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Ion Mobility-Mass Spectrometry Coupled with RAPTOR Structure Prediction and CID for Probing Chemokine Conformation and Stability

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Abstract

Unique to ion mobility-mass spectrometry (IM-MS) is the ability to provide collision cross-section (CCS) data and the capacity to delineate any dissociation and/or unfolding of protein complexes. The strong correlation of the experimentally determined CCS with theory is indicative of the retention of native structure in the gas phase, which in turn, qualifies as a means in evaluating the IM-MS data. The assessment of IM-MS data, however, is currently impeded due to the lack of appropriate structural coordinates to use as input in the *in silico* calculation of theory. To address this issue, this study involves the use of rapid protein threading predictor (RAPTOR) to generate tertiary structures of closely related monomeric chemokines (MCP-1, MCP-3, MCP-4 and eotaxin), and subsequently, utilize these models to estimate the theoretical values. Experimental CCS of both the model proteins and chemokines correlate well with theory generated by RAPTOR. All conformations for $z = 5+$ of chemokines fall within theoretical limits. Of the four chemokines, MCP-4 with $z = 6+$ appears to adopt an extended conformation, while eotaxin gradually unfolds, and the extended structures of MCP-1 and MCP-3 increase in abundance upon activation. Combining RAPTOR with IM-MS and collision-induced dissociation (CID) enables us to interrogate the conformations of homologous proteins with very similar tertiary structures.

INTRODUCTION

Mass spectrometry (MS) has evolved as a useful tool in structural biology and it can be used to analyze intact protein assemblies that, when prepared and electrosprayed properly, can retain their native structures upon transfer from solution into the gas phase.^{1–5} Stoichiometry, topology, dynamics, stability and composition can therefore be determined using these novel “native” mass spectrometry techniques. Over the past several years, MS has been demonstrated to complement or even surpass at times, some of the more traditional biophysical methods, such as cryo-electron microscopy, X-ray crystallography and nuclear magnetic resonance (NMR).

Recently, MS has been coupled to ion mobility permitting simultaneous examination of a protein’s mass, conformation and composition. Separation of mass, charge and shape in ion mobility mass spectrometry (IM-MS) using a stacked ring ion guide cell geometry, is based on the ion’s capacity to traverse a gas-filled cell under the influence of repeating pulsed voltages.⁶ Traveling wave ion mobility mass spectrometry (TWIM-MS) has the ability to provide collision cross-section (CCS) data, accomplished by calibrating the T-wave drift times using protein calibrants with known CCS.⁷ Correlation between the experimental and theoretical CCS is an indication that the folded structure of a protein is retained in the gas phase, and therefore, critical in the data evaluation.

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As a general practice, the atomic coordinates in a database (RCSB Protein Data Bank (PDB)) are widely used as input in the theoretical calculation, which are mainly dependent on either NMR or X-ray crystallography methods. Although there has been a considerable increase in the protein structures deposited in the database (~77,000 to date), a significant number of structures have yet to be unambiguously elucidated. Limitations of the current techniques include: (1) the need for a substantial amount of highly purified sample for NMR, (2) financial cost, (3) time requirement (months to years), and (4) difficulty in obtaining good quality crystals for X-ray crystallography. Oftentimes, PDB files from the database are composed of incomplete coordinates and in some cases, the structural data from X-ray crystallography and NMR are not in good agreement. Consequently, the assessment of IM-MS data is hampered by the lack of well-suited models for theoretical calculations.

A recent review emphasizes a need for more accurate structures that can be used to determine theoretical cross-sections.⁸ Clearly, obtaining accurate models is paramount to the improvement of proper assessment and data interpretation and consequently, in the advancement of the applications of IM-MS for studying protein structures and dynamics. As IM-MS is still gaining momentum, this laboratory and others are developing strategies to establish new methodologies that can be used as a dependable tool in structural biology. With the advances in structure prediction,⁹ IM-MS is becoming a promising tool in reliably generating three-dimensional (3D) protein structures. For example, homology modeling combined with IM-MS was used to produce atomic resolution models of protein complexes.¹⁰ More recently, a method integrating computational methods, IM-MS and incomplete X-ray structures was implemented to generate models of multimeric protein complexes.¹¹

