	0.84	0.006	1.99E-11	1.38E-12		
ARG[153]	TM5	53.20	0.88	0.005	4.01E-10	2.24E-10		
ASP[154]	TM2	53.10	0.90	0.006	2.02E-11	2.28E-12		
THR[155]	TM5	43.50	0.77	0.010	1.02E-09	8.66E-11		
THR[156]	TM5	28.20	0.78	0.010	2.42E-09	2.64E-10		
MET[157]	TM5	81.00	0.85	0.007	1.23E-09	2.47E-10		
ALA[159]	TM5	73.50	0.86	0.007	2.63E-10	1.16E-10		
ALA[160]	TM5	207.00	0.89	0.006	4.47E-10	2.81E-10		
MET[161]	TM5	61.90	0.87	0.007	8.45E-10	9.34E-11		
ALA[162]	TM5	182.00	0.93	0.006	4.73E-10	3.51E-10		

THR[163]	TM5	122.00	0.75	0.006	1.24E-09	9.56E-11		
THR[164]	TM5	66.00	0.84	0.005	9.21E-10	6.52E-11		
LEU[165]	TM5	49.00	0.87	0.007	1.22E-09	1.10E-10		
ARG[166]	TM5	53.60	0.83	0.007	1.28E-09	1.91E-10		
LYS[167]	TM5	42.20	0.87	0.006	2.20E-14	2.23E-04		
LEU[168]	TM5	33.10	0.90	0.008	4.47E-10	1.35E-10		
LEU[169]	TM5	25.20	0.76	0.012	9.54E-10	7.05E-11		
THR[170]	-	-	-	-	-	-	-	-
GLY[171]	TM5	32.80	0.72	0.006	1.15E-09	3.38E-11		
GLU[172]	TM5	257.00	0.81	0.006	4.30E-11	9.98E-11		
LEU[173]	TM5	694.00	0.74	0.006	2.44E-09	1.24E-07		
LEU[174]	TM5	65.00	0.82	0.009	9.81E-10	1.04E-10		
THR[175]	TM5	51.30	0.75	0.007	1.13E-09	9.12E-11		
LEU[176]	TM2	47.00	0.85	0.006	1.37E-11	9.45E-13		
ALA[177]	TM2	45.70	0.88	0.006	1.70E-11	1.63E-12		
SER[178]	TM2	13.40	0.88	0.010	1.45E-11	2.52E-12		
ARG[179]	TM5	50.90	0.85	0.013	9.61E-10	9.20E-11		
GLN[180]	TM5	27.00	0.90	0.006	3.22E-10	1.73E-10		
GLN[181]	TM4	25.20	0.92	0.011	2.55E-11	4.04E-12	0.41	0.21
LEU[182]	TM5	31.80	0.87	0.009	7.16E-10	7.32E-11		
ASP[184]	-	-	-	-	-	-	-	-
TRP[185]	TM3	43.80	0.97	0.021	9.09E-01	6.59E-01	0.91	0.66
MET[186]	-	-	-	-	-	-	-	-
GLU[187]	TM4	277.00	0.97	0.007	3.47E-10	1.59E-10	0.89	0.21
ALA[188]	TM2	30.30	0.93	0.007	6.64E-12	2.71E-12		
ASP[189]	TM4	19.90	0.89	0.015	2.44E-11	4.78E-12	4.24	0.42
LYS[190]	TM3	79.10	0.92	0.021			6.53	0.49
VAL[191]	-	-	-	-	-	-	-	-
ALA[192]	TM5	64.90	0.81	0.008	1.52E-09	1.84E-10		
GLY[193]	TM5	584.00	0.75	0.007	2.39E-09	3.40E-10		
LEU[195]	-	-	-	-	-	-	-	-
LEU[196]	TM4	485.00	0.93	0.010	2.13E-11	5.01E-12	1.32	0.21
ARG[197]	TM5	20.40	0.84	0.013	1.72E-09	5.30E-10		
SER[198]	TM5	34.90	0.72	0.013	2.12E-09	2.81E-10		
ALA[199]	TM5	37.00	0.86	0.005	3.38E-10	7.72E-11		
LEU[200]	TM2	108.00	0.90	0.004	4.30E-11	2.97E-12		
ALA[202]	TM4	34.40	0.83	0.011	1.84E-11	1.52E-12	0.36	0.21
GLY[203]	TM2	81.10	0.80	0.007	9.04E-12	9.95E-13		
TRP[204]	TM5	27.70	0.86	0.012	1.26E-09	1.94E-10		

PHE[205]	TM2	61.50	0.89	0.005	2.33E-11	2.32E-12		
ILE[206]	TM5	66.60	0.88	0.005	3.21E-10	6.58E-11		
ALA[207]	TM5	42.30	0.86	0.014	4.63E-10	4.85E-10		
ASP[208]	TM5	115.00	0.87	0.011	4.76E-10	1.21E-10		
LYS[209]	TM5	480.00	0.86	0.008	9.55E-10	2.19E-10		
GLY[211]	TM5	79.20	0.89	0.017	4.89E-10	2.38E-10		
GLY[213]	TM2	37.70	0.84	0.008	7.97E-12	1.17E-12		
GLU[214]	TM5	105.00	0.92	0.010	8.86E-10	2.09E-10		
ARG[215]	TM5	46.10	0.90	0.007	5.52E-10	1.36E-10		
GLY[216]	TM2	54.30	0.94	0.008	4.54E-10	8.71E-11		
SER[217]	TM5	472.00	0.81	0.006	9.78E-10	1.15E-10		
ARG[218]	TM2	50.80	0.88	0.009	1.19E-11	1.82E-12		
GLY[219]	TM2	33.10	0.91	0.005	1.98E-11	2.83E-12		
ILE[220]	TM5	1540.00	0.86	0.004	5.79E-10	3.29E-10		
ILE[221]	TM3	201.00	0.89	0.011			0.64	0.25
ALA[222]	-	-	-	-	-	-	-	-
ALA[223]	TM4	65.50	0.83	0.009	4.82E-12	9.68E-13	0.47	0.17
LEU[224]	TM5	102.00	0.84	0.005	6.99E-10	8.14E-11		
GLY[225]	TM2	12.00	0.89	0.007	2.70E-11	2.52E-12		
ASP[227]	TM4	288.00	0.87	0.010	1.71E-11	1.92E-12	1.92	0.22
GLY[228]	TM2	162.00	0.81	0.008	1.26E-11	1.19E-12		
LYS[229]	TM4	661.00	0.86	0.006	1.40E-11	1.09E-12	2.57	0.14
SER[231]	TM2	35.80	0.87	0.007	3.51E-11	3.05E-12		
ARG[232]	TM2	42.50	0.88	0.007	2.20E-11	2.24E-12		
ILE[233]	TM2	57.20	0.86	0.005	2.51E-12	1.24E-12		
VAL[234]	TM5	102.00	0.87	0.011	9.94E-10	1.65E-09		
VAL[235]	TM5	209.00	0.82	0.008	8.50E-10	2.04E-10		
ILE[236]	TM2	89.30	0.85	0.008	1.20E-11	1.07E-12		
TYR[237]	TM2	83.60	0.88	0.007	1.39E-11	1.69E-12		
THR[238]	TM2	34.30	0.85	0.007	1.36E-11	1.54E-12		
THR[239]	TM4	7.60	0.91	0.026	4.60E-12	2.33E-12	3.16	0.51
GLY[240]	TM3	51.40	0.92	0.017			2.11	0.37
SER[241]	TM2	18.80	0.84	0.006	1.88E-11	1.62E-12		
GLN[242]	TM5	79.30	0.81	0.007	1.73E-09	2.20E-10		
ALA[243]	TM5	326.00	0.65	0.008	1.98E-09	1.18E-10		
THR[244]	TM5	126.00	0.84	0.006	4.37E-10	6.33E-11		
MET[245]	TM5	76.10	0.75	0.007	1.57E-09	1.91E-10		
ASP[246]	TM5	37.40	0.85	0.006	1.45E-09	2.10E-10		
GLU[247]	TM5	40.00	0.89	0.008	1.15E-09	4.12E-10		

ARG[248]	TM5	24.70	0.88	0.009	1.27E-09	2.53E-10		
ASN[249]	-	-	-	-	-	-	-	-
ARG[250]	TM1	436.00	0.93	0.006				
GLN[251]	TM5	510.00	0.79	0.006	8.00E-10	8.79E-11		
ILE[252]	TM4	32.20	0.89	0.020	1.63E-11	3.69E-12	1.24	0.41
GLU[254]	TM5	154.00	0.90	0.006	4.84E-10	5.03E-10		
ILE[255]	TM5	37.00	0.80	0.011	1.04E-09	7.80E-11		
GLY[256]	TM2	22.30	0.87	0.010	1.06E-11	1.60E-12		
ALA[257]	TM5	31.30	0.89	0.006	3.98E-10	1.14E-10		
SER[258]	TM2	33.10	0.87	0.006	2.12E-11	1.78E-12		
LEU[259]	TM5	27.10	0.87	0.006	9.19E-10	1.19E-10		
ILE[260]	TM4	42.50	0.93	0.011	5.15E-12	3.26E-12	0.54	0.26
LYS[261]	TM5	118.00	0.91	0.008	1.49E-09	2.75E-10		
HIS[262]	TM5	74.50	0.82	0.012	1.75E-09	3.41E-10		
TRP[263]	TM5	87.80	0.81	0.006	6.30E-10	5.80E-11		

Table S2. Per-residue model-free analysis parameters for Holo_{TEM-1}, including residue-specific generalized order parameter (S^2), local correlation time (τ_e), and conformational exchange rate (R_{ex}).

Residue	Chosen Model	AIC	S2	S2 Error	TauE(s)	TauE Error	Rex(s-1)	Rex Error
GLU[3]	TM2	13.00	0.86	0.007	1.68E-11	1.64E-12		
THR[4]	TM4	19.90	0.87	0.022	1.15E-11	2.71E-12	1.15	0.80
LEU[5]	TM2	30.40	0.93	0.007	3.38E-11	4.78E-12		
VAL[6]	TM5	60.00	0.88	0.005	4.39E-10	2.38E-10		
LYS[7]	TM2	25.70	0.92	0.007	2.96E-11	3.70E-12		
VAL[8]	TM5	39.30	0.85	0.009	1.83E-09	5.68E-10		
LYS[9]	TM2	77.40	0.88	0.010	1.65E-11	2.25E-12		
ASP[10]	TM4	24.90	0.89	0.016	1.83E-11	3.48E-12	1.32	0.59
ALA[11]	TM4	23.70	0.90	0.021	5.90E-12	2.65E-12	0.91	0.73
GLU[12]	TM2	33.40	0.92	0.007	1.74E-11	2.95E-12		
ASP[13]	TM5	9.36	0.86	0.008	4.57E-11	2.64E-09		
GLN[14]	TM5	128.00	0.77	0.005	8.12E-10	4.88E-11		
LEU[15]	TM5	25.90	0.81	0.010	1.26E-09	1.29E-10		
GLY[16]	TM3	18.00	0.91	0.027			1.49	0.95
ALA[17]	TM2	67.30	0.85	0.008	1.58E-11	1.30E-12		
ARG[18]	TM5	128.00	0.63	0.009	6.21E-10	2.18E-11		
VAL[19]	TM4	34.20	0.81	0.017	5.59E-12	1.19E-12	2.51	0.64
GLY[20]	TM2	14.90	0.83	0.009	1.28E-11	1.50E-12		

TYR[21]	TM3	32.10	0.89	0.024			3.33	0.82
ILE[22]	TM5	6.78	0.85	0.015	4.94E-10	2.25E-10		
GLU[23]	TM4	10.90	0.81	0.026	1.22E-11	2.35E-12	4.05	0.91
LEU[24]	TM4	11.50	0.78	0.012	8.97E-12	1.12E-12	4.07	0.65
ASP[25]	TM4	54.30	0.81	0.012	1.12E-11	1.27E-12	3.31	0.65
LEU[26]	TM4	52.50	0.80	0.014	1.20E-11	1.28E-12	3.29	0.47
ASN[27]	-	-	-	-	-	-	-	-
SER[28]	TM2	30.70	0.87	0.013	3.42E-11	4.83E-12		
GLY[29]	TM4	26.40	0.91	0.032	2.61E-11	1.12E-11	3.14	1.22
LYS[30]	TM2	142.00	0.95	0.006	7.40E-11	1.36E-11		
ILE[31]	TM4	50.90	0.76	0.012	1.04E-11	8.01E-13	5.71	0.39
LEU[32]	TM4	31.70	0.85	0.016	1.50E-11	2.24E-12	1.19	0.67
GLU[33]	TM4	20.30	0.85	0.018	1.20E-11	1.98E-12	0.87	0.58
SER[34]	-	-	-	-	-	-	-	-
PHE[35]	TM5	46.10	0.86	0.005	3.03E-10	1.89E-10		
ARG[36]	TM2	29.50	0.90	0.006	2.70E-11	2.37E-12		
GLU[38]	TM4	83.00	0.87	0.017	2.85E-12	1.52E-12	2.05	0.59
GLU[39]	TM2	11.50	0.92	0.013	1.91E-11	4.49E-12		
ARG[40]	TM5	1180.00	0.72	0.006	4.51E-10	5.90E-11		
PHE[41]	TM4	25.50	0.84	0.018	8.88E-12	1.40E-12	2.40	0.66
MET[43]	TM2	49.20	0.95	0.013	2.73E-11	9.83E-12		
MET[44]	TM2	86.60	0.86	0.022	9.25E-12	3.30E-12		
GLY[53]	TM5	22.40	0.84	0.032	4.38E-10	5.22E-10		
ALA[54]	TM5	9.93	0.83	0.023	2.39E-09	1.01E-09		
VAL[59]	TM5	9.92	0.92	0.010	1.56E-14	6.65E-06		
ASP[60]	TM5	12.90	0.85	0.010	8.92E-10	1.26E-10		
ALA[61]	TM4	6.64	0.87	0.018	7.01E-12	1.56E-12	3.46	0.66
GLY[62]	TM2	29.20	0.90	0.010	1.35E-11	2.12E-12		
GLN[63]	TM5	53.20	0.85	0.007	4.91E-11	8.73E-11		
GLU[64]	TM5	179.00	0.81	0.008	5.48E-10	6.02E-11		
GLN[65]	TM5	37.00	0.80	0.008	7.17E-10	1.32E-10		
LEU[66]	TM5	34.50	0.82	0.007	7.52E-10	7.67E-11		
GLY[67]	TM2	7.97	0.90	0.022	1.67E-11	4.31E-12		
ARG[68]	TM2	81.40	0.94	0.005	2.29E-11	2.90E-12		
ARG[69]	TM5	21.40	0.77	0.010	1.40E-09	1.02E-10		
ILE[70]	TM5	70.20	0.83	0.009	1.09E-09	1.95E-10		
HIS[71]	TM4	21.70	0.78	0.010	8.86E-12	6.42E-13	3.10	0.34
TYR[72]	TM2	85.50	0.88	0.004	2.30E-11	1.39E-12		
SER[73]	TM4	38.20	0.81	0.026	1.12E-11	2.00E-12	5.64	0.90
GLN[74]	TM2	115.00	0.85	0.006	1.71E-11	1.24E-12		

ASN[75]	TM2	34.40	0.90	0.011	3.32E-11	5.02E-12		
ASP[76]	TM4	32.50	0.86	0.022	1.46E-11	2.78E-12	5.44	0.83
LEU[77]	TM4	32.40	0.88	0.028	1.96E-11	5.64E-12	3.09	0.96
VAL[78]	TM4	31.40	0.79	0.016	1.13E-11	1.34E-12	3.27	0.59
GLU[79]	TM5	19.00	0.86	0.016	7.13E-10	1.51E-10		
TYR[80]	TM2	15.40	0.86	0.011	2.39E-11	2.36E-12		
SER[81]	TM2	41.00	0.85	0.007	1.56E-11	1.61E-12		
VAL[83]	TM4	62.70	0.83	0.016	8.85E-12	1.85E-12	7.17	0.61
GLU[85]	TM1	30.50	0.94	0.008				
LYS[86]	TM2	143.00	0.95	0.009	5.20E-11	1.23E-11		
HIS[87]	TM2	123.00	0.91	0.011	3.20E-11	4.73E-12		
LEU[88]	TM2	9.75	0.86	0.006	1.92E-11	1.37E-12		
THR[89]	TM2	13.30	0.89	0.015	1.79E-11	3.73E-12		
ASP[90]	TM2	65.40	0.85	0.009	2.08E-11	2.04E-12		
GLY[91]	TM2	36.00	0.85	0.009	1.41E-11	1.44E-12		
MET[92]	TM5	69.10	0.91	0.009	3.09E-10	9.64E-11		
THR[93]	TM4	156.00	0.88	0.018	6.27E-12	1.47E-12	1.64	0.57
VAL[94]	TM2	16.00	0.88	0.008	1.14E-11	1.59E-12		
ARG[95]	TM5	49.40	0.91	0.007	6.73E-10	1.70E-10		
GLU[96]	TM5	108.00	0.88	0.007	8.94E-10	1.59E-10		
LEU[97]	TM5	26.60	0.87	0.022	6.95E-10	1.56E-10		
CYS[98]	TM5	19.90	0.80	0.014	4.71E-09	2.96E-09		
SER[99]	TM5	15.80	0.83	0.019	1.26E-09	4.73E-10		
ALA[100]	TM5	39.10	0.72	0.066	1.06E-08	2.21E-08		
ALA[101]	TM5	59.90	0.91	0.009	3.52E-10	1.49E-10		
ILE[102]	TM5	17.80	0.85	0.013	7.63E-10	1.48E-10		
THR[103]	TM5	60.70	0.89	0.011	6.33E-10	2.19E-10		
MET[104]	TM5	69.00	0.93	0.016	4.37E-10	5.03E-10		
SER[105]	TM5	25.10	0.88	0.016	6.05E-10	1.21E-10		
ASP[106]	TM5	10.30	0.88	0.010	1.11E-09	2.01E-10		
ASN[107]	TM5	32.40	0.83	0.011	1.77E-09	4.61E-10		
THR[108]	TM5	272.00	0.79	0.024	7.12E-09	2.06E-08		
ALA[109]	TM5	32.00	0.88	0.012	1.55E-09	3.61E-10		
ALA[110]	TM5	13.70	0.87	0.009	5.17E-10	2.65E-10		
LEU[112]	TM5	27.60	0.84	0.007	7.63E-10	2.18E-10		
LEU[113]	TM1	4.36	0.90	0.016				
LEU[114]	TM1	16.80	0.87	0.008				
THR[115]	TM4	15.20	0.85	0.031	7.52E-12	2.12E-12	4.79	1.00
THR[116]	TM2	109.00	0.84	0.013	2.87E-11	3.18E-12		
GLY[118]	TM2	25.80	0.89	0.009	1.15E-11	1.97E-12		

GLY[119]	TM5	165.00	0.90	0.008	2.16E-10	8.10E-09		
LYS[121]	TM5	18.70	0.87	0.011	6.19E-10	7.57E-11		
GLU[122]	TM5	6.61	0.88	0.019	3.89E-10	1.86E-10		
LEU[123]	TM4	17.80	0.89	0.015	9.13E-12	2.38E-12	1.08	0.52
THR[124]	TM2	31.50	0.87	0.008	1.22E-11	1.95E-12		
ALA[125]	TM5	42.70	0.86	0.008	1.18E-09	1.69E-10		
PHE[126]	TM5	66.60	0.87	0.008	9.03E-10	9.45E-11		
LEU[127]	TM5	46.80	0.86	0.011	1.44E-09	2.27E-10		
HIS[128]	TM5	13.70	0.90	0.007	4.89E-10	9.84E-11		
ASN[129]	TM4	12.10	0.88	0.018	1.77E-11	3.33E-12	4.22	0.77
MET[130]	TM4	29.20	0.85	0.027	1.59E-11	3.56E-12	1.53	0.85
GLY[131]	TM2	19.90	0.95	0.015	6.25E-11	2.46E-11		
ASP[132]	TM2	8.86	0.94	0.008	2.93E-11	5.07E-12		
HIS[133]	TM5	36.20	0.81	0.008	7.45E-10	8.48E-11		
VAL[134]	TM4	31.50	0.88	0.014	2.08E-11	3.16E-12	2.07	0.56
THR[135]	TM2	17.00	0.89	0.009	8.33E-12	3.75E-12		
ARG[136]	TM2	77.20	0.89	0.005	1.83E-11	1.61E-12		
ALA[137]	-	-	-	-	-	-	-	-
ASP[138]	TM2	8.32	0.86	0.017	1.38E-11	2.99E-12		
ARG[139]	TM5	8.82	0.76	0.014	1.03E-09	1.55E-10		
TRP[140]	TM5	227.00	0.67	0.010	1.20E-09	1.03E-10		
GLU[141]	TM5	144.00	0.76	0.007	7.42E-10	6.88E-11		
ILE[142]	-	-	-	-	-	-	-	-
GLU[143]	TM4	32.90	0.92	0.017	3.26E-11	7.81E-12	0.87	0.58
LEU[144]	TM2	20.20	0.91	0.014	2.46E-11	4.69E-12		
ASN[145]	TM5	30.30	0.89	0.011	1.46E-09	5.40E-10		
GLU[146]	TM2	24.00	0.94	0.008	3.03E-11	5.85E-12		
ALA[147]	TM2	9.09	0.92	0.009	4.01E-11	6.20E-12		
ILE[148]	TM4	24.50	0.83	0.014	9.65E-12	1.45E-12	4.30	0.51
ASN[150]	TM4	30.90	0.79	0.027	1.76E-11	2.90E-12	3.20	0.85
ASP[151]	TM4	36.40	0.86	0.020	2.40E-11	4.03E-12	4.17	0.76
GLU[152]	TM4	38.60	0.79	0.016	1.06E-11	1.24E-12	1.94	0.57
ARG[153]	TM2	32.70	0.92	0.008	2.02E-11	3.03E-12		
ASP[154]	TM4	70.70	0.76	0.025	5.94E-12	1.03E-12	7.07	0.92
THR[155]	TM2	26.10	0.88	0.032	1.06E-11	3.84E-12		
THR[156]	TM5	32.50	0.82	0.008	1.90E-09	3.67E-10		
MET[157]	TM5	18.90	0.84	0.012	1.26E-09	2.97E-10		
ALA[159]	TM5	10.70	0.90	0.009	3.87E-10	2.08E-10		
ALA[160]	TM5	38.80	0.86	0.010	3.33E-11	5.02E-10		
MET[161]	TM4	12.80	0.83	0.017	6.40E-12	1.51E-12	3.36	0.60

LEU[162]	-	-	-	-	-	-	-	-
THR[163]	TM5	15.70	0.81	0.016	3.89E-10	5.90E-11		
THR[164]	TM2	14.90	0.91	0.013	1.81E-11	4.95E-12		
LEU[165]	TM1	36.70	0.92	0.008				
LEU[169]	TM4	55.80	0.89	0.026	1.77E-11	5.53E-12	2.49	0.88
THR[170]	TM2	12.10	0.88	0.007	2.67E-11	2.36E-12		
GLY[171]	TM5	107.00	0.60	0.008	1.79E-09	1.23E-10		
GLU[172]	TM4	7.78	0.85	0.033	1.18E-11	3.11E-12	1.77	1.13
LEU[173]	TM2	127.00	0.66	0.008	2.12E-11	8.65E-13		
LEU[174]	TM2	66.50	0.83	0.004	3.11E-11	1.37E-12		
THR[175]	TM2	9.69	0.84	0.008	8.64E-12	8.42E-13		
LEU[176]	TM5	30.10	0.86	0.009	2.13E-09	1.18E-09		
ALA[177]	TM5	47.10	0.89	0.008	1.06E-13	0.00468		
SER[178]	TM5	33.10	0.90	0.008	4.43E-10	2.36E-10		
GLU[187]	TM4	14.30	0.80	0.022	2.24E-12	1.27E-12	5.25	0.80
ALA[188]	TM2	43.50	0.92	0.011	1.88E-11	3.37E-12		
ASP[189]	TM4	35.90	0.86	0.030	1.59E-11	4.25E-12	6.44	1.10
LYS[190]	TM4	62.70	0.84	0.018	8.50E-12	1.42E-12	2.92	0.61
VAL[191]	TM2	13.90	0.88	0.008	1.22E-11	1.49E-12		
ALA[192]	TM2	17.10	0.87	0.010	1.31E-11	2.36E-12		
GLY[193]	TM1	72.20	0.94	0.009				
LEU[195]	TM5	49.20	0.89	0.008	9.16E-10	2.12E-10		
SER[198]	TM2	31.70	0.91	0.015	9.75E-12	3.93E-12		
ALA[199]	TM2	31.70	0.90	0.010	2.88E-11	4.37E-12		
LEU[200]	TM5	19.50	0.81	0.009	9.63E-10	1.82E-10		
ALA[202]	TM4	23.80	0.88	0.012	1.45E-11	1.90E-12	1.10	0.43
GLY[203]	TM2	57.10	0.84	0.010	1.64E-11	1.68E-12		
TRP[204]	TM5	88.40	0.86	0.010	1.14E-09	2.25E-10		
PHE[205]	TM2	40.10	0.88	0.009	2.41E-11	2.66E-12		
ILE[206]	TM5	563.00	0.88	0.009	2.72E-11	2.61E-10		
ALA[207]	TM4	17.30	0.80	0.028	7.49E-12	1.56E-12	4.96	0.95
ASP[208]	TM4	23.50	0.81	0.011	9.00E-12	9.33E-13	4.52	0.46
LYS[209]	TM4	46.50	0.80	0.017	8.22E-12	1.22E-12	5.85	0.63
GLY[211]	TM4	12.70	0.88	0.033	1.66E-11	5.77E-12	5.21	1.26
GLY[213]	TM2	30.10	0.86	0.009	8.06E-12	1.47E-12		
GLU[214]	TM5	88.60	0.91	0.009	8.59E-10	1.22E-10		
ARG[215]	TM2	6.60	0.95	0.007	4.07E-11	7.24E-12		
GLY[216]	TM2	53.30	0.96	0.007	2.91E-10	1.13E-10		
SER[217]	TM5	18.00	0.88	0.008	1.64E-09	2.65E-10		
ARG[218]	TM5	52.00	0.91	0.015	3.42E-10	2.06E-10		

GLY[219]	TM5	38.70	0.88	0.008	2.90E-10	1.32E-10		
ILE[220]	TM4	28.80	0.81	0.020	1.22E-12	1.33E-12	9.48	0.82
ILE[221]	TM4	14.00	0.85	0.021	8.28E-12	2.15E-12	5.75	0.89
ALA[222]	TM4	165.00	0.85	0.016	3.05E-11	4.13E-12	2.77	0.56
ALA[223]	TM4	57.00	0.76	0.012	5.43E-12	7.49E-13	3.32	0.35
LEU[224]	TM3	121.00	0.82	0.014			5.75	0.49
GLY[225]	TM4	18.90	0.81	0.019	8.18E-12	1.40E-12	6.04	0.89
GLY[228]	TM2	70.50	0.87	0.014	1.58E-11	2.51E-12		
LYS[229]	TM2	13.00	0.92	0.010	3.14E-11	4.54E-12		
SER[231]	TM5	18.80	0.83	0.009	2.55E-10	8.32E-11		
ARG[232]	TM2	52.00	0.94	0.010	2.69E-11	6.42E-12		
ILE[233]	TM2	25.00	0.90	0.009	1.14E-11	2.61E-12		
VAL[234]	TM2	20.80	0.89	0.012	1.25E-11	2.54E-12		
VAL[235]	TM2	26.00	0.85	0.004	2.44E-12	2.04E-12		
ILE[236]	TM2	11.70	0.89	0.014	8.86E-12	2.57E-12		
TYR[237]	-	-	-	-	-	-	-	-
THR[238]	TM5	32.50	0.86	0.012	9.16E-10	1.71E-10		
THR[239]	TM4	151.00	0.84	0.011	1.94E-11	1.71E-12	2.44	0.40
GLY[240]	TM4	68.40	0.86	0.023	2.13E-11	4.47E-12	3.43	0.80
SER[241]	TM4	59.60	0.83	0.018	2.05E-11	3.05E-12	0.99	0.59
GLN[242]	TM5	12.60	0.80	0.010	8.07E-10	7.62E-11		
ALA[243]	TM2	31.90	0.85	0.007	1.46E-11	1.25E-12		
THR[244]	TM5	17.10	0.81	0.009	1.02E-09	9.77E-11		
ASP[246]	TM2	30.00	0.89	0.009	9.85E-12	2.05E-12		
GLU[247]	TM2	19.80	0.90	0.006	2.25E-11	2.08E-12		
ASN[249]	TM4	108.00	0.86	0.012	1.06E-11	1.54E-12	1.41	0.38
ARG[250]	TM2	10.10	0.88	0.007	6.80E-12	1.62E-12		
GLN[251]	TM5	19.30	0.87	0.010	8.07E-10	1.28E-10		
ILE[252]	TM5	28.10	0.88	0.010	1.15E-13	7.76E-05		
ALA[253]	TM5	22.80	0.84	0.007	9.96E-10	2.08E-10		
GLU[254]	TM5	23.90	0.86	0.007	8.55E-10	1.14E-10		
ILE[255]	TM5	16.80	0.85	0.007	7.42E-10	1.01E-10		
GLY[256]	TM2	5.74	0.91	0.014	5.74E-12	2.33E-12		
ALA[257]	TM3	55.30	0.91	0.017			1.59	0.54
SER[258]	TM5	53.90	0.75	0.014	3.07E-09	1.30E-09		
LEU[259]	TM5	18.00	0.85	0.012	8.94E-10	1.32E-10		
ILE[260]	-	-	-	-	-	-	-	-
LYS[261]	TM4	22.60	0.85	0.018	1.17E-11	1.98E-12	2.70	0.63
HIS[262]	TM5	11.00	0.83	0.008	5.07E-10	7.41E-11		
TRP[263]	TM5	16.40	0.79	0.010	2.84E-10	4.99E-11		

Backbone Dynamics on the μ s-ms timescale**Table S3** Complete set of residue-specific parameters obtained from two-state fits of ^{15}N D-CEST profiles for Apo_{TEM-1}, including ground-state chemical shifts δ_A , chemical-shift differences between ground and excited states ($\Delta\omega_{AB}$), and longitudinal and transverse relaxation rates R_1 and R_2 respectively.

