

# Metal-binding properties of the N-terminal intrinsically disordered region of the membrane protein PcoB involved in bacterial copper homeostasis

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## ABSTRACT

Intrinsically disordered regions (IDRs) participate in many protein functions, yet their contribution to metal resistance remains mostly unexplored. In *Caulobacter vibrioides*, the copper (Cu) efflux protein PcoB harbors a disordered N-terminal domain (NTD). Here, we have investigated the metal-binding properties of the NTD to elucidate its role in *C. vibrioides* Cu(II) regulation. To this end, AlphaFold3 modeling and sequence-based analysis along with biophysical characterization were performed. The structural properties of recombinant NTD were assessed by circular dichroism (CD), size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS), and nuclear magnetic resonance (NMR) spectroscopy. PcoB NTD was shown to be monomeric in solution, highly disordered, and retaining a highly populated transient 13-residue  $\alpha$ -helix near its C-terminus. Interactions with seven divalent metal cations were evaluated at pH 7.5, 5.5, and 4.5 using intrinsic tyrosine fluorescence (ITyRF), isothermal titration calorimetry (ITC), and NMR. At pH 7.5, the NTD weakly bound Cu(II), Co(II), Ni(II), and Zn(II) ions, whereas under acidic conditions, only Cu(II) binding persisted with a reduced stoichiometry. These results suggest a pH-dependent modulation of the metal-coordination mechanism, Cu(II) displaying a distinct behavior likely driven by the protonation sensitivity of histidine residues and contribution from acidic residues distributed along the sequence. We propose that the disordered NTD acts as a catch-and-deliver arm that captures Cu(II) ions in the periplasm and guides them towards the  $\beta$ -barrel for export. These findings provide a mechanistic framework for PcoB NTD metal handling and, more broadly, how membrane IDRs mediate environment-sensitive periplasmic interactions.

## 1. Introduction

Copper (Cu) is a trace element crucial to vital redox processes and protein functions across all living organisms. Despite its key role, Cu(II) becomes cytotoxic when present in excess as a result of protein aggregation, metalloproteins mismetallation, and reactive oxygen species (ROS) production, leading to DNA degradation and lipid oxidation. Therefore, the capacity of Cu(II) to induce such cellular damage underlies its antimicrobial properties [1–3].

To take advantage of these properties, Cu(II)-containing surfaces — such as alloys or polymers embedded with nanoparticles — are used in

healthcare facilities, reducing pathogen persistence and limiting contact transmission [4–6]. To survive in Cu(II)-rich environments, some bacteria have evolved regulatory pathways to carefully maintain Cu(II) homeostasis and prevent its toxicity. These systems, although slightly variable across species, share key functions including Cu(II) sensing, chelation, oxidation, and export. In Gram-negative bacteria, the most extensively described systems are Cus, Cue, and Pco/Cop [7–9]. The latter is found in several pathogenic microorganisms from the ESKAPEE group — including *Escherichia coli* (*E. coli*) [10], *Pseudomonas aeruginosa* [11], *Klebsiella pneumoniae* [9], *Staphylococcus aureus* [10], and *Acinetobacter baumannii* [9] — known for causing the majority of hospital-

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acquired infections [12,13].

The plasmid-encoded Pco system is also found in non-pathogenic alphaproteobacteria, such as *Caulobacter vibrioides* (*C. vibrioides*, formerly designated as *Caulobacter crescentus*), a free-living organism typically found in oligotrophic aquatic environments [14,15]. While this Pco system, originally found in *E. coli*, consists of seven proteins (PcoABCDERS) [16], it is limited to PcoAB in *C. vibrioides*. PcoA is a periplasmic multicopper oxidase homologous to *E. coli* CueO and oxidizing Cu(I) to Cu(II) [14,15], whilst PcoB is a putative Cu(II)-efflux outer membrane  $\beta$ -barrel porin [15]. Albeit being involved in copper detoxification systems in several bacteria, the specific role of PcoB remains poorly described.

As we previously reported [17,18], the 302-residues long *C. vibrioides* PcoB (UniProt code: A0A0H3C699) features a 193-residues long  $\beta$ -barrel that has been previously crystallized for two other PcoB homologs [19,20] and a 109-residues long N-terminal domain (NTD) oriented towards the periplasm. Interestingly, this NTD contains 17 histidine (His) residues, which represents almost 16% of its amino acid composition. According to the classification proposed by Deiana, A. et al. [21], a terminal region is considered as intrinsically disordered (IDR) if it contains 40 or more consecutive residues predicted as disordered. Such definition qualifies the NTD as an IDR, and PcoB as a hybrid intrinsically disordered protein (IDP) [17]. Furthermore, in vivo site-directed mutations or total deletion of the entire NTD reduce Cu(II) resistance in *C. vibrioides*, supporting a critical role for the NTD in the export function [14,18]. Finally, the NTD exhibits low sequence conservation among bacterial homologs, as the only shared features is a recurrent IDR at the N-terminus [18]. The present study addresses the hypothesis that the flexibility and intrinsic disorder of the His-rich NTD allow PcoB to capture Cu(II) ions from the periplasm and facilitate their passive export across the outer membrane into the extracellular medium, thereby enhancing bacterial survival under high Cu(II) stress [4,10].

We first demonstrate the disordered and monomeric nature of PcoB NTD using complementary spectroscopic techniques, including circular dichroism (CD), intrinsic tyrosine fluorescence (ITyrf), size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS), and nuclear magnetic resonance (NMR) spectroscopy. Subsequently, to investigate the role of the NTD in the PcoAB system involved in Cu(II) regulation in *C. vibrioides*, we assess its interaction with Cu(II) cations using ITyrf, isothermal titration calorimetry (ITC), and NMR. Finally, we evaluate its metal-binding selectivity through comparative interaction analyses with six other bioavailable divalent cations: Ca(II), Co(II), Mg(II), Mn(II), Ni(II), and Zn(II).

## 2. Materials and methods

### 2.1. Bioinformatic analysis

Sequence-based analyses were performed from the amino acid sequence of the PcoB N-terminal domain (UniProt ID: A0A0H3C699), encompassing residues 1 to 109. Amino acid sequence content was evaluated with the ProtParam tool from the ExPASy server (<https://web.expasy.org/protparam/>) [22] and comparison with established databases was performed using the Platform of Tools for Low Complexity (PlaToLoCo) web server (<https://platoloco.aei.polsl.pl/#!/query>) [23], which integrates five different state-of-the-art tools to identify low-complexity regions (LCRs) and calculate amino acid frequencies.

### 2.2. Cloning and plasmid transformation

The *pcoB* NTD gene, corresponding to the N-terminal domain of PcoB (PcoB NTD, residues 1–109 of the full-length PcoB protein), was amplified from the *Caulobacter vibrioides* NA1000 genomic DNA (CCNA\_01016) by polymerase chain reaction (PCR) using Q5 polymerase and gene-specific primers, prior insertion into a pET24b vector

carrying a kanamycin resistance gene. The resulting plasmid was transformed into *E. coli* DH10B and *E. coli* BL21(DE3)pLysS strains for long-term storage and protein expression, respectively.

### 2.3. Protein overexpression and purification

PcoB NTD was expressed without any tag using a pET24b vector in *E. coli* BL21(DE3)pLysS competent cells. Single colonies of *pcoB* over-expressing strain were precultured at 37 °C for 16 h to 18 h in 20 g/L of lysogeny Lennox broth (LB) supplemented with 0.3 mg/mL kanamycin. From 5 mL of preculture, cultures were grown at 37 °C in 20 g/L LB supplied with 0.3 mg/mL kanamycin until the optical density recorded at 600 nm reached a value in the range of 0.5–0.8. Protein expression was induced at 37 °C for 3 h by the addition of 1 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG). After induction, cultures were centrifuged, supernatants discarded, and pellets stored at –20 °C.

Before purification, cell pellets were thawed, resuspended in lysis buffer (phosphate-buffered saline (PBS) pH 7.3, 0.5% (v/v) Triton X-100, and 200 mM KCl), and lysed by sonication in an ice-water bath. Lysates were centrifuged, and supernatants were subjected to a heat-treatment step by incubation at 95 °C for 20 min in the presence of 750 mM NaCl. Following heat shock, the solution was centrifuged at 12,000  $\times$ g for 15 min at 4 °C, and the resulting supernatant was subsequently filtered through a 0.45  $\mu$ m polyethersulfone (PES) syringe filter.

Protein was then purified using an ÄKTA Purifier fast protein liquid chromatography (FPLC) system equipped with a 5 mL HisTrap FF pre-packed column (Cytiva). The equilibration buffer was composed of 20 mM HEPES pH 7.5, 500 mM NaCl, and 5 mM imidazole, while the elution buffer used to recover the protein of interest was 20 mM HEPES pH 7.5, 200 mM NaCl, and 500 mM imidazole. Elution fractions were pooled, and buffer exchange was performed using a desalting column. For this study, experiments involving metal cations were carried out in 20 mM HEPES buffer, while all other assays were conducted in 20 mM phosphate buffer pH 7.5.

Expression and purification of  $^{15}\text{N}$  and  $^{15}\text{N},^{13}\text{C}$ -labeled PcoB NTD followed the same protocol here using  $^{15}\text{NH}_4\text{Cl}$  and  $^{12}\text{C}$  or  $^{13}\text{C}_6$ -glucose as source, and grown in M9 minimal media, supplemented with trace solution.

### 2.4. Assessment of sample purity

The purity of the samples was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and bands of interest were excised, destained to remove Coomassie blue, and subjected to in-gel trypsin digestion. Peptides were subsequently analyzed by mass spectrometry at the Proteomics and Mass Spectrometry Technology Platform (MaSUN), University of Namur using a nano-LC-ESI-MS/MS maXis Impact UHR-TOF (Bruker, Bremen, Germany) coupled with a nanoLC UltiMate 3000 (Thermo). The digests were separated by reverse-phase liquid chromatography using a 75  $\mu$ m  $\times$  250 mm reverse phase Thermo column (Acclaim PepMap 100 C18) in an Ultimate 3000 liquid chromatography system. Mobile phase A was 95% water/5% acetonitrile with 0.1% formic acid, while mobile phase B was 20% water/80% acetonitrile with 0.1% formic acid. The digest (18  $\mu$ L) was injected, and the organic content of the mobile phase was linearly increased for 90 min from 5% B to 40% B, and from 40% B to 100% B in 10 more minutes. The column effluent was connected to a Captive Spray (Bruker). Peak lists were created using DataAnalysis 4.2 (Bruker) and compared to the *Caulobacter vibrioides* PcoB NTD sequence. Scaffold 5.3 (Proteome Software) was used to validate the MS/MS protein identification based on peptides.

