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Gas-Phase Protein Salt Bridge Stabilities from Collisional Activation and Electron Transfer Dissociation

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Abstract

The gas phase structures of several proteins have been studied by electron transfer dissociation (ETD) with and without prior collisional heating after electrospraying these proteins from native-like solutions into a quadrupole ion trap mass spectrometer. Without prior collisional heating, we find that ETD fragmentation is mostly limited to regions of the protein that are not spanned by the salt bridges known to form in solution. When protein ions are collisionally heated before ETD, new product ions are observed, and in almost all cases, these new ions arise from protein regions that are spanned by the salt bridges. Together these results confirm the existence of salt bridges in protein ions and demonstrate that a sufficient amount energy is required to disrupt these salt bridges in the gas phase. More interestingly, we also show that different salt bridges require different collisional activation voltages to be disrupted, suggesting that they have variable stabilities in the gas phase. These stabilities appear to be influenced by the gas-phase basicities of the involved residues and the presence of nearby charged residues. We also find that higher collisional activation voltages are needed to enable the formation of new product from sites spanned by multiple salt bridges.

Graphical Abstract

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Keywords

Electron transfer dissociation; Collisional activation; Native electrospray ionization; Gas phase; Protein ions; Salt bridges

1. Introduction

Electrospraying protein solutions under native-like conditions can allow protein ions to maintain some features of their solution phase structure in the gas phase. Several methods and protein systems have been explored to provide evidence for this idea. An early example was the work by Chait and co-workers that demonstrated different charge-state distributions for myoglobin ions when they were electrosprayed from native-like or denaturing solutions [1]. Numerous subsequent studies have confirmed that proteins that are more folded in solution give rise to spectra dominated by lower charge states, while unfolded proteins in solutions result in higher charge states [2–4]. More recently, ion mobility coupled with mass spectrometry has been used to understand the relationship between protein gas phase and solution phase structures [5–7]. For instance, Loo and co-workers noted that the 20S proteasome maintains a gas phase structure whose diameter is similar to the value calculated from its crystallographic structure [8].

In contrast to charge-state distributions and ion mobility, which are somewhat passive indicators of gas-phase structure, gas-phase dissociation methods have also been used to provide insight into protein ion structure. Electron-based dissociation methods have been the most useful in this regard because they tend to break covalent bonds while maintaining non-covalent interactions. Loo and co-workers found that electron capture dissociation (ECD) was able to localize protein-ligand binding sites, suggesting that non-covalent interactions are maintained in gas phase [9]. Gross and co-workers showed the existence of non-covalent interactions of hemoglobin ions in the gas phase by using ECD combined with collision induced unfolding (CIU) and ion mobility measurements [10]. Activated-ion ETD was

similarly used to study the structures of human hemoglobin and other protein complexes [11, 12]. Breuker and co-workers have investigated gas-phase protein structure and unfolding using ECD [13–17]. Our group recently used electron transfer dissociation together with collision-induced dissociation (CID) to support the idea that protein ions can maintain known solution-phase salt bridges in the gas phase [18]. In that work, product ions from ETD of protein ions were found to arise only from regions not spanned by salt bridges. CID of undissociated charge-reduced ions confirmed that salt bridges were intact as CID disrupted the noncovalent salt bridges to generate new product ions in the regions spanned by these interactions.

The possibility that electrostatic interactions, such as salt bridges, can be maintained in the gas phase is not obvious because barriers for proton transfer between a protonated base and a deprotonated acid are low in the gas phase [19], even though the low dielectric in the gas-phase strengthens electrostatic interactions. Several studies, however, have provided evidence that salt bridges can exist in the gas phase. For example, using blackbody infrared radiative dissociation, Williams and co-workers showed bradykinin and its methylester have different activation energies, suggesting the presence of a salt bridge in one case but not the other [20]. In addition, the same group argued that peptides with more than one basic residue gave rise to more stable salt bridge structures, indicating that salt bridges can be stabilized by a nearby charge or dipole. Breuker and co-workers have also shown the importance of electrostatic interactions for stabilizing the native three-helix bundle of the KIX protein in the gas phase [13, 14]. In other work, this group also demonstrated that salt bridges could hinder ubiquitin from unfolding in the gas phase, thereby influencing its dissociation during ECD [14].