Residue	δ_A (ppm)			$\Delta\omega_{AB}$ (ppm)			R_1 (s ⁻¹)			R_2 (s ⁻¹)		
R43	115.00	±	0.01	2.54	±	0.01	0.73	±	0.01	12.92	±	0.17
V44	128.00	±	0.01	-2.43	±	0.02	0.75	±	0.01	17.70	±	0.29
L169	113.00	±	0.01	1.77	±	0.01	0.85	±	0.01	16.84	±	0.21
N170	120.00	±	0.01	-2.21	±	0.02	0.86	±	0.01	18.51	±	0.29
A172	118.00	±	0.01	4.66	±	0.01	0.80	±	0.01	17.87	±	0.27
I173	121.00	±	0.01	3.17	±	0.01	0.79	±	0.01	18.18	±	0.26
W210	120.00	±	0.01	-0.44	±	0.23	0.86	±	0.03	18.06	±	0.69
E212	125.00	±	0.01	-0.30	±	0.45	0.90	±	0.03	29.04	±	0.78
A213	119.00	±	0.01	10.56	±	0.06	0.82	±	0.02	16.08	±	0.34
D214	116.00	±	0.01	1.41	±	0.05	0.91	±	0.03	23.01	±	0.71
K215	127.00	±	0.01	-0.37	±	0.57	0.92	±	0.04	36.24	±	1.48
V216	110.00	±	0.02	0.56	±	0.49	0.92	±	0.05	48.28	±	1.87
A217	126.00	±	0.01	11.54	±	0.05	0.85	±	0.03	16.89	±	0.59
G218	114.00	±	0.01	-1.86	±	0.05	0.91	±	0.04	26.39	±	0.98
G236	103.00	±	0.02	5.00	±	0.03	0.76	±	0.03	23.52	±	0.79
E240	117.00	±	0.01	6.57	±	0.02	0.89	±	0.02	17.76	±	0.43
R241	118.00	±	0.01	-2.45	±	0.03	0.90	±	0.02	16.92	±	0.43
G242	103.00	±	0.00	6.56	±	0.01	0.81	±	0.01	17.59	±	0.15
S243	119.00	±	0.03	-5.92	±	0.06	0.68	±	0.07	19.21	±	1.33
G245	111.00	±	0.01	-5.53	±	0.02	0.85	±	0.02	18.19	±	0.39
Y264	122.00	±	0.01	4.00	±	0.03	0.78	±	0.03	17.69	±	0.49
G267	114.00	±	0.01	-7.48	±	0.02	0.79	±	0.02	19.34	±	0.36

Table S4. Complete set of residue-specific parameters obtained from two-state fits of ^{15}N CPMG profiles for Apo_{TEM-1}, including ground-state chemical shifts δ_A , chemical-shift differences between ground and excited states ($\Delta\omega_{AB}$), and transverse relaxation rates and R_2 .

Residue	δ_A (ppm)			$\Delta\omega_{AB}$ (ppm)			R_2 (s ⁻¹)		
E37	117.42	±	0.03	0.75	±	0.06	23.50	±	0.15
E48	123.17	±	0.03	0.62	±	0.05	22.30	±	0.11
M69	120.40	±	0.03	0.91	±	0.07	25.40	±	0.16
T71	111.94	±	0.03	1.49	±	0.16	27.61	±	0.27
Y105	120.99	±	0.03	0.72	±	0.04	20.46	±	0.08
S106	119.78	±	0.03	0.70	±	0.05	20.46	±	0.10
T128	109.31	±	0.03	1.11	±	0.13	34.63	±	0.26
S130	115.43	±	0.03	2.14	±	0.23	26.44	±	0.26
D131	120.61	±	0.03	1.45	±	0.14	24.71	±	0.25
H153	120.61	±	0.03	0.72	±	0.06	23.86	±	0.13
H158	121.00	±	0.03	0.64	±	0.05	22.55	±	0.10
E171	120.59	±	0.03	0.75	±	0.06	24.88	±	0.12
S203	118.38	±	0.03	0.88	±	0.07	24.68	±	0.17
L225	120.71	±	0.03	0.87	±	0.06	23.33	±	0.11
I231	123.19	±	0.03	0.77	±	0.05	23.22	±	0.11
I287	117.85	±	0.03	0.86	±	0.08	23.47	±	0.19

Table S5. Complete set of residue-specific parameters obtained from two-state fits of ^{15}N CPMG profiles for Apo_{TEM-1}, including ground-state chemical shifts δ_A , chemical-shift differences between ground and excited states ($\Delta\omega_{AB}$), and transverse relaxation rates and R_2 .

Residue	δ_A (ppm)			$\Delta\omega_{AB}$ (ppm)			R_2 (s ⁻¹)		
R43	115.92	±	0.02	0.50	±	0.02	25.20	±	0.07
M68	119.55	±	0.02	0.82	±	0.06	40.72	±	0.25
Y105	121.69	±	0.02	0.62	±	0.08	35.23	±	0.31
S106	120.15	±	0.03	0.64	±	0.07	32.89	±	0.30
S130	116.07	±	0.01	3.31	±	0.32	42.59	±	0.95
D131	121.09	±	0.02	2.06	±	0.11	39.46	±	0.50
L169	113.39	±	0.03	0.75	±	0.06	34.64	±	0.26
A213	118.28	±	0.01	0.84	±	0.04	39.69	±	0.17
D214	120.87	±	0.02	0.85	±	0.07	40.59	±	0.32
K234	116.36	±	0.02	0.92	±	0.06	38.88	±	0.26
G238	107.89	±	0.03	0.70	±	0.07	36.27	±	0.30
G245	118.71	±	0.02	0.85	±	0.08	41.16	±	0.35
Q269	112.95	±	0.05	0.72	±	0.04	33.77	±	0.17

IX.3. Resistance Mutation Enhances TEM β -Lactamase Dynamics (manuscript in preparation)

In this paper, the X-ray crystallographic analyses and kinetic studies were performed by Dr. Frédéric Cawez and are included for contextual purposes only; they do not form part of my original contributions.

Resistance Mutation Amplifies Multi-Timescale Flexibility in TEM β -Lactamase

Marius Wanko Nembot[†], Frédéric Cawez[‡], Maximiliano Figueroa[§], Alain Brans^{||}, Ulric B. le Paige[#], Frédéric Kerff^{†*}, Moreno Galleni^{†*}, Guillaume Bouvignies[#] and Christian Damblon^{†*}

[†]Structural Biological Chemistry Laboratory, MolSys Research Unity, University of Liege, Liège, Belgium.

[‡]InBioS, Center for Protein Engineering, Biological Macromolecules, Department of Life Sciences, University of Liège, Liège, Belgium

[§]Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Biológicas, Universidad de Concepción, Concepción, Chile

^{||}Center for Protein Engineering, Protein Factory, Department of Life Sciences, University of Liège, Liège, Belgium

[#]Chimie Physique et Chimie du Vivant (CPCV), Département de Chimie, École normale supérieure, PSL University, Sorbonne Université, CNRS, Paris, France

Corresponding Authors

Christian Damblon: C.Damblon@uliege.be

Marius Wanko Nembot: amwanko@uliege.be

Keywords: Enzyme, TEM-1, TEM-121, Conformational exchange, NMR, CPMG, CEST

INTRODUCTION

The rapid evolution of β -lactamase enzymes continues to undermine the clinical efficacy of β -lactam antibiotics. Among them, TEM β -lactamases represent one of the most extensively studied and widely disseminated resistance determinants in Gram-negative pathogens (Bush and Jacoby 2010). The class A enzyme TEM-1 β -lactamase efficiently hydrolyses penicillins and early cephalosporins, and its evolutionary diversification has produced hundreds of variants with expanded-spectrum β -lactamase (ESBL) or inhibitor-resistant (IRT) phenotypes. Despite extensive structural and kinetic characterization, the mechanistic basis by which mutations reconfigure catalytic specificity and inhibitor resistance remains incompletely understood (Knox 1995). Traditional structural biology has provided high-resolution snapshots of TEM variants. However, the minimal structural differences observed among variants often fail to fully rationalize their divergent catalytic specificities and inhibitor susceptibilities. Increasing evidence suggests that enzyme function is governed not solely by ground-state structure but by the distribution and interconversion of conformational substates across multiple timescales. In this view, mutations may not simply alter active-site geometry; instead, they may redistribute conformational populations, modulate dynamic exchange processes, and reshape the underlying energy landscape that governs catalysis and recognition.

The ESBL/IRT variant TEM-121 provides an opportunity to interrogate this dynamic dimension of resistance evolution. TEM-121 differs from TEM-1 by six amino acid substitutions (Q39K, E104K, R164S, A237T, E240K, and R244S). The Q39K substitution (Davies 1994), which is not a canonical determinant of ESBL or IRT, is positioned distal to the active site and may instead enhance molecular dynamics or modulate the structural flexibility of the enzyme (Shcherbinin et al. 2017). The substitutions E104K, R164S, A237T, and E240K are characteristic of ESBL variants, whereas R244S is associated with reduced susceptibility to β -lactamase inhibitors and is commonly observed in IRT enzymes (Poirel et al. 2004). While crystallographic structures of TEM variants frequently reveal only subtle structural differences relative to TEM-1, their kinetic and inhibitory properties differ markedly. Such discrepancies suggest that resistance-conferring mutations may exert their effects through modulation of conformational flexibility, potentially influencing both fast backbone motions (ps–ns) and slower conformational exchange processes (μ s–ms) that are often linked to functional transitions. Solution Nuclear Magnetic Resonance (NMR) spectroscopy is uniquely positioned to quantify protein dynamics across these timescales, enabling residue-specific characterization of fast internal motions and slower exchange processes. When combined with enzymatic kinetics and crystallography, a more complete description of the conformational ensemble can be achieved. Furthermore, ligand or binding-partner recognition offers a controlled perturbation to probe how conformational equilibria respond to binding-induced perturbations.

Here, we employ an integrated structural and biophysical strategy to compare TEM-1 and the ESBL/IRT variant TEM-121 in both their free and antibody-bound states. Enzymatic kinetics reveal that the antibody cAb_{TEM-13} VHH (hereafter referred to as cAb_{TEM-13} or VHH) exerts an inhibitory effect that depends on both the enzymes and the substrate. In contrast, high-resolution crystallographic analysis shows no discernible structural differences between the cAb_{TEM-13} complexes with TEM-1 and TEM-121. In marked

contrast to these static and ensemble-averaged observations, NMR relaxation measurements demonstrate that TEM-121 exhibits enhanced flexibility relative to TEM-1 across both fast (ps–ns) and slow (μ s–ms) timescales, indicating mutation-induced amplification of conformational plasticity. VHH binding further redistributes these dynamic processes through cooperative coupling, differentially modulating fast and slow motions in the two enzymes.

Together, these results suggest that resistance-associated mutations do not primarily manifest through detectable structural rearrangements but instead through reweighting of conformational dynamics across multiple timescales. Our findings identify multi-timescale flexibility as a central determinant in the functional divergence of TEM variants and establish a dynamic framework for understanding how mutation and molecular recognition cooperatively modulate β -lactamase activity.

RESULTS AND DISCUSSION

Kinetic Characterization and X-ray Crystallographic Analysis. In his work, Dr. Frédéric Cawez compared the effect of cAb_{TEM-13} binding on the activity of TEM-1 and TEM-121. To further characterize this interaction, the influence of cAb_{TEM-13} on enzyme activity was evaluated using four β -lactam reporter substrates: one penicillin (benzylpenicillin) and three cephalosporins (nitrocefin, cefalotin, and cephaloridine). Kinetic measurements revealed that the inhibitory effect of the cAb_{TEM-13} varied depending on both the enzyme variant and the substrate (**Figure 1 and Table 1**). Overall, the residual activities of the TEM-1/cAb_{TEM-13} complex hereafter referred as holo_{TEM-1}, were consistently lower than those of the holo_{TEM-121} complex across all substrates.

Table 1. Kinetic parameters describing the inhibitory effect of cAb_{TEM-13}

Cefalotin ^a	v_0 ($\mu\text{mol min}^{-1} \text{mg}^{-1}$) TEM	v_0 ($\mu\text{mol min}^{-1} \text{mg}^{-1}$) TEM/ cAb _{TEM-13}	Residual activity [%]
TEM-1	130 $\pm$ 20	26 $\pm$ 5	20 $\pm$ 1
TEM-121	3.0 $\pm$ 0.3	3.6 $\pm$ 0.3	117 $\pm$ 1
Benzylpenicillin ^b	v_0 ($\mu\text{mol min}^{-1} \text{mg}^{-1}$) TEM	v_0 ($\mu\text{mol min}^{-1} \text{mg}^{-1}$) TEM/ cAb _{TEM-13}	Residual activity [%]
TEM-1	3473 $\pm$ 520	1016 $\pm$ 438	29 $\pm$ 8
TEM-121	36 $\pm$ 7	22 $\pm$ 4	60 $\pm$ 0.3
Nitrocefin ^c	v_0 ($\mu\text{mol min}^{-1} \text{mg}^{-1}$) TEM	v_0 ($\mu\text{mol min}^{-1} \text{mg}^{-1}$) TEM/ cAb _{TEM-13}	Residual activity [%]
TEM-1	1262 $\pm$ 557	7 $\pm$ 1	1 $\pm$ 0.1
TEM-121	133 $\pm$ 11	48 $\pm$ 7	36 $\pm$ 2
Cephaloridin ^d	v_0 ($\mu\text{mol min}^{-1} \text{mg}^{-1}$) TEM	v_0 ($\mu\text{mol min}^{-1} \text{mg}^{-1}$) TEM/ cAb _{TEM-13}	Residual activity [%]
TEM-1	568 $\pm$ 335	21 $\pm$ 1	4 $\pm$ 2
TEM-121	45 $\pm$ 8	21 $\pm$ 1	48 $\pm$ 12

^a Initial rates of cefalotin hydrolysis for 5 nM of TEM-1 and 40 nM of TEM-121. Measures were performed for the apo-enzymes and for complexes in one saturating cAb_{TEM-13} concentration (1 μM). Rates of hydrolysis are expressed in μmol of cefalotin (200 μM) hydrolyzed per minute and per milligram of enzyme.

^b Initial rates of benzylpenicillin hydrolysis for 5 nM of TEM-1 and 40 nM of TEM-121. Measures were performed for the apo-enzymes and for complexes in one saturating cAb_{TEM-13} concentration (1 μM).

^c Initial rates of nitrocefin hydrolysis for 5 nM of TEM-1 and 40 nM of TEM-121. Measures were performed for the apo-enzymes and for complexes in one saturating cAb_{TEM-13} concentration (1 μM).

^d Initial rates of Cephaloridin hydrolysis for 2 nM of TEM-1 and 20 nM of TEM-121. Measures were performed for the apo-enzymes and for complexes in one saturating cAb_{TEM-13} concentration (1 μM).

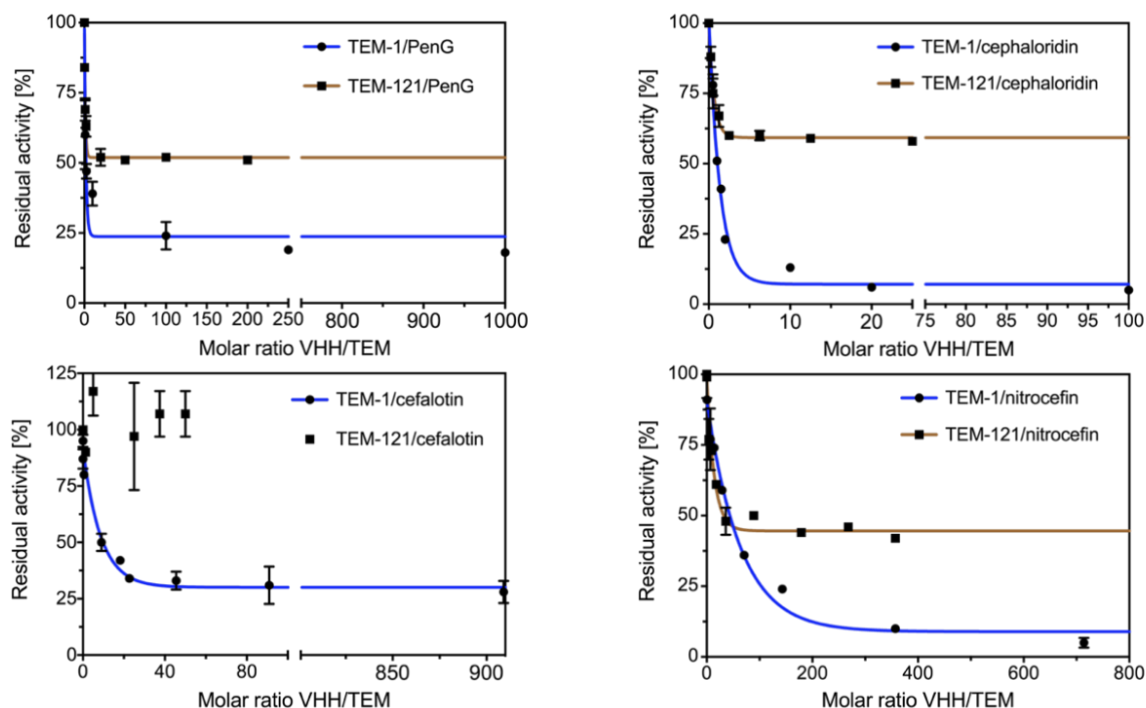


Figure 1. Binding effect of the cAb_{TEM-13} on TEM activity for 300 μ M of benzylpenicillin (A), 200 μ M of cefalotin (B), 200 μ M of cephalexin (C) and 100 of nitrocefin (D). Concentrations of TEM-1 and TEM-121 used for each substrate described above were 0.1 and 5 nM, 1.1 and 20 nM, 0.5 and 4 nM, 0.07 and 2.8 nM, respectively. All data were fitted on a one phase exponential decay equation. Each experiment was realized twice independently. These results indicate that cAb_{TEM-13} exerts an inhibitory effect on TEM-1 and TEM-121, with inhibition depending on both the enzyme and the substrate. Overall, a stronger inhibition of TEM-1 activity is observed compared to TEM-121 for all substrates tested.

Quantitative binding measurements, including the association (k_{on}) and dissociation (k_{off}) rate constants, as well as the equilibrium dissociation constant (K_D), were determined for the interaction between cAb_{TEM-13} and TEM-121 (**Figure 2**) and compared to those obtained for TEM-1 (**Figure 2** and **Table 2**). The association rate constants of the cAb_{TEM-13} with both β -lactamases were very similar, with values on the order of 10^5 $M^{-1}\cdot s^{-1}$, indicating rapid binding of the VHH to its antigen. Furthermore, the dissociation rate constants were low ($\sim 10^{-3}$ s^{-1}), reflecting relatively stable complexes and resulting in high affinities with K_D values of 13 and 6 nM, respectively.

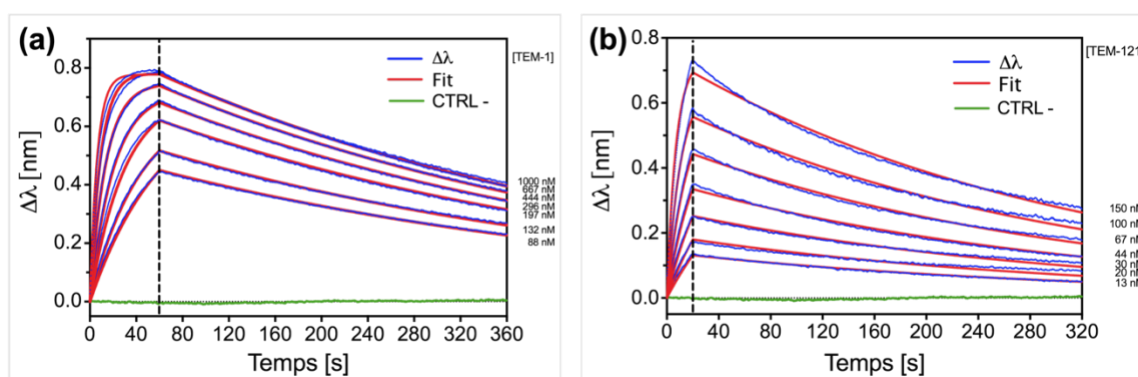


Figure 2. Binding characterization of cAb_{TEM-13}. (a) Quantitative analysis of the interaction between cAb_{TEM-13} and TEM-1. (b) Quantitative analysis of the interaction between cAb_{TEM-13} and TEM-121. The negative control (CTRL-) corresponds to the loading the analyte directly onto the sensor.

Table 2. Association (k_{on}), dissociation (k_{off}), and equilibrium dissociation (K_D) constants derived from global fitting of bio-layer interferometry data obtained at a minimum of seven TEM concentrations, using a 1:1 binding model.

	k_{on} ($10^5 \text{ M}^{-1} \text{ s}^{-1}$)	k_{off} (10^{-3} s^{-1})	K_D (nM)
TEM-1	1.7 ± 0.1	2.2 ± 0.2	13 ± 2
TEM-121	6.1 ± 2.2	3.2 ± 0.1	6 ± 1

The three-dimensional structures of the holot_{TEM-1} and holot_{TEM-121} complexes were therefore determined by X-ray crystallography. Surprisingly, no significant structural differences were observed between the two complexes (**Figure 3**).

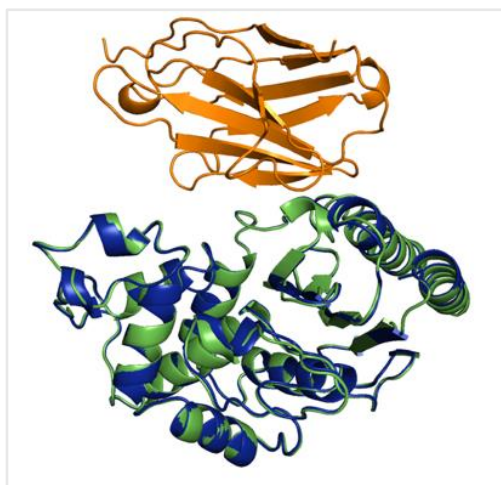


Figure 3. Comparison of the holot_{TEM-1} (green) and holot_{TEM-121} (blue) X-ray structures. holot_{TEM-1} and holot_{TEM-121} structures were determined at 1.6 Å and 2.2 Å resolution, respectively and show no significant structural differences.

Mutation-Induced Chemical Shift Perturbations in apo_{TEM-121}. To assess the structural consequences of the six mutations distinguishing TEM-121 from TEM-1, we first compared their backbone amide chemical shifts in the apo state. Backbone chemical shifts are highly sensitive to the local electronic environment and secondary structure and thus provide residue-specific reporters of mutation-induced perturbations. Both apo_{TEM-1} and apo_{TEM-121} display well-dispersed ^1H - ^{15}N HSQC spectra indicative of properly folded proteins. However, substantial differences in peak positions are observed across the spectrum (**Figure 4 (a)**), revealing widespread mutation-induced perturbations to the backbone amide chemical environments.

Chemical shift perturbations (CSPs) were quantified as the combined amide shift difference, $\Delta\delta_{\text{NH}}$ (ppm), calculated as the weighted root-mean-square of the proton and nitrogen chemical shift differences ($\Delta\delta_{\text{H}}$ and $\Delta\delta_{\text{N}}$, respectively), according to

$$\Delta\delta_{\text{NH}} = \sqrt{\Delta\delta_{\text{H}}^2 + 0.14 \cdot \Delta\delta_{\text{N}}^2}$$

where the factor of 0.14 accounts for differences in sensitivity of the amide proton and nitrogen backbone resonances (Mielke and Krishnan 2009). These are summarized in **Figure 4 (c)**.

The most pronounced chemical shift perturbations are clustered within three distinct regions: the N-terminal segment, the Ω -loop, and the region surrounding the active-site substitution A237T. Perturbations in the N-terminal region are consistent with the presence of the Q39K substitution and suggest local alterations in the chemical environment extending beyond the immediate mutation site. Notably, substantial CSPs are also observed within the Ω -loop, a structural element that plays a critical role in shaping the active site and modulating substrate accommodation (Knox 1995; Meneksedag et al. 2013; Knoverek et al. 2021). The CSPs detected in the Ω -loop may reflect the influence of substitutions clustered in and around this region (**Figure 4 (b)**).

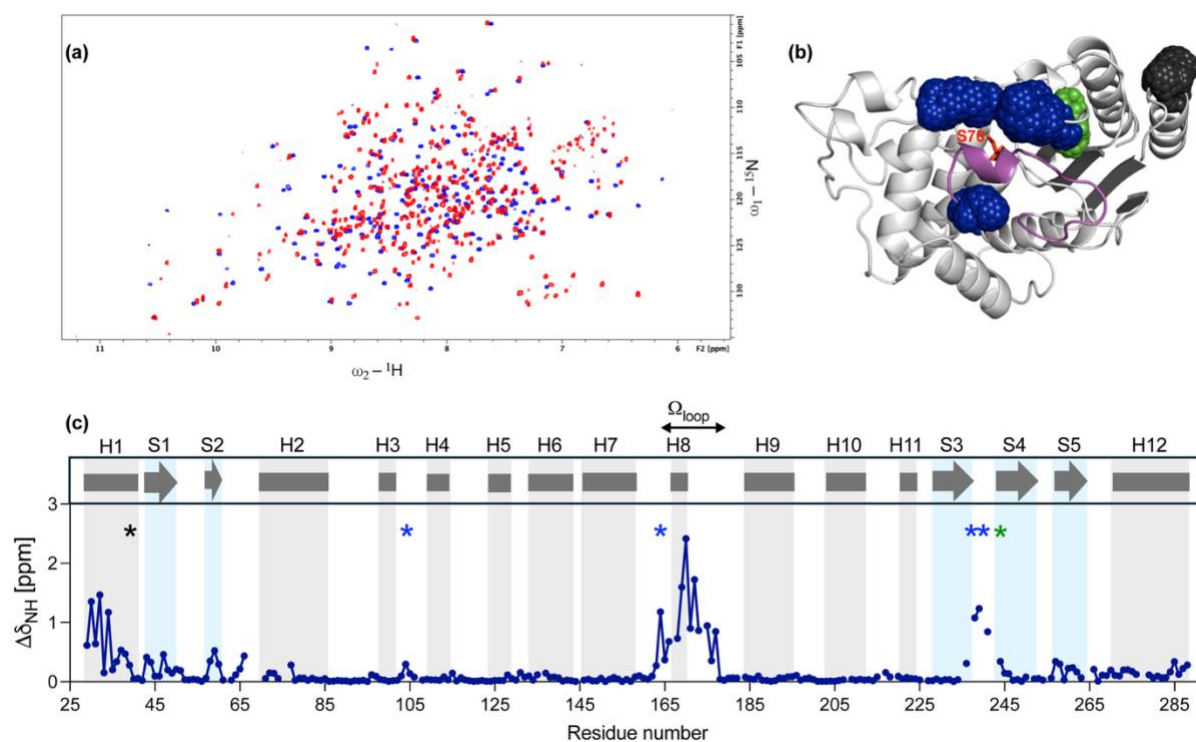


Figure 4. Structural and chemical shift differences between apoTEM-1 and apoTEM-121. (a) Two-dimensional ^1H - ^{15}N HSQC spectra of uniformly [^2H , ^{15}N]-labeled apoTEM-1 (blue) and apoTEM-121 (red) acquired at 700 MHz and 303 K in 50 mM sodium phosphate buffer (pH 6.0) at a protein concentration of 500 μM . (b) Spatial localization of the six mutations in TEM-121 mapped onto the TEM-121 structure. The Ω -loop (residues 164–179) is highlighted in magenta. Mutation sites are color-coded as follows: Q39K (black), ESBL mutations (blue), and the IRT mutation (green). (c) Residue-specific average amide chemical shift perturbations ($\Delta\delta_{\text{NH}}$, ppm) between apoTEM-1 and apoTEM-121. Asterisks indicate mutation sites. Color scheme is identical to that shown in panel (b).

In addition, pronounced perturbations are observed for residues surrounding the active-site substitution A237T (Knox 1995), including those adjacent to E240K and R244S. These substitutions are located within the substrate-binding pocket (Maveyraud et al. 1996; Jelsch et al. 1993), and the associated CSPs may reflect local rearrangements of the active-site environment. Notably, several residues within this region could not be assigned in TEM-121 (resulting in a gap in the **Figure 4 (c)**), suggesting either significant chemical shift changes or line broadening. The absence of assignments is consistent with substantial alterations in the local chemical environment and may indicate enhanced conformational heterogeneity in this region. Although E104K involves a glutamate to lysine charge reversal, no detectable backbone CSPs are observed near residue 104, indicating that the substitution does not substantially alter the local backbone chemical environment in the apo state.

Combined Mutational Effects Enhance ps–ns Backbone Dynamics. Site-specific $\{^{15}\text{N}\}$ - R_1 , $\{^{15}\text{N}\}$ - R_2 and $^1\text{H}\{-^{15}\text{N}\}$ NOE values were obtained for 237 residues in apo_{TEM-121} out of 251 non-proline backbone amide resonances, at magnetic field strengths of 16.4 and 11.7 T. These data, summarized in **Figure 5**, provide residue-specific insight into fast protein dynamics (Kay and Torchia 1998). The $\{^{15}\text{N}\}$ - R_1 profiles exhibit the same residue-specific pattern at 16.4 and 11.7 T, and, as expected, $\{^{15}\text{N}\}$ - R_1 (11.7 T) > $\{^{15}\text{N}\}$ - R_1 (16.4 T). The $\{^{15}\text{N}\}$ - R_2 values are highly similar at 16.4 and 11.7 T, with no significant differences observed in the residue-specific profiles. Although the CSA contribution to $\{^{15}\text{N}\}$ - R_2 is expected to increase with magnetic field strength (scaling with ω_N^2), the near-identical behavior at both fields indicates minimal field-dependent contributions under the present conditions (Palmer et al. 2001). Notably, residues in the hinge region (212–224) display elevated $\{^{15}\text{N}\}$ - R_2 values in apo_{TEM-121}, indicative of conformational exchange on the microsecond-to-millisecond (μs -ms) timescale. The $^1\text{H}\{-^{15}\text{N}\}$ NOE profiles display essentially identical residue-specific patterns at 16.4 and 11.7 T, with negative values observed in the N-terminal region, near the Q39K mutation. Such negative NOE values are characteristic of enhanced fast-timescale (ps–ns) backbone flexibility, indicating that this region undergoes large-amplitude internal motions relative to the overall molecular tumbling (Kay et al. 1989; Tjandra, Kuboniwa, et al. 1995). In agreement with the $^1\text{H}\{-^{15}\text{N}\}$ NOE data, the longitudinal and transverse relaxation rates further support enhanced flexibility in the N-terminal region, as evidenced by elevated $\{^{15}\text{N}\}$ - R_1 values and reduced $\{^{15}\text{N}\}$ - R_2 values relative to the average values. These relaxation signatures support the previous reports suggesting that the Q39K substitution contributes to increased fast-timescale mobility within the N-terminal region (Shcherbinin et al. 2017).

