INVESTIGATION OF STRUCTURE-STABILIZING ELEMENTS IN PROTEINS BY ION MOBILITY MASS SPECTROMETRY AND COLLISION-INDUCED UNFOLDING

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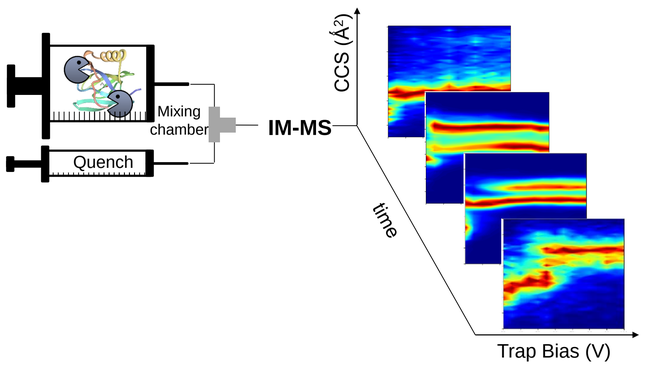
Introduction

A proteolytic reactor, recently designed by our group for structural investigation of proteins[1] was coupled to ion mobility mass spectrometry to follow the evolution of collision cross section (CCS) values of the residual parts of proteins subjected to mono enzymatic digestion (trypsin digest), Upon the progressive loss of various peptides during digestion, the CCS of the remaining sequence of the protein can either comply or diverge from the classical 2/3 power of the CCS-mass relationship (i.e., for spherical structures). Indeed, proteins are generally considered to adopt a globular shape in the gas phase, which correlates CCS to mass via the equation[2] $CCS = A \times m^{2/3}$. Upon the loss of stabilizing elements during digestion, the residual structure can be disrupted and the corresponding CCS value stop complying to the aforementioned trend. In complement to the determination of their CCS values, the residual structures have also been characterized using collision induced unfolding (CIU) to probe their respective resilience towards collisional activation with a neutral gas. The characterized resilience can then be linked to the presence of structure stabilizing element(s) within the proteins and related residual structures. In this study, *β*-lactoglobulin (2 disulfide bridges), cytochrome c (heme) and calmodulin (Ca^{2+} coordination cation) were used to probe the effect of various commonly found structuring elements in proteins on the CCS values. In addition, CIU was performed on protein related residual structures to assess their resilience in the gas phase upon the loss of these various structuring elements. In the gas phase two factor are responsible of the protein unfolding: the charge and the energy barrier. By applying CIU on the structure with or without structure stabilizing elements, it is expected to have to supply more energy on the structure with stabilizing elements. The energy barrier increased in the presence of structure stabilizing elements. $^{TW}CCS_{N2\rightarrow He}$ of the studied species were plotted as a function of their masses and compared to two trend curves describing the CCS/mass relation: (1) a trend curve established by Ruotolo et al.[2] $CCS = 2.45 \times m^{2/3}$ and (2) a similar trend curve established by our group using the trypsin digest of cytochrome c and *β*-lactoglobulin sprayed in non-denaturing conditions $CCS = 2.39 \times m^{2/3}$. It is generally admitted that $^{TW}CCS_{N2\rightarrow He}$ located below or on the trend curve reflects the presence of more compact structures while those located above the trend curve are related to more extended species. Our study shows that proteins and residual structures bearing structure stabilizing elements such as disulfide bridges, a heme or coordination cations systematically present lower $^{TW}CCS_{N2\rightarrow He}$ and a higher resilience towards CIU than protein and residual structures lacking these particular elements. These results confirm the crucial role of intramolecular non-peptidic bonds to the shape of the ions in the gas phase.