## 2.5. Protein concentration determination and UV-visible absorption spectroscopy

UV-visible absorption spectra were recorded with a UV-63000PC spectrophotometer (VWR), using a 10 mm pathlength quartz QS cell (Hellma). The molar extinction coefficient at 280 nm of the N-terminal mutant was computed using the ProtParam tool on the ExPASy Server [24] ( $\epsilon_{280\text{ nm}} = 1490\text{ M}^{-1}\cdot\text{cm}^{-1}$ ), and the final concentration was determined by measuring the absorbance value at 280 nm.

## 2.6. Far-UV circular dichroism (FCD) spectroscopy

FCD spectra were recorded in the range of 260–190 nm with a MOS-500 spectropolarimeter at 20 °C in 20 mM phosphate buffer pH 7.5, using a 1 mm optical path length quartz Suprasil cell (Hellma). The following parameters were used: 30 nm/min scanning rate, 2 nm bandwidth, 0.5 nm data pitch, 1 s digital integration time. Four scans were averaged, and buffer baselines were identically recorded and subtracted; corrected spectra were smoothed.

## 2.7. Size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS)

SEC-MALS experiments were performed using the Shimadzu HPLC separation module, equipped with a UV detector (SPD-20 A, Shimadzu), connected to the 3 angle miniDAWN TREOS II (LS, Wyatt Technology) and a refractive index detector (RID-20 A, Shimadzu). A 24 mL SEC Superdex 75-Increased column was used (Cytiva). The analyses were performed using a flow rate of 0.7 mL/min with 20 mM HEPES buffer pH 7.5 as the mobile phase. The HPLC system was controlled via the Lab Solution software (Shimadzu) to provide loading and injection commands. The Wyatt Technology's ASTRA software was used to record and process the light scattering signals. For each run, 200  $\mu\text{L}$  of 130  $\mu\text{M}$  PcoB NTD in 20 mM HEPES buffer pH 7.5 was injected onto the column.

## 2.8. Intrinsic tyrosine fluorescence (ITyrF) spectroscopy

ITyrF spectra were recorded from 275 to 600 nm with a Shimadzu RF-6000 Spectro fluorophotometer using the LabSolution RF software version 1.18, at 20 °C in 20 mM HEPES buffer pH 7.5 (except for Mn(II) and Zn(II) where pH was 6.9 for solubility issues), using a 10 mm optical pathlength quartz GS cell (Hellma). The data pitch was set to 1.0 nm, the excitation and emission slit widths to 10 nm each, and the excitation wavelength ( $\lambda_{\text{ex}}$ ) to 275 nm. The selected protein-to-metal ratios ranged from 1/0.25 to 1/25 and the following metals salts were used:  $\text{CaCl}_2$  ( $\geq 93.0\%$ , Sigma-Aldrich),  $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$  ( $\geq 99\%$ , Carl Roth)  $\text{CuCl}_2$  (99%, Sigma-Aldrich),  $\text{MgCl}_2$  ( $\geq 98\%$ , Sigma-Aldrich),  $\text{MnCl}_2$  (98%, Sigma-Aldrich),  $\text{NiCl}_2\cdot 6\text{H}_2\text{O}$  ( $\geq 98\%$ , Carl Roth), and  $\text{ZnCl}_2$  ( $\geq 99\%$ , Sigma-Aldrich).

## 2.9. Isothermal titration calorimetry (ITC)

Most ITC experiments were performed using 40  $\mu\text{M}$  PcoB NTD (280  $\mu\text{L}$  in the cell) titrated with 2 mM divalent cations (40  $\mu\text{L}$  in the syringe) in 20 mM HEPES buffer pH 7.5 (except for Mn(II) and Zn(II) where pH was 6.9 for solubility issues), pH 5.5 and pH 4.5. While HEPES no longer provides effective buffering capacity below pH 6.9, the samples were checked before the experiments to ensure that their measured values matched the intended pH. In certain cases, the protein concentration was increased to 100  $\mu\text{M}$  or the metal concentration reduced to 1 mM to enhance the heat release signal and/or better accommodate the different stoichiometries observed for the various metals. The titrations were carried out using a MicroCal i200 ITC microcalorimeter (GE Healthcare) at 25 °C. For each experiment, an initial injection of 0.5  $\mu\text{L}$  (data discarded) was followed by 19 injections of 2  $\mu\text{L}$  titrant using a 120-s interval. Water-water, protein titrated with buffer, and buffer titrated with

ion solution were performed as controls. All data sets were analyzed using the MicroCal PEAQ-ITC Analysis Software (Malvern Panalytical) and fitted using the standard “One Set of Sites” mathematical model. Three additional fitting models (“Two Sets of Sites” and “Sequential Binding Sites” with  $n = 2$  or  $n = 3$ ) were also applied. The Levenberg-Marquardt algorithm was used to achieve convergence for all models, and their mean reduced chi-squares were plotted to identify the best-performing one for each condition where binding is observed.

## 2.10. Nuclear magnetic resonance (NMR) spectroscopy

$^{15}\text{N}$ ,  $^{13}\text{C}$ -PcoB NTD or  $^{15}\text{N}$  PcoB NTD was concentrated using 3 K Mini Amicon Ultra filter in 20 mM HEPES buffer pH 7.5 to concentration between 50 and 200  $\mu\text{M}$  and added 5% (v/v)  $\text{D}_2\text{O}$  for field locking and 125  $\mu\text{M}$  sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) as a  $^1\text{H}$  chemical shift reference. The sample was centrifuged 13.000 g for 5 min and transferred to a 5 mm Shigemi tube. For backbone resonance assignments, a series of multidimensional NMR experiments were acquired at 5 °C on a Bruker Avance III HD 750 MHz equipped with a Bruker proton-optimized triple-resonance 5 mm TCI cryoprobe, including  $^1\text{H}$ ,  $^{15}\text{N}$ -HSQC, HNCACB, HN(CO)CACB, HN(CA)CO spectra. 2D Spectra were processed using TopSpin 4.17 (Bruker), while 3D spectra were recorded with 25% non-uniform sampling and processed using qMDD3 [25] and nmrDraw [26]. Manual assignments were performed with CcpNmr Analysis (v2.5.0) [27]. Secondary chemical shifts (SCSs) were calculated using the assigned  $\text{C}^\alpha$  chemical shifts and the random coil values predicted by POTENCI [28], specifying the experimental conditions (20 mM HEPES, pH 7.5, 5 °C).

Metal ion binding was assessed by NMR recording  $^1\text{H}$ ,  $^{15}\text{N}$  HSQC spectra at 5 °C on a Bruker Avance Neo 800 MHz spectrometer with a CPTXO cryoprobe, using 60  $\mu\text{M}$   $^{15}\text{N}$ -labeled PcoB-NTD in 20 mM HEPES buffer with 5% (v/v)  $\text{D}_2\text{O}$  and 125  $\mu\text{M}$  DSS. Concentrated stock solution of  $\text{ZnCl}_2$ ,  $\text{CuSO}_4$ ,  $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ , and  $\text{MgSO}_4$  were added at various protein/metal molar ratios providing titration from 0.5 to 10 molar ratio, reaching a maximum protein dilution of 2%, which is considered negligible. Experiments were performed at different pH values (pH 7.5, 6.5, 5.5 and 4.5) to assess pH-dependent effects. As for ITC, the samples were checked before the experiments to ensure that their measured values matched the intended pH. Peak intensities were extracted using CcpNmr Analysis, and intensity ratios were calculated as the signal intensity of the protein in the presence of metal ions divided by the one of the free protein.

Diffusion measurements were carried out on a Bruker Avance Neo 800 MHz spectrometer using 200  $\mu\text{M}$   $^{13}\text{C}$ ,  $^{15}\text{N}$ -PcoB-NTD in 20 mM HEPES buffer pH 6.5. After acquisition,  $\text{ZnCl}_2$  was added to a final concentration of 400  $\mu\text{M}$ . Gradient strengths ranged from 2.4 to 47.2 G/cm. Diffusion coefficients ( $D$ ) were obtained by fitting the attenuation of the NMR signal intensity as a function of gradient strength using Dynamics Center (Bruker) and the Stejskal-Tanner equation. Hydrodynamic radius ( $R_h$ ) was calculated from the  $D$  of the protein and from 1,4-dioxane, an internal reference for which  $R_h$  of  $2.17 \pm 0.04\text{ \AA}$  and  $D$  of  $2.04 \times 10^{-8} \pm 3.31 \times 10^{-11}\text{ m}^2/\text{s}$  are well-known [29], using the following Stokes-Einstein derived equation  $R_h(\text{protein}) = [R_h(\text{Dioxane}) \times D(\text{Dioxane})] / D(\text{protein})$ .

## 3. Results and discussion

### 3.1. Sequence-based analysis

While the predicted disordered nature of PcoB NTD was already reported in our previous work [17], we complement such predictions by modeling the 3D-structure of the NTD using AlphaFold3 [30]. As expected due to its flexible properties, the sequence primarily displays low confident predicted local distance difference test (pLDDT) scores, which reflect the model confidence in local structural accuracy [30]. Interestingly, an  $\alpha$ -helix and a single turn structure are found at the C-

terminus. These two regions exhibit high pLDDT scores ( $>90$ ), suggesting partial structuring in the NTD (Fig. 1A).