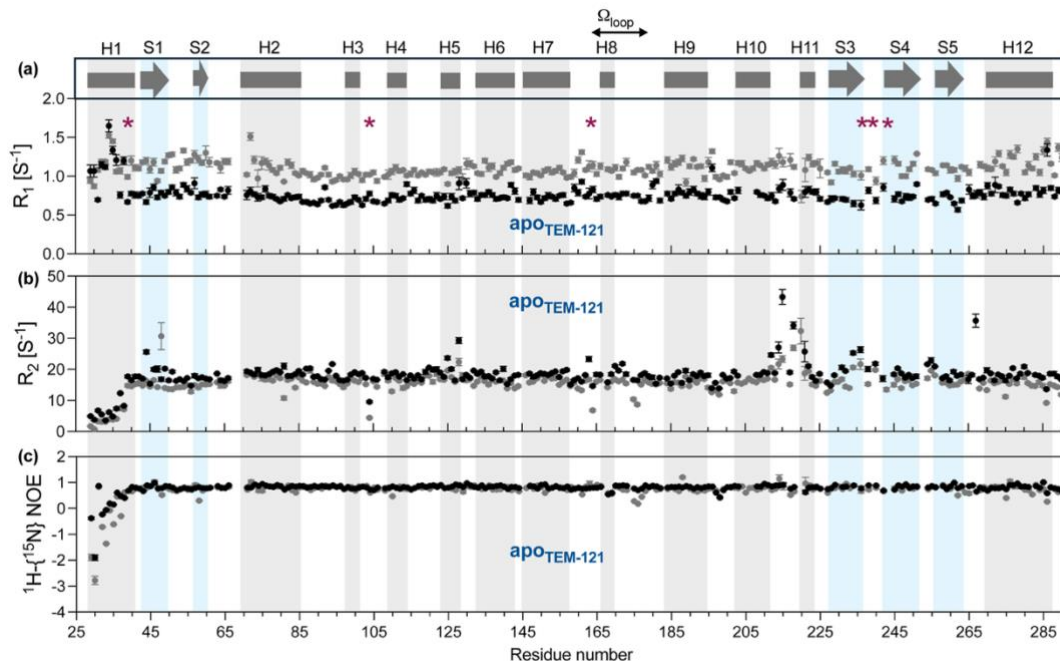


Figure 5. Experimental $\{^{15}\text{N}\}$ - R_1 , $\{^{15}\text{N}\}$ - R_2 and $^1\text{H}\{-^{15}\text{N}\}$ NOE values for free TEM-121 β -lactamase at 16.4 T (color code: black) and 11.7 T (color code: steel), recorded at 30° C. (a, b, c) apo_{TEM-121} $\{^{15}\text{N}\}$ - R_1 , $\{^{15}\text{N}\}$ - R_2 and $^1\text{H}\{-^{15}\text{N}\}$ NOE respectively. Asterisks indicate mutation sites.

Given the strong agreement of the apo_{TEM-121} NSR data acquired at 16.4 and 11.7 T, and the convergence of residue-specific dynamic profiles, model-free analysis was deemed unnecessary for this enzyme. Instead, the three ^{15}N relaxation parameters ($\{^{15}\text{N}\}\text{-R}_1$, $\{^{15}\text{N}\}\text{-R}_2$, and $^1\text{H}\text{-}\{^{15}\text{N}\}$ NOE) measured at 16.4 T (700 MHz) were used for subsequent comparison between apo_{TEM-1} and apo_{TEM-121}.

Comparison of the relaxation parameters between apo_{TEM-1} and apo_{TEM-121} reveals largely similar residue-specific profiles across the enzyme, with the notable exception of the N-terminal region of apo_{TEM-121}, which exhibits enhanced flexibility, potentially associated with the Q39K substitution (**Figure 6**). In contrast, residues within the hinge region display elevated $\{^{15}\text{N}\}\text{-R}_2$ values in both enzymes, consistent with the presence of slower conformational fluctuations occurring on the microsecond-to-millisecond timescale.

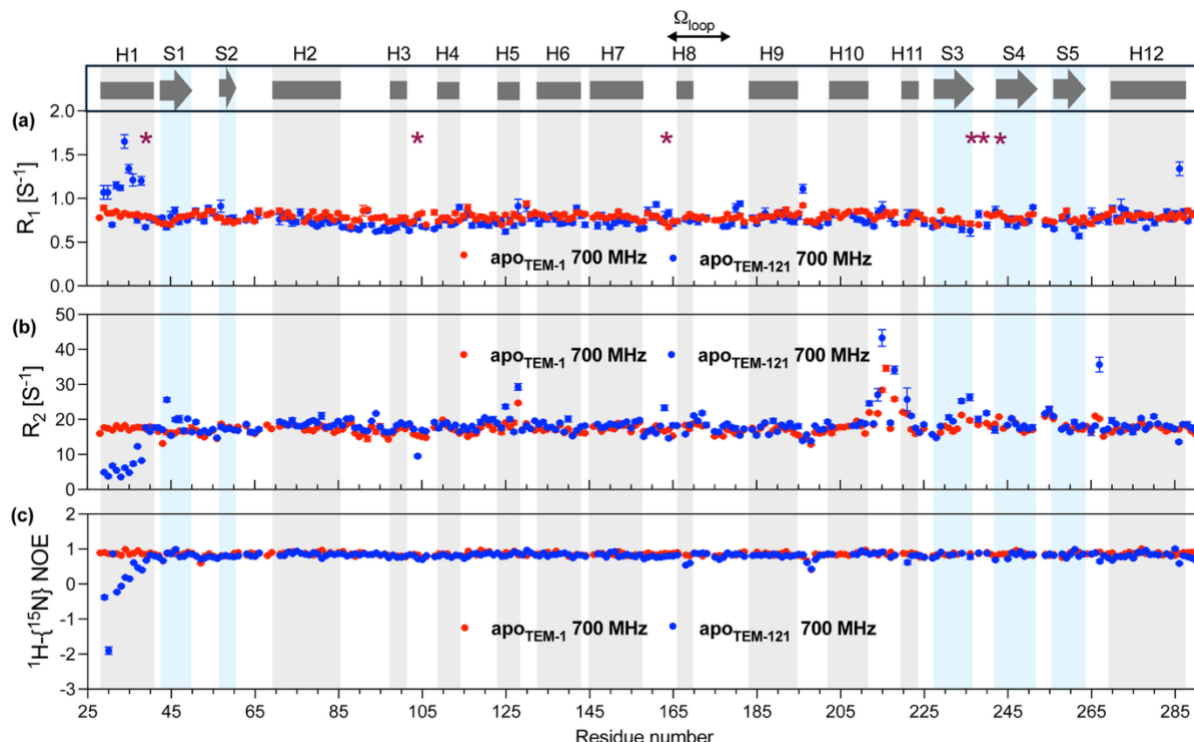


Figure 6. Comparison of NSR data for TEM-1 (color code: red), and TEM-121 (color code: blue), recorded at 16.4 T (700 MHz) and 30° C. (a, b, c) $\{^{15}\text{N}\}\text{-R}_1$, $\{^{15}\text{N}\}\text{-R}_2$ and $^1\text{H}\text{-}\{^{15}\text{N}\}$ NOE respectively. Asterisks indicate mutation sites.

In contrast to apo_{TEM-121}, NMR relaxation measurements for the holo_{TEM-121} (TEM-121/cAb_{TEM-13} complex) form were acquired only at 16.4 T. Comparison of the relaxation parameters between the apo and cAb_{TEM-13}-bound states reveals the expected behavior: $\{^{15}\text{N}\}\text{-R}_1$ values decrease upon complex formation, whereas $\{^{15}\text{N}\}\text{-R}_2$ values increase in the holo state, consistent with the longer overall rotational correlation time of the cAb_{TEM-13}-bound complex (**Figure 7**). Notably, the elevated $\{^{15}\text{N}\}\text{-R}_2$ values observed in the apo state within the hinge region are no longer present in the holo form, suggesting that VHH binding attenuates this local exchange process. The $^1\text{H}\text{-}\{^{15}\text{N}\}$ NOE profiles remain largely unchanged between the two functional states, except for the N-terminal region, near

Q39K mutation, where increased $^1\text{H}\{-^{15}\text{N}\}$ NOE values are detected in the holo enzyme. This behavior indicates reduced fast-timescale flexibility in this region upon VHH binding.

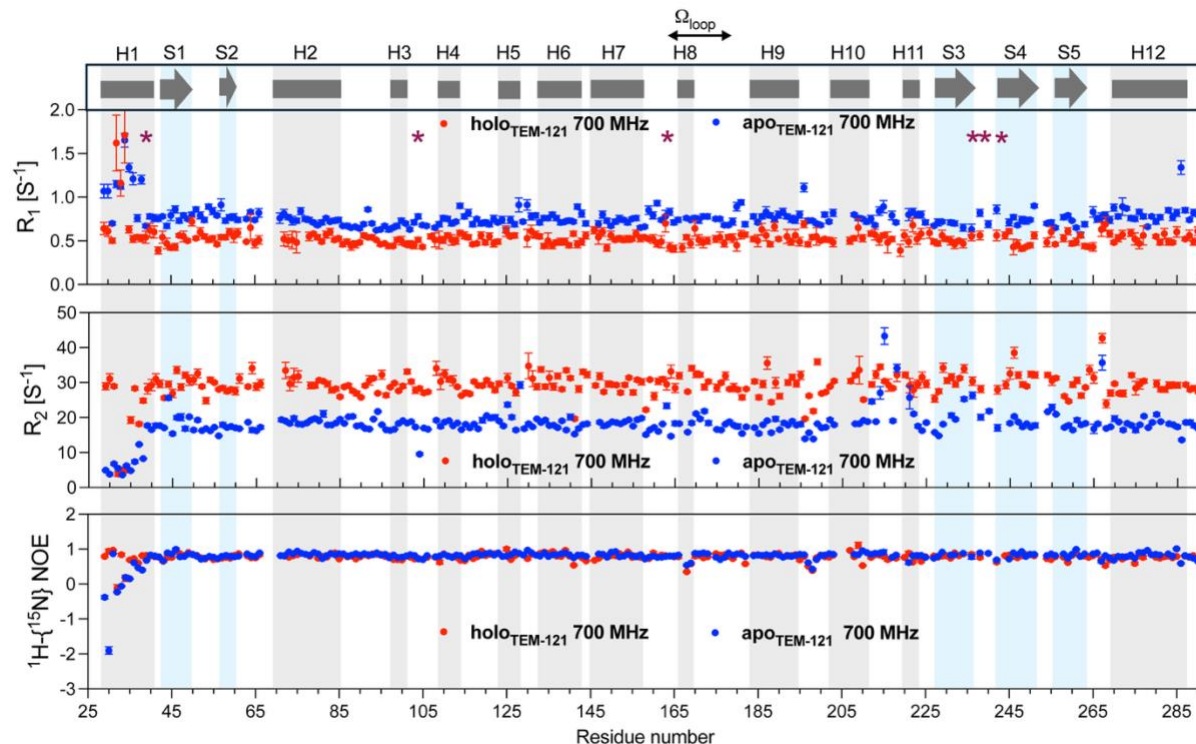


Figure 7. Comparison of NSR data for apo_{TEM-121} (color code: blue), and holo_{TEM-121} (color code: red), recorded at 16.4 T (700 MHz) and 30° C. (a, b, c) $\{^{15}\text{N}\}$ - R_1 , $\{^{15}\text{N}\}$ - R_2 and $^1\text{H}\{-^{15}\text{N}\}$ NOE respectively. Asterisks indicate mutation sites.

Resistance-Associated Mutations Reshape the μs – ms Energy Landscape of TEM β -Lactamases. To gain insight into how resistance-conferring mutations modulate conformational flexibility, and how VHH cAb_{TEM-13} binding influences TEM-121 inhibition, we investigated conformational exchange processes on the microsecond-to-millisecond timescale in both apo and holo TEM-121, with the aim of directly comparing these results to the corresponding TEM-1 states.

To investigate potential conformational exchange on the μs – ms timescale in apo_{TEM-121}, we employed a comprehensive set of chemical exchange-based NMR methods, combining ^{15}N CPMG relaxation dispersion (Hansen et al. 2007), ^{15}N D-CEST (Gareth A. Morris and Freeman 2011; Bodenhausen, Freeman, et al. 1976), and ^{15}N visible peak-position constraints (Khandave et al. 2024; Skrynnikov et al. 2002). All datasets were analysed jointly using a global two-state exchange model ($A \rightleftharpoons B$) implemented in ChemEx (<https://github.com/gbouvignies/chemex>), combining CPMG, D-CEST and peak-position constraints. In this framework, k_{ex} and p_B were shared across residues, while residue-specific parameters included $\Delta\omega_{AB}$ and relaxation rates. The analysis revealed that a significant number of residues undergo conformational exchange in the intermediate-to-fast exchange regime, where ^{15}N D-CEST profiles no longer display well-separated dips corresponding to distinct ground and excited state resonances (Khandave

et al. 2024; Rangadurai et al. 2020; Tiwari et al. 2024). Instead, a single asymmetric dip is observed (**Figure 8 (a–d)**) that can still be analysed to extract exchange parameters and minor-state chemical shifts. The fitted ground-state chemical shifts agree closely with the observed peak positions (**Figure 8 (e)**). Consistent with the ^{15}N D-CEST results, the ^{15}N CPMG relaxation dispersion profiles also indicate exchange processes occurring in the intermediate-to-fast regime (Y. Jin et al. 2025). Together, these data provide direct evidence for microsecond–millisecond conformational exchange in apoTEM-121 using a combined ^{15}N CPMG and D-CEST approach.

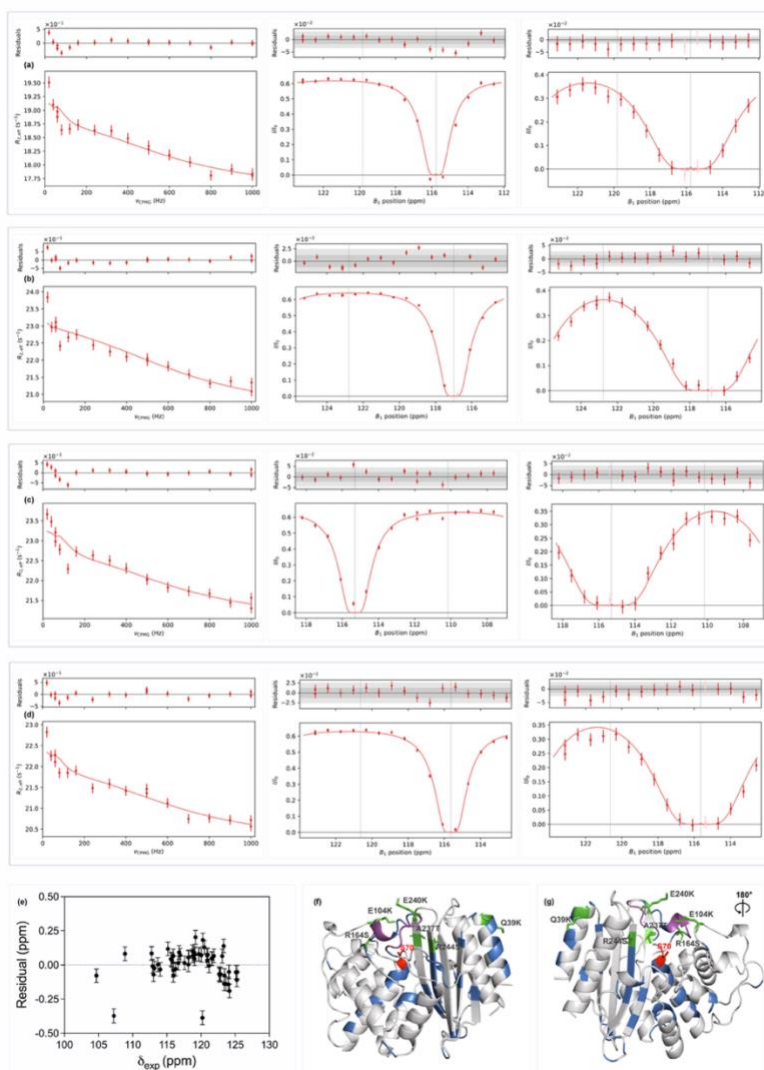


Figure 8. ^{15}N CPMG and ^{15}N D-CEST profiles for residues R43, W165, D179 and M211 (panels a–d respectively) of the apoTEM-121 recorded at 30 °C, $B_0 = 16.4$ T, with SW_{CEST} of 800 Hz and $B_1^{D\text{-CEST}}$ field strengths of 20 Hz and 60 Hz. (e) Residuals from the linear regression between experimental and predicted ground-state chemical shifts. (f–g) Structural representation of apoTEM-121 showing residues undergoing conformational exchange. These residues are color-coded in marine and mutations in green.

Global analysis identified 50 residues including V31, K34, D35, D38, G41, R43, Y46, D50, N52, S59, K53, F60, E63, E64, R65, T71, F72, K73, L77, V80, R83, S106, D115, R120, E121, D163, W165, N170, E171, I173, D179, E197, L198, W210, M211, E212, G218, L221, R222, L225, W229, I231, D233, G245, I246, I247, A248, D254, K256 and S258 undergoing two-state exchange with $k_{ex} = 3449 \pm 364 \text{ s}^{-1}$ and $p_B = 0.23 \pm 0.06\%$. The agreement between the experimental data and the simplest two-state exchange model supports the presence of a sparsely populated excited state ($p_B < 0.5\%$) that exists in dynamic equilibrium with the dominant ground state. The residues exhibiting conformational exchange are broadly distributed throughout the TEM-121 enzyme, including regions proximal to the mutation sites as well as key functional elements of the active-site architecture. Notably, exchange is detected near several core catalytic residues S70, E166 and K234 (T71, F72, K73, W165, N170 and D233), which are integral to nucleophile activation, stabilization of catalytic intermediates, and proton transfer during β -lactam hydrolysis. Additional exchanging residues are observed adjacent to the substrate-positioning residue R244 (G245 and I246), a region that contributes to substrate and inhibitor recognition. Furthermore, dynamic residues cluster within the Ω -loop (W165, N170, E171, I173 and D179), a structural element that shapes the active-site cavity and modulates substrate accommodation. The widespread μs – ms conformational exchange observed across the enzyme, including within key functional regions, suggests that catalysis may be dynamically coupled to coordinated motions within the active-site network. Such collective conformational motions could transiently reorganize the spatial arrangement of catalytic and substrate-positioning residues, thereby modulating the spatial arrangement of catalytic residues and substrate accommodation. Complete residue-specific parameters, including minor-state ^{15}N chemical shifts, are provided in **Table S1**.

Because the extracted exchange parameters place the process in the intermediate-to-fast exchange regime, parameter degeneracies can arise in single-field CPMG analyses. In addition, identifiability of CEST-derived parameters may be limited when ω_1 is below k_{ex} (Vallurupalli et al. 2017; Khandave et al. 2024). To evaluate the extent to which the additional observables provide orthogonal constraints, we repeated the analysis using only ^{15}N CPMG data in combination with visible-state peak positions, with parameter uncertainties estimated from 1000 Monte Carlo simulations. This reduced analysis yielded $k_{ex} = 3353 \pm 300 \text{ s}^{-1}$ and $p_B = 0.23 \pm 0.04 \%$, values comparable to those obtained from the full joint fit (**Figure 9**). The close agreement between the reduced and full joint analyses indicates that incorporation of ^{15}N D-CEST data and peak-position information does not introduce significant additional constraints on k_{ex} and p_B under the present experimental conditions. In the absence of a second magnetic field and ω_1 below k_{ex} , the combined CPMG and D-CEST datasets do not substantially improve parameter identifiability.

Nevertheless, despite the inherent limitations in parameters (k_{ex} and p_B) precision, the ^{15}N CPMG relaxation dispersion and D-CEST profiles robustly establish the presence of conformational exchange in the intermediate-to-fast regime on the μs – ms timescale. The moderate dispersion amplitudes observed in the ^{15}N CPMG profiles, together with a single asymmetric dip in the D-CEST experiments, are characteristic of exchange processes approaching the fast-exchange limit relative to the underlying chemical shift differences.

Importantly, although precise determination of exchange parameters k_{ex} and p_B is limited under these conditions, the combined CPMG and D-CEST data demonstrate the existence of a sparsely populated excited state and support its characteristic timescale. Collectively, these findings provide a dynamic framework for understanding how resistance-associated mutations modulate β -lactamase activity through redistribution of conformational states rather than large-scale structural rearrangements.

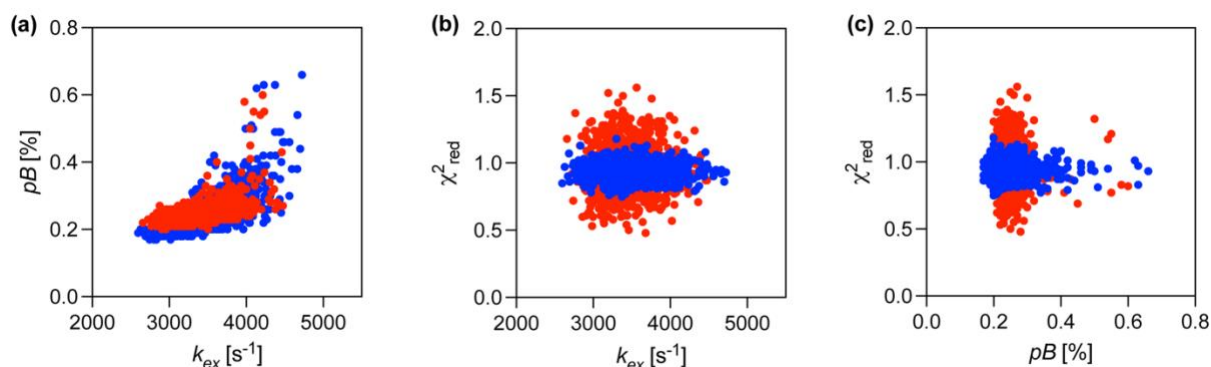


Figure 9. Evaluation of exchange parameters derived from ^{15}N CPMG-only and combined ^{15}N CPMG/D-CEST analyses. (a) Superposition of the $k_{ex} - p_B$ correlation obtained from the two fitting strategies, illustrating the similarity of the extracted exchange parameters. (b) Superposition of the $k_{ex} - \chi^2_{red}$ correlation profiles, highlighting comparable parameter behavior between the two analyses. (c) Superposition of the $p_B - \chi^2_{red}$ correlation profiles, demonstrating similar parameter sensitivity and identifiability. Color scheme: blue, parameters obtained from combined ^{15}N CPMG and D-CEST fits including visible-state peak positions; red, parameters derived from ^{15}N CPMG fits including visible-state peak positions.

Interestingly, regions exhibiting pronounced chemical shift perturbations also display signatures of μs – ms conformational exchange. In particular, residues K34, D35, D38, G41 and R43 within the N-terminal segment; including the Q39K substitution site; undergo detectable exchange, indicating that this region not only experiences altered local chemical environments but also enhanced dynamic motions. These observations are consistent with previous molecular dynamics simulations suggesting that the Q39K substitution reduces local stability and increases conformational mobility within the N-terminal region (Shcherbinin et al. 2017). Similarly, multiple residues within the Ω -loop (W165, N170, E171, I173 and D179) participate in exchange processes, underscoring the dynamic nature of this structural element that shapes the active-site cavity and modulates substrate accommodation. In contrast, no exchange was directly detected for residues surrounding the active-site substitution A237T. However, several residues in this region could not be assigned, likely due to exchange-induced line broadening, suggesting that conformational dynamics may be present but remain experimentally inaccessible under the current conditions. Thus, the apparent absence of exchange signatures near position 237 should be interpreted cautiously.

NMR relaxation studies of apo_{TEM-1} revealed that its conformational dynamics can be described by two independent two-state exchange processes, both originating from a common ground state: one operating in the slow regime ($k_{ex} = 112 \pm 3 \text{ s}^{-1}$ and $p_B = 1.85 \pm 0.03 \%$) and another in the intermediate exchange regime ($k_{ex} = 772 \pm 3 \times 10^{-12} \text{ s}^{-1}$ and $p_B = 2.32 \pm 1.04 \times 10^{-14} \%$) on the μs – ms timescale (**Figure 10**).

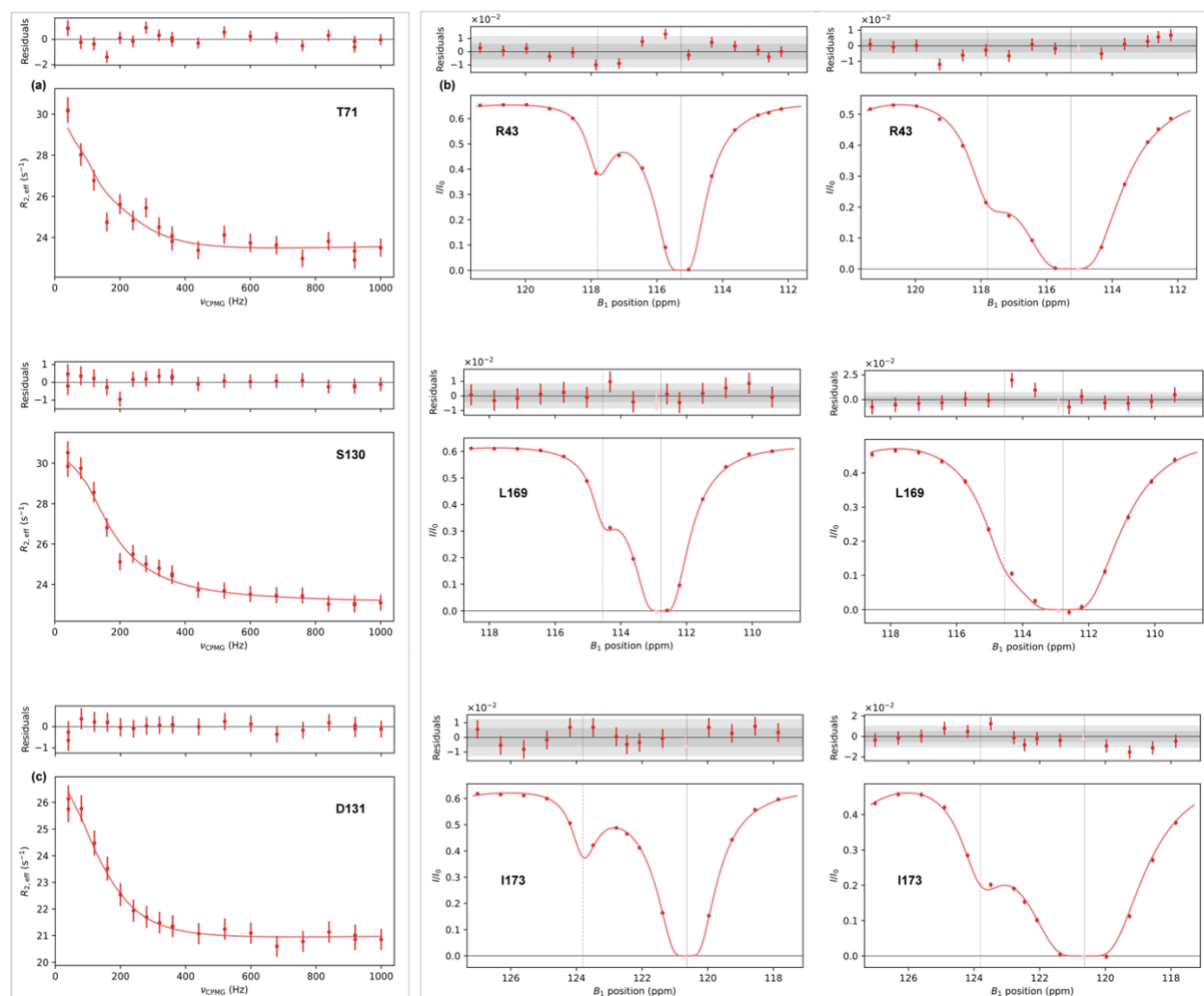


Figure 10. Conformational exchange in apo_{TEM-1} recorded at 30 °C and $B_0 = 16.4$ T. Panel (a) ¹⁵N CPMG profiles for residues R43, S130 and D131 with $T_{EX} = 50$ ms. Panel (b) ¹⁵N D-CEST profiles for residues R43, L169, I173, and D214, respectively with $SW_{CEST} = 700$ Hz and B_1^{D-CEST} field strengths of 20 Hz and 42 Hz.

In contrast, the exchange behavior observed for apo_{TEM-121} shifts toward the intermediate-to-fast exchange regime, as evidenced by the characteristics of the ¹⁵N CPMG and D-CEST profiles. This shift in exchange regime indicates that the resistance-associated substitutions in apo_{TEM-121} modify the intrinsic dynamic behavior of the enzyme relative to apo_{TEM-1}, altering the kinetics and populations of transiently populated excited states.

Rather than introducing new structural features detectable by structural study, the mutations appear to redistribute the rates and amplitudes of conformational fluctuations within functionally important regions, including the catalytic core and Ω -loop. Such mutation-dependent modulation of dynamic exchange supports a model in which resistance evolution is accompanied by modulating of the conformational energy landscape, not through large-scale structural rearrangements, but through altered interconversion kinetics between pre-existing substates. Together, these findings underscore that the functional divergence between apo_{TEM-1} and apo_{TEM-121} is dynamically encoded. By shifting the balance and kinetics of conformational exchange processes, resistance-associated mutations modulate the accessibility and lifetime of catalytically relevant conformations, thereby influencing enzyme activity and inhibitor susceptibility.

