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Native-like and Denatured Cytochrome *c* Ions Yield Cation-to-Anion Proton-Transfer Reaction Products with Similar Collision Cross Sections

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Abstract

The relationship between structures of protein ions, their charge states, and their original structures prior to ionization remains challenging to decouple. Here, we use cation-to-anion proton-transfer reactions (CAPTR) to reduce the charge states of cytochrome *c* ions in the gas phase and ion mobility to probe their structures. Ions were formed using a new temperature-controlled, nanoelectrospray ionization source at 25 °C. Characterization of this source demonstrates that the temperature of the liquid sample is decoupled from that of the atmospheric-pressure interface, which is heated during CAPTR experiments. Ionization from denaturing conditions yields 18+ to 8+ ions, which were each isolated and reacted with monoanions to generate all CAPTR products with charge states of at least 3+. The highest, intermediate, and lowest charge-state products exhibit collision cross section distributions that are unimodal, multimodal, and unimodal, respectively. These distributions depend strongly on the charge state of the product, although those for the intermediate charge-state products also depend on that of the precursor. The distributions of the 3+ products are all similar, with averages that are less than half that of the 18+ precursor ions. Ionization of cytochrome *c* from native-like conditions yields 7+ and 6+ ions. The 3+ CAPTR products from these precursors have slightly more compact collision cross section distributions that are indistinguishable from those for the 3+ CAPTR products from denaturing conditions. More broadly, these results indicate that the collision cross sections of ions of this single domain protein depend strongly on charge state for charge states greater than ~4.

Graphical abstract

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Electronic Supplementary Material. Figures S1 to S3 and method for converting arrival-time distributions to Ω distributions.

Introduction

Ion mobility (IM), mass spectrometry (MS), and allied methods have emerged as powerful tools for characterizing the structures of biological molecules and their noncovalent complexes [1–4]. IM separates ions in a neutral gas based primarily on their charge and shape, which can be quantified by determining the collision cross section (Ω) of the ion-neutral pair [5]. For example, Ω of complexes that contain 18, 40, 60, and 80 copies of the capsid protein of norovirus are consistent with sheet-like intermediates that are capable of forming capsids, rather than assembly-incompetent aggregates [6]. These approaches can also be extended to study unfolded states, intermediates, and other forms of structural heterogeneity [7]. For example, IM spectra of $[\text{Pro}_{13} + 2\text{H}]^{2+}$ ions generated from a sample that was transferred from mostly propanol to mostly water exhibit a total of eight features with different Ω whose abundances depend on time since solvent transfer [8]. The time-dependence of the IM results is strongly correlated with that for an orthogonal analysis using capillary electrophoresis, which suggests that both experiments are sensitive to the same structural transitions in solution [9].

Despite the effort and progress in using gas-phase measurements to probe the structures of biological molecules in solution, a robust understanding of the relationship between the charge state of the gas-phase ion, the structure of the gas-phase ion, and the original structure in solution remains elusive. Electrospray ionization of large, globular proteins and protein complexes typically yields ions that have a relatively narrow range of charge states, relative to their denatured counterparts. The Ω of those ions can depend weakly on charge state [10–13] and polarity [12], but the range of Ω values for each analyte usually exceeds the precision of those measurements. Electrospray ionization of proteins from acidic, denaturing solutions [14–16], as well as prion [17] and intrinsically disordered [18] proteins

from neutral, aqueous solutions, yields ions with a broad range of charge states and whose Ω depend strongly on their charge state. An improved understanding of the phenomena underlying these observations is important for maximizing the structural information that can be drawn from IM experiments. Two of the ongoing challenges include (1) the relationship between the structure(s) in solution and the resulting charge-state distribution after ionization [12, 19] and (2) the extent to which structure(s) in solution are retained or evolve in the corresponding gas-phase ions [20, 21].