Based on the amino acids most commonly involved in metal binding and considering the predominant role of His in Cu coordination, their occurrence was emphasized in PcoB NTD primary structure (Fig. 1B) [31,32]. No cysteine (Cys) residue is present, whereas the combined abundance of histidine (His, 15.6%), aspartate, (Asp, 4.6%), and glutamate (Glu, 3.7%) residues reaches 23.9%. Three distinct His-rich regions within the sequence can be highlighted as potential metal-binding sites:  ${}^7\text{HEHHHH}^{12}$ ,  ${}^{29}\text{HAHH}^{32}$ , and  ${}^{67}\text{HAGHHMH}^{74}$ . In these short segments, some common metal-binding motifs can be found, such as HH, HXHH, or  $\text{HX}_2\text{H}$  [33–37]. Another known metal-binding motif  ${}^{49}\text{HX}_2\text{H}^{52}$  is also present outside these three regions. Together, such features support a potential interaction of PcoB NTD with Cu(II) ions and suggest an affinity for other divalent metal cations as well (Fig. 1C). Particularly, the His enrichment is further highlighted by comparing the NTD sequence to other proteins using the PlaToLoCo web server [38], calculating amino acid composition against reference databases including DisProt (only IDPs), PDB (experimentally resolved 3D structures), and SwissProt (high-quality manually curated proteins). Sequence analysis revealed an additional high content in alanine (23.9%) and proline (19.3%) residues, above the average content observed for IDPs, consistent with a highly flexible and intrinsically disordered region (Fig. 1D). Lastly, modeling the PcoB NTD with three Cu(II) ions using AlphaFold3 supports the sequence-based analysis: the metal ions are predominantly located near the His-rich regions and the short His motif. In addition, the NTD retains its disordered character, with little to no formation of ordered secondary structures (Fig. 1E; Fig. S1). Given the disordered nature of the NTD, all AlphaFold3 models presented here are illustrative and primarily serve as a visual support for the sequence-based analysis and experimental observations.

### 3.2. In-solution state of the disordered PcoB NTD

Pure PcoB NTD samples were obtained through a two-step purification protocol taking advantage of its two intrinsic properties: i) the IDP character enabled the use of a heat-shock step, previously shown to be effective for other IDPs such as  $\alpha$ -synuclein [39,40] to precipitate unwanted and thermally sensitive bacterial proteins, while ii) the unusually high histidine content allowed binding to the nickel-chelated IMAC column without the addition of a 6xHis-tag. The purification yield amounted to 5.82 mg per 100 mL of culture, which is relatively high for an IDP expressed in a heterologous system [41].

Two major bands with apparent molecular masses of  $\sim 16$ – $17$  kDa were observed on the SDS-PAGE gel (Fig. S2A). Mass spectrometry confirmed that both bands correspond to PcoB NTD, with 64% sequence coverage and three unique detected peptides (Fig. S2B). The apparent molecular mass is higher than the theoretical value expected from the sequence (11.3 kDa), a behavior commonly observed for IDPs. Indeed, their high net charge and low hydrophobicity may reduce SDS binding and thus, delay their migration [42,43]. It has also been suggested that their larger Stokes radius further hinders their migration through the gel pores, and these factors often result in apparent molecular mass that are 1.2–1.8 times higher than the predicted values [42,44]. The unusual appearance of two distinct major bands for PcoB NTD might be caused by its high proline content. Proline-rich proteins often migrate as two or more bands, which may result from conformational differences arising from *cis-trans* isomerization [42,45,46]. Additional low-molecular-mass bands likely arose from partial proteolysis, another commonly observed phenomenon for IDPs due to their higher solvent, hence protease, accessibility [42,47]. Mass spectrometry analysis confirmed that all these fragments correspond to PcoB NTD (Fig. S3).

The FCD spectrum of PcoB NTD is characteristic of an IDP, showing a broad negative band around 204 nm and lacking signatures of ordered secondary structures (Fig. 2A). This fingerprint substantiates that PcoB NTD does not seem to form ordered assemblies or aggregates after

purification and remains stable in a disordered state. The broad nature of the band and the wavelength minimum at 204 nm suggest some dynamic or transient structures present in PcoB NTD.

NMR spectroscopy was used to further characterize the structure of PcoB NTD in solution at a residue level. The assigned  ${}^1\text{H}$ ,  ${}^{15}\text{N}$ -HSQC spectrum of PcoB NTD (Fig. 2B) displays a narrow chemical shift dispersion along the  ${}^1\text{H}$  dimension, consistent with a lack of stable tertiary structure and typically indicative of disorder. Nonetheless, to assess the presence of transient structural elements, the backbone resonances were assigned and a secondary chemical shift (SCS) analysis performed based on the assigned  $\text{C}^\alpha$  chemical shifts, with consecutive positive and negative values indicating  $\alpha$ -helical propensity and  $\beta$ -structures or extended conformations, respectively. The SCS profile (Fig. 2C) reveals a helical region spanning residues Pro91 to Glu104, with an estimated population of 42%, and two turn structures (His83-Arg87 and Pro5-Glu8) populated around 7%. In that respect, the two C-terminal structured segments are in good agreement with their predicted location by AlphaFold3 (Fig. 1A). These results confirm the presence of a transient short helical structure within the prevalent disordered state of PcoB NTD.

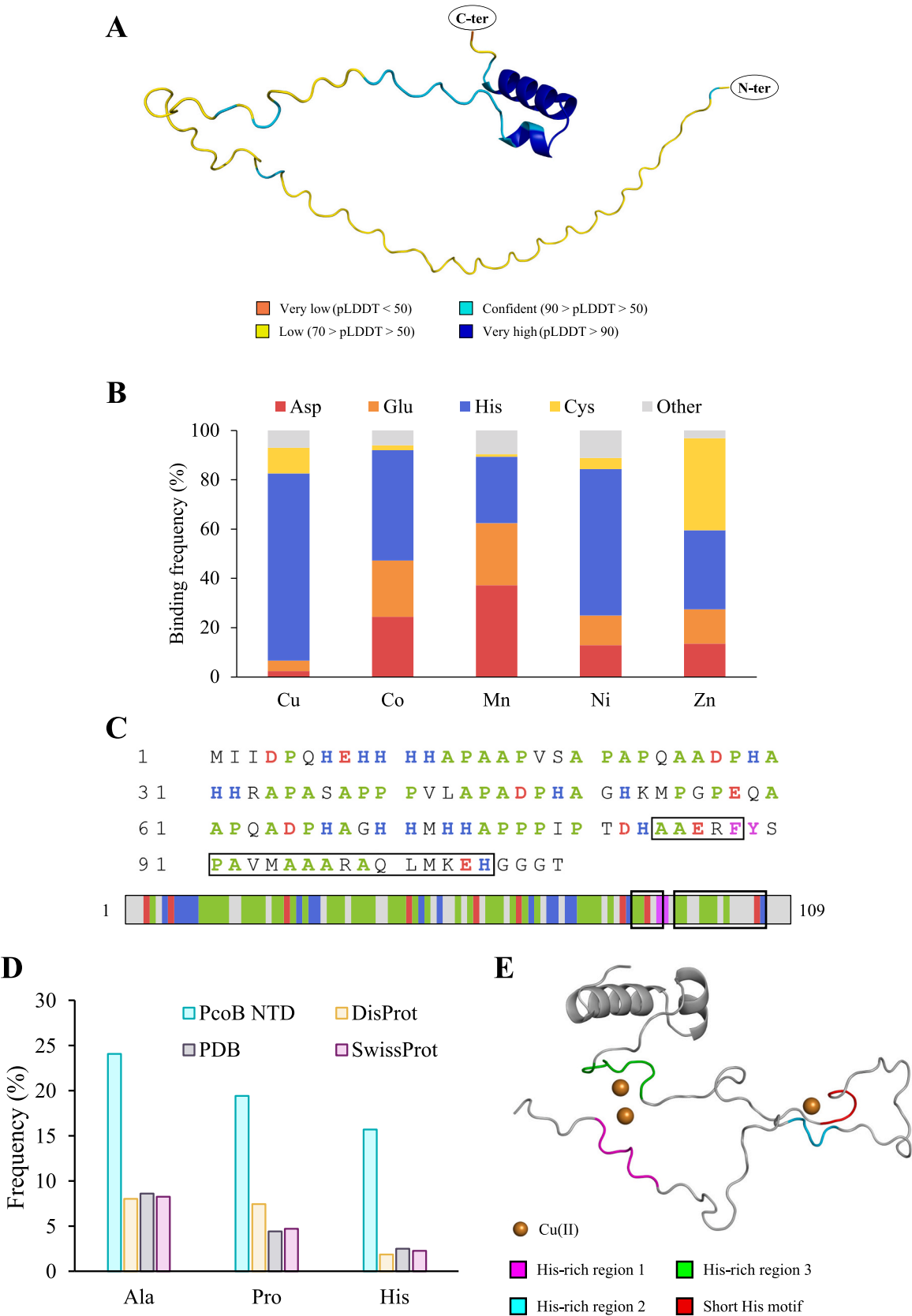
Because some IDPs are prone to self-association and oligomerization, we first verified the in-solution oligomeric state of PcoB NTD using size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) as well as NMR diffusion measurements. The SEC-MALS chromatogram displays three distinct elution bands. The first peak corresponds to the column exclusion limit and is attributed to large protein aggregates or inorganic particles (e.g. dust) present in solution. The second peak displays a symmetric elution profile with an experimental molecular mass of  $11.72 \pm 0.23$  kDa and a polydispersity index of  $1.000 \pm 0.028$ , consistent with a monomeric and highly homogenous PcoB NTD sample. The final band is associated with small non-protein entities, such as salts and buffer present in the injected sample (Fig. 3A). The hydrodynamic radius ( $R_h$ ) extracted from NMR diffusion experiments amounts to  $21.3 \pm 0.4$  Å, in good agreement with the value expected for a monomeric IDP of comparable size [48]. All these results further indicate that PcoB NTD remains monomeric in solution (Fig. 3B).

### 3.3. Impact of divalent metal cation binding on PcoB NTD

To investigate the metal-binding properties of PcoB NTD, several biophysical techniques were employed using various bioavailable divalent metal cations. As the protein sequence lacks a tryptophan residue and contains only one tyrosine residue (Tyr89) located between the two transient helical structures of the C-terminal region (Fig. 1C), intrinsic Tyr fluorescence (ITyrF) was monitored for all spectroscopic assays.