For holo_{TEM-121}, neither ^{15}N CPMG relaxation dispersion (**Figures 11**) nor ^{15}N D-CEST experiments (**Figures 12**) revealed residues with detectable μs – ms exchange under the conditions examined. This apparent absence of exchange signatures contrasts with the distributed exchange processes observed in apo_{TEM-121} and suggests that cAb_{TEM-13} binding attenuates the exchange contributions detectable by these experiments. Importantly, this result does not necessarily imply global rigidification; rather, VHH binding may stabilize the dominant conformational state and/or shift exchange processes outside the sensitivity window of the CPMG and D-CEST measurements (e.g., to faster regime). However, it should be noted that the absence of detectable exchange may also reflect limitations in sensitivity and signal-to-noise ratio, particularly for a system of this size (holo_{TEM-121}, ~ 45 kDa), and therefore does not exclude the presence of low-population or weak exchange processes. Thus, within the μs – ms timescale probed here, molecular recognition by cAb_{TEM-13} reduces the detectability of conformational exchange in TEM-121.

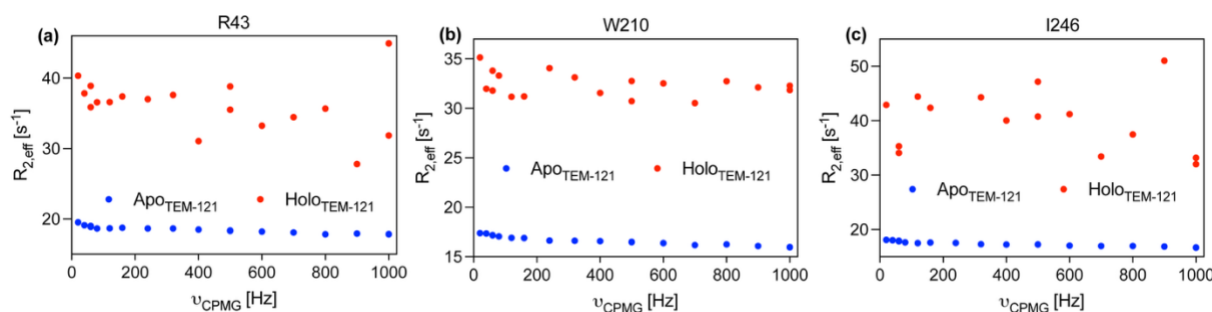


Figure 11. Comparison of ^{15}N CPMG profiles for apo_{TEM-121} (color code: blue), and holo_{TEM-121} (color code: red), recorded at 16.4 T (700 MHz) and 30° C. (a, b, c) R43, W210 and I246 profiles respectively.

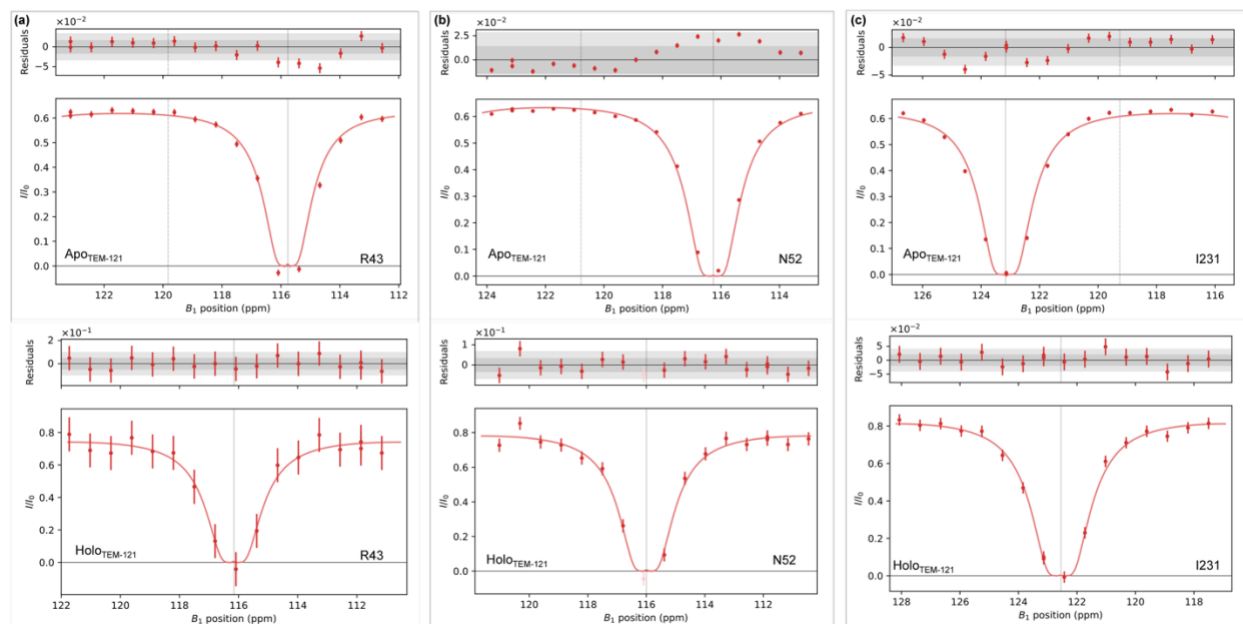


Figure 12. Comparison of ^{15}N D-CEST profiles for apo_{TEM-121} and holo_{TEM-121} recorded at 16.4 T (700 MHz) and 30° C with $\text{SW}_{\text{CEST}} = 800$ Hz and $B_1^{D\text{-CEST}} = 20$ Hz. (a, b, c) R43, 52 and I231 profiles respectively. Holo_{TEM-121} profiles were fitted using one-state kinetic model

These findings contrast with the direct comparison of apo_{TEM-1} and holo_{TEM-1}, for which increased exchange rates and higher minor-state populations were observed in the cAb_{TEM-13}-bound form, indicative of enhanced conformational sampling upon cAb_{TEM-13} binding. In striking contrast, VHH binding to TEM-121 may reduce detectable μs – ms exchange under the present conditions, revealing fundamentally different dynamic responses of the TEM-1 and TEM-121 enzymes to the same molecular recognition event. This opposite dynamic behavior parallels the substrate-dependent inhibitory effect of cAb_{TEM-13}, despite the absence of substantial differences in the X-ray structures of holo_{TEM-1} and holo_{TEM-121}.

Collectively, these results establish a direct connection between resistance-associated substitutions and altered functional dynamics in TEM-121 relative to TEM-1. Although crystallographic snapshots of the holo complexes are highly similar, NMR relaxation measurements demonstrate that mutations and VHH binding reshape the conformational landscapes of the two enzymes in fundamentally distinct ways. These dynamic differences provide a mechanistic basis for their contrasting dynamic responses to VHH binding. By revealing mutation-dependent redistribution of conformational fluctuations within catalytically important regions, this study highlights how resistance evolution can be encoded in changes to molecular motion rather than in detectable structural rearrangements. Thus, functional diversification in TEM β -lactamases may arise not from gross alterations of the ground-state structure, but from modulation of the underlying conformational ensemble that governs catalytic competence and molecular recognition.

CONCLUSIONS

In this work, we combined enzymatic kinetics, crystallography, molecular dynamics simulations, and multi-timescale NMR relaxation measurements to dissect the dynamic consequences of resistance-associated substitutions in TEM-121 relative to TEM-1. Although the crystal structures of the free and cAb_{TEM-13}-bound enzymes are highly similar, solution NMR reveals pronounced differences in conformational dynamics across ps–ns and μ s–ms timescales. Whereas TEM-1 exhibits two independent exchange processes originating from a common ground state, TEM-121 displays a redistribution of exchange toward the intermediate-to-fast regime, particularly within catalytically important regions. Notably, VHH binding produces opposite dynamic effects in the two enzymes: enhanced conformational sampling in TEM-1 but reduction of detectable μ s–ms exchange in TEM-121. These contrasting responses occur despite minimal structural differences in the holo complexes, underscoring that the functional divergence between the two enzymes is dynamically encoded rather than structurally apparent.

Collectively, our results demonstrate that resistance-associated mutations reshape the conformational energy landscape of TEM β -lactamase by modulating the kinetics and populations of transiently sampled states. This study highlights the power of solution NMR to uncover functionally relevant dynamic alterations that remain invisible to structural studies and establishes conformational plasticity as a central determinant of β -lactamase evolution and inhibitor response. More broadly, these findings illustrate how subtle mutations can reprogram enzyme function through redistribution of conformational dynamics, providing a dynamic framework for understanding the evolution of antibiotic resistance.

ACKNOWLEDGMENTS

This study made use of NMRbox: National Center for Biomolecular NMR Data Processing and Analysis, a Biomedical Technology Research Resource (BTRR), which is supported by NIH grant P41GM111135 (NIGMS)

This work was supported by the The Walloon Region and the Fond Européen de Développement Régional (FEDER) [Grant portfolio PHENIX n°244, project PHENIX_Analytique_ULiège n°397]

This work was supported by the Belgian Federal Public Service Health, Food Chain Safety and Environment (grant no. RF 17/6317 RU-BLA-ESBL-CPE) and the Fonds National de la Recherche Scientifique (CDR grant no. J0081.20).

Electronic Supplementary Material

MATERIALS AND METHODS

Sample Preparation.

Uniformly [U- ^{15}N , ^2H]-labeled apo and holo enzymes were expressed and purified as described previously (Tomanicek et al. 2010; Meilleur et al. 2009; Tomanicek et al. 2011; Cawez et al. 2018) with minor modifications. Cells were grown in minimal medium prepared in 100% D_2O and supplemented with $^{15}\text{NH}_4\text{Cl}$ (1 g/L) and $\{^{13}\text{C}, ^1\text{H}\}$ -glucose (4 g/L) as the sole nitrogen and carbon sources, respectively. NMR samples were prepared at a final protein concentration of approximately 500 μM in 50 mM potassium phosphate buffer (pH 6.6), containing 95% H_2O /5% D_2O (v/v). Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, 40 μM) was included as an internal chemical shift reference. For complex formation, apo enzymes were mixed with nanobody at a 1:1.1 molar ratio (enzyme:antibody) to ensure complete saturation, yielding the holo complexes.

NMR Spectroscopy.

^{15}N Nuclear Spin Relaxation (NSR). ^{15}N spin relaxation experiments ($\{^{15}\text{N}\}$ - R_1 , $\{^{15}\text{N}\}$ - R_2 , and ^1H - $\{^{15}\text{N}\}$ heteronuclear NOE) were acquired as described previously for TEM-1.

^{15}N Relaxation Dispersion. ^{15}N CPMG relaxation dispersion experiments were acquired as described previously for CspA, with the exception that parameter uncertainties were estimated using 1000 Monte Carlo simulations.

^{15}N CEST Experiments and Visible peaks-positions. ^{15}N chemical exchange saturation transfer (CEST) experiments on apo and holo_{TEM-1} were performed as described previously. Corresponding experiments on apo and holo_{TEM-121} were conducted following the protocol established for CspA, with the exception that parameter uncertainties were estimated using 1000 Monte Carlo simulations.

Visible peaks-positions. Visible peak positions were extracted from the ^{15}N D-CEST spectra ($\text{SW}_{\text{CEST}} = 700$ and 800 Hz) by referencing the peak positions obtained in control experiments recorded without the two $B_1^{D-\text{CEST}}$ irradiation periods. Uncertainties in the visible peak positions were estimated from these spectra.

Data Analysis.

All NSR spectra of apo and holo TEM-121 were processed and analyzed according to the procedure established for TEM-1.

^{15}N CPMG and ^{15}N D-CEST spectra were processed and analyzed according to the procedure established for CspA.

Backbone Dynamics on the μ s-ms timescale**Table S1.** Complete set of residue-specific parameters obtained from two-state fits of ^{15}N D-CEST profiles for Apo_{TEM-121}, including ground-state chemical shifts δ_{A} , chemical-shift differences between ground and excited states ($\Delta\omega_{\text{AB}}$), and longitudinal and transverse relaxation rates R_1 and R_2 respectively.

Residue	δ_{A} (ppm)	$\Delta\omega_{\text{AB}}$ (ppm)	R_1 (s^{-1})	R_2 (s^{-1})
V31	121.47 $\pm$ 0.02	-3.20 $\pm$ 0.29	0.97 $\pm$ 0.03	15.29 $\pm$ 0.14
K34	125.14 $\pm$ 0.00	-3.78 $\pm$ 0.45	1.42 $\pm$ 0.03	5.38 $\pm$ 0.28
D35	121.90 $\pm$ 0.02	-2.98 $\pm$ 0.31	0.00 $\pm$ 0.41	2.16 $\pm$ 0.18
D38	120.98 $\pm$ 0.01	3.40 $\pm$ 0.30	1.45 $\pm$ 0.04	7.51 $\pm$ 0.17
G41	113.02 $\pm$ 0.02	-3.95 $\pm$ 0.26	0.90 $\pm$ 0.03	18.89 $\pm$ 0.14
R43	115.76 $\pm$ 0.02	4.06 $\pm$ 0.18	0.88 $\pm$ 0.03	17.51 $\pm$ 0.09
Y46	123.35 $\pm$ 0.04	9.99 $\pm$ 1.73	0.93 $\pm$ 0.08	18.00 $\pm$ 0.74
D50	125.01 $\pm$ 0.01	-2.32 $\pm$ 0.46	0.85 $\pm$ 0.01	21.55 $\pm$ 0.18
N52	116.26 $\pm$ 0.01	4.53 $\pm$ 0.50	0.79 $\pm$ 0.02	21.31 $\pm$ 0.27
S59	112.93 $\pm$ 0.01	4.05 $\pm$ 0.31	0.92 $\pm$ 0.02	17.32 $\pm$ 0.17
K53	112.63 $\pm$ 0.02	-2.20 $\pm$ 0.26	0.87 $\pm$ 0.04	20.86 $\pm$ 0.10
F60	121.66 $\pm$ 0.03	-3.37 $\pm$ 0.22	0.99 $\pm$ 0.06	22.49 $\pm$ 0.11
E63	116.55 $\pm$ 0.02	4.27 $\pm$ 0.22	0.83 $\pm$ 0.03	17.83 $\pm$ 0.12
E64	117.77 $\pm$ 0.02	-4.90 $\pm$ 0.25	0.90 $\pm$ 0.04	17.32 $\pm$ 0.13
R65	117.02 $\pm$ 0.01	3.90 $\pm$ 0.20	0.78 $\pm$ 0.02	18.34 $\pm$ 0.10
T71	112.17 $\pm$ 0.03	3.64 $\pm$ 0.32	0.91 $\pm$ 0.06	20.85 $\pm$ 0.16
F72	115.88 $\pm$ 0.02	-5.16 $\pm$ 0.20	0.89 $\pm$ 0.04	21.66 $\pm$ 0.11
K73	121.53 $\pm$ 0.01	-5.17 $\pm$ 0.47	0.80 $\pm$ 0.02	21.71 $\pm$ 0.25
L77	113.70 $\pm$ 0.03	7.38 $\pm$ 0.44	0.90 $\pm$ 0.06	19.53 $\pm$ 0.22
V80	119.06 $\pm$ 0.02	4.97 $\pm$ 0.33	1.11 $\pm$ 0.03	18.28 $\pm$ 0.18
R83	121.58 $\pm$ 0.01	3.84 $\pm$ 0.45	0.81 $\pm$ 0.01	20.92 $\pm$ 0.24
S106	119.73 $\pm$ 0.02	4.69 $\pm$ 0.75	0.73 $\pm$ 0.04	18.54 $\pm$ 0.41
D115	116.87 $\pm$ 0.01	4.58 $\pm$ 0.36	0.89 $\pm$ 0.01	19.00 $\pm$ 0.19
R120	118.13 $\pm$ 0.01	-5.17 $\pm$ 0.69	0.78 $\pm$ 0.02	21.24 $\pm$ 0.37
E121	118.24 $\pm$ 0.01	4.81 $\pm$ 0.47	0.81 $\pm$ 0.01	20.88 $\pm$ 0.25
D163	125.56 $\pm$ 0.03	-6.13 $\pm$ 0.62	0.75 $\pm$ 0.06	21.88 $\pm$ 0.33
W165	116.99 $\pm$ 0.02	5.79 $\pm$ 0.67	0.79 $\pm$ 0.02	20.31 $\pm$ 0.36
N170	120.87 $\pm$ 0.07	9.78 $\pm$ 3.34	0.82 $\pm$ 0.16	21.04 $\pm$ 1.43
E171	106.98 $\pm$ 0.03	-1.08 $\pm$ 1.03	0.97 $\pm$ 0.07	21.81 $\pm$ 0.21
I173	119.03 $\pm$ 0.02	5.34 $\pm$ 0.40	0.85 $\pm$ 0.02	21.14 $\pm$ 0.22
D179	115.30 $\pm$ 0.02	-5.14 $\pm$ 0.26	0.82 $\pm$ 0.03	20.93 $\pm$ 0.14
E197	116.67 $\pm$ 0.07	-3.24 $\pm$ 0.10	0.85 $\pm$ 0.12	16.48 $\pm$ 0.05

L198	122.52 $\pm$ 0.00	3.46 $\pm$ 0.57	0.74 $\pm$ 0.01	16.95 $\pm$ 0.32
W210	120.34 $\pm$ 0.02	3.79 $\pm$ 0.20	0.97 $\pm$ 0.04	15.90 $\pm$ 0.11
M211	115.63 $\pm$ 0.01	5.00 $\pm$ 0.21	0.83 $\pm$ 0.02	20.12 $\pm$ 0.11
E212	124.91 $\pm$ 0.04	7.52 $\pm$ 1.53	1.15 $\pm$ 0.07	22.59 $\pm$ 0.77
G218	114.02 $\pm$ 0.04	-6.12 $\pm$ 0.60	1.19 $\pm$ 0.07	22.41 $\pm$ 0.32
L221	123.79 $\pm$ 0.08	-8.77 $\pm$ 4.19	0.84 $\pm$ 0.23	21.63 $\pm$ 1.95
R222	115.92 $\pm$ 0.02	-6.39 $\pm$ 0.68	0.91 $\pm$ 0.04	21.95 $\pm$ 0.35
L225	120.90 $\pm$ 0.02	8.98 $\pm$ 2.24	0.81 $\pm$ 0.03	17.51 $\pm$ 1.02
W229	120.55 $\pm$ 0.02	6.53 $\pm$ 0.20	0.86 $\pm$ 0.02	19.20 $\pm$ 0.11
I231	123.16 $\pm$ 0.01	-3.90 $\pm$ 0.40	0.89 $\pm$ 0.03	21.62 $\pm$ 0.21
D233	123.88 $\pm$ 0.02	-2.71 $\pm$ 0.26	1.18 $\pm$ 0.07	9.64 $\pm$ 0.13
G245	109.03 $\pm$ 0.02	2.54 $\pm$ 0.36	1.01 $\pm$ 0.04	15.37 $\pm$ 0.15
I246	118.16 $\pm$ 0.03	3.94 $\pm$ 0.24	0.98 $\pm$ 0.05	16.48 $\pm$ 0.12
I247	122.58 $\pm$ 0.01	3.59 $\pm$ 0.40	0.79 $\pm$ 0.02	22.13 $\pm$ 0.20
A248	122.85 $\pm$ 0.01	-4.53 $\pm$ 1.41	0.80 $\pm$ 0.02	27.76 $\pm$ 0.76
D254	114.02 $\pm$ 0.01	10.47 $\pm$ 2.63	0.87 $\pm$ 0.03	17.55 $\pm$ 1.07
K256	119.16 $\pm$ 0.01	4.98 $\pm$ 0.53	0.83 $\pm$ 0.02	22.21 $\pm$ 0.28
S258	112.88 $\pm$ 0.02	6.14 $\pm$ 0.73	0.93 $\pm$ 0.03	16.16 $\pm$ 0.40

Part V.
General Conclusions
&
Perspectives

Chapter X.

General Conclusions

This thesis is situated within the field of biomolecular nuclear magnetic resonance (NMR) spectroscopy and aims to advance our understanding of the intimate relationship between protein structure, conformational dynamics, and biological function at atomic resolution. By integrating complementary experimental and analytical NMR approaches, this work provides detailed insights into dynamic processes occurring over a wide range of timescales, many of which remain inaccessible to static structural techniques such as X-ray crystallography. In particular, NMR uniquely enables the direct observation of conformational exchange, transient states, and molecular flexibility under near-physiological conditions, thereby offering a dynamic view of biomolecular function.

The studies presented in this manuscript enabled the characterization of low-populated conformational states and exchange processes spanning a broad range of timescales, from the intermediate-to-fast to slower, functionally relevant structural states. The combined analysis of NMR relaxation techniques, including ^{15}N CPMG relaxation dispersion and ^{15}N CEST experiments, highlights the complementarity of these methods in providing a reliable framework for relating molecular dynamics to protein function.

Application of these methodologies to biologically relevant systems, including enzymes (TEM-1 and TEM-121), their complexes with the VHH (TEM-1/cAb_{TEM13} and TEM-121/cAb_{TEM13}) and a mesophilic cold shock protein CspA, revealed that conformational dynamics play a central role in regulating activity, molecular recognition, and functional adaptability. In particular, the investigation of the CspA molecular dynamic, enzymes– or partner–induced effects highlighted extensive reshaping of protein energy landscapes, emphasizing the importance of coupled mechanisms and structural plasticity in protein function.

Interestingly, our study of CspA highlights that fast timescale motions (ps–ns), which are often overlooked because they are not directly associated to protein function, may nevertheless play an important role in protein function. Using a multi-field relaxation analysis combined with model-free fitting, we showed that the $\beta 3$ – $\beta 4$ and $\beta 4$ – $\beta 5$ loop regions undergo complex internal dynamics. Specifically, residues within these loops exhibit reduced ^{15}N – $\{^1\text{H}\}$ NOE values while maintaining high generalized order parameters ($S^2 \sim 0.8$), indicating the presence of fast local motions superimposed on an overall well-ordered backbone. Because these loop regions form part of the nucleic acid–binding interface, their dynamic behavior may reflect a functional plasticity that enables CspA to accommodate and stabilize diverse ssDNA or ssRNA conformations. Together, these results suggest that loop flexibility on the ps–ns timescale is an intrinsic property of CspA and may contribute directly to its chaperone-like activity during cold-shock adaptation.

Moreover, we characterized the camelid antibody fragment cAb_{TEM-13} targeting TEM-1 β -lactamase, a widely disseminated class A enzyme that significantly impacts both human

and veterinary health. Beyond evaluating its inhibitory properties, our objective was to elucidate the molecular mechanism by which nanobody binding modulates enzyme function. Our results indicate that cAb_{TEM-13} may act through an allosteric mechanism. Binding at a distal site modulates the conformational landscape of TEM-1 β -lactamase, with dynamic perturbations propagating from the binding interface to distant regions, including catalytically relevant sites, thereby modulating enzymatic activity.

We further employed an integrated structural and biophysical approach to compare TEM-1 and the ESBL/IRT variant TEM-121 in both their free and nanobody-bound states. Our analyses demonstrate that resistance-associated mutations reshape the conformational energy landscape of TEM β -lactamase by altering the kinetics and populations of transiently sampled conformational states, thereby providing a dynamic framework for understanding the molecular basis of antibiotic resistance. These mutations do not merely affect static structural features but reprogram the enzyme's dynamic equilibrium, thereby influencing both catalytic efficiency and inhibitor response.

Collectively, these findings highlight the power of solution NMR spectroscopy to reveal functionally relevant dynamic alterations that remain inaccessible to static structural methods. They establish conformational plasticity as a central determinant of β -lactamase evolution, functional diversification, and inhibitor sensitivity. More broadly, this work provides a dynamic framework for understanding how subtle sequence variations can modulate enzyme function through redistribution of conformational ensembles, offering new perspectives on the molecular basis of antibiotic resistance.

This work highlights the value of integrated analyses of NMR relaxation data, combining complementary techniques to characterize protein dynamics across different timescales. It underscores the importance of moving beyond purely static representations of protein structures toward a dynamic perspective that more accurately reflects the behavior of biomolecules in solution.

In addition to these fundamental studies, this research also addresses the three-dimensional structure determination of venom peptides, a class of small biomolecules with potent and selective biological activities. NMR spectroscopy is especially well suited for the structural characterization of venom peptides, as it does not require crystallization and can accurately resolve structures in solution, where these peptides adopt their biologically relevant conformations.

We determined well-defined solution NMR structures of two peptides, Leiurotoxin I and Leiurotoxin-Dab7 (Lei-Dab7), in which the methionine residue at position 7 of Leiurotoxin I is replaced by the unnatural amino acid diaminobutanoic acid (Dab). When combined with *in vitro* binding studies [realized by Hayen Jean-Luc], this work enables a direct structural and functional comparison of the two peptides and provides a direct insight into the molecular determinants governing their interactions with human SK channels.

Considering the importance of protein dynamics in molecular recognition, the difference in affinity between Leiurotoxin I and Lei-Dab7 may arise from differences in their dynamic behavior.

Taken together, this research highlights the power of biomolecular NMR spectroscopy to integrate high-resolution structural information with dynamic and functional insights, thereby deepening our understanding of protein and peptide mechanisms underlying biological activity.

Chapter XI.

Perspectives

Several perspectives can be envisaged to further improve and refine research presented in this thesis. In particular, the NMR relaxation analysis of the TEM-1/cAb_{TEM13} complex could be significantly enhanced. Due to the absence of uniformly [²H, ¹⁵N]-labeled TEM-1/cAb_{TEM13} samples, it was not possible to further refine the ¹⁵N CPMG relaxation dispersion experiments or to perform ¹⁵N D-CEST measurements on this enzyme complex. Such experiments would provide additional and highly valuable information, allowing a more detailed characterization of the conformational dynamics of TEM-1/cAb_{TEM-1}, comparable to what was achieved for the free TEM-1 enzyme.

In addition, our analysis of the free TEM-121 enzyme demonstrated that incorporating ¹⁵N D-CEST data and peak-position information to complement single-field CPMG relaxation dispersion measurements does not impose significant additional constraints on the determination of the exchange rate (k_{ex}) and the minor-state population (p_B). Therefore, reliable k_{ex} and p_B values can instead be obtained by performing CPMG experiments at a second magnetic field strength, combined with D-CEST measurements acquired using a radiofrequency field (ω_1) exceeding the determined k_{ex} .

Moreover, this research included the study of a mesophilic cold shock protein, CspA. Initially, the broader objective was to investigate three homologous cold shock proteins originating from organisms adapted to different temperature regimes: mesophilic, psychrophilic, and thermophilic. Extending this work to the psychrophilic and thermophilic counterparts would be of particular interest, as it would enable a systematic comparison of how cold shock proteins adapt to extreme thermal environments. Such comparative studies could reveal the dynamic strategies employed by proteins to maintain stability and function under cold stress, including differences in flexibility, conformational exchange processes, and energy landscape reshaping. Ultimately, these insights would contribute to a deeper understanding of thermal adaptation mechanisms at the molecular level.

Finally, this thesis highlights the importance of adopting a dynamic perspective when studying biological systems. Although motions on the ps–ns timescale are often overlooked because they are not always directly linked to biological function, our study of CspA demonstrates that such fast dynamics can nevertheless contribute to protein function. In addition, we have shown the complementarity of μ s–ms timescale NMR experiments, particularly D-CEST and CPMG relaxation dispersion.

We believe that combining these approaches provides a powerful strategy to obtain a more comprehensive description of protein dynamics. In particular, the joint use of ¹⁵N CPMG and D-CEST experiments allows the characterization of conformational exchange processes over a broad range of kinetic regimes, while rotating-frame experiments may further extend the accessible timescales when required. The integration of these complementary techniques therefore offers a valuable framework for linking structural

dynamics to biological function and should contribute to a more complete understanding of the molecular mechanisms governing protein activity.

In light of recent advances in computational structural biology, particularly the emergence of AlphaFold (Abramson et al. 2024; Bryant et al. 2022; Jumper et al. 2021), the landscape of protein structure determination has been profoundly transformed, enabling the rapid and accurate prediction of protein structures. However, despite the presence of confidence metrics such as pLDDT that may highlight regions of uncertainty, predicted structures remain essentially static, hypothesis-driven models that require experimental validation. In this context, NMR spectroscopy provides a unique and complementary perspective by offering insights into protein motions across a wide range of timescales at residue-level resolution. The present work highlights the importance of complementing these predictions with experimental approaches capable of capturing protein dynamics. In particular, the characterization of flexibility and conformational exchange equilibria presented here underscores the critical role of dynamics in modulating protein function. These results illustrate how NMR spectroscopy may contribute to an integrated structural biology framework by providing time-resolved insights that remain largely inaccessible to current *in silico* methods. In this perspective, future developments may rely on the synergistic integration of computational predictions and experimental characterization of dynamics to achieve a more complete understanding of biomolecular function.