In the current work, we set out to further investigate proteins' ability to retain their solution-phase electrostatic interactions and study the extent to which these interactions can be perturbed in the gas phase. In our previous work, we found that salt bridges known to form in solution cause ETD product ions of native-like protein ions to be observed mainly in regions that are not spanned by salt bridges [18]. Based on these previous observations, we hypothesized that native-like salt bridges that persist in the gas-phase could be disrupted by collisional heating/unfolding and subsequent ETD would then release new product ions from regions spanned by the residues involved in these salt bridges. In this work, we test this hypothesis using three model proteins and find that the salt bridges can be disrupted by collisional heating/unfolding, generating the predicted new product ions. Moreover, we find that different salt bridges require different levels of collisional activation to be disrupted, suggesting that they have different gas-phase stabilities.

2. Experimental

2.1 Materials

Ubiquitin (8.6 kDa) from bovine erythrocytes and azurin (13.9 kDa) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trp-cage (2.2 kDa) was purchased from AnaSpec, Inc. (Fremont, CA, USA). Ammonium acetate, water, and acetic acid were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Protein solutions for ESI-MS analysis were prepared by diluting stock solutions in deionized water to a final concentration of 5 μ M in 5

mM ammonium acetate. The pH of the solution was then adjusted with acetic acid to the level used to obtain the NMR or X-ray crystal structures of the protein of interest.

2.2 Instrumentation and ETD and CA/ETD experiments

All mass spectral measurements were carried out on a Bruker AmaZon (Billerica, MA, USA) quadrupole ion trap mass spectrometer equipped with an electrospray ionization source. Typically, the needle voltage was kept at around 4.0 kV, and the capillary temperature was set to 100 °C. In an MS/MS experiment, ETD reagent anions from fluoranthene were generated in a chemical ionization source and injected into the ion trap to react with the stored ions for 100–130 ms. In collisional activation (CA)/ETD experiments, protein ions were collisionally heated before ETD. Collisional heating/unfolding was accomplished by varying the CID voltage by steps of 0.02 or 0.03 V from 0 V to voltages at which 10–15% of the protein ions started to dissociate. For all ETD and CA/ETD experiments, the ions were isolated using a very wide isolation width (~10 Da) to minimize any isolation induced collisional activation.

In evaluating the resulting ETD and CA/ETD spectra, only ions with abundances at least three times greater than the noise were chosen. Ion assignments were made using a mass tolerance of ± 0.1 m/z unit. In addition, because a wide isolation width was used, only product ions containing the predicted isotopic distribution were considered as properly identified. In some cases, the charge states and, thus, identities of more highly charged product ions were confirmed using proton transfer reactions [21].

2.3 Activation energy onset determination

During the CA/ETD experiments, the onset voltages for newly formed product ions were determined by increasing the collision voltage in steps of 0.02 or 0.03 V and finding the minimum voltage at which the new product ion was consistently measured. A new product ion was considered consistently measured when its signal-to-noise ratio exceeded 3 and when its normalized ion abundance was at least three times greater than its abundance at a voltage that was 0.02 or 0.03 V lower. Three replicates of each voltage setting were acquired, and typically 400 spectra were averaged at each voltage setting. The onset voltages were determined for each replicate and then averaged. To account for signal fluctuations over time, the ion abundances of the newly formed product ions were normalized to the most abundant product ion in the spectrum. In almost all cases, the most abundant product was the same ion regardless of collisional activation voltage used.

2.4 Protein structures

The structures of each studied protein, including salt bridge interactions and secondary structural elements, were obtained from either NMR or X-ray crystal structures present in the Protein Data Bank (PDB). The PDB files that were used for the studied proteins were 1AZU for azurin, 2JZZ for ubiquitin, and 1L2Y for Trp-cage. A constraint of 4 Å between the positive and negative charge centers on side chains was used to identify salt bridges. The identified salt bridge patterns were also verified using the appropriate tools from Proteopedia (<http://www.Proteopedia.org/>).