One strategy to probe the relationship between the structures and charge states of protein ions is to manipulate their charge states in the gas phase. For example, in ion/neutral proton-transfer reactions a multiply charged precursor cation is reacted with a neutral molecule, A, which has a higher gas-phase basicity [22, 23]:

Foundational results combining ion/neutral proton-transfer reactions in tandem with IM-MS showed that mixtures of protein ions that have high-charge states and unfolded structures can yield product ions that have lower charge states and partially folded structures [24]. Alternatively, the charge states of multiply charged protein cations can be reduced using ion/ion proton-transfer reactions with monoanions:

Relative to ion/neutral reactions, ion/ion reactions benefit from more favorable kinetics and thermodynamics [25–27]. Ion/ion proton-transfer reactions were pioneered by McLuckey and coworkers [26, 27], who perform these reactions under pseudo first-order kinetics with effectively constant anion abundance [26–28]. Recently, we reported an approach that we refer to as cation-to-anion proton-transfer reactions (CAPTR) [29], in which the abundance of anions depletes during individual experiments and as a result a wide range of product ion charge states are formed from different numbers of sequential proton-transfer events [29]. Electron transfer can also be used to reduce the charge states of protein ions (electron transfer no dissociation) [30], but electron transfer can also result in bond cleavage [31, 32].

Recently, we reported the analysis of the CAPTR products of m/z -selected ions of denatured ubiquitin [33]. In those experiments, each subsequent CAPTR event resulted in the formation of a charge-reduced product ion that had a more compact Ω . The Ω of the CAPTR product ions depended on their charge state, and were independent of the charge state of the precursor ion. One particularly interesting observation from that work was that the lowest-charge-state product ion observed (3+) exhibited a Ω similar to that determined experimentally for native-like ions of ubiquitin [34, 35] and determined computationally for an energy-minimized version of an NMR structure [35]. One limitation of that work was that Ω of some precursor ions depended on the temperature of the MS interface, which is heated during CAPTR experiments to prevent the buildup of byproducts of glow-discharge

ionization on electrodes in the atmospheric-pressure interface. Those changes in Ω were attributed to heat transfer from the MS interface to the sample capillary [33].

Here we use IM-MS to characterize the CAPTR products of ions of cytochrome *c*, which is a single domain protein bound to a heme prosthetic group. Ions were formed using a new temperature-controlled, nanoelectrospray ionization source, which enables independent temperature control of the sample capillary and MS interface. In these experiments, the sample capillary was set to 25 °C, and ions of cytochrome *c* were generated from either denaturing or native-like conditions. These results show that the CAPTR product ions with the lowest charge state (3+) all have similar Ω distributions, regardless of the Ω distribution of the precursor ion or whether the precursors were formed from denaturing or native-like conditions.

Methods

Samples and ionization

Cytochrome *c* from equine heart (Sigma-Aldrich, St. Louis, MO) was dissolved into either a denaturing solution of 70:30 water:methanol with 0.1% trifluoroacetic acid at pH 2 (denaturing conditions) or 200 mM aqueous ammonium acetate at pH 7 (native-like conditions). Ubiquitin (Boston Biochem, Cambridge, MA) was prepared using denaturing conditions. All cations were generated by electrokinetic nanoelectrospray ionization from borosilicate glass capillaries that were pulled to a 1 to 3 μ m tip on one end using a Sutter Instruments Model P-97 micropipette puller (Novato, CA) [36].