Bibliography

- A T Brünger. 1992. "Free R Value: A Novel Statistical Quantity for Assessing the Accuracy of Crystal Structures." *Nature* 355 (January). <https://doi.org/DOI:%2010.1038/355472a0>.
- Abdi, Mohamad, Xue Feng, Changyu Sun, Kenneth C. Bilchick, Craig H. Meyer, and Frederick H. Epstein. 2021. "Suppression of Artifact-generating Echoes in Cine DENSE Using Deep Learning." *Magnetic Resonance in Medicine* 86 (4): 2095–104. <https://doi.org/10.1002/mrm.28832>.
- Abia, A., C. D. Lobatón, A. Moreno, and J. García-Sancho. 1986. "Leiurus Quinquestriatus Venom Inhibits Different Kinds of Ca^{2+} -Dependent K^{+} Channels." *Biochimica Et Biophysica Acta* 856 (2): 2. [https://doi.org/10.1016/0005-2736\(86\)90054-4](https://doi.org/10.1016/0005-2736(86)90054-4).
- Abraham, A. 1983. *The Principles of Nuclear Magnetism*. International Series of Monographs on Physics. Oxford University Press.
- Abramov, Gili, Algirdas Velyvis, Enrico Rennella, Leo E. Wong, and Levitt E. Kay. 2020. "A Methyl-TROSY Approach for NMR Studies of High-Molecular-Weight DNA with Application to the Nucleosome Core Particle." *PNAS*. <https://doi.org/10.1073/pnas.2004317117>.
- Abramson, Josh, Jonas Adler, Jack Dunger, et al. 2024. "Accurate Structure Prediction of Biomolecular Interactions with AlphaFold 3." *Nature* 630 (8016): 493–500. <https://doi.org/10.1038/s41586-024-07487-w>.
- Alderson, T. Reid, and Levitt E. Kay. 2020. "Unveiling Invisible Protein States with NMR Spectroscopy." *Current Opinion in Structural Biology, Folding and Binding Proteins*, vol. 60 (February): 39–49. <https://doi.org/10.1016/j.sbi.2019.10.008>.
- Alderson, T. Reid, and Levitt E. Kay. 2021. "NMR Spectroscopy Captures the Essential Role of Dynamics in Regulating Biomolecular Function." *Cell* 184 (3): 577–95. <https://doi.org/10.1016/j.cell.2020.12.034>.
- Allerhand, Adam, and H. S. Gutowsky. 1965. "Spin-Echo Studies of Chemical Exchange. II. Closed Formulas for Two Sites." *The Journal of Chemical Physics* 42 (5): 1587–99. <https://doi.org/10.1063/1.1696165>.
- Andrec, Michael, Gaetano T. Montelione, and Ronald M. Levy. 1999. "Estimation of Dynamic Parameters from NMR Relaxation Data Using the Lipari–Szabo Model-Free Approach and Bayesian Statistical Methods." *Journal of Magnetic Resonance* 139 (2): 408–21. <https://doi.org/10.1006/jmre.1999.1839>.
- Armstrong, David A., Ai-Hua Jin, Nayara Braga Emidio, Richard J. Levitt, Paul F. Alewood, and K. Johan Rosengren. 2021. "Chemical Synthesis and NMR Solution Structure of Conotoxin GXIA from *Conus Geographus*." *Marine Drugs* 19 (2): 60. <https://doi.org/10.3390/md19020060>.
- Auguste, P., M. Hugues, B. Gravé, et al. 1990. "Leiurotoxin I (Scyllatoxin), a Peptide Ligand for Ca^{2+} -Activated K^{+} Channels. Chemical Synthesis, Radiolabeling, and Receptor Characterization." *The Journal of Biological Chemistry* 265 (8): 8.

- Auvergne, Edward J. d', and Paul R. Gooley. 2003. "The Use of Model Selection in the Model-Free Analysis of Protein Dynamics." *Journal of Biomolecular NMR* 25 (1): 25–39. <https://doi.org/10.1023/A:1021902006114>.
- Avci, Fatma Gizem, Fatma Ece Altinisik, Didem Vardar Ulu, Elif Ozkirimli Olmez, and Berna Sariyar Akbulut. 2016. "An Evolutionarily Conserved Allosteric Site Modulates Beta-Lactamase Activity." *Journal of Enzyme Inhibition and Medicinal Chemistry* 31 (sup3): 33–40. <https://doi.org/10.1080/14756366.2016.1201813>.
- Avery, Chris, Lonnie Baker, and Donald J. Jacobs. 2022. "Functional Dynamics of Substrate Recognition in TEM Beta-Lactamase." *Entropy* 24 (5): 729. <https://doi.org/10.3390/e24050729>.
- Bahia, Parmvir K., Rie Suzuki, David C. H. Benton, et al. 2005. "A Functional Role for Small-Conductance Calcium-Activated Potassium Channels in Sensory Pathways Including Nociceptive Processes." *Cellular/Molecular. Journal of Neuroscience* 25 (14): 3489–98. <https://doi.org/10.1523/JNEUROSCI.0597-05.2005>.
- Bain, Alex D. 2003. "Chemical Exchange in NMR." *Progress in Nuclear Magnetic Resonance Spectroscopy* 43 (3–4): 63–103. <https://doi.org/10.1016/j.pnmrs.2003.08.001>.
- Bain, Alex D., Christopher Kumar Anand, and Zhenghua Nie. 2011. "Exact Solution of the CPMG Pulse Sequence with Phase Variation down the Echo Train: Application to R2 Measurements." *Journal of Magnetic Resonance* 209 (2): 183–94. <https://doi.org/10.1016/j.jmr.2011.01.009>.
- Baldwin, Andrew J., and Levitt E. Kay. 2009. "NMR Spectroscopy Brings Invisible Protein States into Focus." *Nature Chemical Biology* 5 (11): 808–14. <https://doi.org/10.1038/nchembio.238>.
- Barbato, Gaetano, Mitsuhiro Ikura, and Levitt E. Kay. 1992. "Backbone Dynamics of Calmodulin Studied by 15 N Relaxation Using Inverse Detected Two-Dimensional NMR Spectroscopy: The Central Helix Is Flexible." *Biochemistry* 31 (23): 5269–78. <https://doi.org/10.1021/bi00138a005>.
- Barfod, E. T., A. L. Moore, and S. D. Lidofsky. 2001. "Cloning and Functional Expression of a Liver Isoform of the Small Conductance Ca²⁺-Activated K⁺ Channel SK3." *American Journal of Physiology. Cell Physiology* 280 (4): C836–842. <https://doi.org/10.1152/ajpcell.2001.280.4.C836>.
- Bartels, Christian, Tai-he Xia, Martin Billeter, Peter Güntert, and Kurt Wüthrich. 1995. "The Program XEASY for Computer-Supported NMR Spectral Analysis of Biological Macromolecules." *Journal of Biomolecular NMR* 6 (1): 1–10. <https://doi.org/10.1007/BF00417486>.
- Bax, Ad, G. Marius Clore, and Angela M. Gronenborn. 1990. "1H-1H Correlation via Isotropic Mixing of 13C Magnetization, a New Three-Dimensional Approach for Assigning 1H and 13C Spectra of 13C-Enriched Proteins." *Journal of Magnetic Resonance* 88 (2): 425–31. [https://doi.org/10.1016/0022-2364\(90\)90202-K](https://doi.org/10.1016/0022-2364(90)90202-K).
- Bax, Ad, and Stephan Grzesiek. 1993. "Methodological Advances in Protein NMR." *Accounts of Chemical Research* 26 (4): 131–38. <https://doi.org/10.1021/ar00028a001>.

- Behl, Ankita, Vikash Kumar, Maxim Shevtsov, and Shailja Singh. 2020. "Pleiotropic Roles of Cold Shock Proteins with Special Emphasis on Unexplored Cold Shock Protein Member of Plasmodium Falciparum." *Malaria Journal* 19 (October): 382. <https://doi.org/10.1186/s12936-020-03448-6>.
- Bhattacharya, Aneerban, Roberto Tejero, and Gaetano T. Montelione. 2007. "Evaluating Protein Structures Determined by Structural Genomics Consortia." *Proteins: Structure, Function, and Bioinformatics* 66 (4): 778–95. <https://doi.org/10.1002/prot.21165>.
- Bhattacharyya, Rangeet, and Anil Kumar. 2004. "A Fast Method for the Measurement of Long Spin–Lattice Relaxation Times by Single Scan Inversion Recovery Experiment." *Chemical Physics Letters* 383 (1–2): 99–103. <https://doi.org/10.1016/j.cplett.2003.10.133>.
- Billeter, Martin, Gerhard Wagner, and Kurt Wüthrich. 2008. "Solution NMR Structure Determination of Proteins Revisited." *Journal of Biomolecular NMR* 42 (3): 155–58. <https://doi.org/10.1007/s10858-008-9277-8>.
- Blackledge, Martin, Florence Cordier, Patrice Dosset, and Dominique Marion. 1998. "Precision and Uncertainty in the Characterization of Anisotropic Rotational Diffusion by ^{15}N Relaxation." *Journal of the American Chemical Society* 120 (18): 4538–39. <https://doi.org/10.1021/ja9742646>.
- Blanc, E., C. Lecomte, J. Van Rietschoten, J. M. Sabatier, and H. Darbon. 1997. "Solution Structure of TsKapa, a Charybdotoxin-like Scorpion Toxin from Tityus Serrulatus with High Affinity for Apamin-Sensitive Ca^{2+} -Activated K^{+} Channels." *Proteins: Structure, Function, and Genetics* 29 (3): 359–69. [https://doi.org/10.1002/\(SICI\)1097-0134\(199711\)29:3%3C359::AID-PROT9%3E3.0.CO;2-5](https://doi.org/10.1002/(SICI)1097-0134(199711)29:3%3C359::AID-PROT9%3E3.0.CO;2-5).
- Bloch, F. 1946. "Nuclear Induction." *Physical Review* 70 (7–8): 460–74. <https://doi.org/10.1103/PhysRev.70.460>.
- Bloch, F., W. W. Hansen, and M. Packard. 1946. "The Nuclear Induction Experiment." *Physical Review* 70 (7–8): 474–85. <https://doi.org/10.1103/PhysRev.70.474>.
- Bloch, F., and A. Siegert. 1940. "Magnetic Resonance for Nonrotating Fields." *Physical Review* 57 (6): 522–27. <https://doi.org/10.1103/PhysRev.57.522>.
- Bloembergen, N., E. M. Purcell, and R. V. Pound. 1948. "Relaxation Effects in Nuclear Magnetic Resonance Absorption." *Physical Review* 73 (7): 679–712. <https://doi.org/10.1103/PhysRev.73.679>.
- Bloom, M., L. W. Reeves, and E. J. Wells. 1965. "Spin Echoes and Chemical Exchange." *The Journal of Chemical Physics* 42 (5): 1615–24. <https://doi.org/10.1063/1.1696168>.
- Bodenhausen, Geoffrey, G. A. Morris, and Ray Freeman. 1976. "A Simple Pulse Sequence for Selective Excitation in Fourier Transform NMR." *Journal of Magnetic Resonance* 23 (1): 171–75. [https://doi.org/10.1016/0022-2364\(76\)90150-5](https://doi.org/10.1016/0022-2364(76)90150-5).
- Boehr, David D., Dan McElheny, H. Jane Dyson, and Peter E. Wright. 2006. "The Dynamic Energy Landscape of Dihydrofolate Reductase Catalysis." *Science* 313 (5793): 1638–42. <https://doi.org/10.1126/science.1130258>.

- Bond, C. T. 2004. "Small Conductance Ca^{2+} -Activated K^{+} Channel Knock-Out Mice Reveal the Identity of Calcium-Dependent Afterhyperpolarization Currents." *Journal of Neuroscience* 24 (23): 5301–6. <https://doi.org/10.1523/JNEUROSCI.0182-04.2004>.
- Bonneau, Richard, Jerry Tsai, Ingo Ruczinski, et al. 2001. "Rosetta in CASP4: Progress in Ab Initio Protein Structure Prediction." *Proteins: Structure, Function, and Genetics* 45 (S5): 119–26. <https://doi.org/10.1002/prot.1170>.
- Bouvignies, Guillaume, and Levitt E. Kay. 2012. "Measurement of Proton Chemical Shifts in Invisible States of Slowly Exchanging Protein Systems by Chemical Exchange Saturation Transfer." *The Journal of Physical Chemistry B* 116 (49): 14311–17. <https://doi.org/10.1021/jp311109u>.
- Bowie, James U., Roland L  thy, and David Eisenberg. 1991. "A Method to Identify Protein Sequences That Fold into a Known Three-Dimensional Structure." *Science* 253 (5016): 164–70. <https://doi.org/10.1126/science.1853201>.
- Bradley, Philip, Dylan Chivian, Jens Meiler, et al. 2003. "Rosetta Predictions in CASP5: Successes, Failures, and Prospects for Complete Automation." *Proteins: Structure, Function, and Genetics* 53 (S6): 457–68. <https://doi.org/10.1002/prot.10552>.
- Br  hler, Sebastian, Anuradha Kaistha, Volker J. Schmidt, et al. 2009. "Genetic Deficit of SK3 and IK1 Channels Disrupts the Endothelium-Derived Hyperpolarizing Factor Vasodilator Pathway and Causes Hypertension." *Circulation* 119 (17): 2323–32. <https://doi.org/10.1161/CIRCULATIONAHA.108.846634>.
- Brown, Brandon M., Heesung Shim, Palle Christophersen, and Heike Wulff. 2020. "Pharmacology of Small- and Intermediate-Conductance Calcium-Activated Potassium Channels." *Annual Review of Pharmacology and Toxicology* 60 (Volume 60, 2020): 219–40. <https://doi.org/10.1146/annurev-pharmtox-010919-023420>.
- Br  nger, A. T., P. D. Adams, G. M. Clore, et al. 1998. "Crystallography & NMR System: A New Software Suite for Macromolecular Structure Determination." *Acta Crystallographica. Section D, Biological Crystallography* 54 (Pt 5): 905–21. <https://doi.org/10.1107/s0907444998003254>.
- Bryant, Patrick, Gabriele Pozzati, and Arne Elofsson. 2022. "Improved Prediction of Protein-Protein Interactions Using AlphaFold2." *Nature Communications* 13 (1): 1265. <https://doi.org/10.1038/s41467-022-28865-w>.
- Budisa, Nediljko. 2025. "Introduction: 'Noncanonical Amino Acids.'" *Chemical Reviews* 125 (4): 1659–62. <https://doi.org/10.1021/acs.chemrev.5c00065>.
- Burd, Christopher G., and Gideon Dreyfuss. 1994. "Conserved Structures and Diversity of Functions of RNA-Binding Proteins." *Science* 265 (5172): 615–21. <https://doi.org/10.1126/science.8036511>.
- Bush, Karen. 2010. "Alarming β -Lactamase-Mediated Resistance in Multidrug-Resistant Enterobacteriaceae." *Current Opinion in Microbiology* 13 (5): 558–64. <https://doi.org/10.1016/j.mib.2010.09.006>.

- Bush, Karen. 2018. "Past and Present Perspectives on β -Lactamases." *Antimicrobial Agents and Chemotherapy* 62 (10): e01076-18. <https://doi.org/10.1128/AAC.01076-18>.
- Bush, Karen, and Patricia A. Bradford. 2020. "Epidemiology of β -Lactamase-Producing Pathogens." *Clinical Microbiology Reviews* 33 (2): e00047-19. <https://doi.org/10.1128/CMR.00047-19>.
- Bush, Karen, and George A. Jacoby. 2010. "Updated Functional Classification of β -Lactamases." *Antimicrobial Agents and Chemotherapy* 54 (3): 969–76. <https://doi.org/10.1128/AAC.01009-09>.
- Butler, Andrew S., Jules C. Hancox, and Neil V. Marrion. 2023. "Preferential Formation of Human Heteromeric SK2:SK3 Channels Limits Homomeric SK Channel Assembly and Function." *Journal of Biological Chemistry* 299 (1): 102783. <https://doi.org/10.1016/j.jbc.2022.102783>.
- Carr, H. Y., and E. M. Purcell. 1954. "Effects of Diffusion on Free Precession in Nuclear Magnetic Resonance Experiments." *Physical Review* 94 (3): 630–38. <https://doi.org/10.1103/PhysRev.94.630>.
- Carver, J. P., and R. E. Richards. 1972. "A General Two-Site Solution for the Chemical Exchange Produced Dependence of T₂ upon the Carr-Purcell Pulse Separation." *Journal of Magnetic Resonance (1969)* 6 (1): 89–105. [https://doi.org/10.1016/0022-2364\(72\)90090-X](https://doi.org/10.1016/0022-2364(72)90090-X).
- Cavanagh, J., Fairbrother, W. J., Palmer G. A., and Skelton, N. J. 2006. *Protein NMR Spectroscopy*. <https://shop.elsevier.com/books/protein-nmr-spectroscopy/cavanagh/978-0-12-164491-8>.
- Cawez, F., E. Duray, Y. Hu, et al. 2018. "Combinatorial Design of a Nanobody That Specifically Targets Structured RNAs." *Journal of Molecular Biology* 430 (11): 1652–70. <https://doi.org/10.1016/j.jmb.2018.03.032>.
- Cepeda, Diego Sebastián Insuasty, Héctor Manuel Pineda Castañeda, Andrea Verónica Rodríguez Mayor, et al. 2019. "Synthetic Peptide Purification via Solid-Phase Extraction with Gradient Elution: A Simple, Economical, Fast, and Efficient Methodology." *Molecules* 24 (7): 1215. <https://doi.org/10.3390/molecules24071215>.
- Champdoré, Marcella de, Maria Staiano, Mosè Rossi, and Sabato D'Auria. 2007. "Proteins from Extremophiles as Stable Tools for Advanced Biotechnological Applications of High Social Interest." *Journal of the Royal Society Interface* 4 (13): 183–91. <https://doi.org/10.1098/rsif.2006.0174>.
- Chatterjee, Soumya Deep, Marcellus Ubbink, and Hugo Van Ingen. 2018. "Removal of Slow-Pulsing Artifacts in in-Phase 15N Relaxation Dispersion Experiments Using Broadband 1H Decoupling." *Journal of Biomolecular NMR* 71 (2): 69–77. <https://doi.org/10.1007/s10858-018-0193-2>.
- Chen, Jianhan, Charles L. Brooks, and Peter E. Wright. 2004. "Model-Free Analysis of Protein Dynamics: Assessment of Accuracy and Model Selection Protocols Based on Molecular Dynamics Simulation." *Journal of Biomolecular NMR* 29 (3): 243–57. <https://doi.org/10.1023/B:JNMR.0000032504.70912.58>.
- Chen, Kang, and Nico Tjandra. 2008. "Extended Model Free Approach To Analyze Correlation Functions of Multidomain Proteins in the Presence of Motional Coupling." *Journal of the American Chemical Society* 130 (38): 12745–51. <https://doi.org/10.1021/ja803557t>.

- Choy, Wing-Yiu, Zheng Zhou, Yawen Bai, and Levitt E. Kay. 2005. "An¹⁵ N NMR Spin Relaxation Dispersion Study of the Folding of a Pair of Engineered Mutants of Apocytochrome *b*₅₆₂." *Journal of the American Chemical Society* 127 (14): 5066–72. <https://doi.org/10.1021/ja042560u>.
- Claridge, Timothy D. W. 2016. *High-Resolution NMR Techniques in Organic Chemistry*. Elsevier.
- Clore, G. Marius, and Charles D. Schwieters. 2006. "Concordance of Residual Dipolar Couplings, Backbone Order Parameters and Crystallographic B-Factors for a Small α/β Protein: A Unified Picture of High Probability, Fast Atomic Motions in Proteins." *Journal of Molecular Biology* 355 (5): 879–86. <https://doi.org/10.1016/j.jmb.2005.11.042>.
- Clore, G. Marius, Attila Szabo, Ad Bax, Levitt E. Kay, Paul C. Driscoll, and Angela M. Gronenborn. 1990. "Deviations from the Simple Two-Parameter Model-Free Approach to the Interpretation of Nitrogen-15 Nuclear Magnetic Relaxation of Proteins." *Journal of the American Chemical Society* 112 (12): 4989–91. <https://doi.org/10.1021/ja00168a070>.
- Collins, Tony, and Georges Feller. 2023. "Psychrophilic Enzymes: Strategies for Cold-Adaptation." *Essays in Biochemistry* 67 (4): 701–13. <https://doi.org/10.1042/EBC20220193>.
- Conrath, Katja E., Marc Lauwereys, Moreno Galleni, and Andr E. Matagne. 2001. " β -Lactamase Inhibitors Derived from Single-Domain Antibody Fragments Elicited in the Camelidae." *Antimicrobial Agents and Chemotherapy* 45 (10): 2807–12. <https://doi.org/10.1128/AAC.45.10.2807-2812.2001>.
- Conrath, Katja, Cécile Vincke, Benoît Stijlemans, et al. 2005. "Antigen Binding and Solubility Effects upon the Veneering of a Camel VHH in Framework-2 to Mimic a VH." *Journal of Molecular Biology* 350 (1): 112–25. <https://doi.org/10.1016/j.jmb.2005.04.050>.
- Cornilescu, Gabriel, Frank Delaglio, and Ad Bax. 1999. "Protein Backbone Angle Restraints from Searching a Database for Chemical Shift and Sequence Homology." *Journal of Biomolecular NMR* 13 (3): 289–302. <https://doi.org/10.1023/A:1008392405740>.
- Cruickshank, D. W. J. 1999. "Remarks about Protein Structure Precision." *Acta Crystallographica Section D Biological Crystallography* 55 (3): 583–601. <https://doi.org/10.1107/S09074444998012645>.
- Czisch, M., G. C. King, and A. Ross. 1997. "Removal of Systematic Errors Associated with Off-Resonance Oscillations in T2Measurements." *Journal of Magnetic Resonance (1997)* 126 (1): 154–57. <https://doi.org/10.1006/jmre.1997.1168>.
- Daniele-Silva, Alessandra, Suedson De Carvalho Silva Rodrigues, Elizabeth Cristina Gomes Dos Santos, et al. 2021. "NMR Three-Dimensional Structure of the Cationic Peptide Stigmurin from Tityus Stigmurus Scorpion Venom: In Vitro Antioxidant and in Vivo Antibacterial and Healing Activity." *Peptides* 137 (March): 170478. <https://doi.org/10.1016/j.peptides.2020.170478>.
- Davies, Julian. 1994. "Inactivation of Antibiotics and the Dissemination of Resistance Genes." *Science* 264 (5157): 375–82. <https://doi.org/10.1126/science.8153624>.
- Delaglio, Frank, Stephan Grzesiek, Geerten W. Vuister, Guang Zhu, John Pfeifer, and Ad Bax. 1995. "NMRPipe: A Multidimensional Spectral Processing System Based on UNIX Pipes." *Journal of Biomolecular NMR* 6 (3): 277–93. <https://doi.org/10.1007/BF00197809>.

- Desai, Heta S., Tianyang Yan, Fengchao Yu, et al. 2022. "SP3-Enabled Rapid and High Coverage Chemoproteomic Identification of Cell-State-Dependent Redox-Sensitive Cysteines." *Molecular & Cellular Proteomics* 21 (4): 100218. <https://doi.org/10.1016/j.mcpro.2022.100218>.
- Dobrianov, I., C. Caylor, S. G. Lemay, K. D. Finkelstein, and R. E. Thorne. 1999. "X-Ray Diffraction Studies of Protein Crystal Disorder." *Journal of Crystal Growth* 196 (2–4): 511–23. [https://doi.org/10.1016/S0022-0248\(98\)00833-1](https://doi.org/10.1016/S0022-0248(98)00833-1).
- Dosset, P., J. C. Hus, M. Blackledge, and D. Marion. 2000. "Efficient Analysis of Macromolecular Rotational Diffusion from Heteronuclear Relaxation Data." *Journal of Biomolecular NMR* 16 (1): 23–28. <https://doi.org/10.1023/a:1008305808620>.
- Doucet, Nicolas, Pierre-Yves Savard, Joelle N. Pelletier, and Stéphane M. Gagné; 2007. "NMR Investigation of Tyr105 Mutants in TEM-1 β -Lactamase." *Journal of Biological Chemistry* 282 (29): 21448–59. <https://doi.org/10.1074/jbc.M609777200>.
- Dubovskii, Peter V., Alexander A. Vassilevski, Sergey A. Kozlov, Alexey V. Feofanov, Eugene V. Grishin, and Roman G. Efremov. 2015. "Latacins: Versatile Spider Venom Peptides." *Cellular and Molecular Life Sciences* 72 (23): 4501–22. <https://doi.org/10.1007/s00018-015-2016-x>.
- Ermolenko, D. N., and G. I. Makhatadze. 2002. "Bacterial Cold-Shock Proteins." *Cellular and Molecular Life Sciences: CMLS* 59 (11): 1902–13. <https://doi.org/10.1007/PL00012513>.
- Ernst, Richard, Geoffrey Bodenhausen, and Alexander Wokaun. 1990. *Principles of Nuclear Magnetic Resonance in One and Two Dimensions*. International Series of Monographs on Chemistry. Oxford University Press.
- Evans, Philip R., and Garib N. Murshudov. 2013. "How Good Are My Data and What Is the Resolution?" *Acta Crystallographica Section D Biological Crystallography* 69 (7): 1204–14. <https://doi.org/10.1107/S0907444913000061>.
- Farrow, Neil A., Ranjith Muhandiram, Alex U. Singer, et al. 1994. "Backbone Dynamics of a Free and a Phosphopeptide-Complexed Src Homology 2 Domain Studied by ^{15}N NMR Relaxation." *Biochemistry* 33 (19): 5984–6003. <https://doi.org/10.1021/bi00185a040>.
- Farrow, Neil A., Ouwen Zhang, Attila Szabo, Dennis A. Torchia, and Levitt E. Kay. 1995. "Spectral Density Function Mapping Using ^{15}N Relaxation Data Exclusively." *Journal of Biomolecular NMR* 6 (2): 153–62. <https://doi.org/10.1007/BF00211779>.
- Fawzi, Nicolas L., Jinfa Ying, Rodolfo Ghirlando, Dennis A. Torchia, and G. Marius Clore. 2011. "Atomic-Resolution Dynamics on the Surface of Amyloid- β Protofibrils Probed by Solution NMR." *Nature* 480 (7376): 268–72. <https://doi.org/10.1038/nature10577>.
- Feng, Wenqing, Roberto Tejero, Diane E. Zimmerman, Masayori Inouye, and Gaetano T. Montelione. 1998. "Solution NMR Structure and Backbone Dynamics of the Major Cold-Shock Protein (CspA) from *Escherichia Coli*: Evidence for Conformational Dynamics in the Single-Stranded RNA-Binding Site." *Biochemistry* 37 (31): 10881–96. <https://doi.org/10.1021/bi980269j>.

- Fernandez, C. 2003. "TROSY in NMR Studies of the Structure and Function of Large Biological Macromolecules." *Current Opinion in Structural Biology* 13 (5): 570–80. <https://doi.org/10.1016/j.sbi.2003.09.009>.
- Ferrage, Fabien, David Cowburn, and Ranajeet Ghose. 2009. "Accurate Sampling of High-Frequency Motions in Proteins by Steady-State¹⁵N–{¹H} Nuclear Overhauser Effect Measurements in the Presence of Cross-Correlated Relaxation." *Journal of the American Chemical Society* 131 (17): 6048–49. <https://doi.org/10.1021/ja809526q>.
- Ferrage, Fabien, Andrea Piserchio, David Cowburn, and Ranajeet Ghose. 2008. "On the Measurement of ¹⁵N–{¹H} Nuclear Overhauser Effects." *Journal of Magnetic Resonance* 192 (2): 302–13. <https://doi.org/10.1016/j.jmr.2008.03.011>.
- Fesik, Stephen W., Hugh L. Eaton, Edward T. Olejniczak, Erik R. P. Zuiderweg, Lawrence P. McIntosh, and Frederick W. Dahlquist. 1990. "2D and 3D NMR Spectroscopy Employing Carbon-13/Carbon-13 Magnetization Transfer by Isotropic Mixing. Spin System Identification in Large Proteins." *Journal of the American Chemical Society* 112 (2): 886–88. <https://doi.org/10.1021/ja00158a069>.
- Fisette, Olivier, Sébastien Morin, Pierre-Yves Savard, Patrick Lagüe, and Stéphane M. Gagné. 2010. "TEM-1 Backbone Dynamics—Insights from Combined Molecular Dynamics and Nuclear Magnetic Resonance." *Biophysical Journal* 98 (4): 637–45. <https://doi.org/10.1016/j.bpj.2009.08.061>.
- Forsén, Sture, and Ragnar A. Hoffman. 1963. "Study of Moderately Rapid Chemical Exchange Reactions by Means of Nuclear Magnetic Double Resonance." *The Journal of Chemical Physics* 39 (11): 2892–901. <https://doi.org/10.1063/1.1734121>.
- Fosgerau, Keld, and Torsten Hoffmann. 2015. "Peptide Therapeutics: Current Status and Future Directions." *Drug Discovery Today* 20 (1): 122–28. <https://doi.org/10.1016/j.drudis.2014.10.003>.
- Freeman, Ray, and Eriks Kupče. 1997. "Decoupling: Theory and Practice I. Current Methods and Recent Concepts." *NMR in Biomedicine* 10 (8): 372–80. [https://doi.org/10.1002/\(SICI\)1099-1492\(199712\)10:8%3C372::AID-NBM478%3E3.0.CO;2-Z](https://doi.org/10.1002/(SICI)1099-1492(199712)10:8%3C372::AID-NBM478%3E3.0.CO;2-Z).
- Frère, Jean-Marie. 1995. "Beta-lactamases and Bacterial Resistance to Antibiotics." *Molecular Microbiology* 16 (3): 385–95. <https://doi.org/10.1111/j.1365-2958.1995.tb02404.x>.
- Fukushima, Eiichi. 1981. *Experimental Pulse NMR: A Nuts and Bolts Approach*. With Internet Archive. Reading, Mass.: Addison-Wesley Pub. Co., Advanced Book Program. <http://archive.org/details/experimentalpuls0000fuku>.
- Fushman, David, and David Cowburn. 2001. "Nuclear Magnetic Resonance Relaxation in Determination of Residue-Specific ¹⁵N Chemical Shift Tensors in Proteins in Solution: Protein Dynamics, Structure, and Applications of Transverse Relaxation Optimized Spectroscopy." In *Methods in Enzymology*, edited by Thomas L. James, Volker Dötsch, and Uli Schmitz, vol. 339. Nuclear Magnetic Resonance of Biological Macromolecules - Part B. Academic Press. [https://doi.org/10.1016/S0076-6879\(01\)39312-6](https://doi.org/10.1016/S0076-6879(01)39312-6).