In addition to providing CCS data, IM-MS can furnish additional information about the protein's conformational stability and detailed structural features through collision-induced dissociation (CID). It is now recognized that the CID mechanism of non-covalently bound assemblies generally entails the gradual unfolding of a single subunit, followed by its ejection from the complex.^{12–15} Proton transfer occurs concomitantly as the subunit unfolds, resulting in highly charged subunits proportional to surface area yet disproportionate to mass. Dissociation behaviors and specific non-covalent features of protein ensembles have been successfully studied using IM-MS coupled with CID.^{16–18}

In this report, we demonstrate a strategy employing rapid protein threading predictor (RAPTOR) structure prediction to obtain structural models that strongly correlate with experimental CCS. RAPTOR is capable of providing 3D structures based on homology modeling and protein threading.¹⁹ Homology modeling relies on sequence alignment only, while protein threading is based upon structure-sequence homology. It has three algorithms: no core, non-pairwise (NP) core and integer programming (IP). RAPTOR's approach essentially involves the following: given the amino acid sequence of a protein, it evaluates the sequence to identify conserved and probable secondary structures. It then scans its built-in PDB-based template library, aligns the query sequence against the library and finally, ranks and identifies the best template using rigid statistical measures. Part of the scoring mechanism of RAPTOR incorporates sequence homology, secondary structure, the exposed/buried state and the interaction between two residues in 3D space.

RAPTOR structure prediction in combination with IM-MS and CID has permitted us to interrogate the conformations of closely related monomeric chemokines (MCP-1, MCP-3, MCP-4 and eotaxin). We show that RAPTOR provides accurate coordinates from which to calculate theoretical CCS, which in turn match well with experimentally measured values. Comparison with other prediction algorithms favors RAPTOR while CID allowed us to assess stability of this important class of cytokines. Utilization of IM-MS, CID and

RAPTOR as an ensemble of techniques will allow investigators to make assessments on how these chemokines present themselves to glycosaminoglycans (GAG) thus enabling signaling through the corresponding receptors.

EXPERIMENTAL SECTIONS

Chemokines Preparation

MCP-1, MCP-3, MCP-4 and eotaxin were expressed, isolated and purified as described previously.²⁰ Protein stock solutions (~60 – 100 pmol/μL) were buffer-exchanged twice in 100 mM ammonium acetate (NH₄OAc), pH 6.8 using micro-bio spin columns prepacked with Bio-Gel P-6 (BioRad, Hercules, CA, USA) prior to nanoelectrospray ionization (nano-ESI) TWIM/MS analyses. Protein concentrations were estimated using Nanodrop UV spectrophotometer, absorbance measurement at 280 nm (Thermo Fisher Scientific, Wilmington, DE, USA). Native-like solutions (8 – 15 pmol/μL) were kept on ice until analysis.

Nano-ESI-TWIM/MS Analysis and Collisional Activation

All nano-ESI-TWIM/MS analyses were performed using a T-wave ion mobility mass spectrometry system, Synapt G2 HDMS (Waters Corp., Manchester, UK).^{21–23} The system was equipped with a nanolockspray source, a borosilicate glass capillary sprayer, a T-wave ion guide, a quadrupole and a tri T-wave region (trap, IM and transfer). The tri T-wave region was embedded between the quadrupole and the orthogonal acceleration reflectron time-of-flight (oa-TOF) mass analyzer. For each analysis, a 3 – 6 μL aliquot of the protein solution was loaded onto custom-made borosilicate glass nanotips (1.0 mm OD, 0.78 mm ID), pulled and gold-coated as described previously.²⁴ Nano-ESI was accomplished by application of 0.6–0.8 kV capillary voltages via a conductive elastomer. The acquisition parameters were adjusted to maintain the native state of ions without compromising the transmission efficiency and mass accuracy: cone voltages: 5 – 7 V, extractor voltages: 1 – 2 V, trap direct current (DC) bias: 40 V, trap collision energy (CE): 3 V, transfer CE: 0 V. IM separation was carried-out using a wave velocity of 700 m/s, a wave height of 40 V and nitrogen as the buffer gas (flow rate: 90 mL/min, IM cell pressure: ~3 mbar). Mass spectra were externally calibrated using 2 μg/μL cesium iodide in 50% (v/v) aqueous acetonitrile. Acquisitions were performed in the positive ion mode for 3 min from *m/z* 500 – 5000 at 1 scan/sec.