Workflow

Preservation of structuring elements after enzymatic digestion
- Avoid collapse/complete structural loss of the residual structure (CCS values)
- Provides higher resiliance towards activation in the gas phase (CIU heatmaps)



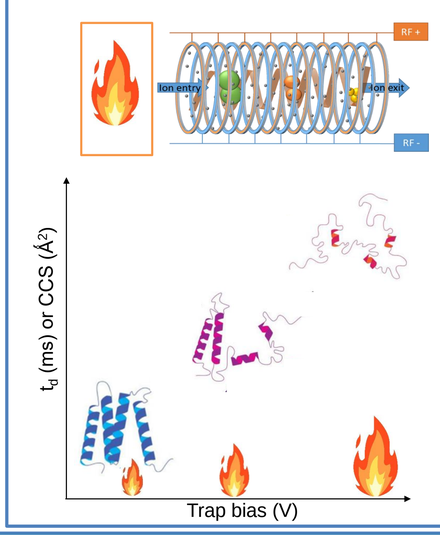
CCS at low trap bias voltage
→ Structural effect of structuring elements

CIU Heatmap
→ Resiliance towards collisional activation in the gas phase

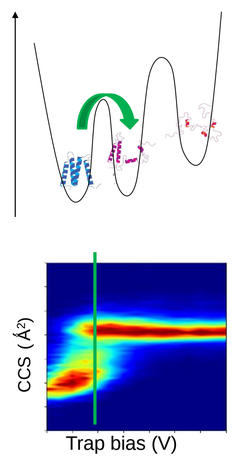
Model proteins

β-lactoglobulin (disulfide bridges)
Calmodulin (coordinating cation)
Cytochrome *c* (heme)

Collision induced unfolding (CIU)



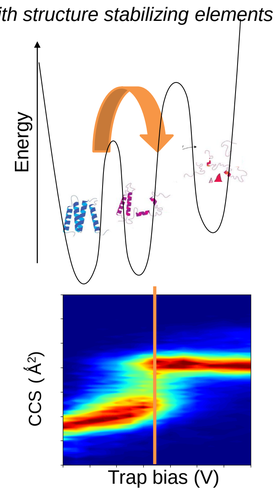
Without structure stabilizing elements



Energy barrier increasing

V1 < V2

With structure stabilizing elements



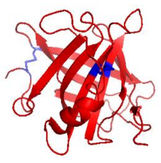
Results

β-lactoglobuline

	$^{TW}CCS_{N_2\rightarrow He}$ 30V	$^{TW}CCS_{N_2\rightarrow He}$ 70V	Disulfide bridges
Native *β*-lactoglobulin	1,726.58 Å²	2468,62 Å²	2
Reduced *β*-lactoglobulin	1,726.48 Å² 1,967.80 Å²	2,690.02 Å² 3,108.86 Å²	0

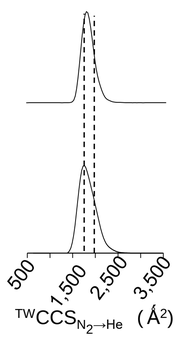
-S-S- prevent the formation of highly extended structures and impact energy barriers