- Fushman, David, Rong Xu, and David Cowburn. 1999. "Direct Determination of Changes of Interdomain Orientation on Ligation: Use of the Orientational Dependence of ^{15}N NMR Relaxation in Abl SH(32)." *Biochemistry* 38 (32): 10225–30. <https://doi.org/10.1021/bi990897g>.
- Gagné, Stéphane M., Sakae Tsuda, Leo Spyropoulos, Levitt E. Kay, and Brian D. Sykes. 1998. "Backbone and Methyl Dynamics of the Regulatory Domain of Troponin C: Anisotropic Rotational Diffusion and Contribution of Conformational Entropy to Calcium Affinity." *Journal of Molecular Biology* 278 (3): 667–86. <https://doi.org/10.1006/jmbi.1998.1723>.
- Galdadas, Ioannis, Shen Qu, Ana Sofia F. Oliveria, et al. 2021. *Allosteric Communication in Class A β -Lactamases Occurs via Cooperative Coupling of Loop Dynamics*. <https://doi.org/10.7554/elif66567>.
- Geddes, Alasdair M., Keith P. Klugman, and George N. Rolinson. 2007. "Introduction: Historical Perspective and Development of Amoxicillin/Clavulanate." *International Journal of Antimicrobial Agents* 30 (December): 109–12. <https://doi.org/10.1016/j.ijantimicag.2007.07.015>.
- Ghanshani, Sanjiv, Heike Wulff, Mark J. Miller, et al. 2000. "Up-Regulation of the IKCa1 Potassium Channel during T-Cell Activation." *Journal of Biological Chemistry* 275 (47): 37137–49. <https://doi.org/10.1074/jbc.M003941200>.
- Ghose, Ranajeet, David Fushman, and David Cowburn. 2001. "Determination of the Rotational Diffusion Tensor of Macromolecules in Solution from NMR Relaxation Data with a Combination of Exact and Approximate Methods—Application to the Determination of Interdomain Orientation in Multidomain Proteins." *Journal of Magnetic Resonance* 149 (2): 204–17. <https://doi.org/10.1006/jmre.2001.2295>.
- Ghuysen, J. M. 1991. "Serine Beta-Lactamases and Penicillin-Binding Proteins." *Annu. Rev. Microbial* 45: 37–67. <https://doi.org/10.1146/annurev.mi.45.100191.000345>.
- Gil, R., and A. Navarro-Vázquez. n.d. *Chapter 1. Application of the Nuclear Overhauser Effect to the Structural Elucidation of Natural Products*. Accessed October 22, 2025. <https://www.scienceopen.com/book?vid=60aff547-be24-40f7-9103-208b25419fd2>.
- Gong, Qingguo, and Rieko Ishima. 2007. " ^{15}N – $\{^1\text{H}\}$ NOE Experiment at High Magnetic Field Strengths." *Journal of Biomolecular NMR* 37 (2): 147–57. <https://doi.org/10.1007/s10858-006-9125-7>.
- Grissmer, S., R. S. Levitt, and M. D. Cahalan. 1992. "Ca(2+)-Activated K⁺ Channels in Human Leukemic T Cells." *The Journal of General Physiology* 99 (1): 63–84. <https://doi.org/10.1085/jgp.99.1.63>.
- Gronwald, Wolfram, and Hans Robert Kalbitzer. 2004. "Automated Structure Determination of Proteins by NMR Spectroscopy." *Progress in Nuclear Magnetic Resonance Spectroscopy* 44 (1–2): 33–96. <https://doi.org/10.1016/j.pnmrs.2003.12.002>.
- Grzesiek, S., J. Anglister, and A. Bax. 1993. "Correlation of Backbone Amide and Aliphatic Side-Chain Resonances in $^{13}\text{C}/^{15}\text{N}$ -Enriched Proteins by Isotropic Mixing of ^{13}C Magnetization." *Journal of Magnetic Resonance, Series B* 101 (1): 114–19. <https://doi.org/10.1006/jmrb.1993.1019>.

- Grzesiek, Stephan, and Ad Bax. 1992. "An Efficient Experiment for Sequential Backbone Assignment of Medium-Sized Isotopically Enriched Proteins." *Journal of Magnetic Resonance (1969)* 99 (1): 201–7. [https://doi.org/10.1016/0022-2364\(92\)90169-8](https://doi.org/10.1016/0022-2364(92)90169-8).
- Grzesiek, Stephan, and Frank Delaglio. n.d. *Measurement of HN-Ha J Couplings in Calcium-Free Calmodulin Using New 2D and 3D Water-Flip-Back Methods*.
- Guenneugues, Marc, Patrick Berthault, and Hervé Desvaux. 1999. "A Method for Determining B1 Field Inhomogeneity. Are the Biases Assumed in Heteronuclear Relaxation Experiments Usually Underestimated?" *Journal of Magnetic Resonance* 136 (1): 118–26. <https://doi.org/10.1006/jmre.1998.1590>.
- Gullion, Terry, David B. Baker, and Mark S. Conradi. 1990. "New, Compensated Carr-Purcell Sequences." *Journal of Magnetic Resonance (1969)* 89 (3): 479–84. [https://doi.org/10.1016/0022-2364\(90\)90331-3](https://doi.org/10.1016/0022-2364(90)90331-3).
- Güntert, P., C. Mumenthaler, and K. Wüthrich. 1997. "Torsion Angle Dynamics for NMR Structure Calculation with the New Program Dyana1." *Journal of Molecular Biology* 273 (1): 283–98. <https://doi.org/10.1006/jmbi.1997.1284>.
- Güntert, Peter. 2004. "Automated NMR Structure Calculation With CYANA." In *Protein NMR Techniques*, edited by A. Kristina Downing. Humana Press. <https://doi.org/10.1385/1-59259-809-9:353>.
- Güntert, Peter. 2009. "Automated Structure Determination from NMR Spectra." *European Biophysics Journal* 38 (2): 129–43. <https://doi.org/10.1007/s00249-008-0367-z>.
- Güntert, Peter, Werner Braun, and Kurt Wüthrich. 1991. "Efficient Computation of Three-Dimensional Protein Structures in Solution from Nuclear Magnetic Resonance Data Using the Program DIANA and the Supporting Programs CALIBA, HABAS and GLOMSA." *Journal of Molecular Biology* 217 (3): 517–30. [https://doi.org/10.1016/0022-2836\(91\)90754-T](https://doi.org/10.1016/0022-2836(91)90754-T).
- Güntert, Peter, and Lena Buchner. 2015. "Combined Automated NOE Assignment and Structure Calculation with CYANA." *Journal of Biomolecular NMR* 62 (4): 453–71. <https://doi.org/10.1007/s10858-015-9924-9>.
- Gutowsky, H. S., D. W. McCall, and C. P. Slichter. 1951. "Coupling among Nuclear Magnetic Dipoles in Molecules." *Physical Review* 84 (3): 589–90. <https://doi.org/10.1103/PhysRev.84.589.2>.
- Hahn, E. L. 1950. "Spin Echoes." *Physical Review* 80 (4): 580–94. <https://doi.org/10.1103/PhysRev.80.580>.
- Hall, Jennifer B., and David Fushman. 2003. "Characterization of the Overall and Local Dynamics of a Protein with Intermediate Rotational Anisotropy: Differentiating between Conformational Exchange and Anisotropic Diffusion in the B3 Domain of Protein G." *Journal of Biomolecular NMR* 27 (3): 261–75. <https://doi.org/10.1023/a:1025467918856>.
- Hansen, D. Flemming, Pramodh Vallurupalli, and Levitt E. Kay. 2008. "An Improved¹⁵ N Relaxation Dispersion Experiment for the Measurement of Millisecond Time-Scale Dynamics in Proteins." *The Journal of Physical Chemistry B* 112 (19): 5898–904. <https://doi.org/10.1021/jp074793o>.

- Hansen, D. Flemming, Pramodh Vallurupalli, Patrik Lundström, Philipp Neudecker, and Levitt E. Kay. 2008. "Probing Chemical Shifts of Invisible States of Proteins with Relaxation Dispersion NMR Spectroscopy: How Well Can We Do?" *Journal of the American Chemical Society* 130 (8): 2667–75. <https://doi.org/10.1021/ja078337p>.
- Hansen, D. Flemming, Daiwen Yang, Haniqiao Feng, et al. 2007. "An Exchange-Free Measure of ^{15}N Transverse Relaxation: An NMR Spectroscopy Application to the Study of a Folding Intermediate with Pervasive Chemical Exchange." *Journal of the American Chemical Society* 129 (37): 11468–79. <https://doi.org/10.1021/ja072717t>.
- Harrison, Paul M., and Michael J. E. Sternberg. 1996. "The Disulphide B-Cross: From Cystine Geometry and Clustering to Classification of Small Disulphide-Rich Protein Folds." *Journal of Molecular Biology* 264: 603–23. <https://doi.org/10.1006/jmbi.1996.0664>.
- Hazes, B., and B. W. Dijkstra. 1988. "Model Building of Disulfide Bonds in Proteins with Known Three-Dimensional Structure." *Protein Engineering* 2 (2): 119–25. <https://doi.org/10.1093/protein/2.2.119>.
- Heinemann, Udo. 1994. "Crystal Structure of CspA, the Major Cold Shock Protein of Escherichia Coli." *Biochemistry* 91 (May): 5119–23.
- Henzler-Wildman, Katherine, and Dorothee Kern. 2007. "Dynamic Personalities of Proteins." *Nature* 450 (7172): 964–72. <https://doi.org/10.1038/nature06522>.
- Herrmann, Torsten, Peter Güntert, and Kurt Wüthrich. 2002. "Protein NMR Structure Determination with Automated NOE Assignment Using the New Software CANDID and the Torsion Angle Dynamics Algorithm DYANA." *Journal of Molecular Biology* 319 (1): 209–27. [https://doi.org/10.1016/S0022-2836\(02\)00241-3](https://doi.org/10.1016/S0022-2836(02)00241-3).
- Hobbs, Billy, Jack Drant, and Mike P. Williamson. 2022. "The Measurement of Binding Affinities by NMR Chemical Shift Perturbation." *Journal of Biomolecular NMR* 76 (4): 153–63. <https://doi.org/10.1007/s10858-022-00402-3>.
- Hoch, Jeffrey C., Kumaran Baskaran, Harrison Burr, et al. 2023. "Biological Magnetic Resonance Data Bank." *Nucleic Acids Research* 51 (D1): D368–76. <https://doi.org/10.1093/nar/gkac1050>.
- Hoey, Robert J., Hyeyoung Eom, and James R. Horn. 2019. "Structure and Development of Single Domain Antibodies as Modules for Therapeutics and Diagnostics." *Experimental Biology and Medicine* 244 (17): 1568–76. <https://doi.org/10.1177/1535370219881129>.
- Horn, G., R. Hofweber, W. Kremer, and H. R. Kalbitzer. 2007. "Structure and Function of Bacterial Cold Shock Proteins." *Cellular and Molecular Life Sciences: CMLS* 64 (12): 1457. <https://doi.org/10.1007/s00018-007-6388-4>.
- Horn, James R., and Brian K. Shoichet. 2004. "Allosteric Inhibition Through Core Disruption." *Journal of Molecular Biology* 336 (5): 1283–91. <https://doi.org/10.1016/j.jmb.2003.12.068>.
- Huang, Liwen, Pui-Kin So, Yu Wai Chen, Yun-Chung Leung, and Zhong-Ping Yao. 2020. "Conformational Dynamics of the Helix 10 Region as an Allosteric Site in Class A β -Lactamase Inhibitory Binding."

- Journal of the American Chemical Society* 142 (32): 13756–67. <https://doi.org/10.1021/jacs.0c04088>.
- Hughes, D. G. 1977. "Errors in T_2 Values Measured with the Carr-Purcell-Meiboom-Gill Pulsed NMR Sequence." *Journal of Magnetic Resonance (1969)* 26 (3): 481–89. [https://doi.org/10.1016/0022-2364\(77\)90099-3](https://doi.org/10.1016/0022-2364(77)90099-3).
- Hürlimann, M. D. 2001. "Optimization of Timing in the Carr-Purcell-Meiboom-Gill Sequence." *Magnetic Resonance Imaging* 19 (3–4): 375–78. [https://doi.org/10.1016/S0730-725X\(01\)00251-X](https://doi.org/10.1016/S0730-725X(01)00251-X).
- Idiyatullin, Djaudat, Vladimir A. Daragan, and Kevin H. Mayo. 2001. "Improved Measurement of ^{15}N – $\{^1\text{H}\}$ NOEs in the Presence of H(N)-Water Proton Chemical Exchange." *Journal of Magnetic Resonance* 153 (1): 138–43. <https://doi.org/10.1006/jmre.2001.2412>.
- Ikura, Mitsuhiko, and Levitt E. Kay. 1990. "A Novel Approach for Sequential Assignment of ^1H , ^{13}C , and ^{15}N Spectra of Larger Proteins: Heteronuclear Triple-Resonance Three-Dimensional NMR Spectroscopy. Application to Calmodulin." *Biochemistry* 29: 4659–67. <https://doi.org/https://doi.org/10.1021/bi00471a022>.
- Ishii, Takahiro M., Christopher Silvia, Birgit Hirschberg, Chris T. Bond, John P. Adelman, and James Maylie. 1997. "A Human Intermediate Conductance Calcium-Activated Potassium Channel." *Proceedings of the National Academy of Sciences* 94 (21): 11651–56. <https://doi.org/10.1073/pnas.94.21.11651>.
- Ishima, Rieko, John M. Louis, and Dennis A. Torchia. 1999. "Transverse ^1H Cross Relaxation in ^1H – ^{15}N Correlated ^1H CPMG Experiments." *Journal of Magnetic Resonance* 137 (1): 289–92. <https://doi.org/10.1006/jmre.1998.1672>.
- Ishima, Rieko, and Dennis A. Torchia. 2003. "Extending the Range of Amide Proton Relaxation Dispersion Experiments in Proteins Using a Constant-Time Relaxation-Compensated CPMG Approach." *Journal of Biomolecular NMR* 25: 243–48. <https://doi.org/10.1023/a:1022851228405>.
- Ishima, Rieko, and Dennis A. Torchia. 2005. "Error Estimation and Global Fitting in Transverse-Relaxation Dispersion Experiments to Determine Chemical-Exchange Parameters." *Journal of Biomolecular NMR* 32 (1): 41–54. <https://doi.org/10.1007/s10858-005-3593-z>.
- Jacob, F., B. Joris, S. Lepage, J. Dusart, and J. M. Frère. 1990. "Role of the Conserved Amino Acids of the 'SDN' Loop (Ser130, Asp131 and Asn132) in a Class A β -Lactamase Studied by Site-Directed Mutagenesis." *Biochemical Journal* 271 (2): 399–406. <https://doi.org/10.1042/bj2710399>.
- Jarymowycz, Virginia A., and Martin J. Stone. 2006. "Fast Time Scale Dynamics of Protein Backbones: NMR Relaxation Methods, Applications, and Functional Consequences." *Chemical Reviews* 106 (5): 1624–71. <https://doi.org/10.1021/cr040421p>.
- Jelsch, Christian, Lionel Mourey, Jean-Michel Masson, and Jean-Pierre Samama. 1993. "Crystal Structure of *Escherichia Coli* TEM1 β -lactamase at 1.8 Å Resolution." *Proteins: Structure, Function, and Bioinformatics* 16 (4): 364–83. <https://doi.org/10.1002/prot.340160406>.

- Jiang, Bin, Binhan Yu, Xu Zhang, Maili Liu, and Daiwen Yang. 2015. "A 15N CPMG Relaxation Dispersion Experiment More Resistant to Resonance Offset and Pulse Imperfection." *Journal of Magnetic Resonance* 257 (August): 1–7. <https://doi.org/10.1016/j.jmr.2015.05.003>.
- Jin, Bonghwan, Ki-Woong Jeong, and Yangmee Kim. 2014. "Structure and Flexibility of the Thermophilic Cold-Shock Protein of *Thermus Aquaticus*." *Biochemical and Biophysical Research Communications* 451 (3): 402–7. <https://doi.org/10.1016/j.bbrc.2014.07.127>.
- Jin, Yangzhuoyue, Yingxian Cui, and Tairan Yuwen. 2025. "NMR Methods for Investigating Functionally Relevant Biomolecular Dynamics." *Magnetic Resonance Letters*, March, 200195. <https://doi.org/10.1016/j.mrl.2025.200195>.
- Johnson, Bruce A. 2004. "Using NMRView to Visualize and Analyze the NMR Spectra of Macromolecules." In *Protein NMR Techniques*, edited by A. Kristina Downing. Humana Press. <https://doi.org/10.1385/1-59259-809-9:313>.
- Jumper, John, Richard Evans, Alexander Pritzel, et al. 2021. "Highly Accurate Protein Structure Prediction with AlphaFold." *Nature* 596 (7873): 583–89. <https://doi.org/10.1038/s41586-021-03819-2>.
- Karplus, M., and J. Kuriyan. 2005. "Molecular Dynamics and Protein Function." *Proceedings of the National Academy of Sciences* 102 (19): 6679–85. <https://doi.org/10.1073/pnas.0408930102>.
- Karplus, Martin. 1959. "Contact Electron-Spin Coupling of Nuclear Magnetic Moments." *The Journal of Chemical Physics* 30 (1): 11–15. <https://doi.org/10.1063/1.1729860>.
- Kay, Levitt E., A. Bax, and D. A. Torchia. 1992. "Pulse Sequences for Removal of the Effects of Cross Correlation between Dipolar and Chemical-Shift Anisotropy Relaxation Mechanisms on the Measurement of Heteronuclear T1 and T2 Values in Proteins." *Journal of Magnetic Resonance* 97: 359–75.
- Kay, Levitt E., D. A. Torchia, and Ad Bax. 1989a. "Backbone Dynamics of Proteins as Studied by 15N Inverse Detected Heteronuclear NMR Spectroscopy: Application to Staphylococcal Nuclease." *Biochemistry*.
- Kay, Levitt E., and Dennis A. Torchia. 1998. "Protein Dynamics from NMR." *Nature Structural Biology* 5 (7): 513–17. <https://doi.org/10.1038/755>.
- Kay, Levitt E., Dennis A. Torchia, and Ad Bax. 1989b. "Backbone Dynamics of Proteins as Studied by Nitrogen-15 Inverse Detected Heteronuclear NMR Spectroscopy: Application to Staphylococcal Nuclease." *Biochemistry* 28 (23): 8972–79. <https://doi.org/10.1021/bi00449a003>.
- Keeler, James. 2002. "Understanding NMR Spectroscopy 2nd." *John Wiley & Sons Inc.*
- Kellman, Peter, Andrew E. Arai, Elliot R. McVeigh, and Anthony H. Aletras. 2002. "Phase-sensitive Inversion Recovery for Detecting Myocardial Infarction Using Gadolinium-delayed Hyperenhancement†." *Magnetic Resonance in Medicine* 47 (2): 372–83. <https://doi.org/10.1002/mrm.10051>.
- Khandave, Nihar Pradeep, D. Flemming Hansen, and Pramodh Vallurupalli. 2024. "Increasing the Accuracy of Exchange Parameters Reporting on Slow Dynamics by Performing CEST Experiments with 'High'

- B1 Fields." *Journal of Magnetic Resonance* 363 (June): 107699. <https://doi.org/10.1016/j.jmr.2024.107699>.
- Khandave, Nihar Pradeep, Ashok Sekhar, and Pramodh Vallurupalli. 2023. "Studying Micro to Millisecond Protein Dynamics Using Simple Amide ^{15}N CEST Experiments Supplemented with Major-State R_2 and Visible Peak-Position Constraints." *Journal of Biomolecular NMR* 77 (4): 165–81. <https://doi.org/10.1007/s10858-023-00419-2>.
- Kharchenko, Vladlena, Michal Nowakowski, Mariusz Jaremko, Andrzej Ejchart, and Łukasz Jaremko. 2020. "Dynamic $^{15}\text{N}\{^1\text{H}\}$ NOE Measurements: A Tool for Studying Protein Dynamics." *Journal of Biomolecular NMR* 74 (12): 707–16. <https://doi.org/10.1007/s10858-020-00346-6>.
- Kim, Seho, Kuen-Phon Wu, and Jean Baum. 2013. "Fast Hydrogen Exchange Affects ^{15}N Relaxation Measurements in Intrinsically Disordered Proteins." *Journal of Biomolecular NMR* 55 (3): 249–56. <https://doi.org/10.1007/s10858-013-9706-1>.
- King, Glenn F. 2011. "Venoms as a Platform for Human Drugs: Translating Toxins into Therapeutics." *Expert Opinion on Biological Therapy* 11 (11): 1469–84. <https://doi.org/10.1517/14712598.2011.621940>.
- Kleckner, Ian R., and Mark P. Foster. 2011. "An Introduction to NMR-Based Approaches for Measuring Protein Dynamics." *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* 1814 (8): 942–68. <https://doi.org/10.1016/j.bbapap.2010.10.012>.
- Kleywegt, Gerard J., and T. Alwyn Jones. 1997. "[11] Model Building and Refinement Practice." In *Methods in Enzymology*, vol. 277. Elsevier. [https://doi.org/10.1016/S0076-6879\(97\)77013-7](https://doi.org/10.1016/S0076-6879(97)77013-7).
- Knoverek, Catherine R., Upasana L. Mallimadugula, Sukrit Singh, et al. 2021. "Opening of a Cryptic Pocket in β -Lactamase Increases Penicillinase Activity." *Proceedings of the National Academy of Sciences* 118 (47): e2106473118. <https://doi.org/10.1073/pnas.2106473118>.
- Knox, James R. 1995. "Extended-Spectrum and Inhibitor-Resistant TEM-Type β -Lactamases: Mutations, Specificity, and Three-Dimensional Structure." *Antimicrobial Agents and Chemotherapy* 39 (12): 2593–601. <https://doi.org/10.1128/AAC.39.12.2593>.
- Köhler, M., B. Hirschberg, C. T. Bond, et al. 1996. "Small-Conductance, Calcium-Activated Potassium Channels from Mammalian Brain." *Science (New York, N.Y.)* 273 (5282): 1709–14. <https://doi.org/10.1126/science.273.5282.1709>.
- Koradi, Reto, Martin Billeter, Max Engeli, Peter Güntert, and Kurt Wüthrich. 1998. "Automated Peak Picking and Peak Integration in Macromolecular NMR Spectra Using AUTOPSY." *Journal of Magnetic Resonance* 135 (2): 288–97. <https://doi.org/10.1006/jmre.1998.1570>.
- Korzhev, Dmitry M., Xavier Salvatella, Michele Vendruscolo, et al. 2004. "Low-Populated Folding Intermediates of Fyn SH3 Characterized by Relaxation Dispersion NMR." *Nature* 430 (6999): 586–90. <https://doi.org/10.1038/nature02655>.
- Korzhev, Dmitry M., Eugene V. Tischenko, and Alexander S. Arseniev. 2000. "Off-Resonance Effects in ^{15}N T₂ CPMG Measurements." *Journal of Biomolecular NMR* 17: 231–37. <https://doi.org/10.1023/a:1008348827208>.

- Kuhlmann, Karl F., and David M. Grant. 1971. "Carbon-13 Relaxation and Internal Rotation in Mesitylene and *o*-Xylene." *The Journal of Chemical Physics* 55 (6): 2998–3007. <https://doi.org/10.1063/1.1676529>.
- Kuhlmann, Karl F., David M. Grant, and Robin K. Harris. 1970. "Nuclear Overhauser Effects and ¹³C Relaxation Times in ¹³C–{H} Double Resonance Spectra." *The Journal of Chemical Physics* 52 (7): 3439–48. <https://doi.org/10.1063/1.1673508>.
- Kumari, Pratibha, Lukas Frey, Alexander Sobol, Nils-Alexander Lakomek, and Roland Riek. 2018. "15N Transverse Relaxation Measurements for the Characterization of Ms–Ms Dynamics Are Deteriorated by the Deuterium Isotope Effect on 15N Resulting from Solvent Exchange." *Journal of Biomolecular NMR* 72 (3–4): 125–37. <https://doi.org/10.1007/s10858-018-0211-4>.
- Kuschert, Sarah, Martin Stroet, Yanni Ka-Yan Chin, et al. 2023. "Facilitating the Structural Characterisation of Non-Canonical Amino Acids in Biomolecular NMR." *Magnetic Resonance* 4 (1): 57–72. <https://doi.org/10.5194/mr-4-57-2023>.
- Kuzmenkov, Alexey I., Steve Peigneur, Joshua A. Nasburg, et al. 2022. "Apamin Structure and Pharmacology Revisited." *Frontiers in Pharmacology* 13 (September): 977440. <https://doi.org/10.3389/fphar.2022.977440>.
- Lancaster, B., and P. R. Adams. 1986. "Calcium-Dependent Current Generating the Afterhyperpolarization of Hippocampal Neurons." *Journal of Neurophysiology* 55 (6): 1268–82. <https://doi.org/10.1152/jn.1986.55.6.1268>.
- Land, Henrik, and Maria Svedendahl Humble. 2018. "YASARA: A Tool to Obtain Structural Guidance in Biocatalytic Investigations." In *Protein Engineering: Methods and Protocols*, edited by Uwe T. Bornscheuer and Matthias Höhne. Springer. https://doi.org/10.1007/978-1-4939-7366-8_4.
- Landsman, D. 1992. "RNP-1, an RNA-Binding Motif Is Conserved in the DNA-Binding Cold Shock Domain." *Nucleic Acids Research* 20 (11): 2861–64. <https://doi.org/10.1093/nar/20.11.2861>.
- Laskowski, Roman A., J. Antoon C. Rullmann, Malcolm W. MacArthur, Robert Kaptein, and Janet M. Thornton. 1996. "AQUA and PROCHECK-NMR: Programs for Checking the Quality of Protein Structures Solved by NMR." *Journal of Biomolecular NMR* 8 (4): 477–86. <https://doi.org/10.1007/BF00228148>.
- Lee, Chia-Hsueh, and Roderick MacKinnon. 2018. "Activation Mechanism of a Human SK-Calmodulin Channel Complex Elucidated by Cryo-EM Structures." *Science* 360 (6388): 508–13. <https://doi.org/10.1126/science.aas9466>.
- Lee, Juho, Ki-Woong Jeong, Bonghwan Jin, et al. 2013. "Structural and Dynamic Features of Cold-Shock Proteins of *Listeria Monocytogenes*, a Psychrophilic Bacterium." *Biochemistry* 52 (14): 2492–504. <https://doi.org/10.1021/bi301641b>.
- Lee, Wei-Sheng, Thu Jennifer Ngo-Anh, Andrew Bruening-Wright, James Maylie, and John P. Adelman. 2003. "Small Conductance Ca²⁺-Activated K⁺ Channels and Calmodulin." *Journal of Biological Chemistry* 278 (28): 25940–46. <https://doi.org/10.1074/jbc.M302091200>.

- Lee, Woonghee, Marco Tonelli, and John L. Markley. 2015. "NMRFAM-SPARKY: Enhanced Software for Biomolecular NMR Spectroscopy." *Bioinformatics* 31 (8): 1325–27. <https://doi.org/10.1093/bioinformatics/btu830>.
- Lee, Yeongjoon, Chulhee Kwak, Ki-Woong Jeong, et al. 2018. "Tyr51: Key Determinant of the Low Thermostability of the *Colwellia Psychrerythraea* Cold-Shock Protein." *Biochemistry* 57 (26): 3625–40. <https://doi.org/10.1021/acs.biochem.8b00144>.
- Levitt, Malcolm H. 1982. "Symmetrical Composite Pulse Sequences for NMR Population Inversion. II. Compensation of Resonance Offset." *Journal of Magnetic Resonance (1969)* 50 (1): 95–110. [https://doi.org/10.1016/0022-2364\(82\)90035-X](https://doi.org/10.1016/0022-2364(82)90035-X).
- Levitt, Malcolm H. 2013. *Spin Dynamics: Basics of Nuclear Magnetic Resonance*. John Wiley & Sons.
- Levitt, Malcolm H. 2008. "Spin Dynamics: Basics of Nuclear Magnetic Resonance, 2nd Edition." John Wiley & Sons.
- Lian, Lu-Yun, and Igor L. Barsukov. 2011. "Resonance Assignments." In *Protein NMR Spectroscopy: Practical Techniques and Applications*. John Wiley & Sons, Ltd. <https://doi.org/10.1002/9781119972006.ch3>.
- Linge, Jens P., Michael Habeck, Wolfgang Rieping, and Michael Nilges. 2003. "ARIA: Automated NOE Assignment and NMR Structure Calculation." *Bioinformatics (Oxford, England)* 19 (2): 315–16. <https://doi.org/10.1093/bioinformatics/19.2.315>.
- Linge, Jens P., Mark A. Williams, Christian A. E. M. Spronk, Alexandre M. J. J. Bonvin, and Michael Nilges. 2003. "Refinement of Protein Structures in Explicit Solvent." *Proteins: Structure, Function, and Bioinformatics* 50 (3): 496–506. <https://doi.org/10.1002/prot.10299>.
- Lipari, Giovanni, and Attila Szabo. 1982a. "Model-Free Approach to the Interpretation of Nuclear Magnetic Resonance Relaxation in Macromolecules. 1. Theory and Range of Validity." *Journal of the American Chemical Society* 104 (17): 4546–59. <https://doi.org/10.1021/ja00381a009>.
- Lipari, Giovanni, and Attila Szabo. 1982b. "Model-Free Approach to the Interpretation of Nuclear Magnetic Resonance Relaxation in Macromolecules. 2. Analysis of Experimental Results." *Journal of the American Chemical Society* 104 (17): 4559–70. <https://doi.org/10.1021/ja00381a010>.
- Long, Dong, Maili Liu, and Daiwen Yang. 2008. "Accurately Probing Slow Motions on Millisecond Timescales with a Robust NMR Relaxation Experiment." *Journal of the American Chemical Society* 130 (8): 2432–33. <https://doi.org/10.1021/ja710477h>.
- Loria, J. Patrick, Mark Rance, and Arthur G. Palmer. 1999. "A Relaxation-Compensated Carr–Purcell–Meiboom–Gill Sequence for Characterizing Chemical Exchange by NMR Spectroscopy." *Journal of the American Chemical Society* 121 (10): 2331–32. <https://doi.org/10.1021/ja983961a>.
- Loria, J. Patrick, Mark Rance, and Arthur G. Palmer. 1999. "A TROSY CPMG Sequence for Characterizing Chemical Exchange in Large Proteins." *Journal of Biomolecular NMR* 15 (2): 151–55. <https://doi.org/10.1023/A:1008355631073>.

