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Exploring Salt Bridge Structures of Gas-Phase Protein Ions using Multiple Stages of Electron Transfer and Collision Induced Dissociation

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Abstract

The gas-phase structures of protein ions have been studied by electron transfer dissociation (ETD) and collision-induced dissociation (CID) after electrospraying these proteins from native-like solutions into a quadrupole ion trap mass spectrometer. Because ETD can break covalent bonds while minimally disrupting non-covalent interactions, we have investigated the ability of this dissociation technique together with CID to probe the sites of electrostatic interactions in gas phase protein ions. By comparing spectra from ETD with spectra from ETD followed by CID, we find that several proteins, including ubiquitin, CRABP I, azurin, and β -2-microglobulin, appear to maintain many of the salt bridge contacts known to exist in solution. To support this conclusion, we also performed calculations to consider all possible salt bridge patterns for each protein, and we find that the native salt bridge pattern explains the experimental ETD data better than nearly all other possible salt bridge patterns. Overall, our data suggest that ETD and ETD/CID of native protein ions can provide some insight into approximate location of salt bridges in the gas phase.

Keywords

Electron transfer dissociation; collision-induced dissociation; native electrospray ionization; gas phase; protein ions; salt bridges

Introduction

Electrospray ionization (ESI) can gently transfer proteins and protein complexes into the gas phase. If samples are ionized from native-like solutions, then proteins can often maintain some features of their native structure [1-10]. The removal of solvent as protein ions transition into the gas phase, however, results in the removal of a key driving force that keeps proteins folded in solution, namely the hydrophobic effect. At the same time other interactions, such as hydrogen bonding and electrostatic interactions, can be enhanced.

There has been a lot of interest in understanding the extent to which proteins maintain their native structure in the gas phase. Several lines of evidence have been provided to support the idea that proteins maintain some aspects of their solution structure in the gas phase. Measurements of charge-state distributions produced during ESI were one of the first ways used to support the idea. For instance, Chait and co-workers noted that myoglobin sprayed under native and denatured conditions led to very different charge-state distributions, which reflected compact and unfolded conformations known to exist in solution under those

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conditions [1]. Agreements between solution-phase and gas-phase protein complex stoichiometries have also been used as evidence that proteins maintain aspects of their structure in gas phase. As an example the trp RNA binding protein complex can maintain its 11-membered subunit stoichiometry and even keeps its ring topology as revealed by ion mobility mass spectrometry [2]. Indeed, ion mobility spectrometry has been used quite successfully to support the idea of protein structural maintenance in the gas phase. For example, Bowers and co-workers used ion mobility spectrometry and molecular dynamics to support the idea that small proteins like ubiquitin maintain native-like structures in the gas phase [3]. Similarly, using ion mobility Loo and co-workers provided evidence that large proteins, like the 20S proteasome, maintain a diameter that is similar to the value found by crystallographic methods [4]. Other gas-phase techniques, such as electron capture dissociation (ECD), blackbody infrared radiative dissociation (BIRD), and gas-phase hydrogen deuterium (H/D) exchange measurements have also been used to explore gas-phase protein structure. Loo and co-workers used ECD to localize protein-ligand binding sites, arguing that the non-covalent interactions known to exist in solution are maintained in gas phase [5]. Klassen and co-workers have used BIRD to support the notion that specific lipid-protein [6, 7] and protein-oligosaccharide interactions [8] can be preserved in the gas phase. MS-based H/D exchange measurements have also been used to conclude that some protein interactions that are present in solution appear to be present in the gas phase [9]. Molecular dynamics simulations further suggest that some proteins can retain aspects of their solution-phase hydrogen bonding patterns in the gas phase [10].

While numerous studies have argued that proteins can maintain aspects of their solution structure in the gas phase, other reports suggest that proteins do not retain many aspects of their native structure once transferred into the gas phase. For instance, cytochrome c, one of the most studied proteins in the gas phase, appears to change its structure after transition into the gas phase. Computational results [11], ion mobility cross sections [12] and NECD measurements [13] suggest that this protein undergoes significant structural rearrangements [14]. It has been argued that cytochrome c undergoes significant conformational changes as new electrostatic interactions are formed that lead to reorientation of several residues [11]. It should be noted, though, that this conclusion about cytochrome c has been debated based upon a comparison between ECD data and crystallography B-factors [15]. Substantial changes in the structures of protein complexes have also been indicated as exemplified by the work on the tumor suppressor protein p53 by Robinson and co-workers [16]. The p53 protein was found by ion mobility to undergo a large structural collapse during desolvation.