For most experiments, the sample capillary was held at 25 °C using a new temperature-controlled, nanoelectrospray ionization source, which decouples the temperatures of the sample and the atmospheric-pressure interface. This source will be referred to as the “temperature-controlled source” and is shown in Figures 1. The sample capillary was positioned between three layers of thermally conductive and electrically insulating silicone elastomer (3M 5592 and 5591S, St. Paul, MN) that are each 1 mm thick. A platinum wire was inserted into the wide end of a capillary to apply a voltage to the solution for electrospray (Figure 1b). The thermally conductive elastomer layers and sample capillary were placed on top of a Peltier device (Laird Technologies 430126-503, Milpitas, CA), which moves heat between the elastomer and a ¼” by 1” by 12” bar of copper. The copper bar exchanges heat with a heat sink (Cooler Master Hyper T2, New Taipei City, Taiwan). The copper bar was then inserted into a nylon sleeve placed on top of an aluminum bracket, which was attached to a three-dimensional translational stage (Physik Instrumente, Karlsruhe, Germany). All thermal connections were made using thermal paste (TE Technologies TC-1). The temperature of the Peltier is monitored using a thermistor (TE Technologies MP-3193) placed on top of the Peltier device and next to the capillary (Figure 1b). Based on the thermistor temperature, the voltage supplied to the Peltier device is adjusted by a controller (TE Technologies TC-48-20 OEM). Selected experiments, as indicated, used the Waters NanoLockSpray source and the nanoelectrospray ionization glass capillary assembly (M955213AC1-S), which will be referred to as the “original source.” This assembly holds the capillary near the atmospheric-pressure interface and does not have temperature control. Photographs of both sources are shown in Figure S1.

CAPTR and IM-MS experiments

All experiments were performed on a Waters Synapt G2 HDMS instrument equipped with a radio-frequency (rf) confining ion mobility drift cell [37] and ion/ion reaction capabilities [38]. CAPTR was performed as described previously [33]. Briefly, for 0.1 s the $[M-F]^-$ fragments of perfluoro-1,3-dimethylcyclohexane (PDCH) were produced, quadrupole selected, and accumulated in the Trap Cell of the instrument. $[PDCH-F]^-$ reacts exclusively through proton transfer (Reaction 2) [29, 39, 40]. Following anion accumulation, the instrument was switched into positive mode for 5 to 10 s, during which time a single charge state of cytochrome *c* was quadrupole selected and transferred into the Trap Cell for CAPTR. Every 22 ms, CAPTR products and unreacted precursor ions were injected into the rf-confining drift cell [37] with a 212 V drift voltage and filled with 2.0 mbar helium. Based on the measured arrival-time distributions, the apparent Ω distributions were calculated using a method described in the Electronic Supplementary Material that is identical to that used previously for the CAPTR products of denatured ubiquitin [33].

Calculated Ω

For the native model, Chimera [41] was used to add missing side-chain and hydrogen atoms to an x-ray crystal structure of cytochrome *c* (PDB: 1HRC [42]). The linear and α -helical models lack the heme group and were built using Chimera and the expected dihedral angles. Ω values were calculated using the projection approximation (PA) [43] and the exact hard-sphere scattering approximation (EHSS) [44] as implemented in EHSS2/k [45]. These approximations and their relationship to momentum transfer in IM have been discussed elsewhere [5, 44].

Results

Previously, we reported the analysis of the CAPTR products of m/z -selected ions of ubiquitin from denaturing conditions [33]. The Ω values of the CAPTR product ions depended strongly on the charge state of the product ion. Furthermore, the Ω of the lowest charged state product ions were similar to those for native-like ions of ubiquitin, implying that although the CAPTR product ions had folded in the gas-phase, they had a similar size to the native-like structure of ubiquitin in the gas phase. One limitation of those experiments was that Ω of the precursor ions depended on the temperature of the atmospheric-pressure interface to the mass spectrometer. In order to decouple the temperature of the interface and the liquid sample, we developed a new temperature-controlled, nanoelectrospray ionization source. Using that source, we generated ions of cytochrome *c* from both denaturing and native-like conditions, which were m/z selected prior to CAPTR and IM-MS analysis. These results provide detailed insights into how protein ions from denaturing and native-like conditions respond to changes in their charge state.