- Lovell, Simon C., Ian W. Davis, W. Bryan Arendall III, et al. 2003. "Structure validation by C α geometry: ϕ , ψ and C β deviation." *Proteins: Structure, Function, and Bioinformatics* 50 (3): 437–50. <https://doi.org/10.1002/prot.10286>.
- Lu, Yimei, and Daiwen Yang. 2021. "Conformational Exchange of Fatty Acid Binding Protein Induced by Protein-Nanodisc Interactions." *Biophysical Journal* 120 (21): 4672–81. <https://doi.org/10.1016/j.bpj.2021.09.037>.
- Lundström, Patrik, Hong Lin, and Levitt E. Kay. 2009. "Measuring $^{13}\text{C}\beta$ Chemical Shifts of Invisible Excited States in Proteins by Relaxation Dispersion NMR Spectroscopy." *Journal of Biomolecular NMR* 44 (3): 139–55. <https://doi.org/10.1007/s10858-009-9321-3>.
- Macomber, J. D. 1995. "Basic Principles of Magnetic Resonance." In *Dynamics During Spectroscopic Transitions: Basic Concepts*, edited by E. Lippert and J. D. Macomber. Springer. https://doi.org/10.1007/978-3-642-79407-0_8.
- Madhurima, Kulkarni, Bodhisatwa Nandi, Sneha Munshi, Athi N. Naganathan, and Ashok Sekhar. 2023. "Functional Regulation of an Intrinsically Disordered Protein via a Conformationally Excited State." *SCIENCE ADVANCES*, ahead of print, June. <https://doi.org/10.1126/sciadv.adh4591>.
- Maharjan, S., A. Singh, D. K. Khadka, and M. Aryal. 2017. "Drug Resistance Pattern in Pulmonary Tuberculosis Patients and Risk Factors Associated with Multi-Drug Resistant Tuberculosis." *Journal of Tuberculosis Research* 05 (02): 106–17. <https://doi.org/10.4236/jtr.2017.52012>.
- Malde, Alpeshkumar K., Le Zuo, Matthew Breeze, et al. 2011. "An Automated Force Field Topology Builder (ATB) and Repository: Version 1.0." *Journal of Chemical Theory and Computation* 7 (12): 4026–37. <https://doi.org/10.1021/ct200196m>.
- Mandel, Arthur M., Mikael Akke, and Arthur G. Palmer. 1995. "Backbone Dynamics of Escherichiacoli Ribonuclease HI: Correlations with Structure and Function in an Active Enzyme." *J. Mol. Bio* 246: 144–63. <https://doi.org/10.1006/jmbi.1994.0073>.
- Marion, Dominique, Paul C. Driscoll, Levitt E. Kay, et al. 1989. "Overcoming the Overlap Problem in the Assignment of Proton NMR Spectra of Larger Proteins by Use of Three-Dimensional Heteronuclear Proton-Nitrogen- ^{15}N Hartmann-Hahn-Multiple Quantum Coherence and Nuclear Overhauser-Multiple Quantum Coherence Spectroscopy: Application to Interleukin 1.Beta." *Biochemistry* 28 (15): 6150–56. <https://doi.org/10.1021/bi00441a004>.
- Marion, Dominique, Levitt E. Kay, Steven W. Sparks, Dennis A. Torchia, and Ad Bax. 1989. "Three-Dimensional Heteronuclear NMR of Nitrogen- ^{15}N Labeled Proteins." *Journal of the American Chemical Society* 111 (4): 1515–17. <https://doi.org/10.1021/ja00186a066>.
- Marley, Jonathan, Min Lu, and Clay Bracken. 2001. "A Method for Efficient Isotopic Labeling of Recombinant Proteins." *Journal of Biomolecular NMR* 20 (1): 71–75. <https://doi.org/10.1023/A:1011254402785>.
- Martins, J. C., W. G. Zhang, A. Tartar, M. Lazdunski, and F. A. Borremans. 1990. "Solution Conformation of Leiurotoxin I (Scyllatoxin) by ^1H Nuclear Magnetic Resonance. Resonance Assignment and Secondary Structure." *FEBS Letters* 260 (2): 2. [https://doi.org/10.1016/0014-5793\(90\)80115-y](https://doi.org/10.1016/0014-5793(90)80115-y).

- Martins, José C., Frank J. M. Van De Ven, and Frans A. M. Borremans. 1995. "Determination of the Three-Dimensional Solution Structure of Scyllatoxin by ¹H Nuclear Magnetic Resonance." *Journal of Molecular Biology* 253 (4): 4. <https://doi.org/10.1006/jmbi.1995.0575>.
- MATAGNE, Andre, Josette LAMOTTE-BRASSEUR, and Jean-Marie FRÈRE. 1998. "Catalytic Properties of Class A β -Lactamases : Efficiency and Diversity." *Biochem. J.* 330: 581–98.
- Maudsley, A. A. 1986. "Modified Carr-Purcell-Meiboom-Gill Sequence for NMR Fourier Imaging Applications." *Journal of Magnetic Resonance* (1969) 69 (3): 488–91. [https://doi.org/10.1016/0022-2364\(86\)90160-5](https://doi.org/10.1016/0022-2364(86)90160-5).
- Maveyraud, Laurent, Isabelle Saves, Odile Burlet-Schiltz, et al. 1996. "Structural Basis of Extended Spectrum TEM β -Lactamases." *Journal of Biological Chemistry* 271 (18): 10482–89. <https://doi.org/10.1074/jbc.271.18.10482>.
- Max, Klaas E. A., Markus Zeeb, Ralf Bienert, Jochen Balbach, and Udo Heinemann. 2006. "T-Rich DNA Single Strands Bind to a Preformed Site on the Bacterial Cold Shock Protein Bs-CspB." *Journal of Molecular Biology* 360 (3): 702–14. <https://doi.org/10.1016/j.jmb.2006.05.044>.
- Max, Klaas E. A., Markus Zeeb, Ralf Bienert, Jochen Balbach, and Udo Heinemann. 2007. "Common Mode of DNA Binding to Cold Shock Domains: Crystal Structure of Hexathymidine Bound to the Domain-swapped Form of a Major Cold Shock Protein from *Bacillus Caldolyticus*." *The FEBS Journal* 274 (5): 1265–79. <https://doi.org/10.1111/j.1742-4658.2007.05672.x>.
- McConnell, Harden M. 1958. "Reaction Rates by Nuclear Magnetic Resonance." *The Journal of Chemical Physics* 28 (3): 430–31. <https://doi.org/10.1063/1.1744152>.
- Meiboom, S., and D. Gill. 1958. "Modified Spin-Echo Method for Measuring Nuclear Relaxation Times." *Review of Scientific Instruments* 29 (8): 688–91. <https://doi.org/10.1063/1.1716296>.
- Meilleur, Flora, Kevin L. Weiss, and Dean A. A. Myles. 2009. "Deuterium Labeling for Neutron Structure-Function-Dynamics Analysis." In *Micro and Nano Technologies in Bioanalysis: Methods and Protocols*, edited by Robert S. Foote and James Weifu Lee. Humana Press. https://doi.org/10.1007/978-1-59745-483-4_18.
- Meissner, Axel, and Ole Winneche Sørensen. 2001. "Sequential HNCACB and CBCANH Protein NMR Pulse Sequences." *Journal of Magnetic Resonance* 151 (2): 328–31. <https://doi.org/10.1006/jmre.2001.2374>.
- Meneksedag, Deniz, Asligul Dogan, Pinar Kanlikilicer, and Elif Ozkirimli. 2013. "Communication between the Active Site and the Allosteric Site in Class A Beta-Lactamases." *Computational Biology and Chemistry* 43 (April): 1–10. <https://doi.org/10.1016/j.compbiolchem.2012.12.002>.
- Mielke, Steven P., and V. V. Krishnan. 2009. "Characterization of Protein Secondary Structure from NMR Chemical Shifts." *Progress in Nuclear Magnetic Resonance Spectroscopy* 54 (3–4): 141–65. <https://doi.org/10.1016/j.pnmrs.2008.06.002>.
- Millet, Oscar, J. Patrick Loria, Christopher D. Kroenke, Miquel Pons, and Arthur G. Palmer. 2000. "The Static Magnetic Field Dependence of Chemical Exchange Linebroadening Defines the NMR Chemical

- Shift Time Scale." *Journal of the American Chemical Society* 122 (12): 2867–77. <https://doi.org/10.1021/ja993511y>.
- Mittermaier, Anthony, and Levitt E. Kay. 2006. "New Tools Provide New Insights in NMR Studies of Protein Dynamics." *Science* 312 (5771): 224–28. <https://doi.org/10.1126/science.1124964>.
- Mobli, Mehdi, and Glenn F. King. 2010. "NMR Methods for Determining Disulfide-Bond Connectivities." *Toxicon* 56 (6): 849–54. <https://doi.org/10.1016/j.toxicon.2010.06.018>.
- Morin, Sébastien. 2011. "A Practical Guide to Protein Dynamics from ¹⁵N Spin Relaxation in Solution." *Progress in Nuclear Magnetic Resonance Spectroscopy* 59 (3): 245–62. <https://doi.org/10.1016/j.pnmrs.2010.12.003>.
- Morris, G. A. 1997. "P. J. HORE Nuclear Magnetic Resonance. Oxford Chemistry Primers No. 32 Oxford Science Publications, Oxford University Press, Oxford, 1995, Pp. 90, £4.99. ISBN 0 19 855682 9." *Magnetic Resonance in Chemistry* 35 (4): 281–281. [https://doi.org/10.1002/\(SICI\)1097-458X\(199704\)35:4%3C281::AID-OMR31%3E3.0.CO;2-L](https://doi.org/10.1002/(SICI)1097-458X(199704)35:4%3C281::AID-OMR31%3E3.0.CO;2-L).
- Morris, Gareth A., and Ray Freeman. 2011. "Selective Excitation in Fourier Transform Nuclear Magnetic Resonance. 1978." *Journal of Magnetic Resonance (San Diego, Calif.: 1997)* 213 (2): 214–43. <https://doi.org/10.1016/j.jmr.2011.08.031>.
- Moy, Franklin J., Andrew P. Seddon, Ernest B. Campbell, Peter Böhlen, and Robert Powers. 1995. "¹H, ¹⁵N, ¹³C and ¹³CO Assignments and Secondary Structure Determination of Basic Fibroblast Growth Factor Using 3D Heteronuclear NMR Spectroscopy." *Journal of Biomolecular NMR* 6: 245–54. <https://doi.org/10.1007/BF00197806>.
- Mpari, Bedel, Imed Regaya, Guy Escoffier, and Christiane Mourre. 2005. "DIFFERENTIAL EFFECTS OF TWO BLOCKERS OF SMALL CONDUCTANCE Ca²⁺-ACTIVATED K⁺ CHANNELS, APAMIN AND LEI-DAB7, ON LEARNING AND MEMORY IN RATS." *Journal of Integrative Neuroscience* 04 (03): 381–96. <https://doi.org/10.1142/S0219635205000884>.
- Mulder, Frans A. A., Nikolai R. Skrynnikov, Bin Hon, Frederick W. Dahlquist, and Levitt E. Kay. 2001. "Measurement of Slow (Ms–ms) Time Scale Dynamics in Protein Side Chains by ¹⁵N Relaxation Dispersion NMR Spectroscopy: Application to Asn and Gln Residues in a Cavity Mutant of T4 Lysozyme." *Journal of the American Chemical Society* 123 (5): 967–75. <https://doi.org/10.1021/ja003447g>.
- Myint, Wazo, Yufeng Cai, Celia A. Schiffer, and Rieko Ishima. 2012. "Quantitative Comparison of Errors in ¹⁵N Transverse Relaxation Rates Measured Using Various CPMG Phasing Schemes." *Journal of Biomolecular NMR* 53 (1): 13–23. <https://doi.org/10.1007/s10858-012-9621-x>.
- Neudecker, Philipp, Paul Robustelli, Andrea Cavalli, et al. 2012. "Structure of an Intermediate State in Protein Folding and Aggregation." *Science* 336 (6079): 362–66. <https://doi.org/10.1126/science.1214203>.
- Neuhaus, David. 2011. "Nuclear Overhauser Effect." In *eMagRes*. John Wiley & Sons, Ltd. <https://doi.org/10.1002/9780470034590.emrstm0350.pub2>.

- Newkirk, K., W. Feng, W. Jiang, et al. 1994. "Solution NMR Structure of the Major Cold Shock Protein (CspA) from Escherichia Coli: Identification of a Binding Epitope for DNA." *Proceedings of the National Academy of Sciences* 91 (11): 5114–18. <https://doi.org/10.1073/pnas.91.11.5114>.
- Nielsen, Jakob Toudahl, and Frans A. A. Mulder. 2018. "POTENCI: Prediction of Temperature, Neighbor and pH-Corrected Chemical Shifts for Intrinsically Disordered Proteins." *Journal of Biomolecular NMR* 70 (3): 141–65. <https://doi.org/10.1007/s10858-018-0166-5>.
- Norton, Raymond S. 2018. "Peptide Toxin Structure and Function by NMR." In *Modern Magnetic Resonance*, edited by Graham A. Webb. Springer International Publishing. https://doi.org/10.1007/978-3-319-28388-3_120.
- Ollila, O. H. Samuli, Harri A. Heikkinen, and Hideo Iwai. 2018. "Rotational Dynamics of Proteins from Spin Relaxation Times and Molecular Dynamics Simulations." *The Journal of Physical Chemistry B* 122 (25): 6559–69. <https://doi.org/10.1021/acs.jpcb.8b02250>.
- O'Neill, Jim. 2016. *Tackling Drug-Resistant Infections Globally: Final Report and Recommendations*. Report. Government of the United Kingdom. United Kingdom. <https://apo.org.au/node/63983>.
- Ottiger, Marcel, Frank Delaglio, and Ad Bax. 1998. "Measurement of J and Dipolar Couplings from Simplified Two-Dimensional NMR Spectra." *Journal of Magnetic Resonance* 131 (2): 373–78. <https://doi.org/10.1006/jmre.1998.1361>.
- Padilla, Jennifer E., and Todd O. Yeates. 2003. "A Statistic for Local Intensity Differences: Robustness to Anisotropy and Pseudo-Centering and Utility for Detecting Twinning." *Acta Crystallographica Section D Biological Crystallography* 59 (7): 1124–30. <https://doi.org/10.1107/S0907444903007947>.
- Palmer, Arthur G. 2004. "NMR Characterization of the Dynamics of Biomacromolecules." *Chemical Reviews* 104 (8): 3623–40. <https://doi.org/10.1021/cr030413t>.
- Palmer, Arthur G. 2014. "Chemical Exchange in Biomacromolecules: Past, Present, and Future." *Journal of Magnetic Resonance* 241 (April): 3–17. <https://doi.org/10.1016/j.jmr.2014.01.008>.
- Palmer, Arthur G., Christopher D. Kroenke, and J. Patrick Loria. 2001. "Nuclear Magnetic Resonance Methods for Quantifying Microsecond-to-Millisecond Motions in Biological Macromolecules." In *Methods in Enzymology*, vol. 339. Elsevier. [https://doi.org/10.1016/S0076-6879\(01\)39315-1](https://doi.org/10.1016/S0076-6879(01)39315-1).
- Palmer, Arthur G., and Francesca Massi. 2006. "Characterization of the Dynamics of Biomacromolecules Using Rotating-Frame Spin Relaxation NMR Spectroscopy." *Chemical Reviews* 106 (5): 1700–1719. <https://doi.org/10.1021/cr0404287>.
- Palmer, Arthur G., Nicholas J. Skelton, Walter J. Chazin, Peter E. Wright, and Mark Rance. 1992. "Suppression of the Effects of Cross-Correlation between Dipolar and Anisotropic Chemical Shift Relaxation Mechanisms in the Measurement of Spin-Spin Relaxation Rates." *Molecular Physics* 75 (3): 699–711. <https://doi.org/10.1080/00268979200100511>.

- Parthasarathy, S., and M. R. N. Murthy. 1997. "Analysis of Temperature Factor Distribution in High-resolution Protein Structures." *Protein Science* 6 (12): 2561–67. <https://doi.org/10.1002/pro.5560061208>.
- Pawley, Norma H., M. Daniel Clark, and Ryszard Michalczyk. 2006. "Rectifying System-Specific Errors in NMR Relaxation Measurements." *Journal of Magnetic Resonance* 178 (1): 77–87. <https://doi.org/10.1016/j.jmr.2005.08.017>.
- Pedarzani, P., and M. Stocker. 2008. "Molecular and Cellular Basis of Small- and Intermediate-Conductance, Calcium-Activated Potassium Channel Function in the Brain." *Cellular and Molecular Life Sciences* 65 (20): 3196–217. <https://doi.org/10.1007/s00018-008-8216-x>.
- Pedarzani, Paola, Dieter D'hoedt, Kevina B. Doorty, et al. 2002. "Tamapin, a Venom Peptide from the Indian Red Scorpion (Mesobuthus Tamulus) That Targets Small Conductance Ca²⁺-Activated K⁺ Channels and Afterhyperpolarization Currents in Central Neurons." *The Journal of Biological Chemistry* 277 (48): 46101–9. <https://doi.org/10.1074/jbc.M206465200>.
- Pengt, Jeffrey W., and Gerhard Wagner. 1992. "Mapping of the Spectral Densities of N-H Bond Motions in Eglin c Using Heteronuclear Relaxation Experiments." *Biochemistry* 31: 8571–86. <https://doi.org/10.1021/bi00151a027>.
- Pervushin, Konstantin, Roland Riek, Gerhard Wider, and Kurt Wüthrich. 1997. "Attenuated T2 Relaxation by Mutual Cancellation of Dipole–Dipole Coupling and Chemical Shift Anisotropy Indicates an Avenue to NMR Structures of Very Large Biological Macromolecules in Solution." *Proceedings of the National Academy of Sciences* 94 (23): 12366–71. <https://doi.org/10.1073/pnas.94.23.12366>.
- Poirel, Laurent, Hedi Mammeri, and Patrice Nordmann. 2004. "TEM-121, a Novel Complex Mutant of TEM-Type β -Lactamase From." *Antimicrobial Agents and Chemotherapy* 48 (12): 4528–31. <https://doi.org/10.1128/AAC.48.12.4528-4531.2004>.
- Poppe, Leszek, John O. Hui, Joseph Ligutti, Justin K. Murray, and Paul D. Schnier. 2012. "PADLOC: A Powerful Tool to Assign Disulfide Bond Connectivities in Peptides and Proteins by NMR Spectroscopy." *Analytical Chemistry* 84 (1): 262–66. <https://doi.org/10.1021/ac203078x>.
- Possani, Lourival D., Baltazar Becerril, Jan Tytgat, and Muriel Delepierre. 2001. "High Affinity Scorpion Toxins for Studying Potassium and Sodium Channels." In *Ion Channel Localization: Methods and Protocols*, edited by Anatoli N. Lopatin and Colin G. Nichols. Humana Press. <https://doi.org/10.1385/1-59259-118-3:145>.
- Press, William H., ed. 2007. *Numerical Recipes: The Art of Scientific Computing*. 3. ed. Cambridge University Press.
- Prieur, Daniel. 1997. "Microbiology of Deep-Sea Hydrothermal Vents." *Trends in Biotechnology* 15 (7): 242–44. [https://doi.org/10.1016/S0167-7799\(97\)01052-4](https://doi.org/10.1016/S0167-7799(97)01052-4).
- Purcell, E. M. 1953. "Research in Nuclear Magnetism." *Science* 118 (3068): 431–36. <https://doi.org/10.1126/science.118.3068.431>.

- Purcell, E. M., H. C. Torrey, and R. V. Pound. 1946. "Resonance Absorption by Nuclear Magnetic Moments in a Solid." *Physical Review* 69 (1–2): 37–38. <https://doi.org/10.1103/PhysRev.69.37>.
- Rabi, I. I., S. Millman, P. Kusch, and J. R. Zacharias. 1939. "The Molecular Beam Resonance Method for Measuring Nuclear Magnetic Moments. The Magnetic Moments of $\text{Li } 6 \text{ } ^3$, $\text{Li } 7 \text{ } ^3$ and $\text{F } 19 \text{ } ^9$." *Physical Review* 55 (6): 526–35. <https://doi.org/10.1103/PhysRev.55.526>.
- Rangadurai, Atul, Honglue Shi, and Hashim M. Al-Hashimi. 2020. "Extending the Sensitivity of CEST NMR Spectroscopy to Micro-to-Millisecond Dynamics in Nucleic Acids Using High-Power Radio-Frequency Fields." *Angewandte Chemie International Edition* 59 (28): 11262–66. <https://doi.org/10.1002/anie.202000493>.
- Rangadurai, Atul, Eric S. Szymaski, Isaac J. Kimsey, Honglue Shi, and Hashim M. Al-Hashimi. 2019. "Characterizing Micro-to-Millisecond Chemical Exchange in Nucleic Acids Using off-Resonance $R_{1\rho}$ Relaxation Dispersion." *Progress in Nuclear Magnetic Resonance Spectroscopy* 112–113 (June): 55–102. <https://doi.org/10.1016/j.pnmrs.2019.05.002>.
- Ranjbar, Reza, and Mostafa Alam. 2024. "Antimicrobial Resistance Collaborators (2022). Global Burden of Bacterial Antimicrobial Resistance in 2019: A Systematic Analysis." *Health Promotion and Public Health. Evidence-Based Nursing* 27 (1): 16–16. <https://doi.org/10.1136/ebnurs-2022-103540>.
- Redfield, A. G. 1957. "On the Theory of Relaxation Processes." *IBM Journal of Research and Development* 1 (1): 19–31. <https://doi.org/10.1147/rd.11.0019>.
- Renner, Christian, Luis Moroder, and Tad A. Holak. 2001. "Analytical Solution to the Lipari–Szabo Model Based on the Reduced Spectral Density Approximation Offers a Novel Protocol for Extracting Motional Parameters." *Journal of Magnetic Resonance* 151 (1): 32–39. <https://doi.org/10.1006/jmre.2001.2346>.
- Ridgway, John P. 2010. "Cardiovascular Magnetic Resonance Physics for Clinicians: Part I." *Journal of Cardiovascular Magnetic Resonance* 12 (1): 71. <https://doi.org/10.1186/1532-429X-12-71>.
- Rieping, Wolfgang, Michael Habeck, Benjamin Bardiaux, Aymeric Bernard, Thérèse E. Malliavin, and Michael Nilges. 2007. "ARIA2: Automated NOE Assignment and Data Integration in NMR Structure Calculation." *Bioinformatics* 23 (3): 381–82. <https://doi.org/10.1093/bioinformatics/btl589>.
- Robinson, Samuel D., Eivind A. B. Undheim, Beatrix Ueberheide, and Glenn F. King. 2017. "Venom Peptides as Therapeutics: Advances, Challenges and the Future of Venom-Peptide Discovery." *Expert Review of Proteomics* 14 (10): 931–39. <https://doi.org/10.1080/14789450.2017.1377613>.
- Rohl, Carol A., Charlie E. M. Strauss, Kira M. S. Misura, and David Baker. 2004. "Protein Structure Prediction Using Rosetta." In *Methods in Enzymology*, vol. 383. Elsevier. [https://doi.org/10.1016/S0076-6879\(04\)83004-0](https://doi.org/10.1016/S0076-6879(04)83004-0).
- Ross, A., M. Czirisch, and G. C. King. 1997. "Systematic Errors Associated with the CPMG Pulse Sequence and Their Effect on Motional Analysis of Biomolecules." *Journal of Magnetic Resonance* 124 (2): 355–65. <https://doi.org/10.1006/jmre.1996.1036>.

- Roy, Alankar, and Sujay Ray. 2024. "Traversing DNA-Protein Interactions Between Mesophilic and Thermophilic Bacteria: Implications from Their Cold Shock Response." *Molecular Biotechnology* 66 (4): 824–44. <https://doi.org/10.1007/s12033-023-00711-4>.
- Sachs, Rolf, Klaas E. A. Max, Udo Heinemann, and Jochen Balbach. 2012. "RNA Single Strands Bind to a Conserved Surface of the Major Cold Shock Protein in Crystals and Solution." *RNA* 18 (1): 65–76. <https://doi.org/10.1261/rna.02809212>.
- Sacolick, Laura I., Florian Wiesinger, Ileana Hancu, and Mika W. Vogel. 2010. " B_1 Mapping by Bloch-Siebert Shift." *Magnetic Resonance in Medicine* 63 (5): 1315–22. <https://doi.org/10.1002/mrm.22357>.
- Sah, Pankaj. 1996. "Ca²⁺-Activated K⁺ Currents in Neurones: Types, Physiological Roles and Modulation." *Trends in Neurosciences* 19 (4): 150–54. [https://doi.org/10.1016/S0166-2236\(96\)80026-9](https://doi.org/10.1016/S0166-2236(96)80026-9).
- Sakhrani, Varun V., Rittik K. Ghosh, Eduardo Hilario, Kevin L. Weiss, Leighton Coates, and Leonard J. Mueller. 2021. "Toho-1 β -Lactamase: Backbone Chemical Shift Assignments and Changes in Dynamics upon Binding with Avibactam." *Journal of Biomolecular NMR* 75 (8–9): 303–18. <https://doi.org/10.1007/s10858-021-00375-9>.
- Saljic, Arnala, Jordi Heijman, and Dobromir Dobrev. 2024. "From Atrial Small-Conductance Calcium-Activated Potassium Channels to New Antiarrhythmics." *European Cardiology Review* 19 (December). <https://doi.org/10.15420/ecr.2024.41>.
- Salzmann, Michael, Konstantin Pervushin, Gerhard Wider, Hans Senn, and Kurt Wüthrich. 1998. "TROSY in Triple-Resonance Experiments: New Perspectives for Sequential NMR Assignment of Large Proteins." *Proceedings of the National Academy of Sciences* 95 (23): 13585–90. <https://doi.org/10.1073/pnas.95.23.13585>.
- Sanchez-Campos, Noemi, Johanna Bernaldez-Sarabia, and Alexei F. Licea-Navarro. 2022. "Conotoxin Patenting Trends in Academia and Industry." *Marine Drugs* 20 (8): 531. <https://doi.org/10.3390/md20080531>.
- Sarkar, Jaydeep. 2014. "Ferromagnetic Sputtering Targets and Thin Films for Silicides and Data Storage." In *Sputtering Materials for VLSI and Thin Film Devices*. Elsevier. <https://doi.org/10.1016/B978-0-8155-1593-7.00007-2>.
- Sattler, M. 1999. "Heteronuclear Multidimensional NMR Experiments for the Structure Determination of Proteins in Solution Employing Pulsed Field Gradients." *Progress in Nuclear Magnetic Resonance Spectroscopy* 34 (2): 93–158. [https://doi.org/10.1016/S0079-6565\(98\)00025-9](https://doi.org/10.1016/S0079-6565(98)00025-9).
- Savard, Pierre-Yves, and Stéphane M. Gagné. 2006. "Backbone Dynamics of TEM-1 Determined by NMR: Evidence for a Highly Ordered Protein." *Biochemistry* 45 (38): 11414–24. <https://doi.org/10.1021/bi060414q>.
- Savchenko, Alexei, Adelinda Yee, Anna Khachatryan, et al. 2003. "Strategies for Structural Proteomics of Prokaryotes: Quantifying the Advantages of Studying Orthologous Proteins and of Using Both NMR and X-ray Crystallography Approaches." *Proteins: Structure, Function, and Bioinformatics* 50 (3): 392–99. <https://doi.org/10.1002/prot.10282>.

- Schägger, Hermann. 2006. "Tricine-SDS-PAGE." *Nature Protocols* 1 (1): 16–22. <https://doi.org/10.1038/nprot.2006.4>.
- Schindler, Thomas, Peter L. Graumann, Dieter Perl, Saufung Ma, Franz X. Schmid, and Mohamed A. Marahiel. 1999. "The Family of Cold Shock Proteins of *Bacillus Subtilis*." *Journal of Biological Chemistry* 274 (6): 3407–13. <https://doi.org/10.1074/jbc.274.6.3407>.
- Schmidtke, Achim, Jörn Lötsch, Rainer Freynhagen, and Gerd Geisslinger. 2010. "Ziconotide for Treatment of Severe Chronic Pain." *The Lancet* 375 (9725): 1569–77. [https://doi.org/10.1016/S0140-6736\(10\)60354-6](https://doi.org/10.1016/S0140-6736(10)60354-6).
- Schröder, Katja, Peter Graumann, Arndt Schnuchel, Tad A. Holak, and Mohamed A. Marahiel. 1995. "Mutational Analysis of the Putative Nucleic Acid-binding Surface of the Cold-shock Domain, CspB, Revealed an Essential Role of Aromatic and Basic Residues in Binding of Single-stranded DNA Containing the Y-box Motif." *Molecular Microbiology* 16 (4): 699–708. <https://doi.org/10.1111/j.1365-2958.1995.tb02431.x>.
- Schroeder, Christina I., and K. Johan Rosengren. 2020. "Three-Dimensional Structure Determination of Peptides Using Solution Nuclear Magnetic Resonance Spectroscopy." In *Snake and Spider Toxins: Methods and Protocols*, edited by Avi Priel. Springer US. https://doi.org/10.1007/978-1-4939-9845-6_7.
- Schubert, Mario, Dirk Labudde, Hartmut Oschkinat, and Peter Schmieder. 2002. "A Software Tool for the Prediction of Xaa-Pro Peptide Bond Conformations in Proteins Based on ¹³C Chemical Shift Statistics." *Journal of Biomolecular NMR* 24: 149–54. <https://doi.org/10.1023/a:1020997118364>.
- Schumacher, Maria A., Andre F. Rivard, Hans Peter Bächinger, and John P. Adelman. 2001. "Structure of the Gating Domain of a Ca²⁺-Activated K⁺ Channel Complexed with Ca²⁺/Calmodulin." *Nature* 410 (6832): 1120–24. <https://doi.org/10.1038/35074145>.
- Schwieters, Charles D., John J. Kuszewski, Nico Tjandra, and G. Marius Clore. 2003. "The Xplor-NIH NMR Molecular Structure Determination Package." *Journal of Magnetic Resonance* 160 (1): 65–73. [https://doi.org/10.1016/S1090-7807\(02\)00014-9](https://doi.org/10.1016/S1090-7807(02)00014-9).
- Sedaghat Doost, Ali, Marzieh Akbari, Christian V. Stevens, Arima Diah Setiowati, and Paul Van Der Meeren. 2019. "A Review on Nuclear Overhauser Enhancement (NOE) and Rotating-Frame Overhauser Effect (ROE) NMR Techniques in Food Science: Basic Principles and Applications." *Trends in Food Science & Technology* 86 (April): 16–24. <https://doi.org/10.1016/j.tifs.2019.02.001>.
- Sekhar, Ashok, and Levitt E. Kay. 2019. "An NMR View of Protein Dynamics in Health and Disease." *Annual Review of Biophysics* 48 (1): 297–319. <https://doi.org/10.1146/annurev-biophys-052118-115647>.
- Sekhar, Ashok, Jessica Ao Rumfeldt, Helen R. Broom, et al. 2015. "Thermal Fluctuations of Immature SOD1 Lead to Separate Folding and Misfolding Pathways." *eLife* 4 (June): e07296. <https://doi.org/10.7554/eLife.07296>.
- Shaka, A. J., S. P. Rucker, and A. Pines. 1988. "Iterative Carr-Purcell Trains." *Journal of Magnetic Resonance* (1969) 77 (3): 606–11. [https://doi.org/10.1016/0022-2364\(88\)90022-4](https://doi.org/10.1016/0022-2364(88)90022-4).