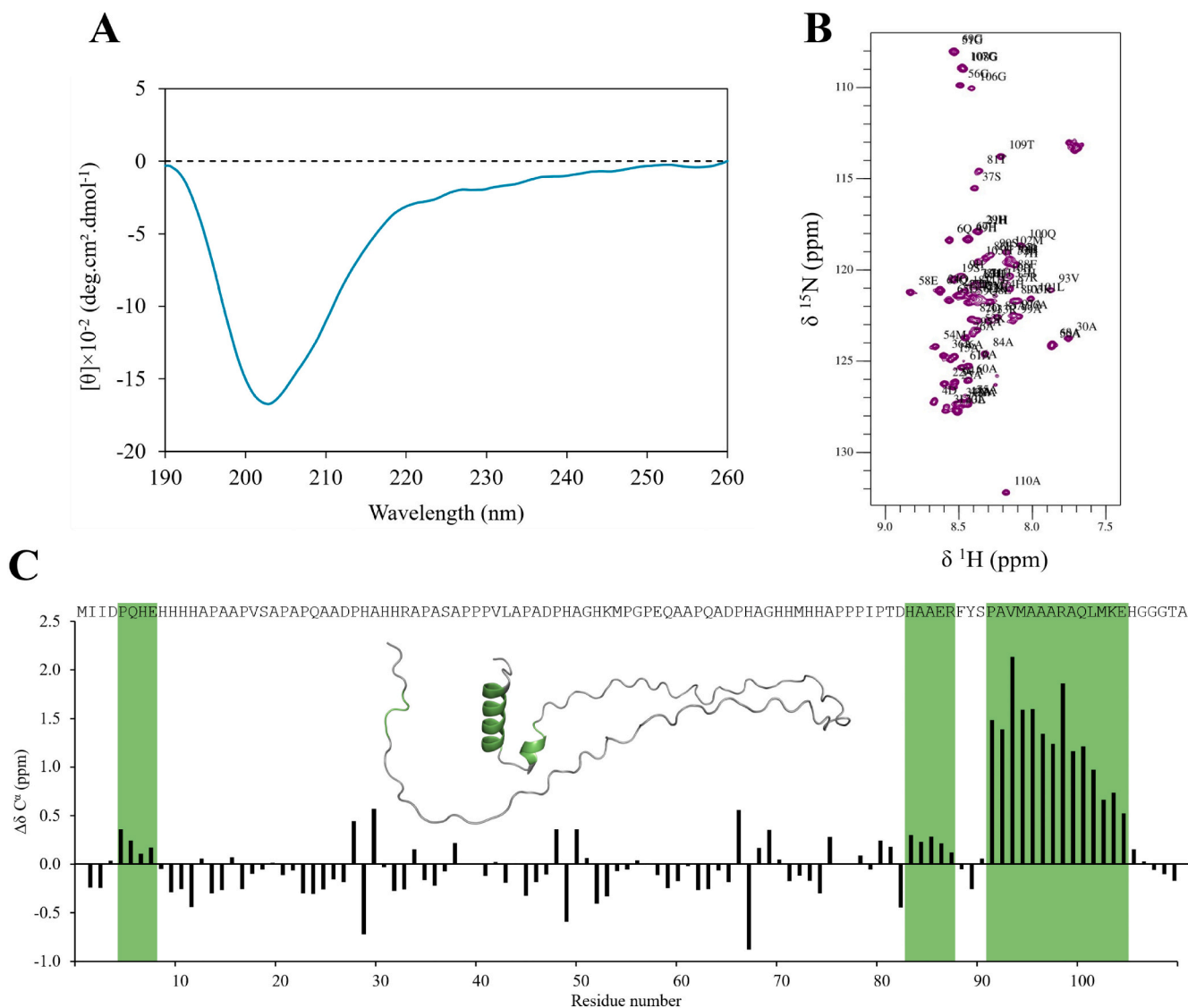
First, the impact of metal cations on PcoB NTD conformation was assessed by recording ITyrF spectra upon increasing the cation concentration at pH 7.5. These experiments were conducted as a qualitative screening to evaluate whether divalent metal cations affect the fluorescence signal of PcoB NTD. While no wavelength shift was observed, fluorescence intensity of Tyr was quenched in the presence of Cu(II) ions at increasing ratios (Fig. 4A). The same measurements were performed with free L-Tyr in solution as a control to evaluate the contribution of non-specific Tyr-Cu(II) interactions to the signal (Fig. 4B). No significant fluorescence intensity quenching was observed for free Tyr, while control measurements of buffer and metal solutions alone showed no detectable fluorescence signal under the experimental conditions. Therefore, the variations observed upon addition of Cu(II) are most likely caused by changes in the local microenvironment of Tyr89 within the disordered conformational ensemble resulting from one or more interactions between PcoB NTD and metal cations, rather than direct Tyr-Cu(II) contacts.

To evaluate the metal-binding behavior of PcoB NTD, the same protocol was applied to six other bioavailable cations: Ca(II), Co(II), Mg(II), Mn(II), Ni(II), and Zn(II) (Fig. S4). Interestingly, Co(II), Ni(II), and



(caption on next page)

**Fig. 1.** AlphaFold3 and bioinformatic sequence analysis of the PcoB N-terminal domain (NTD). (A) PcoB NTD conformation predicted by AlphaFold3. The per-residue confidence is shown using the pLDDT score (0–100), which estimates the accuracy of local atomic distances based on polymer contacts. Colors indicate prediction confidence: navy blue (very high, >90), cyan (confident, 70–90), yellow (low, 50–70), and orange (very low, <50). (B) Residue preferences for the different metal with aspartate (Asp, red), glutamate (Glu, orange), histidine (His, blue), cysteine (Cys, yellow) and other (grey). Adapted from Barber-Sucker S., et al. [32]. (C) Amino acid sequence of the PcoB NTD with key residues highlighted: acidic residues (aspartate and glutamate, red), histidines (blue), enriched disorder-promoting residues (alanine and proline, green), and aromatic residues (phenylalanine and tyrosine, magenta). Regions predicted by AlphaFold3 to form  $\alpha$ -helices are outlined in black. (D) PlaToLoCo residue frequency profile of PcoB NTD (cyan) compared with several reference protein databases. DisProt (beige, only intrinsically disordered proteins), PDB (grey, experimentally resolved 3D structures), and SwissProt (purple, high-quality manually curated proteins). (E) AlphaFold3-predicted conformation of PcoB NTD in the presence of three Cu(II) cations. His-rich regions 1–3 and the short His motif are highlighted in magenta, cyan, green, and red, respectively.

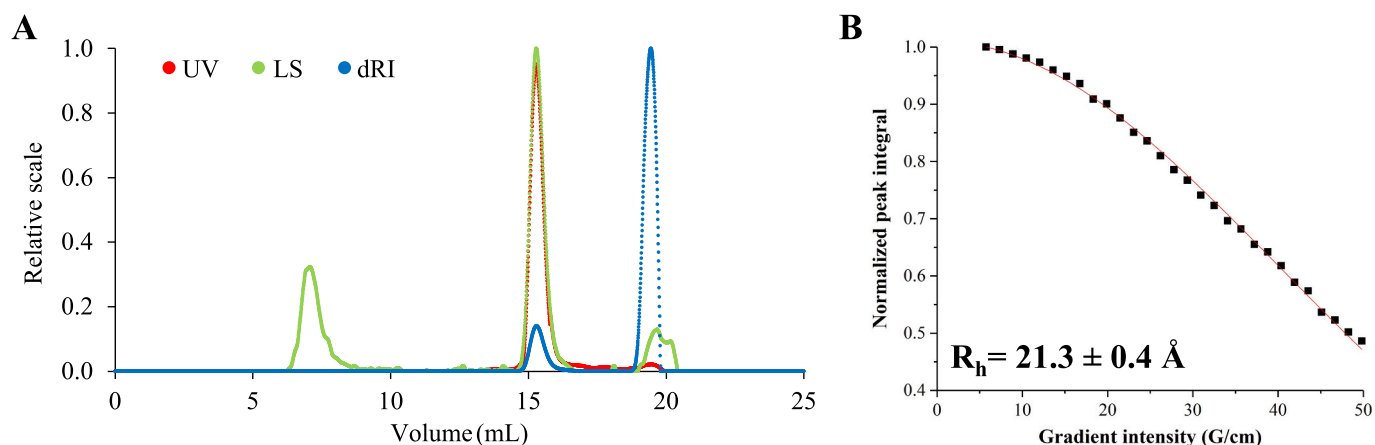


**Fig. 2.** Far-UV circular dichroism (FCD) spectrum and nuclear magnetic resonance (NMR) spectroscopy experiments of PcoB NTD. (A) Measurements were performed at a protein concentration of 10  $\mu$ M in 20 mM phosphate buffer pH 7.5. (B) Assigned  $^{15}\text{N}$ – $^1\text{H}$  HSQC spectrum of 170  $\mu$ M PcoB NTD in 20 mM HEPES buffer pH 7.5. (C) SCS of  $\text{C}^\alpha$ -atoms along PcoB NTD sequence and its AlphaFold3 model (helical and turn regions in green).

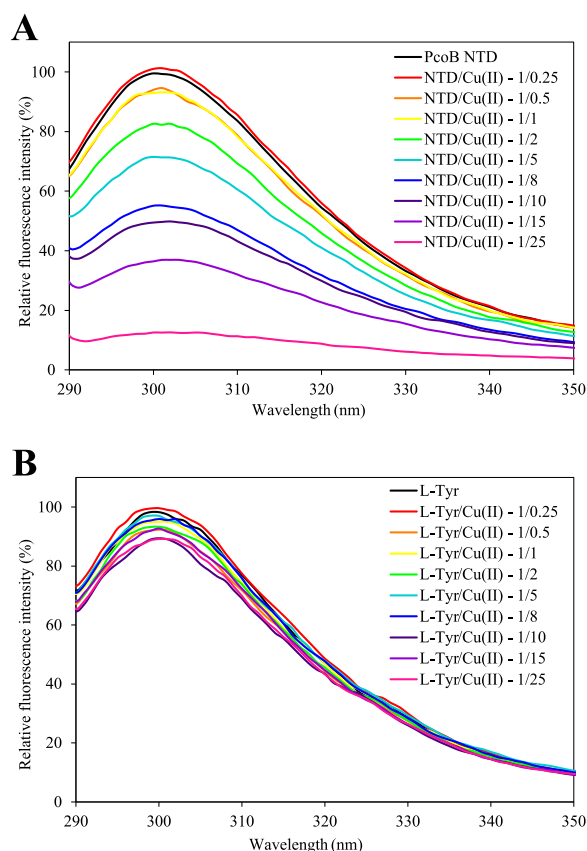
Zn(II) also induced detectable fluorescence changes, whereas no significant effects were observed with Ca(II), Mg(II), and Mn(II). These results suggest that PcoB NTD, initially presumed to exclusively interact with Cu(II), would also be capable of interacting with Zn(II), Ni(II), and Co(II). While the interaction with Ni(II) cations was anticipated, given that PcoB NTD can be purified without fusion tags using a Ni-chelated affinity column, the binding to Zn(II) and Co(II) ions was unexpected.