Effect of the temperature of the atmospheric-pressure interface on the temperature of the sample

Anions for these ion/ion chemistry experiments are generated using glow-discharge ionization performed immediately after the sample cone, which is also used to transfer analyte cations from atmospheric pressure to vacuum. During these experiments, this region

(the “MS interface”) is resistively heated to 120 °C to minimize the buildup of byproducts of glow-discharge ionization on the interface electrodes. Previously, we reported that the Ω distributions of denatured 7+ ubiquitin using the original source depended on the temperature of the MS interface [33]. Those results are replotted in Figure 2a. At ambient temperature, the Ω distribution was bimodal with features centered around 13 and 15 nm². However, when the interface temperature was increased to 40 °C, the distribution shifted to a single feature centered near 15 nm². The latter distribution persisted as the temperature of the MS interface was increased to 120 °C.

In order to characterize how the temperature of the MS interface affects the temperature of samples in the original source, we positioned a thermocouple inside a sample capillary containing deionized water. Temperature measurements were made using the conditions used for CAPTR experiments, but without a platinum wire electrode. Figure 3a shows the temperature measured inside of a capillary as a function of the temperature of the MS interface (*black triangles*). Using the original source, the temperature of the sample depends strongly on the temperature of the MS interface, which is consistent with the convective heat transfer. As the interface temperature increases from 28 to 120 °C, the temperature of the sample in the original source increases from 27 to 48 °C, which may induce thermal melting of proteins in solution.

Performance of the temperature-controlled source

In order to control the temperature of samples prior to ionization, we constructed a temperature-controlled source that is described in the *Methods*, shown using a diagram in Figure 1, and shown using photographs in Figure S1. Figure 3a also shows the temperature of samples using the temperature-controlled source as a function of the temperature of the MS interface and the Peltier device (*colored circles*). Unlike those of the original source, the temperature of the sample in the temperature-controlled source is independent of the temperature of the MS interface.

Figure 3b shows the temperature of the sample as a function of the temperature of the Peltier device, using interface temperatures of 28, 60, and 120 °C. As indicated in Figure 3a, the temperature in the capillary depends strongly on the temperature of the Peltier device and is independent of that of the MS interface. Figure S2 shows that the differences between the temperatures of the Peltier device and the sample capillary are less than 1 °C for Peltier device temperatures from 20 to 30 °C and all MS-interface temperatures. Over the full range of Peltier device temperatures of 5 to 70 °C, the magnitude of the differences between the set temperatures of the Peltier device and the sample capillary span from +2 to –3 °C with increasing temperature. These small differences are consistent with inefficient heat transfer between the sample and the Peltier device.

Several approaches for controlling the temperature of samples for nanoelectrospray ionization have been reported, including the use of forced air in proximity to the sample capillary [46, 47] or flowing the sample through a temperature-controlled device [48]. The present implementation is most similar to those reported by Robinson and coworkers [49] and later by Heeren and coworkers [50], in which a gold-plated sample capillary was enclosed in a stainless-steel capillary sleeve. The capillary sleeve was in thermal and

electrical contact with the gold-plated capillary, which was electrically biased to provide the electrospray potential and was in thermal contact with the Peltier device. The present design uses thermally conductive and electrically insulating elastomer to establish thermal contact with the capillary, and a separate platinum wire inserted into the capillary to provide the electrospray potential. This approach provides a facile solution to ensuring independent thermal and electrical contact with the sample.

In order to further evaluate this temperature-controlled source, we measured the Ω distributions of 7+ ubiquitin from a denaturing solution as a function of the temperature of the interface using a Peltier-device temperature of 25 °C (Figure 2b). With the interface at ambient temperature, the Ω distribution is bimodal and similar to that measured using that interface temperature and the original source (bottom distribution in Figure 2a). As the interface temperature was increased up to 120 °C, the more compact feature centered around 12 nm² persists at approximately the same relative intensity. The retention of the compact form of 7+ ubiquitin ions when using an interface temperature of 120 °C suggests that the elevated interface temperature for CAPTR is decoupled from the remainder of the experiment when using this temperature-controlled source. Note that controlling the temperature of the sample does not preclude structural isomerization in the gas phase aided by elevated gas temperatures in the interface, although no evidence for that was observed here.