3. Results and Discussion

3.1 Azurin

The first protein that we investigated was azurin, which we had previously shown maintained its native pattern of salt bridges in the gas phase [18]. Upon electrospraying azurin from a native-like solution, the 8+ and 7+ charge states of the protein were observed. As we demonstrated previously, the ETD spectrum of the protein ions (Figure 1a) shows backbone dissociations mainly from regions that are not spanned by salt bridges (Figure 1c). Based on the PDB structure of azurin, salt bridges exist in the solution phase between K24 and E104, D62 and R79, D62 and K74, and D77 and R79. In the ETD spectrum, the most abundant product ions are a series of z ions from z_3^+ to z_{24}^{2+} corresponding to a protein region that is not spanned by salt bridges, suggesting that the salt bridges in the rest of the protein are preventing ion dissociation. Some ions (e.g. z_{25}^{2+} , z_{32}^{2+} , and z_{43}^{3+}) with low abundance are observed in a region spanned by the salt bridge between K24 and E104, suggesting that the salt bridge may not exist in a small fraction of the azurin ions in the gas phase, but there is no product ion resulting from the disruption of any of the other salt bridges. Interestingly, if the K24-E104 salt bridge is missing in some protein ions, it is somewhat surprising that no product ions are observed between C26 and T61. One possible reason for the lack of product ions between C26 and T61 could be the fact that there are only 2 basic residues in this span of 35 amino acids, which would limit electron transfer and thus dissociation in this region of the protein. Another interesting observation in the ETD spectrum of azurin is the appearance of a c_9^+ product ion of low intensity that must form by cleavage of the disulfide bond and associated cleavage of an N-C $_{\alpha}$ bond. Cleavage of the disulfide bonds during ETD has been previously reported [22, 23], but it is intriguing that disulfides appeared to be cleaved in some cases before salt bridges.

To further confirm the presence of salt bridges in azurin ions and to investigate their stability in the gas phase, we collisionally activated (but not dissociated) the protein ions by resonantly exciting them in the quadrupole ion trap prior to ETD. In effect, we sought to “unfold” and/or disrupt the salt bridges in the protein ions via collisional heating in the gas phase. Varying resonance excitation voltages were applied just prior to the ETD step, and the effect on the resulting ETD spectrum was measured. Figure 1b illustrates that a sufficient collisional activation voltage prior to ETD increases the abundance of some products ions, especially the product ions from the region spanned by the aforementioned salt bridge from K24-E104 (e.g. z_{32}^{3+} , z_{35}^{3+} , and z_{40}^{3+} - see Figure 2b) that may only be intact in some of the protein ions. More importantly, sufficient collisional activation generates new product ions. In all cases, these new product ions come from regions of the protein that require the disruption of one or more salt bridges, most notably between D62-R79 (Figure 1c), but also between all of the salt bridges in the protein. Presumably, collisional activation results in protein ion heating and/or unfolding, which disrupts salt bridges in the gas phase and therefore allows ETD to release these new product ions. It is conceivable that CA causes a rearrangement of some of the salt bridges, as suggested in recent work by Loo et al. [24], but we do not have sufficient data to test this hypothesis. Interestingly, the appearance of different product ions requires different extents of collisional activation. For example, Figure 2 shows that the new product ion z_{51}^{3+} is reliably measured only after a collisional activation

voltage of 1.3 V is applied prior to ETD. For this particular product ion to form, three salt bridges (i.e. K24-E104, D62-R79, and D77-R79) must be broken. If the same analysis is done for all of the new product ions that are formed upon CA/ETD of the 8⁺ ion of azurin (Table 1), we find that the collisional activation voltage needed to generate the new product ions depends on the number of salt bridges that need to be broken. For example, a collisional activation voltage of 1.1 V is needed to generate z_{47}^{3+} , c_{79}^{4+} , and c_{82}^{4+} , all of which result from the disruption of the salt bridge between K24 and E104. The increasing activation voltage required for the formation of different product ions implies a greater energy is required to separate protein regions that are stabilized by a greater number of salt bridges.

3.2 Ubiquitin

To determine if prior collisional activation had an effect on the ETD spectra of other proteins that are electrosprayed from native-like solutions, we investigated ubiquitin, which was also previously shown to maintain native-like aspects of its structure, including salt bridges, in the gas phase [14, 18]. ETD of the 6⁺ charge state of ubiquitin results in a series of c ions from c_6^+ to c_{17}^+ and several z ions, including z_3 and z_{17}^{2+} , but no product ions are observed between Glu18 and Asp58, which is a region spanned by several salt bridges (Figure 3), indicating that such electrostatic interactions dictate the dissociation patterns of this protein ion. When ubiquitin is collisionally activated prior to ETD, several new product ions are formed in the region of the protein spanned by the salt bridges (orange lines in Figure 3), implying that cleavage of one or more salt bridges is taking place upon collisional heating/unfolding. Of the four new product ions that are reliably formed, z_{31}^{3+} and z_{34}^{3+} , which require disruption of the salt bridge between K27 and D52, appear at the lowest collisional voltages (Table 2). The other two product ions, c_{55}^{4+} and c_{21}^{2+} , form only after higher collisional voltages are applied (Table 2).