- Shakkottai, Vikram G., Imed Regaya, Heike Wulff, et al. 2001. "Design and Characterization of a Highly Selective Peptide Inhibitor of the Small Conductance Calcium-Activated K⁺Channel, SkCa₂." *Journal of Biological Chemistry* 276 (46): 46. <https://doi.org/10.1074/jbc.M106981200>.
- Sharma, Gagan, Carolyn B. Braga, Kai-En Chen, et al. 2021. "Structural Basis for the Binding of the Cancer Targeting Scorpion Toxin, ClTx, to the Vascular Endothelial Growth Factor Receptor Neuropilin-1." *Current Research in Structural Biology* 3: 179–86. <https://doi.org/10.1016/j.crstbi.2021.07.003>.
- Shcherbinin, D. S., M. Yu. Rubtsova, V. G. Grigorenko, I. V. Uporov, A. V. Veselovsky, and A. M. Egorov. 2017. "The Study of the Role of Mutations M182T and Q39K in the TEM-72 β -Lactamase Structure by the Molecular Dynamics Method." *Biochemistry (Moscow), Supplement Series B: Biomedical Chemistry* 11 (2): 120–27. <https://doi.org/10.1134/S1990750817020056>.
- Shen, Yang, and Ad Bax. 2013. "Protein Backbone and Sidechain Torsion Angles Predicted from NMR Chemical Shifts Using Artificial Neural Networks." *Journal of Biomolecular NMR* 56 (3): 227–41. <https://doi.org/10.1007/s10858-013-9741-y>.
- Shen, Yang, Frank Delaglio, Gabriel Cornilescu, and Ad Bax. 2009. "TALOS+: A Hybrid Method for Predicting Protein Backbone Torsion Angles from NMR Chemical Shifts." *Journal of Biomolecular NMR* 44 (4): 213–23. <https://doi.org/10.1007/s10858-009-9333-z>.
- Shen, Yang, Oliver Lange, Frank Delaglio, et al. 2008. "Consistent Blind Protein Structure Generation from NMR Chemical Shift Data." *Proceedings of the National Academy of Sciences* 105 (12): 4685–90. <https://doi.org/10.1073/pnas.0800256105>.
- Siigur, Jüri, and Ene Siigur. 2022. "Biochemistry and Toxicology of Proteins and Peptides Purified from the Venom of Vipera Berus Berus." *Toxicon: X* 15 (June): 100131. <https://doi.org/10.1016/j.toxcx.2022.100131>.
- Silverstein Robert M, Webster Francis X, and Kiemle David J. 2005. *Silverstein - Spectrometric Identification of Organic Compounds 7th*. John Wiley & Sons.
- Sinchaikul, Supachok, Boonyaras Sookkheo, Suree Phutrakul, Fu-Ming Pan, and Shui-Tein Chen. 2002. "Proteomic Study of Cold Shock Protein in Bacillus Stearothermophilus P1: Comparison of Temperature Downshifts." *PROTEOMICS* 2 (9): 1316–24. [https://doi.org/10.1002/1615-9861\(200209\)2:9%3C1316::AID-PROT1316%3E3.0.CO;2-0](https://doi.org/10.1002/1615-9861(200209)2:9%3C1316::AID-PROT1316%3E3.0.CO;2-0).
- Sippl, Manfred J. 1993. "Recognition of Errors in Three-Dimensional Structures of Proteins." *Proteins: Structure, Function, and Bioinformatics* 17 (4): 355–62. <https://doi.org/10.1002/prot.340170404>.
- Skrynnikov, Nikolai R., Frederick W. Dahlquist, and Levitt E. Kay. 2002. "Reconstructing NMR Spectra of 'Invisible' Excited Protein States Using HSQC and HMQC Experiments." *Journal of the American Chemical Society* 124 (41): 12352–60. <https://doi.org/10.1021/ja0207089>.
- Slichter, Charles P. P. 2010. *Principles of Magnetic Resonance*. Springer.
- Smet, Annemieke, An Martel, Davy Persoons, et al. 2020. "Broad-Spectrum β -Lactamases among Enterobacteriaceae of Animal Origin: Molecular Aspects, Mobility and Impact on Public Health." *FEMS Microbiol* 34: 295–316. <https://doi.org/10.1111/j.1574-6976.2009.00198.x>.

- Smyth, M. S., and J. H. J. Martin. 2000. "X Ray Crystallography." *Molecular Pathology* 53 (1): 8–14. <https://doi.org/10.1136/mp.53.1.8>.
- Snyder, David A., Yang Chen, Natalia G. Denissova, et al. 2005. "Comparisons of NMR Spectral Quality and Success in Crystallization Demonstrate That NMR and X-Ray Crystallography Are Complementary Methods for Small Protein Structure Determination." *Journal of the American Chemical Society* 127 (47): 16505–11. <https://doi.org/10.1021/ja053564h>.
- Solomon, I. 1955. "Relaxation Processes in a System of Two Spins." *Physical Review* 99 (2): 559–65. <https://doi.org/10.1103/PhysRev.99.559>.
- Atta-ur Rahman, Muhammad Iqbal Choudhary and Atia-tul- Wahab. 2015. "Solving Problems with NMR Spectroscopy - Edition 2." Elsevier.
- Srinivasan, N., R. Sowdhamini, C. Ramakrishnan, and P. Balaram. 1990. "Conformations of Disulfide Bridges in Proteins." *International Journal of Peptide and Protein Research* 36 (2): 147–55. <https://doi.org/10.1111/j.1399-3011.1990.tb00958.x>.
- Stables, L. A., R. P. Kennan, A. W. Anderson, and J. C. Gore. 1999. "Density Matrix Simulations of the Effects of J Coupling in Spin Echo and Fast Spin Echo Imaging." *Journal of Magnetic Resonance* 140 (2): 305–14. <https://doi.org/10.1006/jmre.1998.1655>.
- Stanek, Jan, Michał Nowakowski, Saurabh Saxena, Katarzyna Ruszczyńska-Bartnik, Andrzej Ejchart, and Wiktor Koźmiński. 2013. "Selective Diagonal-Free ^{13}C , ^{13}C -Edited Aliphatic–Aromatic NOESY Experiment with Non-Uniform Sampling." *Journal of Biomolecular Nmr* 56 (3): 217–26. <https://doi.org/10.1007/s10858-013-9739-5>.
- Stocker, Martin, Michael Krause, and Paola Pedarzani. 1999. "An Apamin-Sensitive Ca^{2+} -Activated K^{+} Current in Hippocampal Pyramidal Neurons | PNAS." <https://doi.org/https://doi.org/10.1073/pnas.96.8.4662>.
- Stocker, Martin, and Paola Pedarzani. 2000. "Differential Distribution of Three Ca^{2+} -Activated K^{+} Channel Subunits, SK1, SK2, and SK3, in the Adult Rat Central Nervous System." *Molecular and Cellular Neuroscience* 15 (5): 476–93. <https://doi.org/10.1006/mcne.2000.0842>.
- Strøbæk, Dorte, Tino D. Jørgensen, Palle Christophersen, Philip K. Ahring, and Søren-Peter Olesen. 2000. "Pharmacological Characterization of Small-conductance Ca^{2+} -activated K^{+} Channels Stably Expressed in HEK 293 Cells." *British Journal of Pharmacology* 129 (5): 991–99. <https://doi.org/10.1038/sj.bjp.0703120>.
- Sun, Zhen-Yu J., and Gerhard Wagner. 2006. "3D Structure Determination by NMR." In *Encyclopedic Reference of Genomics and Proteomics in Molecular Medicine*. Springer. https://doi.org/10.1007/3-540-29623-9_5010.
- Takai, M., I. Umemura, K. Yamasaki, et al. 1992. "A Potent and Specific Agonist, Suc-[Glu9,Ala11,15]-Endothelin-1(8-21), IRL 1620, for the ETB Receptor." *Biochemical and Biophysical Research Communications* 184 (2): 953–59. [https://doi.org/10.1016/0006-291x\(92\)90683-c](https://doi.org/10.1016/0006-291x(92)90683-c).

- Tang, Yuefeng, William M. Schneider, Yang Shen, et al. 2010. "Fully Automated High-Quality NMR Structure Determination of Small 2H-Enriched Proteins." *Journal of Structural and Functional Genomics* 11 (4): 223–32. <https://doi.org/10.1007/s10969-010-9095-6>.
- Tiwari, Ved Prakash, Debajyoti De, Nemika Thapliyal, Levitt E. Kay, and Pramodh Vallurupalli. 2024. "Beyond Slow Two-State Protein Conformational Exchange Using CEST: Applications to Three-State Protein Interconversion on the Millisecond Timescale." *Journal of Biomolecular NMR* 78 (1): 39–60. <https://doi.org/10.1007/s10858-023-00431-6>.
- Tiwari, Ved Prakash, Subhendu Pandit, and Pramodh Vallurupalli. 2019. "Exchangeable Deuterons Introduce Artifacts in Amide 15N CEST Experiments Used to Study Protein Conformational Exchange." *Journal of Biomolecular NMR* 73 (1–2): 43–48. <https://doi.org/10.1007/s10858-018-00223-3>.
- Tjandra, Nico, Scott E. Feller, Richard W. Pastor, and Ad Bax. 1995. "Rotational Diffusion Anisotropy of Human Ubiquitin from 15N NMR Relaxation." *Journal of the American Chemical Society* 117 (50): 12562–66. <https://doi.org/10.1021/ja00155a020>.
- Tjandra, Nico, Hitoshi Kuboniwa, Hao Ren, and Ad Bax. 1995. "Rotational Dynamics of Calcium-Free Calmodulin Studied by 15N-NMR Relaxation Measurements." *European Journal of Biochemistry* 230 (3): 1014–24. <https://doi.org/10.1111/j.1432-1033.1995.1014g.x>.
- Tollinger, Martin, Nikolai R. Skrynnikov, Frans A. A. Mulder, Julie D. Forman-Kay, and Levitt E. Kay. 2001. "Slow Dynamics in Folded and Unfolded States of an SH3 Domain." *Journal of the American Chemical Society* 123 (46): 11341–52. <https://doi.org/10.1021/ja011300z>.
- Tomanicek, Stephen J., Matthew P. Blakeley, Jonathan Cooper, Yu Chen, Pavel V. Afonine, and Leighton Coates. 2010. "Neutron Diffraction Studies of a Class A β -Lactamase Toho-1 E166A/R274N/R276N Triple Mutant." *Journal of Molecular Biology* 396 (4): 1070–80. <https://doi.org/10.1016/j.jmb.2009.12.036>.
- Tomanicek, Stephen J., Kathy K. Wang, Kevin L. Weiss, et al. 2011. "The Active Site Protonation States of Perdeuterated Toho-1 β -Lactamase Determined by Neutron Diffraction Support a Role for Glu166 as the General Base in Acylation." *FEBS Letters* 585 (2): 364–68. <https://doi.org/10.1016/j.febslet.2010.12.017>.
- Tuteja, Dipika, Danyan Xu, Valeriy Timofeyev, et al. 2005. "Differential Expression of Small-Conductance Ca²⁺-Activated K⁺ Channels SK1, SK2, and SK3 in Mouse Atrial and Ventricular Myocytes." *American Journal of Physiology-Heart and Circulatory Physiology* 289 (6): H2714–23. <https://doi.org/10.1152/ajpheart.00534.2005>.
- Vallurupalli, Pramodh, Guillaume Bouvignies, and Levitt E. Kay. 2011. "Increasing the Exchange Time-Scale That Can Be Probed by CPMG Relaxation Dispersion NMR." *The Journal of Physical Chemistry B* 115 (49): 14891–900. <https://doi.org/10.1021/jp209610v>.
- Vallurupalli, Pramodh, Guillaume Bouvignies, and Levitt E. Kay. 2012. "Studying 'Invisible' Excited Protein States in Slow Exchange with a Major State Conformation." *Journal of the American Chemical Society* 134 (19): 8148–61. <https://doi.org/10.1021/ja3001419>.

- Vallurupalli, Pramodh, Guillaume Bouvignies, and Levitt E. Kay. 2012. "Studying 'Invisible' Excited Protein States in Slow Exchange with a Major State Conformation." *Journal of the American Chemical Society* 134 (19): 8148–61. <https://doi.org/10.1021/ja3001419>.
- Vallurupalli, Pramodh, D. Flemming Hansen, Patrik Lundström, and Levitt E. Kay. 2009. "CPMG Relaxation Dispersion NMR Experiments Measuring Glycine $^1\text{H}\alpha$ and $^{13}\text{C}\alpha$ Chemical Shifts in the 'Invisible' Excited States of Proteins." *Journal of Biomolecular NMR* 45 (1–2): 45–55. <https://doi.org/10.1007/s10858-009-9310-6>.
- Vallurupalli, Pramodh, D. Flemming Hansen, Elliott Stollar, Eva Meirovitch, and Levitt E. Kay. 2007. "Measurement of Bond Vector Orientations in Invisible Excited States of Proteins." *Proceedings of the National Academy of Sciences* 104 (47): 18473–77. <https://doi.org/10.1073/pnas.0708296104>.
- Vallurupalli, Pramodh, Ashok Sekhar, Tairan Yuwen, and Levitt E. Kay. 2017. "Probing Conformational Dynamics in Biomolecules via Chemical Exchange Saturation Transfer: A Primer." *Journal of Biomolecular NMR* 67 (4): 243–71. <https://doi.org/10.1007/s10858-017-0099-4>.
- Van Boeckel, Thomas P., Charles Brower, Marius Gilbert, et al. 2015. "Global Trends in Antimicrobial Use in Food Animals." *Proceedings of the National Academy of Sciences* 112 (18): 5649–54. <https://doi.org/10.1073/pnas.1503141112>.
- Van Zijl, Peter C. M., and Nirbhay N. Yadav. 2011. "Chemical Exchange Saturation Transfer (CEST): What Is in a Name and What Isn't?" *Magnetic Resonance in Medicine* 65 (4): 927–48. <https://doi.org/10.1002/mrm.22761>.
- Verdes, Aida, Prachi Anand, Juliette Gorson, et al. 2016. "From Mollusks to Medicine: A Venomics Approach for the Discovery and Characterization of Therapeutics from Terebridae Peptide Toxins." *Toxins* 8 (4): 117. <https://doi.org/10.3390/toxins8040117>.
- Vögeli, Beat. 2014. "The Nuclear Overhauser Effect from a Quantitative Perspective." *Progress in Nuclear Magnetic Resonance Spectroscopy* 78 (April): 1–46. <https://doi.org/10.1016/j.pnmrs.2013.11.001>.
- Vold, R. L., R. R. Vold, and H. E. Simon. 1973. "Errors in Measurements of Transverse Relaxation Rates." *Journal of Magnetic Resonance (1969)* 11 (3): 283–98. [https://doi.org/10.1016/0022-2364\(73\)90054-1](https://doi.org/10.1016/0022-2364(73)90054-1).
- Von König, Konstanze, Norman Kachel, Hans Robert Kalbitzer, and Werner Kremer. 2020. "RNA and DNA Binding Epitopes of the Cold Shock Protein TmCsp from the Hyperthermophile Thermotoga Maritima." *The Protein Journal* 39 (5): 487–500. <https://doi.org/10.1007/s10930-020-09929-6>.
- Vranken, Wim F., Wayne Boucher, Tim J. Stevens, et al. 2005. "The CCPN Data Model for NMR Spectroscopy: Development of a Software Pipeline." *Proteins: Structure, Function, and Bioinformatics* 59 (4): 687–96. <https://doi.org/10.1002/prot.20449>.
- Walewska, Aleksandra, Jack J. Skalicky, Darrell R. Davis, et al. 2008. "NMR-Based Mapping of Disulfide Bridges in Cysteine-Rich Peptides: Application to the μ -Conotoxin SxIIIA." *Journal of the American Chemical Society* 130 (43): 14280–86. <https://doi.org/10.1021/ja804303p>.

- Walinda, Erik, Daichi Morimoto, and Kenji Sugase. 2018. "Resolving Biomolecular Motion and Interactions by R2 and R1p Relaxation Dispersion NMR." *Methods* 148 (September): 28–38. <https://doi.org/10.1016/j.ymeth.2018.04.026>.
- Walker, Olivier, Ranjani Varadan, and David Fushman. 2004. "Efficient and Accurate Determination of the Overall Rotational Diffusion Tensor of a Molecule from 15N Relaxation Data Using Computer Program ROTDIF." *Journal of Magnetic Resonance* 168 (2): 336–45. <https://doi.org/10.1016/j.jmr.2004.03.019>.
- Wang, Hongzhi, Ming Zhao, Jerome L. Ackerman, and Yiqiao Song. 2017. "Saturation-Inversion-Recovery: A Method for T1 Measurement." *Journal of Magnetic Resonance* 274 (January): 137–43. <https://doi.org/10.1016/j.jmr.2016.11.015>.
- Wangsness, R. K., and F. Bloch. 1953. "The Dynamical Theory of Nuclear Induction." *Physical Review* 89 (4). <https://doi.org/https://doi.org/10.1103/PhysRev.89.728>.
- Wanko Nembot, Marius, Georges Feller, Alexander N. Volkov, Ulric B. Le Paige, Guillaume Bouvignies, and Christian Dambon. 2026. "Structural and Functional Insights into a Mesophilic Cold Shock Protein CspA with Enhanced Precision." *Journal of Magnetic Resonance* 388 (July): 108077. <https://doi.org/10.1016/j.jmr.2026.108077>.
- Ward, K. M., A. H. Aletras, and R. S. Balaban. 2000. "A New Class of Contrast Agents for MRI Based on Proton Chemical Exchange Dependent Saturation Transfer (CEST)." *Journal of Magnetic Resonance* 143 (1): 79–87. <https://doi.org/10.1006/jmre.1999.1956>.
- Warecki, Brenda A., Diego M. Moreno, Robert A. Bonomo, and Alejandro J. Vila. 2025. "Let's Dance: How Protein Dynamics Drive β -Lactamase Evolution and Antibiotic Resistance?" *Biochemistry* 64 (22): 4498–512. <https://doi.org/10.1021/acs.biochem.5c00437>.
- Webb, Graham A., ed. 2018. *Modern Magnetic Resonance*. Springer International Publishing. <https://doi.org/10.1007/978-3-319-28388-3>.
- Whitehead, Brian, C. Jeremy Craven, and Jonathan P. Waltho. 1997. "Double and Triple Resonance NMR Methods for Protein Assignment." In *Protein NMR Techniques*, edited by David G. Reid. Humana Press. <https://doi.org/10.1385/0-89603-309-0:29>.
- Williams, S. H., and A. Constanti. 1988. "Quantitative Effects of Some Muscarinic Agonists on Evoked Surface-negative Field Potentials Recorded from the Guinea-pig Olfactory Cortex Slice." *British Journal of Pharmacology* 93 (4): 846–54. <https://doi.org/10.1111/j.1476-5381.1988.tb11471.x>.
- Williams, S. R., D. G. Gadian, E. Proctor, et al. 1985. "Proton NMR Studies of Muscle Metabolites *in Vivo*." *Journal of Magnetic Resonance (1969)* 63 (2): 406–12. [https://doi.org/10.1016/0022-2364\(85\)90336-1](https://doi.org/10.1016/0022-2364(85)90336-1).
- Williamson, Michael P, Timothy F. Have, and Kurt Wiithrich. 1985. "Solution Conformation of Proteinase Inhibitor IIA from Bull Seminal Plasma by ¹H Nuclear Magnetic Resonance and Distance Geometry." *Journal of Molecular Biology* 182: 295–315. [https://doi.org/10.1016/0022-2836\(85\)90347-x](https://doi.org/10.1016/0022-2836(85)90347-x).

- Williamson, Mike P. 2013. "Using Chemical Shift Perturbation to Characterise Ligand Binding." *Progress in Nuclear Magnetic Resonance Spectroscopy* 73 (August): 1–16. <https://doi.org/10.1016/j.pnmrs.2013.02.001>.
- Wittekind, M., and L. Mueller. 1993. "HNCACB, a High-Sensitivity 3D NMR Experiment to Correlate Amide-Proton and Nitrogen Resonances with the Alpha- and Beta-Carbon Resonances in Proteins." *Journal of Magnetic Resonance, Series B* 101 (2): 201–5. <https://doi.org/10.1006/jmr.b.1993.1033>.
- Woessner, D. E. 1962. "Nuclear Spin Relaxation in Ellipsoids Undergoing Rotational Brownian Motion." *The Journal of Chemical Physics* 37 (3): 647–54. <https://doi.org/10.1063/1.1701390>.
- Wüthrich, Kurt. 1986. "NMR with Proteins and Nucleic Acids." *Europhysics News* 17 (1): 11–13. <https://doi.org/10.1051/epr/19861701011>.
- Wüthrich, Kurt. 2003. "NMR Studies of Structure and Function of Biological Macromolecules (Nobel Lecture)." *Angewandte Chemie International Edition* 42 (29): 3340–63. <https://doi.org/10.1002/anie.200300595>.
- Wüthrich, Kurt, Martin Billeter, and Werner Braun. 1984. "Polypeptide Secondary Structure Determination by Nuclear Magnetic Resonance Observation of Short Proton-Proton Distances." *Journal of Molecular Biology* 180 (3): 715–40. [https://doi.org/10.1016/0022-2836\(84\)90034-2](https://doi.org/10.1016/0022-2836(84)90034-2).
- Wuthrich, Kurt, Gerhard Wider, Gerhard Wagner, and Werner Braun. 1982. "Sequential Resonance Assignments as a Basis for Determination of Spatial Protein Structures by High Resolution Proton Nuclear Magnetic Resonance." *Journal of Biomolecular NMR* 155: 311–19. [https://doi.org/10.1016/0022-2836\(82\)90007-9](https://doi.org/10.1016/0022-2836(82)90007-9).
- "X-Plor Version 3.1: A System for X-Ray Crystallography and NMR." 1993. *Yale University Press*, February. <https://yalebooks.yale.edu/book/9780300054026/x-plor-version-3-1/>.
- Xu, Yanfang, Dipika Tuteja, Zhao Zhang, et al. 2003. "Molecular Identification and Functional Roles of a Ca²⁺-Activated K⁺ Channel in Human and Mouse Hearts." *Journal of Biological Chemistry* 278 (49): 49085–94. <https://doi.org/10.1074/jbc.M307508200>.
- Yamazaki, Toshio, Julie D. Forman-Kay, and Levitt E. Kay. 1993. "Two-Dimensional NMR Experiments for Correlating Carbon-13.Beta. and Proton.Delta./Epsilon. Chemical Shifts of Aromatic Residues in ¹³C-Labeled Proteins via Scalar Couplings." *Journal of the American Chemical Society* 115 (23): 11054–55. <https://doi.org/10.1021/ja00076a099>.
- Yamazaki, Toshio, Weontae Lee, Cheryl H. Arrowsmith, D. R. Muhandiram, and Levitt E. Kay. 1994. "A Suite of Triple Resonance NMR Experiments for the Backbone Assignment of ¹⁵N, ¹³C, ²H Labeled Proteins with High Sensitivity." *Journal of the American Chemical Society* 116 (26): 11655–66. <https://doi.org/10.1021/ja00105a005>.
- Yee, Adelinda A., Alexei Savchenko, Alexandr Ignachenko, et al. 2005. "NMR and X-Ray Crystallography, Complementary Tools in Structural Proteomics of Small Proteins." *Journal of the American Chemical Society* 127 (47): 16512–17. <https://doi.org/10.1021/ja053565+>.

- Yilmaz, Emel Maden, and Peter Güntert. 2015. "NMR Structure Calculation for All Small Molecule Ligands and Non-Standard Residues from the PDB Chemical Component Dictionary." *Journal of Biomolecular NMR* 63 (1): 21–37. <https://doi.org/10.1007/s10858-015-9959-y>.
- Yip, Grover N. B., and Erik R. P. Zuiderweg. 2004. "A Phase Cycle Scheme That Significantly Suppresses Offset-Dependent Artifacts in the R2-CPMG 15N Relaxation Experiment." *Journal of Magnetic Resonance* 171 (1): 25–36. <https://doi.org/10.1016/j.jmr.2004.06.021>.
- Yuwen, Tairan, Guillaume Bouvignies, and Levitt E. Kay. 2018. "Exploring Methods to Expedite the Recording of CEST Datasets Using Selective Pulse Excitation." *Journal of Magnetic Resonance* 292 (July): 1–7. <https://doi.org/10.1016/j.jmr.2018.04.013>.
- Yuwen, Tairan, and Levitt E. Kay. 2019. "Revisiting 1HN CPMG Relaxation Dispersion Experiments: A Simple Modification Can Eliminate Large Artifacts." *Journal of Biomolecular NMR* 73 (10–11): 641–50. <https://doi.org/10.1007/s10858-019-00276-y>.
- Yuwen, Tairan, Levitt E. Kay, and Guillaume Bouvignies. 2018. "Dramatic Decrease in CEST Measurement Times Using Multi-Site Excitation." *ChemPhysChem* 19 (14): 1707–10. <https://doi.org/10.1002/cphc.201800249>.
- Zavrtanik, Uroš, Junoš Lukan, Remy Loris, Jurij Lah, and San Hadži. 2018. "Structural Basis of Epitope Recognition by Heavy-Chain Camelid Antibodies." *Journal of Molecular Biology* 430 (21): 4369–86. <https://doi.org/10.1016/j.jmb.2018.09.002>.
- Zeeb, Markus, and Jochen Balbach. 2005. "NMR Spectroscopic Characterization of Millisecond Protein Folding by Transverse Relaxation Dispersion Measurements." *Journal of the American Chemical Society* 127 (38): 13207–12. <https://doi.org/10.1021/ja051141+>.
- Zeeb, Markus, Klaas E. A. Max, Ulrich Weininger, Christian Löw, Heinrich Sticht, and Jochen Balbach. 2006. "Recognition of T-Rich Single-Stranded DNA by the Cold Shock Protein Bs-CspB in Solution." *Nucleic Acids Research* 34 (16): 4561–71. <https://doi.org/10.1093/nar/gkl376>.
- Zerrouk, H., P. Mansuelle, A. Benslimane, H. Rochat, and M. F. Martin-Eauclaire. 1993. "Characterization of a New Leiurotoxin I-like Scorpion Toxin: PO₅ from *Androctonus Mauretanicus Mauretanicus*." *FEBS Letters* 320 (3): 3. [https://doi.org/10.1016/0014-5793\(93\)80583-G](https://doi.org/10.1016/0014-5793(93)80583-G).
- Zhang, Yan, and Carol A. Gross. 2021. "Cold Shock Response in Bacteria." *Annual Review of Genetics* 55 (1): 377–400. <https://doi.org/10.1146/annurev-genet-071819-031654>.
- Zhang, Yonghong, Changjie Bao, Lijun Shen, et al. 2022. *Microbial Cold Shock Proteins: Overview of Their Function and Mechanism of Action*. 29 (3): 133–42. <https://doi.org/10.2174/0929866528666211118084519>.
- Zhou, Yang, and Daiwen Yang. 2014. "Effects of J Couplings and Unobservable Minor States on Kinetics Parameters Extracted from CEST Data." *Journal of Magnetic Resonance* 249 (December): 118–25. <https://doi.org/10.1016/j.jmr.2014.10.010>.

- Zhu, Guang, Renwick Alistair, and Ad Bax. 1994. "Measurement of Two- and Three-Bond ^1H - ^{13}C J Couplings from Quantitative Heteronuclear J Correlation for Molecules with Overlapping ^1H Resonances, Using t, Noise Reduction." *Journal of Magnetic Resonance*, A 110, 257–61.
- Zhu, Guang, Xiang Ming Kong, and Kong Hung Sze. 1999. "Gradient and Sensitivity Enhancement of 2D TROSY with Water Flip-Back, 3D NOESY-TROSY and TOCSY-TROSY Experiments." *Journal of Biomolecular NMR* 13 (1): 77–81. <https://doi.org/10.1023/A:1008398227519>.
- Zhu, Guang, Youlin Xia, Linda K. Nicholson, and Kong Hung Sze. 2000. "Protein Dynamics Measurements by TROSY-Based NMR Experiments." *Journal of Magnetic Resonance* 143 (2): 423–26. <https://doi.org/10.1006/jmre.2000.2022>.
- Zhu, Guang, and Xuejun Yao. 2008. "TROSY-Based NMR Experiments for NMR Studies of Large Biomolecules." *Progress in Nuclear Magnetic Resonance Spectroscopy* 52 (1): 49–68. <https://doi.org/10.1016/j.pnmrs.2007.10.001>.
- Ziegler, A., M. Decorps, and C. Remy. 1992. " T_2 Measurements with the Carr-Purcell-Meiboom-Gill Sequence in the Inhomogeneous RF Field of Surface Coils." *Journal of Magnetic Resonance (1969)* 96 (3): 589–95. [https://doi.org/10.1016/0022-2364\(92\)90344-7](https://doi.org/10.1016/0022-2364(92)90344-7).
- Zuiderweg, Erik R. P., and Stephen W. Fesik. 1989. "Heteronuclear Three-Dimensional NMR Spectroscopy of the Inflammatory Protein C5a." *Biochemistry* 28 (6): 2387–91. <https://doi.org/10.1021/bi00432a008>.