Ω of cytochrome *c* ions

Using the temperature-controlled source and denaturing conditions, we first measured the arrival-time distributions of cytochrome *c* ions generated directly using nanoelectrospray as a function of *z*. Using the procedure described in the Electronic Supplementary Material, we converted the measured arrival-time distributions to apparent Ω distributions. We then calculated critical Ω values that correspond to 10%, 50%, and 90% of the cumulative distribution function (CDF), which is the integral of the apparent Ω distribution with respect to Ω . A representative Ω distribution (*solid black line*), CDF (*red line*), and the corresponding set of critical Ω values (*dashed black lines*) are shown in Figure 4a. Those critical Ω values for each ion are represented by the lower bars, middle points, and higher bars in Figure 4b, respectively. In agreement with other measurements of Ω for denatured protein ions with helium gas [10, 15, 51], the Ω values of cytochrome *c* ions from denaturing conditions increase with increasing *z*.

The Ω of cytochrome *c* ions from water/methanol/acetic acid (49/49/2%) [10] and water/ acetonitrile/acetic acid (75/25/0–4%) [51] have been reported previously. Compared to the former study [10], the Ω determined here are 0.7% to 3.0% larger. Although these differences are comparable to the absolute errors estimated for rf-confining drift cells [10, 37], there may also be contributions from the CDF data analysis used here versus the centroid of the best-fit Gaussian functions used previously. Compared to the latter study [51], the Ω determined here range from 5.5% smaller to 3.4% larger. Those differences may be attributable to some combination of differences in instrumentation, solution conditions, and data analysis.

Using this approach, we also measured the arrival-time distributions of cytochrome *c* ions from 200 mM aqueous ammonium acetate at pH 7 (Figure 4b, black squares), which will be referred to as native-like ions. The 50% value of the CDF for the 7+ and 6+ native-like ions are 14.0 and 12.7 nm². In comparison to previous results for native-like cytochrome *c* (Figure 4b, magenta diamonds [12]), these values are +2.4 and -7.2% different, respectively. The 2.4% difference for the 7+ ions may not be significant, given the absolute errors estimated for rf-confining drift cells [10, 37] and differences in data analysis. The -7.2% difference of the 6+ ion reported here is much more compact than observed previously, which may be the result of differences in the ionization and extents of activation in the two experiments. Furthermore, the apparent resolving powers for the ion mobility analysis of native-like ions of cytochrome *c* were previously found to be low relative to other native-like ions [37], which may accentuate differences in data analysis.

The projection approximation (PA) [43] and exact hard-sphere scattering (EHSS) [44, 45] Ω for a model of native cytochrome *c* are (i) 10.8 and (ii) 13.5 nm², respectively (Table 1). These calculated values bracket the experimental results for native-like cytochrome *c*, consistent with reports that the EHSS method usually overestimates the experimental Ω of native-like ions [52] and that the PA method usually underestimates the experimental Ω of native-like ions [21, 52]. Because we do not have structures for non-native forms of cytochrome *c*, we instead built linear and α -helical models that do not contain a heme group. The Ω calculated for these models (iii to vi) span a broad range of values. Comparisons between the experimental Ω for cytochrome *c* generated from native-like and denaturing conditions (Figure 4b) and the theoretical Ω for models (Table 1) indicate that these experiments probe a diverse set of structures, from those that are compact and comparable in size to the native structure to those that are extremely extended and have minimal interactions between neighboring amino acids.