In contrast to azurin, lower collisional voltages are required to observe new product ions for ubiquitin. These lower voltages likely reflect the smaller number of degrees of freedom per unit charge in ubiquitin; less of an increase in kinetic energy, and thus less deposited internal energy upon collisions with helium, is needed to disrupt the salt bridges. Like in the case of azurin, disruption of more than one salt bridge, as is required for formation of c_{21}^{2+} , requires a higher collisional activation voltage than is needed for the formation of the other product ions that require only a single salt bridge to be broken. The higher voltage needed to observe the formation of c_{55}^{4+} as compared to the formation of z_{31}^{3+} and z_{34}^{3+} might reflect the greater stability of the R54-D58 salt bridge as compared to the K27-D52 salt bridge. Even though the voltage difference is small, arginine's higher gas-phase basicity might explain why the R54-D58 salt bridge is slightly more stable than the salt bridge containing the less basic lysine residue (i.e. K27-D52). Indeed, a similar argument for the greater stability of arginine-containing salt bridges has been made previously [20]. In addition, McLuckey and co-workers recently found that protonated arginine side chains have stronger electrostatic interactions with anionic sites than protonated lysine side chains [25], further suggesting that arginine-containing salt bridges might have greater stability in the gas phase. The higher activation voltage needed to generate the c_{21}^{2+} ion is likely due to the complex salt bridge between E18, K29, and D21. Previous work has also suggested that the presence of nearby charges and dipoles can stabilize salt bridges in the gas phase [20].

3.3 Trp-cage

Another protein that we investigated was Trp-cage, which adopts a well-defined compact structure in solution. Methods like ECD, infrared multiphoton dissociation (IRMPD), and simulations have been used to provide evidence that Trp-cage maintains much of its solution-phase tertiary structure in gas phase as a 2+ ion [26–28]. After electrospraying Trp-cage from a native-like solution, ETD of the 2+ charge state of Trp-cage results in product ions being formed at the two termini (black lines in Figure 4a). The observation is very similar to results from previous ECD experiments [26]. The presence of terminal product ions is also consistent with the NMR structure of Trp-cage (Figure 4b), which shows a salt bridge between D9 and R16. Interestingly, an α -helix spans most of the sequence from N1 to S14, and this secondary structural feature might also influence product ion formation, although there is no consensus in the literature as to whether or not alpha helices impact dissociation in ECD or ETD. Breuker and coworkers found that dissociation was hindered by α -helices during ECD [13], while Tsybin and coworkers found that periodic preferential cleavage sites could exist in peptides with an α -helical structure [29]. When Trp-cage is collisionally heated/unfolded prior to ETD, several new product ions are observed (Table 3). While many of these new ions (c_9 , c_{12} , and c_{15}) are formed by disruption of the salt bridge between D9 and R16, several more (c_5 , c_8 , z_{16} , and z_{17}) appear in regions not spanned by this salt bridge. The latter ions might be formed after disruption of the N-terminal region of the α -helix that comprises most of the protein's secondary structure. An interesting difference between these sets of new product ions is the onset voltage required for their formation. The product ions from the region spanned by the salt bridge (i.e. c_9 , c_{12} and c_{15}) require collisional activation voltages above 0.4 V to be formed, whereas the other product ions are formed at lower voltages. The higher energy that is required for c_9 , c_{12} and c_{15} might be due to the need to break the salt bridge between D9-R16 and the α -helix, whereas the other new product ions only require disruption of the helix. While the data for Trp-cage is only one example, it is possible that different gas-phase protein structural elements might require different amounts of energy to disrupt.

4. Conclusions

Our results from the ETD and CA/ETD experiments further support the idea that protein ions electrosprayed from native-like solutions can maintain their solution-phase electrostatic interactions in the gas phase. The key experimental support for this contention is that most of the new product ions that are formed upon collisional activation prior to ETD are found in the regions of the protein known to be spanned by salt bridges. Presumably, collisional activation eliminates the salt bridges that prevent product ion formation by ETD in the first place by heating the ions enough that proton transfer between the oppositely-charged side chains is now possible. Moreover, by varying collisional activation voltages during the CA/ETD experiment, we provide evidence that some electrostatic interactions in the gas-phase protein ions are more stable than others. The number of salt bridges that are broken, higher gas-phase basicities of the residues involved in the salt bridge, and the presence of nearby charged residues all appear to increase the activation voltages required to disrupt the salt bridges.