To quantify the metal-binding properties of PcoB NTD, isothermal titration calorimetry (ITC) experiments were conducted with the seven

tested divalent cations. Titrations were carried out at three pH values (7.5, 5.5, and 4.5), except for Zn(II) and Mn(II) where pH 7.5 was replaced with pH 6.9 due to solubility issues. This range of conditions was chosen to probe the contribution of His residues to the metal binding process. Using the Henderson–Hasselbalch relation and considering average pKa values of 6.45, 3.43, and 4.14 for His, Asp and Glu, respectively, the estimated protonated fraction of these residues at each pH values were calculated (Table 1). Thus, compared to pH 7.5, these acidic conditions should significantly attenuate His-mediated



**Fig. 3.** Hydrodynamic properties of PcoB NTD. (A) Size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) of 130  $\mu$ M PcoB NTD in 20 mM HEPES buffer pH 7.5. The dots correspond to absorbance at 280 nm (UV, red), light scattering (LS, green), and differential refractive index (dRI, blue). (B) Nuclear magnetic resonance (NMR) signal intensity decay as a function of gradient strength from diffusion experiment of 200  $\mu$ M PcoB NTD in 20 mM HEPES buffer pH 6.5. Data fitted using the Stejskal-Tanner equation to extract  $D$  and then calculate  $R_h$  via an internal dioxane standard.



**Fig. 4.** Intrinsic tyrosine fluorescence emission spectra of PcoB NTD and L-tyrosine at increasing Cu(II) concentrations. (A) Emission spectra of PcoB NTD (0.8 g/L) in 20 mM HEPES buffer pH 7.5 with increasing Cu(II) concentrations. Spectra were recorded from 290 to 350 nm after excitation at 275 nm, with Cu (II) added at protein-to-metal molar ratios ranging from 1/0.25 to 1/25. Fluorescence intensity was normalized by dividing all values by the maximum emission of the metal-free protein (black line). (B) Emission spectra of L-tyrosine (1 g/L) under identical buffer conditions and measured at the same increasing Cu(II) concentrations, serving as a negative control for nonspecific quenching. Fluorescence intensity was normalized by dividing all values by the maximum emission of the metal-free L-tyrosine (black line).

**Table 1**

Estimated protonated fractions (%) of histidine, aspartate, and glutamates residues at pH 7.5, 5.5 and 4.5. Values were calculated using the Henderson-Hasselbalch relation and the average pKa values cited by Pahari et al. [49].

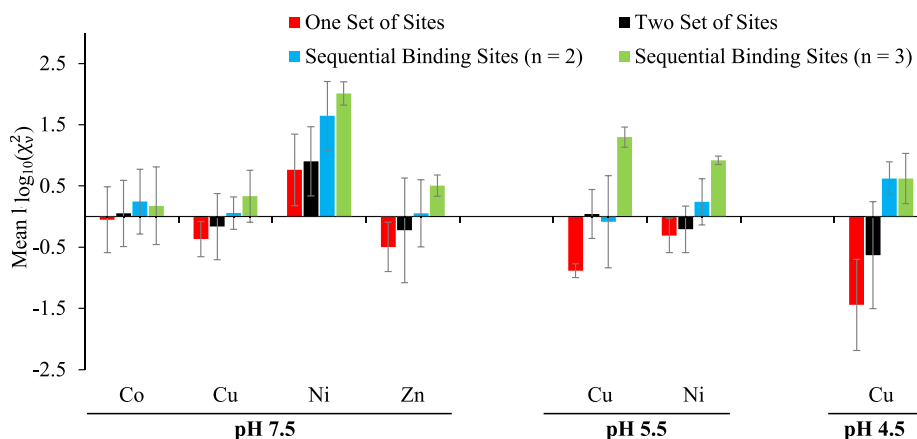
| Residue | Average lateral chain pKa [49] | Estimated protonated fraction (%) |        |        |
|---------|--------------------------------|-----------------------------------|--------|--------|
|         |                                | pH 7.5                            | pH 5.5 | pH 4.5 |
| His     | 6.45                           | 8.2                               | 90     | 99     |
| Asp     | 3.43                           | 0.01                              | 0.8    | 7.8    |
| Glu     | 4.14                           | 0.04                              | 4.2    | 30     |

coordination without abolishing the ones driven by Asp and Glu.

All ITC data have been fitted using the standard “One Set of Sites” mathematical model. While remaining the most appropriate model for these data (Fig. 5), the interpretation of extracted parameters should remain cautious. Raw heat changes and integrated binding isotherms of interacting cations are displayed on Fig. 6 and related metrics extracted from isotherms are listed in Table 2. Thermograms of non-interacting cations are displayed on Fig. 7.

Overall, ITC results are consistent with those obtained by ITyrF. PcoB NTD interacts at pH 7.5 with Cu(II), Co(II), and Ni(II), as well as with Zn (II) at pH 6.9. The measured affinity constants  $K_A$  are all in the range of  $10^4$ – $10^5$   $M^{-1}$ , indicating weak and transient interactions between these metal cations and PcoB NTD. Such affinities are coherent with the putative role of the NTD in the copper efflux: too tight binding would lead to metal sequestration by the protein, rather than efficient transfer and export. Regarding stoichiometries, two patterns can be observed: Cu(II) and Co(II) have decimal stoichiometries of 2.5 and 2.3, respectively, while Ni(II) and Zn(II) have integer stoichiometries of 1.0 and 3.1, respectively. These values seem to hint that, as predicted by the sequence analysis, PcoB NTD has three possible interaction sites that are totally or partially occupied for all metals except Ni(II) (Table 2).

A closer inspection of the thermodynamic parameters reveals two other distinct binding modes. Although ITyrF and stoichiometries have suggested that Cu(II) and Co(II) ions induce similar conformational changes, the ITC-derived variations in enthalpy ( $\Delta H$ ) and entropy ( $\Delta S$ ) changes align Cu(II) more closely with Ni(II). Both cations display a more negative  $\Delta H$  and a  $\Delta S < 0$ . Thus, the binding is enthalpy-driven and likely involves changes in the hydrogen bond network and electrostatic interactions, potentially due to exchanges between disordered conformational states upon metal binding. Conversely, Co(II) and Zn(II) ions exhibit lower  $\Delta H$  values and  $\Delta S > 0$ , indicating an entropy-driven binding process that likely involves desolvation, counter-ion release and/or hydrophobic effects often associated with decreased binding



**Fig. 5.** Mean  $\log_{10}(\chi^2_v)$  for conditions where binding is observed. Four mathematical fitting models (“One Set of Sites”, “Two Sets of Sites”, “Sequential Binding Sites” with  $n = 2$ , and “Sequential Binding Sites” with  $n = 3$ ; colored in red, black, blue, and green, respectively) were compared for all binding metals under the three pH conditions tested. Reduced chi-square ( $\chi^2_v = \chi^2/\text{DoF}$ ) is the chi-square divided by the degrees of freedom (DoF), defined as the number of experimental points minus the parameters floating in the fit ( $\text{DoF} = N_{\text{exp}} - N_{\text{fit}}$ ). Values are reported as mean  $\pm$  standard error. The number of replicates for each measurement was 3.

specificity (Table 2).

Upon acidification, Cu(II) binding was preserved, Ni(II) binding was progressively weakened (i.e., reduced at pH 5.5 and abolished at pH 4.5), and Co(II) and Zn(II) binding was completely lost at both acidic pH values. For Cu(II), moderate acidification (pH 5.5) slightly strengthened the interaction without altering its stoichiometry, while stronger acidification (pH 4.5) weakened the binding and reduced the number of detectable binding sites. Such pattern could result from the ability of metal cations to act as Lewis acids, stabilizing the deprotonated form of histidine residues and effectively lowering its apparent pKa [50–52]. This increases the fraction of binding-competent residues, allowing metal coordination at acidic pH where the protonated form would otherwise dominate according to the theoretical pKa. Among the studied cations, Cu(II) is the strongest Lewis acid and produces the largest pKa shift. Such property likely explains why a similar binding is observed at pH 5.5 compared to the one at pH 7.5. However, the acidic conditions of pH 4.5 should be sufficient to overcome this effect, and residual binding observed could arise from Asp and Glu residues taking over the coordination [51].

Regarding the only other interaction maintained at pH 5.5, namely that with Ni(II), the experimental thermodynamic parameters revealed a binding mode distinct from the one observed at pH 7.5. The  $\Delta H$  value was greatly reduced and  $\Delta S > 0$ , suggesting that under these conditions, the interaction is no longer only enthalpy-driven but arises from a combination of enthalpy and entropy effects which could be caused by less favorable interactions and more conformational flexibility. Noticeably, raw heat changes recorded under acidic conditions appeared more defined and less noisy, likely due to the increased solubility and stability of these metal cations compared to neutral conditions.