Native *β*-lactoglobulin 10+

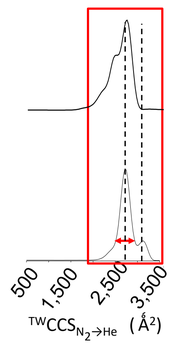


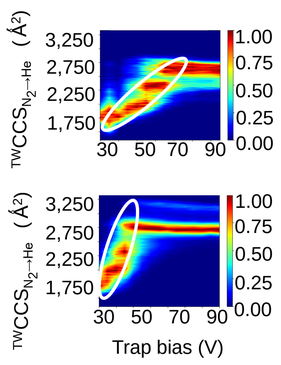
Reduced *β*-lactoglobulin 10+

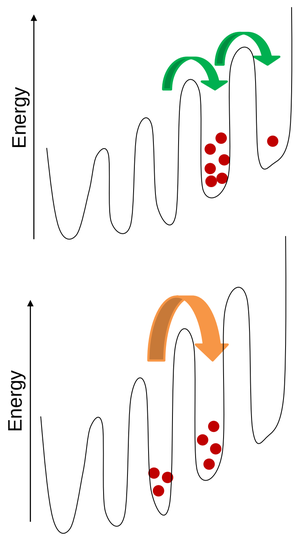
Trap bias = 30V



Trap bias = 70V







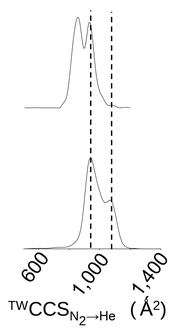
	masses	$^{TW}CCS_{N_2\rightarrow He}$ 30V	$^{TW}CCS_{N_2\rightarrow He}$ 70V	Disulfide bridges
Native Structure 1$_{(\beta)}$	7,120.284 Da	833.99 Å² 935.267 Å²	941.20 Å²	1
Reduced Structure 2$_{(\beta)}$	6,231.250 Da	934.03 Å² 977.82 Å² 1,091.65 Å²	977.82 Å² 1,091.65 Å²	0

-S-S- prevent the formation of highly extended structures

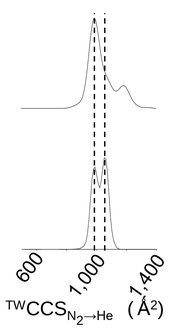
Native structure 1 $_{(\beta)}$ 5+

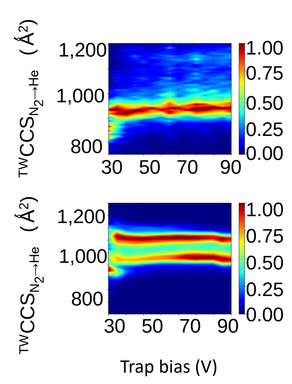
Reduced structure 2 $_{(\beta)}$ 5+

Trap bias = 30V



Trap bias = 70V





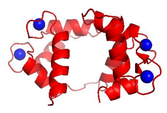
Calmodulin

	$^{TW}CCS_{N_2\rightarrow He}$ 30V	$^{TW}CCS_{N_2\rightarrow He}$ 70V	Calcium ligands
Calmodulin	1,545.92 Å²	2,015.83 Å²	0
Calmodulin – Ca^{2+}	1,507.25 Å²	1,507.25 Å² 2,015.83 Å²	4

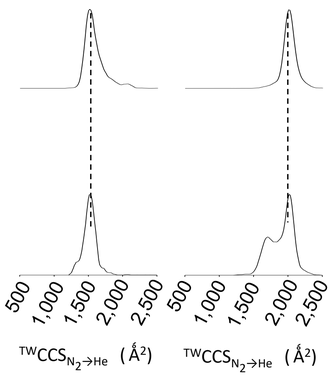
- Preservation of compact conformers up to higher values of trap bias
- Impact the energy barrier

Calmodulin

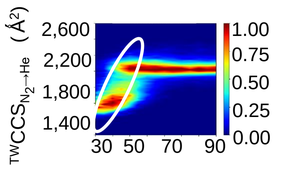
Calmodulin – Ca^{2+}

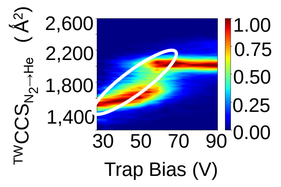


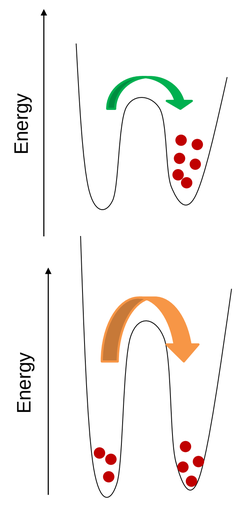
Trap bias = 30V



Trap bias =70V



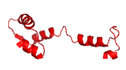




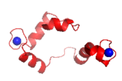
	$^{TW}CCS_{N_2\rightarrow He}$ 30V	$^{TW}CCS_{N_2\rightarrow He}$ 70V	Calcium ligands
M = 8,571.918 Da	1,030.21 Å² 1,188.02 Å² 1,297.48 Å²	1,188.02 Å² 1,297.48 Å²	0
M = 8,652.868 Da	984.96 Å² 1,103.35 Å² 1,229.16 Å²	1,188.02 Å² 1,297.48 Å²	2