CAPTR of cytochrome *c* ions from denaturing conditions

The precursor ions generated using denaturing conditions were each m/z selected and subjected to CAPTR. For the remainder of the discussion, the CAPTR product ions will be referred to as “ $P \rightarrow C$ ”, where P and C are the charge states of the precursor and product ions, respectively. Figure 5a shows the Ω distributions of the $18 \rightarrow C$ ions from denaturing conditions, which are also reproduced in Figure S3a. The Ω of the $18 \rightarrow C$ ions decrease with decreasing charge. From $C = 18$ to 3, there is over a 50% compaction in Ω , which is consistent with the product ions folding to form additional intramolecular interactions as charge is removed. The Ω distributions of the highest- C (18 to 11) and lowest- C (4 and 3) products (Figure 5a) are qualitatively unimodal, *i.e.*, they exhibit a single local maximum and are relatively symmetric. Note that unimodal Ω distributions may be the result of ensembles of ions with closely related structures or more complex structural distributions with similar Ω . In contrast, the intermediate C products (9 to 5) exhibit multimodal Ω distributions, *i.e.*, exhibit multiple local maxima and/or greater asymmetry that are consistent with greater structural diversity.

The Ω distributions for the $9 \rightarrow C$ ions from denaturing conditions are shown in Figure S3b. There are similarities between the distributions for the $18 \rightarrow C$ and $9 \rightarrow C$ ions of a given C ,

particularly for the $P \rightarrow 4$ and $P \rightarrow 3$ products. The Ω distributions for the intermediate charge state products from $P = 18$ and 9 depend on the identity of the precursor. For example, Figures 5c and 5d show the Ω distributions (*black solid lines*) of $18 \rightarrow 9$ and $9 \rightarrow 9$ ions from denaturing conditions, respectively. These Ω distributions span similar ranges and have features centered around similar Ω values, but the relative intensities of the features are clearly different. The differences in the Ω distributions are also apparent in the corresponding critical Ω values (*black dashed lines*) calculated from the CDFs (*red lines*).

Figure 6a shows the critical Ω values for all CAPTR precursor and product ions of cytochrome *c* from denaturing conditions. The identity of each precursor ion, $P = 18$ to 8, is indicated using a different color. The Ω distributions of the $P \rightarrow C$ ions compact to smaller Ω with each subsequent CAPTR event, which is consistent with the ions refolding to form additional intramolecular interactions as charges are removed. Although the Ω distributions depend strongly on C , they can also depend on P as highlighted for the $18 \rightarrow 9$ and $9 \rightarrow 9$ ions. At low C the change in Ω with each subsequent CAPTR event decreases until the difference between the Ω for the $P \rightarrow 4$ and $P \rightarrow 3$ ions is extremely small. This is consistent with the protein providing adequate shielding between those few remaining charges, suggesting that further charge reduction may not result in further compaction.

CAPTR of cytochrome *c* ions from native-like conditions

The precursor ions generated using native-like conditions were each m/z selected and subjected to CAPTR, using the same procedure described for ions from denaturing conditions. Ω distributions for the native-like $7 \rightarrow C$ ions are shown in Figure 5b and reproduced in Figure S3c. The Ω distribution for $7+$ ions from native-like conditions is smaller in magnitude and narrower in width than the distributions for the $P \rightarrow 7$ ions from denaturing conditions (Figures S3a and S3b). This trend continues for the $7 \rightarrow C$ ions from native-like conditions and the $P \rightarrow C$ ions from denaturing conditions, for $C = 5$ and 6. However, the Ω distribution for the $7 \rightarrow 3$ ions from native-like conditions are indistinguishable from those for the $P \rightarrow 3$ ions from denaturing conditions (Figure S3a and S3b). Relative to the Ω distributions for the ions from denaturing conditions (Figure S3a and S3b), the distributions for the ions from native-like conditions span a far narrower range of values.