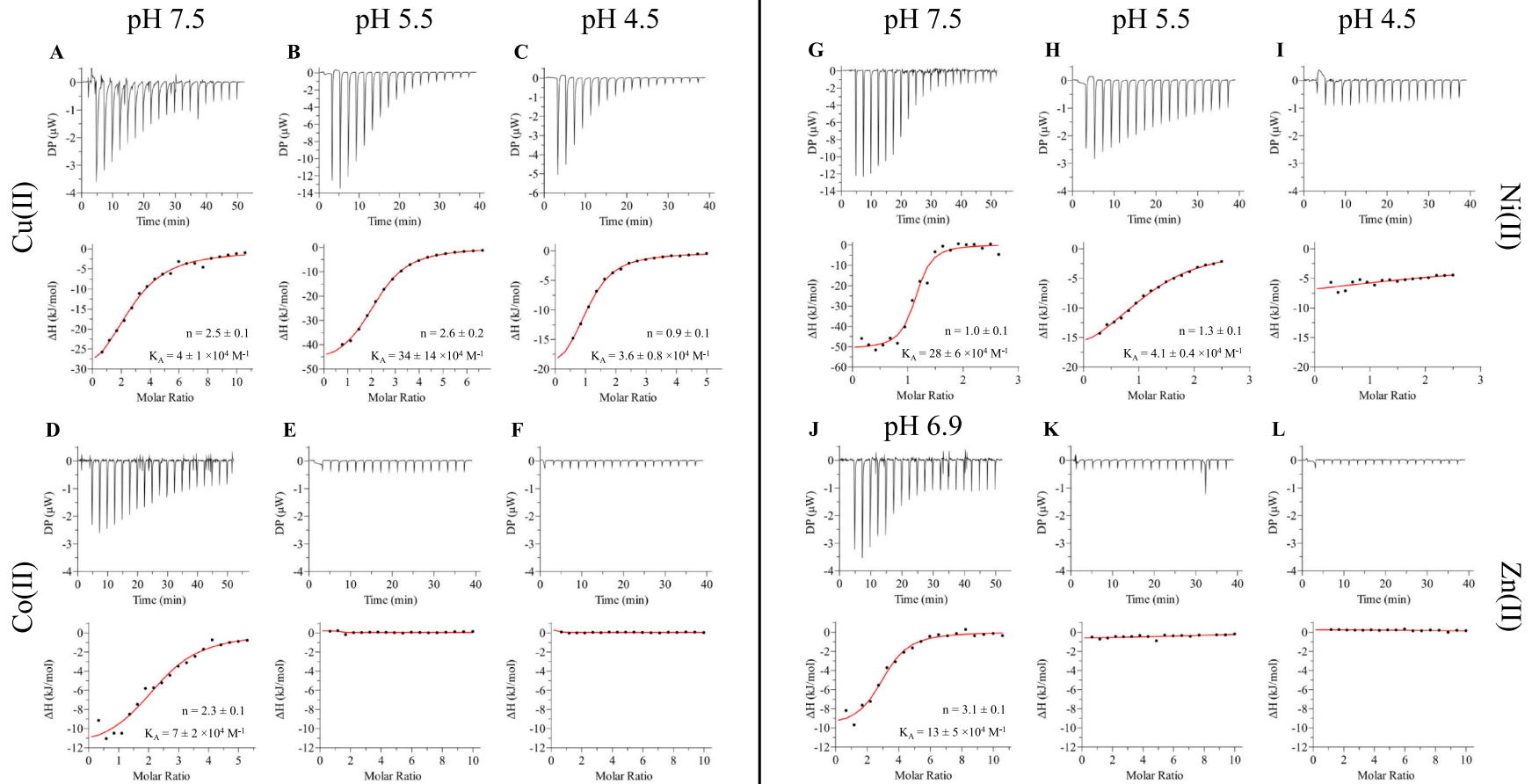
Concerning the non-integer stoichiometries observed, they can arise from multiple factors. Beyond small deviations between theoretical and effective protein or metal concentrations, they could also reflect heterogeneity within the protein sample (aggregated or proteolyzed species) or partial site occupancy prior to the experiment due to trace metals introduced during expression or purification. The use of the “One Set of Sites” fitting model could also yield an average non-integer stoichiometry if the protein has multiple binding sites with similar but non-equivalent affinities, while remaining the most appropriate model for these data (Fig. 5). Moreover, metal speciation issues such as precipitation could reduce the effective free metal concentration, or protein oligomerization upon titrant addition may directly affect the calculated stoichiometry. For these reasons, we remain cautious when interpreting these values in terms of binding mechanisms. Although maintaining long-term stability of divalent metal solutions is challenging, 20 mM

HEPES buffer at pH 7.5 (or pH 6.9 for Mn(II) and Zn(II)) has proved to be adequate for short-term stabilization. In addition, NMR diffusion measurements in the presence of 120  $\mu\text{M}$  Zn(II) indicated a slightly more compact conformation while retaining a monomeric state (Fig. S5). In summary, the observed non-integer stoichiometries are compatible with the weak and transient nature of PcoB NTD–cation interactions, while remaining mindful that several other factors may contribute to these values.

Altogether, these results indicate that PcoB NTD interacts with Cu(II), Co(II), Ni(II), and Zn(II) cations at pH 7.5, whereas acidic conditions partially or completely disrupt this binding, at the exception of Cu(II) cations. While such acidic conditions are unlikely to occur within living bacteria, they emphasize the potential role of pH in modulating the geometries and parameters of protein-metal interactions. Similar pH-dependent effects have been reported for other Cu-binding IDRs/IDPs, such as the N-terminal region of the human Cu transporter hCtr1 [53], the prion protein [54], or the amyloid- $\beta$  [55]. These condition-sensitive changes in apparent affinities suggest that Cu(II) exhibits a distinct binding behavior, likely driven by the abundance of His residues complemented by the presence of Asp and Glu residues distributed all along the protein sequence.

### 3.4. NMR measurements confirm metallic cations binding through His residues

To understand cation binding at the residue level, we used NMR spectroscopy and started with Zn(II), as it is the only diamagnetic binder confirmed by ITC. The absence of paramagnetic effects, such as line broadening or signal loss, makes Zn(II) particularly suitable for detailed NMR investigation [56]. To investigate Zn(II) binding to PcoB NTD, a series of  $^1\text{H}$ ,  $^{15}\text{N}$ -HSQC spectra were recorded at increasing Zn(II) concentrations and at varying pH (Fig. 8A). At pH 7.5, nearly all signals disappear upon reaching a protein/Zn(II) molar ratio of 1/2, indicating interaction. At pH 6.5, fewer peaks vanish, although many show reduced intensity. This effect becomes progressively weaker at lower pH, as only a few peaks disappear or decrease in intensity at pH 5.5, while no spectral changes are observed at pH 4.5 (even at a protein/Zn(II) molar ratio of 1/10). This pH-dependent behavior is in good agreement with the ITC results and consistent with the involvement of His residues in Zn(II) coordination, as protonation of their imidazole side chains at low pH reduces their metal-binding capacity. Binding remains fully reversible, as demonstrated by complete signal recovery upon EDTA addition (Fig. S6), ruling out irreversible aggregation or degradation. Residue-specific peak intensity analysis (Fig. 8B) reveals that the C-terminal



**Fig. 6.** Isothermal titration calorimetry (ITC) experiments of PcoB NTD titrated with four divalent metal cations at three different pH values. All measurements were performed in 20 mM HEPES buffer and each condition (metal and pH combination) is shown in a separate panel: Cu(II) at pH 7.5, 5.5, and 4.5 (A–C), Co(II) (D–F), Ni(II) (G–I), and Zn(II) (J–L). For each condition, raw heat changes (top) and the integrated binding isotherm (bottom) from a representative experiment are displayed. For conditions where binding was observed, the extracted stoichiometry ( $n$ ) and association constant ( $K_A$ ) are also shown. The number of replicates for each measurement was 3.

Table 2

Binding stoichiometry (n), affinity constant ( $K_A$ ,  $\times 10^4$  M<sup>-1</sup>), enthalpy variation ( $\Delta H$ , kJ·mol<sup>-1</sup>), entropy variation ( $-T\Delta S$ , kJ·mol<sup>-1</sup>), and free energy variation ( $\Delta G$ , kJ·mol<sup>-1</sup>) obtained by isothermal titration calorimetry measurements of PcoB NTD in 20 mM HEPES buffer with seven different divalent metal cations (Ca(II), Co(II), Cu(II), Mg(II), Mn(II), Ni(II), and Zn(II)) at pH 7.5, 5.5, and 4.5. Values are reported as mean  $\pm$  standard error. The number of replicates for each measurement was 3. No data available for non-interacting metals returning no ITC binding profiles.

| Metal Cation | pH 7.5        |                                         |                                    |                                      |                                    | pH 5.5        |                                         |                                    |                                      |                                    | pH 4.5        |                                         |                                    |                                      |                                    |
|--------------|---------------|-----------------------------------------|------------------------------------|--------------------------------------|------------------------------------|---------------|-----------------------------------------|------------------------------------|--------------------------------------|------------------------------------|---------------|-----------------------------------------|------------------------------------|--------------------------------------|------------------------------------|
|              | n             | $K_A$ ( $\times 10^4$ M <sup>-1</sup> ) | $\Delta H$ (kJ·mol <sup>-1</sup> ) | $-T\Delta S$ (kJ·mol <sup>-1</sup> ) | $\Delta G$ (kJ·mol <sup>-1</sup> ) | n             | $K_A$ ( $\times 10^4$ M <sup>-1</sup> ) | $\Delta H$ (kJ·mol <sup>-1</sup> ) | $-T\Delta S$ (kJ·mol <sup>-1</sup> ) | $\Delta G$ (kJ·mol <sup>-1</sup> ) | n             | $K_A$ ( $\times 10^4$ M <sup>-1</sup> ) | $\Delta H$ (kJ·mol <sup>-1</sup> ) | $-T\Delta S$ (kJ·mol <sup>-1</sup> ) | $\Delta G$ (kJ·mol <sup>-1</sup> ) |
| Ca(II)       | —             | —                                       | —                                  | —                                    | —                                  | —             | —                                       | —                                  | —                                    | —                                  | —             | —                                       | —                                  | —                                    | —                                  |
| Co(II)       | 2.3 $\pm$ 0.1 | 7 $\pm$ 2                               | -12.3 $\pm$ 0.6                    | -15 $\pm$ 1                          | -27.5 $\pm$ 0.6                    | —             | —                                       | —                                  | —                                    | —                                  | —             | —                                       | —                                  | —                                    | —                                  |
| Cu(II)       | 2.5 $\pm$ 0.1 | 4 $\pm$ 1                               | -30 $\pm$ 8                        | 12 $\pm$ 1                           | -25.7 $\pm$ 0.7                    | 2.6 $\pm$ 0.2 | 34 $\pm$ 14                             | -38 $\pm$ 6                        | 8 $\pm$ 7                            | -29.3 $\pm$ 0.9                    | 0.9 $\pm$ 0.1 | 3.6 $\pm$ 0.8                           | -40 $\pm$ 10                       | 14 $\pm$ 11                          | -25.9 $\pm$ 0.6                    |
| Mg(II)       | —             | —                                       | —                                  | —                                    | —                                  | —             | —                                       | —                                  | —                                    | —                                  | —             | —                                       | —                                  | —                                    | —                                  |
| Mn(II)       | —             | —                                       | —                                  | —                                    | —                                  | —             | —                                       | —                                  | —                                    | —                                  | —             | —                                       | —                                  | —                                    | —                                  |
| Ni(II)       | 1.0 $\pm$ 0.1 | 28 $\pm$ 6                              | -61 $\pm$ 3                        | 30 $\pm$ 3                           | -30.9 $\pm$ 0.6                    | 1.3 $\pm$ 0.1 | 4.1 $\pm$ 0.4                           | -16 $\pm$ 2                        | -10 $\pm$ 3                          | -26.3 $\pm$ 0.3                    | —             | —                                       | —                                  | —                                    | —                                  |
| Zn(II)       | 3.1 $\pm$ 0.1 | 13 $\pm$ 5                              | -12 $\pm$ 2                        | -17 $\pm$ 3                          | -28 $\pm$ 1                        | —             | —                                       | —                                  | —                                    | —                                  | —             | —                                       | —                                  | —                                    | —                                  |

helical region is less affected by the presence of Zn(II) ions, while His and neighboring residues outside the helical regions consistently show stronger signal loss, supporting His-centered metal coordination. The observed signal loss is most likely due to a dynamic and heterogeneous ensemble of conformational binding states with exchange in the intermediate NMR time regime, as diffusion measurements performed with Zn(II) ions (Fig. S5) ascertain that PcoB NTD remains monomeric in the presence of metals, excluding oligomerization as a source of line broadening. Persistence of a disordered ensemble in the bound state is further supported by highly identical FCD spectra at increasing Cu(II) concentrations (Fig. S7), highlighting a lack of folding upon binding.