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Figure 1.

Example ETD and CA/ETD spectra of the $(M+8H)^{8+}$ ion of azurin and a summary of the cleavage sites. (a) ETD spectrum; (b) CA/ETD spectrum; product ions labeled in red indicate ions that are observed only after CA; (c) summary of cleavage sites of ETD and CA/ETD experiments with a collisional activation voltage of 1.3 V. The amino acid sequence of the protein is shown in (c); the Cu-binding amino acids are shown with red letters. The black lines on the top of this sequence indicate the locations of the salt bridges. The blue line on the top of this sequence indicates location of the disulfide bond. The black bars under the sequence represent the cleavage sites from the ETD experiments. The orange bars under the sequence represent the cleavages sites that are associated with the new product ions that are measured during CA/ETD experiment.

Figure 2.

Normalized ion abundance of the z_{51}^{3+} product ion of azurin, which is only formed when the 8+ ion of azurin is collisionally activated prior to ETD. The ion abundance of z_{51}^{3+} was normalized to the ion abundance of z_{10} to compensate for signal fluctuations. Its ion abundance was then compared with its normalized ion abundance, as detailed in section 2.3, at different activation voltages to determine the dissociation onset. Bars with asterisks (*) indicate the collisional activation voltages at which the new product ion is not detectable.

Figure 3. Summary of cleavage sites of ubiquitin ETD and CA/ETD experiments with a collisional activation voltage of 1.15 V. The amino acid sequence of the protein is shown at the top of the figure. The black lines on top of this sequence indicate the locations of the salt bridges. The black bars under the sequence represent the cleavage sites from the ETD experiments. The orange bars under the sequence represent the cleavages sites indicated by the new product ions found in CA/ETD experiment.

Figure 4.

Summary of cleavage sites of Trp-cage and its NMR structure. (a) Cleavage sites after ETD and CA/ETD experiments with a collisional activation voltage of 0.4 V. The amino acid sequence of the peptide is shown at the top of the figure. The black lines on this sequence indicate the locations of the salt bridge. The black bars under the sequence represent the cleavage sites from the ETD experiments. The orange bars under the sequence represent the cleavages sites indicated by the new product ions found in CA/ETD experiment. (b) Ribbon representation of Trp-cage from its NMR structure (PDB ID: 1L2Y).

Table 1

Summary of the newly formed product ions from CA/ETD of azurin with the corresponding salt bridges that are broken and the collisional activation onset voltages needed to form these new product ions.

Product ions	Salt bridge(s) broken	Onset Voltage (V)
z_{42}^{3+} , z_{47}^{3+} , c_{79}^{4+} , c_{82}^{4+}	K24-E104	1.10 ± 0.03
z_{52}^{3+} , z_{53}^{3+}	K24-E104, D62-R79	1.15 ± 0.03
z_{51}^{3+} , c_{78}^{4+}	K24-E104, D62-R79, D77-R79	1.30 ± 0.03
z_{56}^{3+} , z_{60}^{3+} , z_{62}^{3+}	K24-E104, D62-R79, D62-K74	1.30 ± 0.03

Table 2

Summary of the newly formed product ions from CA/ETD of ubiquitin with the corresponding salt bridges that are broken and the collisional activation onset voltages needed to form these new product ions.

Product ions	Salt bridge(s) broken	Onset Voltage (V)
z_{31}^{3+}, z_{34}^{3+}	K27-D52	1.00 ± 0.02
c_{55}^{4+}	R54-D58	1.05 ± 0.02
c_{21}^{2+}	D21-K29, E18-K29	1.15 ± 0.02

Table 3

Summary of the newly formed product ions from CA/ETD of Trp-cage with the corresponding structural elements that are broken and the collisional activation onset voltages needed to form these new product ions.

Product ions	Structural feature broken	Onset Voltage (V)
z_{16}, z_{17}, c_5	α -helix	0.30 ± 0.02
c_8	α -helix	0.35 ± 0.02
c_9, c_{12}, c_{15}	D9-R16 salt bridge and α -helix	0.40 ± 0.02