The critical Ω values of native-like $7 \rightarrow C$ and $6 \rightarrow C$ ions were determined using the same procedure described for the ions from denaturing conditions and are shown in Figure 6b. Just as with the $7 \rightarrow C$ ions, the $6 \rightarrow C$ ions compact with decreasing C . The most compact products for both precursors are the $P \rightarrow 3$ ions, whose median Ω values are 12.4 and 12.5 nm² for the $7 \rightarrow 3$ and $6 \rightarrow 3$ ions, respectively. These values are 10% and 3% smaller than those for the $7+$ and $6+$ precursor ions, respectively. The $6 \rightarrow C$ ions are more compact than the corresponding $7 \rightarrow C$ ions, but those differences decrease with decreasing C until those for the two $P \rightarrow 3$ ions are effectively indistinguishable. This may be due to the ions compacting to similar structural populations, or different structural populations with indistinguishable Ω distributions.

The critical Ω values for the $18 \rightarrow C$ ions from denaturing conditions are also shown in Figure 6b. Although the critical Ω values for the $18 \rightarrow 7$ and $18 \rightarrow 6$ ions from denaturing

conditions are significantly larger than the corresponding values for the $P \rightarrow 7$ and $P \rightarrow 6$ ions from native-like conditions, respectively, those differences decrease with decreasing C . Therefore, all $P \rightarrow 4$ and $P \rightarrow 3$ ions have similar critical Ω values, regardless of the Ω distribution of the precursor ion or whether the precursors were formed from denaturing or native-like conditions.

Conclusions

These experiments used CAPTR and IM to investigate the relationship between the Ω distributions and charge states of cytochrome c ions generated at ambient temperature from denaturing and from native-like conditions. To enable these experiments, we first developed a temperature-controlled, nanoelectrospray ionization source to control the temperature of the liquid samples, which are in close proximity to the heated atmospheric-pressure interface of the mass spectrometer in these experiments. We characterized this source using measurements of both the temperature of a liquid in the sample capillary (Figure 3) and the Ω distributions of 7+ ubiquitin ions from denaturing conditions (Figure 2b), which were sensitive to the temperature of the atmospheric-pressure interface in previous experiments using the original source (Figure 2a, [33]). These results all show that this temperature-controlled source decouples the temperature of the liquid sample from the temperature of the atmospheric-pressure interface.

Comparisons between the experimental (Figure 6) and calculated (Table 1) Ω indicate that CAPTR results in the formation of a diverse set of structures, from those that are compact and native-like to those that are still very extended and have minimal interactions between neighboring amino acids. The Ω of the products depend most strongly on C . Most notably, the Ω distributions of all $P \rightarrow 3$ ions are very similar and are all centered near 12.4 nm², a value that is bracketed by the two estimates for the native model of cytochrome c (Table 1) and is slightly smaller than the Ω measured for native-like ions of cytochrome c without CAPTR.

Several aspects of the IM results for the CAPTR products of cytochrome c ions from denaturing solutions (Figures 5a, S3b, and 6a) share similarities with those reported for ubiquitin from denaturing solutions [33]. Most notably, the magnitude of the Ω distribution decreases by ~50% from the highest C observed to the lowest C observed in both cases. Furthermore, in both cases the products with the highest and lowest values of C exhibit Ω distributions that are unimodal, whereas those with intermediate values of C exhibit multiple features. However, although the relative intensities of the features observed for the $P \rightarrow 6$ ions of ubiquitin depend on P , similar ranges of Ω were observed for all C . In contrast, the ranges and relative intensities of the features in the Ω distributions for the CAPTR products of cytochrome c depend more strongly on P (Figures 5c, 5d, and 6a). Finally, the Ω for the lowest- C CAPTR products of both proteins from denaturing solutions are all consistent with the formation of compact structures. These compact ions have Ω that are similar to those measured for the corresponding native-like ions and those calculated using the corresponding native structure. Future experiments will focus on probing the similarities and differences between these compact protein ions formed through such different mechanisms,

which will require complementary probes of ion structure, *e.g.*, ion chemistry [7, 20, 53], alternative dissociation techniques [20, 31, 32, 54], and spectroscopy [55].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Diagram of the temperature-controlled, nanoelectrospray ionization source. (a) Side view of the source. The sample capillary is held between three layers of electrically insulating and thermally conductive elastomer placed on top of a Peltier device. Heat is moved between the sample side of the Peltier device and a copper bar, which exchanges heat with the atmosphere via a heat sink supplied with forced air by a fan. The Peltier device is controlled by a TE Technologies TC-48-20 OEM controller, based on the temperature measured using the thermistor. (b) Top-down view of the temperature-controlled source, with the top layer of thermally conductive elastomer removed to expose the sample capillary and platinum wire electrode.