Following the Zn(II) experiments, <sup>1</sup>H, <sup>15</sup>N-HSQC spectra were recorded in the presence of increasing Cu(II) concentration at pH 5.5, a condition where Zn(II) binding is still detectable but attenuated, allowing for clearer observation of residue-specific effects. Cu(II) ions induce a stronger overall decrease in peak intensity than Zn(II) ions under the same conditions (Fig. 9A) likely due to the paramagnetic nature of Cu(II) causing line broadening. Despite this effect, the pattern remains consistent: signals from the C-terminal helical region are less affected, while more pronounced signal losses occur in the vicinity of His residues, supporting their role in metal coordination. In an attempt to obtain a more precise description of the binding regions, additional <sup>1</sup>H, <sup>15</sup>N-HSQC spectra were recorded at a Cu(II) concentration corresponding to 5% of the protein concentration (PcoB NTD/Cu(II) molar ratio 1/0.05). However, no improvement in the marking out the Cu(II) specific binding sites was observed (Fig. S8).

As a control, Mg(II), Ca(II) and Mn(II), three divalent metal cations not expected to interact with PcoB NTD based on the previously described results, were also tested at pH 7.5 using NMR. In the presence of Mg(II) or Ca(II) ions, even at a 1/10 protein-to-ion molar ratio, no relevant changes in signal intensity were observed (Fig. 9B,C), confirming the absence of interaction. In contrast, spectra recorded with Mn(II) reveal slight perturbation in signal intensity (Fig. 9D), suggesting a weaker interaction compared to other cations. However, no interaction was detected by ITyrF or ITC under the same conditions used for the other cations. A plausible explanation is that Mn(II) binding does not perturb the local environment of Tyr89, rendering ITyrF insensitive, whereas NMR is sensitive to very weak binding effects.

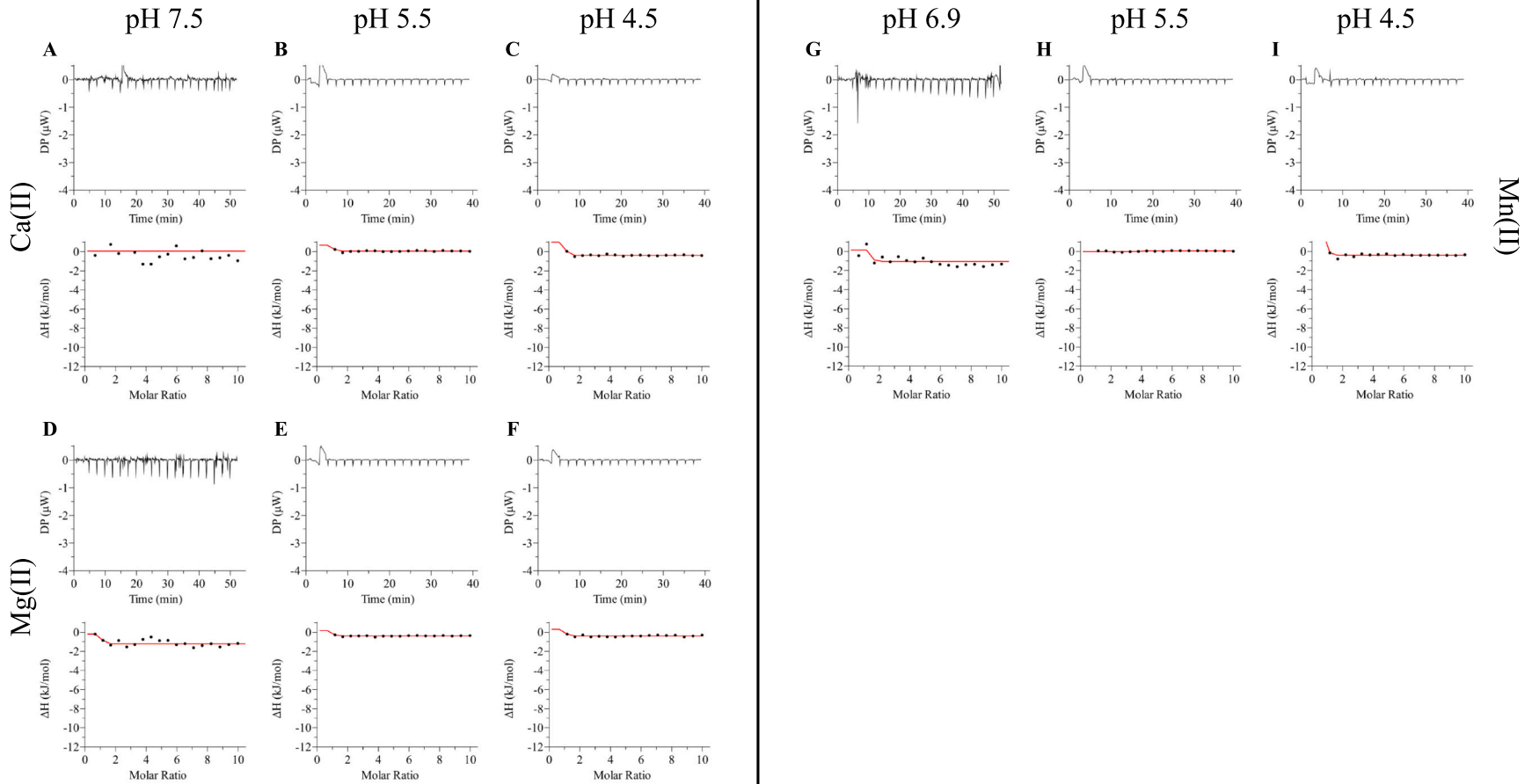
4. Conclusions

Copper (Cu) is an essential trace element for all living organisms but

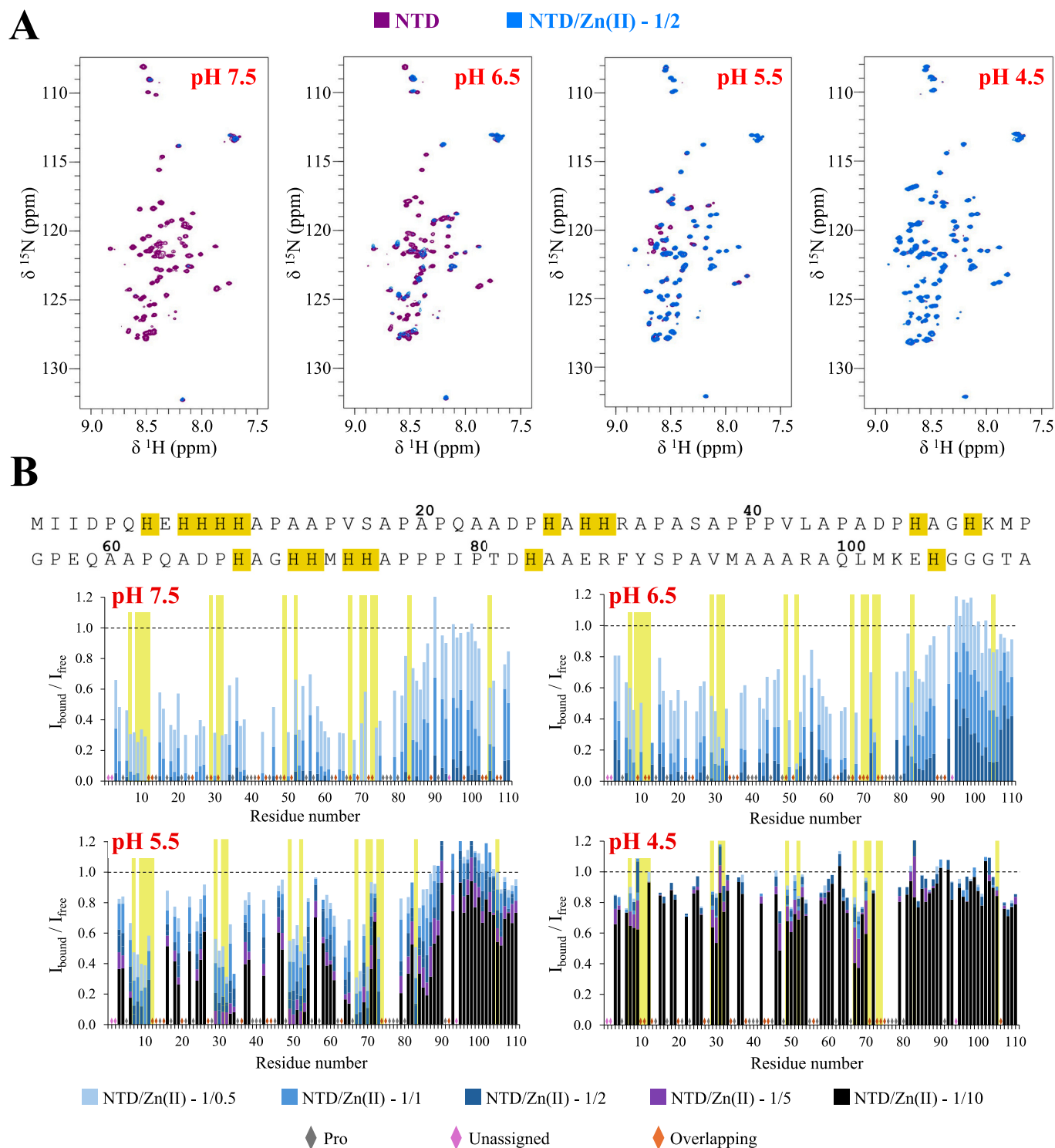
becomes cytotoxic when present in excess, thus harboring antimicrobial properties [1–3]. To survive in Cu-rich environments, some bacteria—including the pathogenic ESKAPEE group responsible for most health-acquired infections—have developed pathways to escape these antimicrobial effects [9–13]. However, associated resistance mechanisms are not yet fully understood. In this context, the metal-binding properties of the N-terminal domain (NTD) of the *Caulobacter vibrioides* membrane efflux protein PcoB were assessed with respect to its intrinsic disorder in the presence of Cu(II) and six other bioavailable cations.