For collisional activation, the mass-to-charge selected precursor ions at the quadrupole region were activated within the trap cell region (argon gas flow rate: 3 mL/min) by gradually increasing the trap CE. The activated ions were then pulsed and collisionally cooled by helium prior to injection into the IM cell. Acquisition parameters were the same as those specified above.

RESULTS AND DISCUSSION

Collision Cross-Section

Ion mobilities and collision cross-sections (Ω) are obtained in drift tube mobility separators using the Mason-Schamp law,²⁵ expressed below:

$$\Omega = \frac{3q}{16N} \left[\frac{2\pi}{\mu k_b T} \right]^{1/2} \frac{1}{K} \quad (1)$$

where K is the ion mobility, q is the ion charge, N is the gas number density, μ is the reduced mass of the ion (M) and buffer gas (m) given by $(mM / (m + M))$, k_b is the Boltzmann's constant, and T is the absolute temperature.

Unlike drift tube mobility systems where drift time correlates linearly to mobility, drift time in traveling wave IM scale quadratically (as the inverse square of mobility).²⁶ Although the traveling wave IM is more complicated than traditional mobility separator systems because of its configuration and the nonuniform field, TWIM-MS has been demonstrated in the literature to provide CCS values that are in excellent agreement with those derived from drift tube mobility system and/or theoretical models after calibration of the T-wave device.^{27–32}

In this study, the calibration was accomplished using denatured myoglobin with known CCS values as calibrant.^{7, 33} The drift times corresponding to each charge state were corrected to consider only the time spent in the cell (reduced mobility, td''). The CCS of calibrant ions were corrected for both the charge state and reduced mass (corrected CCS). The corrected CCS values were plotted against td'' and fitted to a power series curve. The drift times for the target charge state were also normalized followed by the calculation of experimental CCS using the power series equation. Identical parameters were applied for both the calibrant and chemokines. CCS calibration curves covered nearly the entire drift times and mass-to-charge of chemokine ions with correlation coefficients > 0.998 (Figure S1 in the Supporting Information). All measurements were made in triplicate with $< \pm 1\%$ relative standard deviation (RSD). CCS measurements over several months were highly reproducible, within $< \pm 1\%$ RSD among datasets.

Theoretical CCS values were estimated using projection approximation (PA) and exact hard sphere scattering (EHSS) methods. The calculations were implemented in the modified version of MOBCAL program (first implemented in ref. 7).^{7, 34, 35} Briefly, the PDB files were first converted into a readable format prior to calculation. After each calculation, the program provided both the PA and EHSS values.

It is important to note that given the wide range of CCS values between the PA and EHSS theoretical values using MOBCAL, it is possible that multiple conformations of the chemokine are possible. Unfortunately, this field of research has no other accepted programs that allow us to calculate tighter boundaries between the PA and EHSS methods. However, equally important is the observation that RAPTOR at least allows the investigator to produce the “best fit” structure based on the experimental cross section measured, and the experimental CCS is within the PA and EHSS theoretical boundaries as calculated using RAPTOR data.

Structure Prediction and Evaluation

All PDB files used for theoretical calculations were obtained from RAPTOR (Bioinformatics Solutions Inc, Waterloo, ON, Canada). The automatic generation of protein 3D structures was accomplished using a comparative modeling method (OWL), and side chains were modeled via tree-decomposition method. Both homology modeling and NP core methods were carried-out to obtain candidate structures. The candidate structures were evaluated and specifically selected to satisfy the following: (1) general topology consistent with CC chemokines - three antiparallel beta strands, one C-terminal alpha helix (2) generated from the best-fit structural template (3) 100% atomic coverage (4) z-score should be within the range for native proteins, and (5) theoretical and experimental CCS are in agreement.

To further validate the chosen structures, comparison between the RAPTOR-derived and database structures was accomplished using a flexible protein structure alignment algorithm (FATCAT)³⁶ - rigid method, available in the RCSB Protein Data Bank (PDB) web portal as a java application. FATCAT method takes into account the conformational flexibility, providing a more accurate comparison. Generally, the algorithm first identifies compatible aligned fragment pairs (AFP), which are based on the similarities in local geometry prior to all backbone (C_{α}) alignment. The overall similarity between the two structures is given a root mean square deviation (RMSD) value. Structural assessment was also performed using protein structure analysis (ProSA)³⁷ to recognize any errors in the model. The program provides a z-score value to describe the overall quality.