Figure 2.

Ω distributions of 7+ ubiquitin from denaturing conditions determined as a function of the temperature of the MS interface using (a) the original source and (b) the new temperature-controlled source with the Peltier device held at 25 °C. The Ω distributions in (a) were reported previously [33]. The Ω distributions under ambient conditions in (b) are $\sim 0.5 \text{ nm}^2$ more compact than those in (a), which may be due to systematic differences between the two sets of IM experiments.

Figure 3.

(a) Sample temperature measured with a thermocouple probe (Omega TT-T-40, Norwalk, CT) placed inside a capillary containing deionized water using the original source (*black triangles*) and using the temperature-controlled source (*colored circles*) as a function of the measured temperature of the MS interface. For the temperature-controlled source, measurements were also made for a series of set temperature for the Peltier device. (b) Results obtained using the temperature-controlled source from (a), plotted as a function of the set temperature of the Peltier device. The black line indicates when the temperatures of the Peltier device and sample are equal.

Figure 4.

(a) Apparent Ω distribution for 8+ cytochrome *c* (black solid line) from denaturing conditions, and the corresponding cumulative distribution function (CDF, red line), which is the integral of the apparent Ω distribution with respect to Ω . Critical Ω values representing 10%, 50%, and 90% of the CDF are shown using black dashed lines. (b) Critical Ω values for all denatured (colored circles) and native-like (black squares) ions. Ω values for native-like ions that were reported previously [12] are shown using hollow magenta diamonds. The horizontal dashed black lines correspond to Ω values calculated for models of cytochrome *c* (Table 1).

Figure 5.

(a) Apparent Ω distributions for all $18 \rightarrow C$ ions of cytochrome *c* from denaturing conditions. (b) Ω distributions for all $7 \rightarrow C$ ions of cytochrome *c* from native-like conditions. Ω distribution (black solid lines), CDF (red lines) and critical Ω values (black dashed lines) for the (c) $18 \rightarrow 9$ and (d) $9 \rightarrow 9$ ions from denaturing conditions. All experiments probed ions generated using the temperature-controlled source with the temperature of the Peltier device set to 25 °C.

Figure 6.

(a) Critical Ω values, which correspond to 10%, 50%, and 90% of the cumulative distribution function, for the CAPTR precursor and product ions of cytochrome *c* from denaturing conditions. The charge state of the precursor (*P*) for each set of values is indicated using a unique color. (b) Critical Ω values for native-like $7 \rightarrow C$ (black squares), native-like $6 \rightarrow C$ (gray squares) and denatured $18 \rightarrow C$ (blue circles) cytochrome *c* as a function of C . The three series are offset horizontally to aid in visualization, as labeled for the $P \rightarrow 5$ data. Ω values for native-like ions that were reported previously [12] are shown using hollow

magenta diamonds. The horizontal dashed black lines in panels (a) and (b) correspond to Ω values calculated for models of cytochrome *c* (Table 1).

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Table 1

Ω values calculated for models of cytochrome *c*.

	Model	Method	Ω/nm^2
<i>i</i>	native	PA	10.8
<i>ii</i>		EHSS	13.5
<i>iii</i>	α -helix	PA	19.9
<i>iv</i>		EHSS	24.3
<i>v</i>	linear strand	PA	31.4
<i>vi</i>		EHSS	35.8