The disordered NTD of the *C. vibrioides* membrane protein PcoB was shown to be monomeric in solution while maintaining a disordered conformational ensemble that includes a 42% populated short 13-residue  $\alpha$ -helix at its C-terminal end. The NTD can engage in weak and transient interactions with Cu(II), Co(II), Ni(II), and Zn(II) ions at pH 7.5, whereas under acidic conditions, only Cu(II) binding is preserved with a reduced stoichiometry. Binding locates to His-rich regions outside the helical C-terminal, and the cation-bound ensemble appears heterogeneous and dynamic.

Based on these observations, we hypothesize that PcoB NTD acts as a flexible and disordered arm capable of capturing Cu(II) ions within the periplasm and guiding them towards the  $\beta$ -barrel pore for export. Such catch-and-deliver mechanism appears to be pH-dependent, with Cu(II) uniquely maintaining affinity for the NTD under acidic conditions, likely reflecting a distinct coordination behavior driven by histidine protonation states and the presence of acidic residues distributed all along the sequence. Furthermore, we do not exclude potential interactions between PcoB NTD and the multicopper oxidase PcoA, which is the only other member of the *C. vibrioides* Pco copper regulation system. Such interaction could induce conformational rearrangements that facilitate unbound Cu(II) efflux or lead to a spatial alignment of the membrane pore with PcoA, allowing direct export of Cu(I) oxidized to Cu(II). This hypothesis is supported by preliminary in vivo results, indicating a destabilization of PcoB and, more surprisingly, PcoA in bacterial strains lacking the PcoB NTD, suggesting a potentially novel role for an IDR in the homeostasis of a partner protein [18]. The proposed model is supported by a similar mode of action reported in membrane protein transporters containing an IDR, such as the human Cu(I) importer hCtr1, the N-terminal disordered segment of which mediates metal uptake through transient disorder-to-order transitions [57]. As such, PcoB NTD may operate through a similar mechanism, exploiting its structural flexibility to enable Cu(II) recognition and transfer across the



**Fig. 7.** Isothermal titration calorimetry (ITC) experiments of PcoB NTD titrated with three divalent metal cations at three different pH values. All measurements were performed in 20 mM HEPES buffer and each condition (metal and pH combination) is shown in a separate panel: Ca(II) at pH 7.5, 5.5, and 4.5 (A–C), Mg(II) (D–F), and Mn(II) (G–I). For each condition, raw heat changes (top) and the integrated binding isotherm (bottom) are displayed. For conditions where binding was observed, the extracted stoichiometry ( $n$ ) and association constant ( $K_A$ ) are also shown.

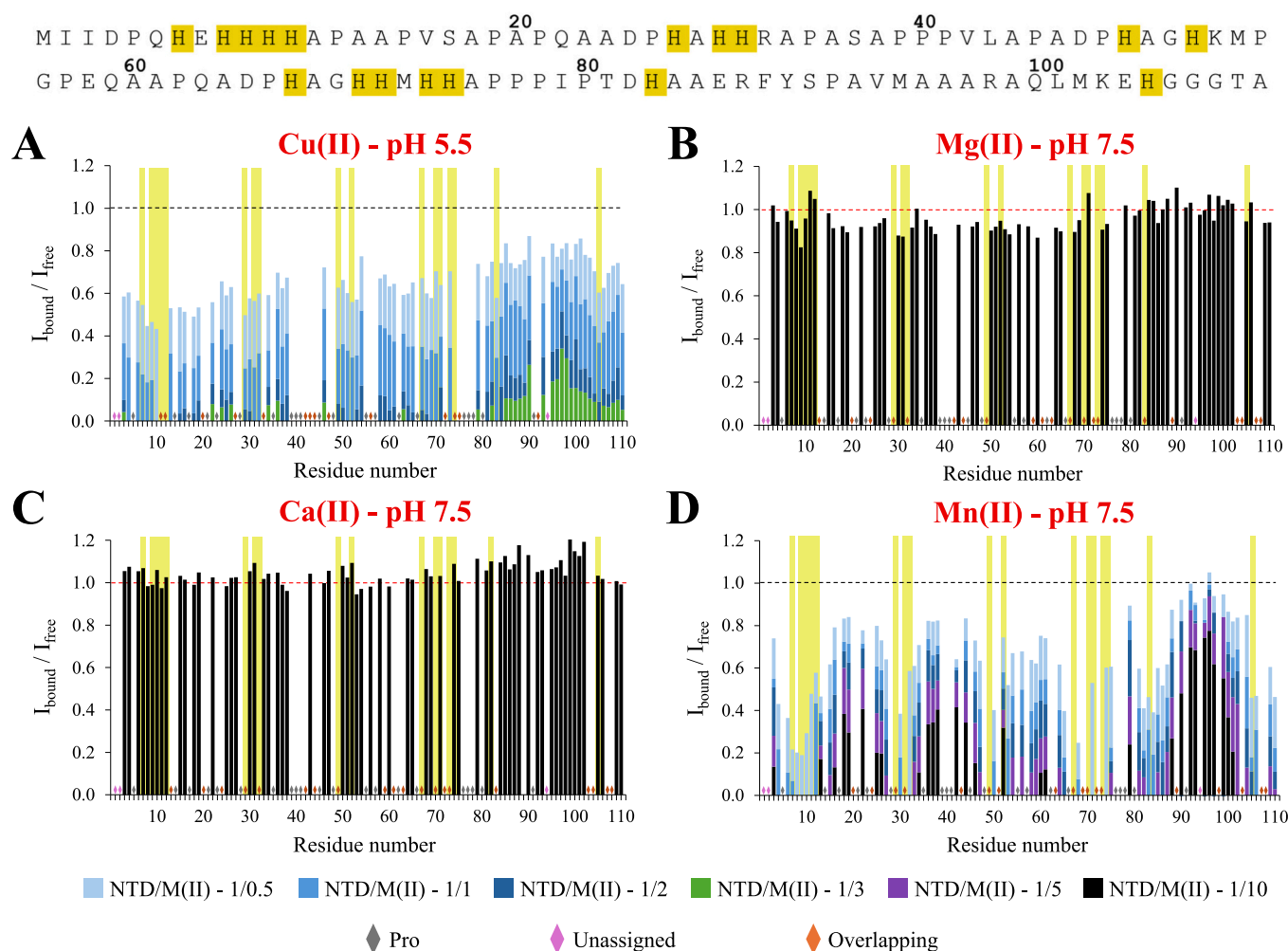


**Fig. 8.** Zinc binding to PcoB NTD at the residue level. NMR experiments of 60  $\mu\text{M}$  PcoB NTD in 20 mM HEPES buffer at different pH in presence of Zn(II) ions. (A)  $^{15}\text{N}$ – $^1\text{H}$  HSQC spectra without (purple) and with 120  $\mu\text{M}$  Zn(II) (blue). (B) Intensity ratio plots for different PcoB NTD/Zn(II) molar ratios with His residues are highlighted in yellow in the PcoB NTD sequence and in the below plots.

membrane. As binding leads to loss of NMR signals, the bound state ensemble reported here remains dynamic and heterogeneous without folding, as supported by CD data, contrasting with what is usually observed for other transporters. As such, the bound state dynamics may facilitate cation transport through mechanisms observed for other dynamic IDP complexes, such as the nuclear pore transporters [58] or the histone-DNA-chaperone system [59,60].

Altogether, our work contributes to deepen our understanding of

how IDPs bind metal ions, which is still a relatively overlooked topic [61,62]. It also provides new insights into the molecular basis of copper export mechanism mediated by the IDR of PcoB, a key protein for bacterial copper resistance. In the longer term, these findings may contribute to the rational design of blocking agents targeting this efflux process, thereby enhancing the antibacterial efficiency of metallic surfaces.



**Fig. 9.** Metal ion binding to PcoB NTD followed by NMR. NMR peak intensity ratios plots of 60  $\mu$ M PcoB NTD in 20 mM HEPES buffer for different PcoB NTD/M(II) molar ratios with His residues highlighted in yellow in the PcoB NTD sequence and in the plots below. (A) M = Cu(II) at pH 5.5. (B) M = Mn(II) at pH 7.5. (C) M = Ca(II) at pH 7.5. (D) M = Mg(II) at pH 7.5.

#### CRediT authorship contribution statement

**Hugo Bâlon:** Writing – original draft, Visualization, Investigation, Formal analysis. **Simon Rouxhet:** Writing – review & editing, Visualization, Investigation. **Julien Mignon:** Writing – review & editing, Visualization, Validation, Formal analysis. **Marylène Vandevenne:** Writing – review & editing, Visualization, Resources. **Andreas Prestel:** Writing – review & editing, Visualization, Resources. **Birthe B. Krage-lund:** Writing – review & editing, Validation, Supervision, Resources. **Jean-Yves Matroule:** Writing – review & editing, Validation, Conceptualization. **Catherine Michaux:** Writing – review & editing, Validation, Supervision, Funding acquisition, Conceptualization.

#### Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used OpenAI's ChatGPT (GPT-5, October 2025 version) to assist solely with the reformulation and refinement of English-language phrasing throughout the manuscript. The tool was used to improve clarity, grammar, and academic tone, without generating or altering scientific content. All ideas, interpretations, and conclusions are entirely the authors' own. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

The assigned chemical shifts of PcoB NTD have been deposited in the BioMagResBank (BMRB), accession number 53466. Other data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2026.151734>.

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