Assessment and Validation of Theoretical CCS

CCS data of standard proteins were also examined to validate the RAPTOR-derived theoretical values. The theoretical CCS listed in Table 1 showed good correlation with experimental (from this laboratory and from literature^{31, 32, 38}) and/or theoretical values calculated using the PDB files from the database. However, as mentioned earlier, it is important to note that a few of these PDB files from the database have either incomplete atomic coordinates or have additional residues arising from cloning artifacts (e.g. in bovine carbonic anhydrase, BCA).

Additionally, theoretical values from RAPTOR-derived models were compared to other structure prediction programs such as Phyre³⁹ and I-TASSER.⁴⁰ Among the three prediction programs, only the CCS values calculated from RAPTOR for chemokines, MCP-1 and eotaxin correlated the best with the experimental CCS as shown in Table S2 (Supporting Information). For BCA, both RAPTOR and I-TASSER-derived structures were in good agreement with experimental CCS.

Tertiary Structures of Chemokines and Model Evaluations

Chemokines are a family of small (8–12 kDa), secreted chemotaxis-inducing proteins involved in many homeostatic, pathological and inflammatory processes.^{41, 42} These proteins (~50 in humans) are generally classified into four subfamilies according to the position of the N-terminal cysteine residues - C, CC, CXC, and CX₃C, where X is any amino acid.⁴³ The proteins' sequence and the predominant receptors are listed in Table S1 (Supporting Information). These chemokines share a significant sequence similarity (>50%); hence, the secondary and tertiary structures are expected to be similar. All RAPTOR predicted structures adopt the typical fold of monomeric CC chemokines; a highly flexible N-terminal domain followed by an extended N-loop, three anti-parallel beta pleated sheets, two intramolecular disulfide bonds and a C-terminal alpha helix.⁴² To evaluate the structures, comparisons were made between the RAPTOR structures and the structures in the RCSB PDB database. The structure prediction for MCP-1 (Figure 1A), when compared to the crystal structure, PDB ID: 3IFD, resulted in an RMSD of 2.40 Å for residues 7–70 (64 equivalent positions). *It is important to note that the structure from the database has ten missing residues, thus, the comparison accounted for 97% of the total protein sequence.* Likewise, structural comparisons of MCP-3 and eotaxin yielded low RMSD values of 2.00 Å and 2.07 Å, respectively. All structural comparisons covered >85% of the total amino acid sequence in each protein. In addition to alignment with structures determined by x-ray or NMR methods, the z-scores of the predicted structures were within the typical range for native proteins. The MCP-1 structure, in particular, was consistent to those structures solved by NMR, z-score = -4.70 (Figure 1B). This observation also holds true for the other three chemokines with z-scores ranging from -3.9 to -4.5. MCP-4, on the other hand, has a single entry in the database (based on its X-ray structure, PDB ID: 2RA4), which consists solely of the dimer - i.e. there is no structure for the monomer. For this particular protein,

RAPTOR is the only source for obtaining good structural information, and consequently, the appropriate theoretical CCS data. In the case of MCP-4, the best structure derived from the RAPTOR output was missing four residues (residues 4 – 8) and therefore, did not satisfy the criteria (i.e. 100% atomic coverage). Thus, we chose the second best model as a candidate structure for MCP-4 (Figure 1C). All models selected fulfilled the above criteria and were derived using the NP core method.

Nano-ESI-TWIM/MS Analysis and CCS Determinations

The mass spectra of all chemokines displayed predominantly monomers with charge states of 5+ and 6+, characteristics typical for folded proteins⁴⁴ - Figure 2A. The appearance of fewer charge states is due to the limited accessible surface area for protonation. MCP-1, by contrast, existed as both monomer-dimer in approximately equal amounts, while MCP-3, MCP-4 and eotaxin showed mainly monomer with some dimer present. These observations correlate with earlier data, which demonstrated that MCP-3, MCP-4 and eotaxin are monomeric under experimental conditions.^{45–47} We have also shown previously that MCP-1 exists in equilibrium between monomer and dimer forms.^{20, 24, 48} Furthermore, the gas phase observed charged states are lower than the predicted, based on the Rayleigh limit predicted charge state model.⁴⁹ The most abundant ion observed, 6+, for MCP-3, MCP-4 and eotaxin is 83% of the predicted charge (7.2+). This finding is consistent with literature,⁵ providing evidence that the native fold of chemokines in solution are preserved upon transfer in the gas phase.