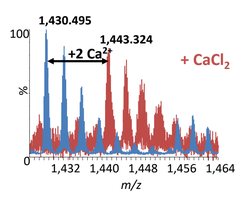
Preservation of compact conformers up to higher values of trap bias.
Impact the energy barrier

M = 8.571,918 Da

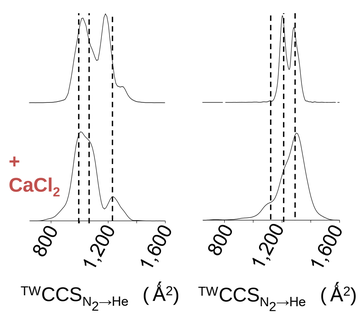


M = 8.652,868 Da

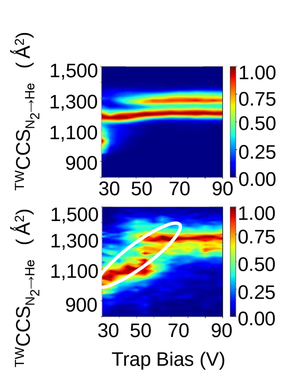




Trap bias = 30V

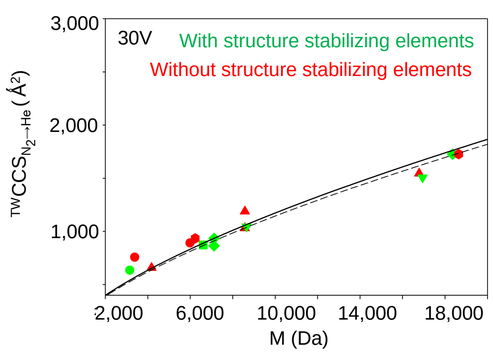


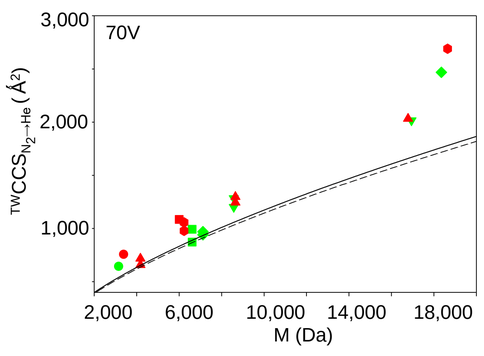
Trap bias =70V



Compact conformer ? Unfolded conformer ?

ModBea
Cytochrome c
Calmodulin
Calmodulin – Ca^{2+}
Native*b*-lactoglobulin
Reduced*b*-lactoglobulin
CCS = 2.45 x $m^{2/3}$ Ruotolo
CCS = 2.39 x $m^{2/3}$ MS Lab





Conclusion

IMS - CIU

CCS < CCS
Resilience > Resilience

In this work, a workflow based on the combination of CIU and IM-MS was evaluated to assess structural features of proteins and residual structures generated during an enzymatic digestion reaction. It was demonstrated that monitoring the CCS value evolution as a function of the collisional activation during a CIU experiment is a powerful approach to highlight the importance of structure-stabilizing elements in proteins.

Prevent the formation of highly extended structure and impact energy barriers

1. Grifnée, E. et al. Label-Free Higher Order Structure and Dynamic Investigation Method of Proteins in Solution Using an Enzymatic Reactor Coupled to Electrospray High-Resolution Mass Spectrometry Detection. *J. Am. Soc. Mass Spectrom.* **2021**, jasms.1c00274. https://doi.org/10.1021/jasms.1c00274.

2. Ruotolo, B. T.et al. Ion Mobility–Mass Spectrometry Analysis of Large Protein Complexes. *Nat Protoc* **2008**, *3* (7), 1139–1152