When the corresponding masses for the 5+ charge state were extracted, the mobility signals exhibited a single conformation as demonstrated in Figure 2B. The extracted mobility signals for $z = 5+$ were near symmetric and approached Gaussian distribution. The experimental CCS of MCP-1, MCP-4 and eotaxin were quite similar and slightly lower than that of MCP-3. Whereas, $z = 6+$ of eotaxin exhibited two major conformations and a minor conformer. The majority of the other three chemokines showed asymmetry and some evidence of protein elongation and unfolding.

The comparison of experimental and theoretical CCS is provided in Figure S2 (Supporting Information). With the exception of MCP-4, $z = 6+$, all conformations of chemokines were within the theoretical limits. These results suggest that the proposed model structures for these chemokines could likely be well correlated to their cross-sections in the gas phase.

Collisional Activation of CC Chemokines

In order to assess the stability of the CC chemokines and to obtain insights on their unfolding/dissociation behaviors, deliberate activation of the proteins was carried-out by gradually increasing the trap CE. Figure 3A shows that the $z = 5+$ of MCP-3 and eotaxin remained stable and unaffected after collisional activation. On the contrary, the minor and more compact structure of MCP-4 at 6.17 ms and MCP-1 at 6.06 ms disappeared at 20 V CE. Additionally, at 20 V CE, the major conformation at 7.72 ms of MCP-4 shifted to a longer drift time at 8.05 ms. The mobility signal for the major 5+ ion at 7.72 ms of MCP-4 at 5 V and 10 V also appeared to be slightly asymmetric compared to MCP-1, MCP-3 and eotaxin (Figure 2B), indicating possible elongation to one final open structure.

After 20 V, this structure remained stable even at higher CE values. It is possible that beyond this voltage, MCP-4 has taken on a maximum elongated structure for this charge state. Consistent with prior observations, the resilience to unfolding and stability of the compact 3D structures of CC chemokines may well be attributed to the presence of the two intramolecular disulfide bonds.^{24, 50} The presence of these disulfide bonds in chemokines maintain the functional domains intact, which are critical for biological activity.⁵¹

Figure 3B shows that the 6+ ion of MCP-4 remained stable during activation. By contrast, the more compact structure of eotaxin gradually unfolded to a more extended conformation. The gradual unfolding of eotaxin follows the same behavior as that of activated protein assemblies assuming partially folded intermediates prior to dissociation.¹⁷ This behavior of eotaxin also conforms to the widely recognized model for activation of protein complexes by CID.^{13, 14} While the more compact structures of MCP-1 and MCP-3 remained stable, the more elongated structures of each, however, observed to have increased in abundance at increasing CE values. On the contrary, the 6+ ion of MCP-4 appears to have adopted an extended state prior to dissociation. This is consistent given the data shown for the 5+ ion mentioned above for MCP-4. Possible explanations for this is that, MCP-4 with the most exposed basic sites (six) at the C-terminal region (61–75 residue) induces Coulomb repulsion, which in turn produces a more elongated structure. MCP-4 has also the least content of neutralizing charges⁵² (five acidic residues) among the four chemokines. The intrinsic properties of MCP-4 clearly influence these distinct differences, which are not observed for the other chemokines studied.

Structural Comparison of CC Chemokines

Having the appropriate models allows us to further examine the link between the structural differences, the gas-phase behaviors, and the putative biological functions of chemokines. The comparison between MCP-1, a well-studied chemokine, and MCP-3, MCP-4, eotaxin shows that their N-terminal regions vary (Figure 4A). The result shown in Figure 4A is fully consistent with this finding where N-terminal motifs are demonstrated to be crucial for receptor binding and signaling^{46, 53} and specifically, the N-terminal regions are also identified as specific determinants for the recognitions of MCP-1 and eotaxin to their receptors.⁵⁴

In addition, the chemokine's activity could also be influenced by GAG binding and oligomerization.^{47, 55, 56} Figure 4B indicates the GAG key binding residues in MCP-1⁵⁵ in comparison to the other three chemokines. In contrast to MCP-1, Arg¹⁸ identified as the most important key residue in MCP-1-GAG interaction, is substituted with Lys¹⁸ in both MCP-3 and MCP-4. These substitutions may likely play a role in the ability of the chemokines to oligomerize. The contribution of the N-termini and other critical residues such as Tyr¹³, for example, may also influence this behavior. From the structural alignment, Tyr¹³ was replaced by phenylalanine in eotaxin and MCP-4. The substitutions of these key residues in signaling, binding and dimerization of chemokines might influence these processes as previously demonstrated.⁵⁵

CONCLUSIONS

The combination of RAPTOR structure prediction with nano-ESI-TWIM/MS and CID allows us to investigate the conformations of a subfamily of monomeric CC chemokines (MCP-1, MCP-3, MCP-4 and eotaxin). The RAPTOR-derived models align well with structures elucidated by well-established methodologies (NMR and X-ray crystallography). Such models could then be used in calculating the theoretical CCS and consequently, in evaluating the IM-MS data. Experimentally determined CCS of both the standard proteins and chemokines are also in excellent agreement with RAPTOR-derived theoretical models. The results convey that these models resemble the gas-phase CCS and that the folded structures are retained upon transfer in the gas phase. Although monomeric chemokines share significant sequence similarity and therefore, the same topology, results from collisional activation reveal distinct gas-phase behaviors and are likely attributable to their intrinsic characteristics. The practical application of the methodology developed here is therefore suitable for comparative structural analysis of other homologous proteins. Recent

advances and developments in this field will greatly enhance the applicability of IM-MS as a tool in structural biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Comparison of MCP-1 structures: RCSB PDB database, PDB ID: 3IFD (yellow) and RAPTOR predicted structure (blue) with RMSD = 2.40 Å (A). The predicted structure falls within the typical range of native protein structures (B). The z-score lies within the limit of scores as those determined by NMR. Shown in C is a RAPTOR generated 3D structure of MCP-4, helix (red) beta-sheet (rainbow), loop (white). Structures are rendered using Swiss-PdbViewer.

Figure 2.

Heat map of chemokines nanoelectrosprayed in 100 mM NH_4OAc - **A**. The color scheme depicts the ion intensity using the square root scale. The insets show the mass spectra with predominantly protonated ions, 5+ and 6+. The expanded signal of $z = 5+$ for eotaxin exhibits 0.20 m/z spacings, confirming the identity of its monomeric form. Extracted ion mobility signals for the $z = 5+$ (left) and 6+ (right) are shown in **B**. Experimental CCS (average) are indicated on top of each peak.

Figure 3. Extracted ion mobility signals for $z = 5+$ (A) and $z = 6+$ (B) of MCP-1, MCP-3, MCP-4 and eotaxin at increasing trap CE from 5 V to 60 V.

Figure 4. Structural alignments of MCP-3, MCP-4 and eotaxin with MCP-1 (**A**) – helix (red), beta-sheet (yellow), loop (white/blue). jFATCAT (rigid) method was used to compare the structures. Glycosaminoglycans key binding residues (from ref. 51) are indicated in **B**. Structural coordinates are generated using RAPTOR and rendered using Swiss-PdbViewer.

Table 1

Collision cross-section determinations of standard proteins.

Standard Protein	Theoretical CCS ($\text{\AA}^2$)*				Experimental CCS ($\text{\AA}^2$)	
	PDB ID	Method	RCSB PDB	RAPTOR	Unpublished data from this lab**	Literature
Bovine Carbonic Anhydrase	1V9I (X-ray)	PA	1893	1880	2060 (9+)	2004 (9+) ³²
		EHSS	2420	2412		
Chicken Lysozyme	1GXX (NMR)	PA	1171	1161	1299 (6+)	1333 (6+) ³¹
		EHSS	1450	1448		
Equine Cytochrome-C	1HRC (X-ray)	PA	1056	1005	1226 (5+)	1238 (5+) ³¹
		EHSS	1318	1249		
Bovine Ubiquitin	1V81 (NMR)	PA	907	763	857 (4+)	791 (4+) ³¹
		EHSS	1127	925		

* CCS are estimated using projection approximation (PA) and exact hard sphere scattering (EHSS) methods. The PDB files used as input are from the RCSB PDB (pdb files are indicated next to the protein) and from RAPTOR structure prediction software.

** CCS are determined using nanoESI-TWIM/MS. Denatured equine myoglobin was used as calibrant.