The ability to retain native-like structure in the gas phase is probably protein dependent and may rely on a protein's ability to form intramolecular electrostatic interactions that are similar to those that exist in solution. Ample evidence has been provided that such electrostatic interactions can exist, and indeed are important, in the gas phase [17, 18]. Williams and co-workers have used a variety of experimental and computational tools to provide evidence for the existence of salt bridges in the gas phase. For example, differences in the activation energies of bradykinin and its methylester from BIRD measurements were used to argue for the existence of salt bridge structures in the gas phase [19]. Breuker and co-workers have also shown that salt bridges and ionic hydrogen bonds are important for stabilizing the native three-helix bundle of the KIX protein in the gas phase [20]. This group also used ECD to study folding of ubiquitin where they found stabilization by salt bridges could prevent ubiquitin unfolding [21]. It can be argued that proton transfer will cause zwitterionic forms, such as salt bridges, to vanish, especially given that energy barriers for such reactions are relatively low [22]; however, the presence of nearby charge sites can stabilize the large dipoles created by salt bridges. Indeed, Williams and co-workers found that salt bridges were more stable in peptides having more than one basic residue [23]. This observation implies that salt bridges in larger polypeptides or proteins are likely to be

stabilized by the presence of numerous basic residues. In addition, carbonyl oxygens in polypeptides could further stabilize such charge sites. The abundance of such charge sites and carbonyl oxygens in gas phase protein ions make it more likely that salt bridges could be maintained in the gas phase.

In this work, we set out to investigate the ability of different proteins to retain their solution-phase electrostatic interactions (e.g. salt bridges and ionic hydrogen bonds) in the gas phase. To do this, we exploited the complementarity of electron transfer dissociation (ETD) and collision-induced dissociation (CID) when dissociating gas-phase protein ions. ETD, via its unique dissociation mechanism, has shown the ability to dissociate biomolecular ions in such a way that non-covalent interactions can often be maintained. In contrast, CID, especially low-energy CID in quadrupole ion trap mass spectrometers, is a slow heating activation method that usually results in the dissociation of the lowest energy pathways, meaning non-covalent interactions are typically disrupted before covalent bonds. By using ETD and CID in sequence, we have developed an approach that can reveal the location of protein salt bridges in the gas phase. Application of this combination of dissociation methods along with statistical evaluations of the resulting dissociation patterns demonstrates that many protein ions maintain their native-like salt bridge structure once transferred into the gas phase.

Experimental

Materials

Ubiquitin from bovine erythrocytes, azurin, and equine heart cytochrome c were purchased from Sigma-Aldrich (St. Louis, MO, USA). β -2-microglobulin (β 2m) was purchased from Fitzgerald Industries International, Inc. (Concord, MA, USA). The His-tagged R131Q mutant of the cellular retinoic acid binding protein I (CRABP I) was a gift from Professor Lila M. Gierasch's group at the University of Massachusetts Amherst. Ammonium acetate, water, methanol 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 de-ionized water to a final concentration of 1-5 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.

Instrumentation and ETD and CID 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 4.5 kV, and the capillary temperature was set to 100 °C. 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 the ETD/CID experiments, ETD was conducted during an MS/MS stage of analysis, and CID was performed during the MS³ stage of analysis on the charge-reduced ion. The CID amplitude chosen for these MS³ experiments was identified by finding the voltage just below the onset of dissociation for the charge state one lower than the initial parent ion. For example, if the 6+ charge state of a protein was subjected to ETD, thereby producing a 5+ charge-reduced ion, then the low amplitude CID voltage was identified by finding the voltage just below the onset of dissociation for the 5+ ion that was produced directly by ESI. The reason for choosing this low CID amplitude was to avoid dissociation of covalent bonds and only facilitate the dissociation of existing non-covalent interactions. When the ETD/CID experiments were performed, the charge reduced ion from ETD was isolated prior to performing CID using a very broad isolation width (> 10 Da) to avoid any isolation-induced collisional activation.

In evaluating the resulting ETD and ETD/CID spectra, only ions with abundances at least five times greater than the noise were chosen. In addition, only product ions containing the predicted isotopic distribution were considered. In some cases, the charge states, and thus identities, of more highly-charged product ions were confirmed using proton transfer reactions [24].

Protein Structures

The structures of each studied protein, including salt bridge and ionic hydrogen bond interactions, 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 2JZZ for ubiquitin, 1CBI for CRABP I, 1AZU for azurin, 3HLA for β 2m, and 1AKK for cytochrome c. A constraint of 4 Å between the positive and negative charge centers on side chains was used to identify salt bridges and ionic hydrogen bonds. The identified salt bridge patterns were also verified using the appropriate tools from Proteopedia (<http://www.Proteopedia.org>).

Statistical Analysis of Salt Bridge Patterns

A computational analysis that took into account all possible salt bridges patterns for each protein was performed using an in-house coded program. The program was designed using object-oriented principles and implemented in Java 1.7 using the Eclipse compiler 0.A48. Details of how the algorithm was designed are provided in the Results and Discussion section and Figure S1 in supporting information.

Results and Discussion

Ubiquitin

The first protein system that we investigated was ubiquitin because there are numerous other gas-phase and solution-phase studies of this protein to which we can compare. Upon electrospraying ubiquitin from a native-like solution (i.e. 5 mM ammonium acetate at pH 7.0), the 6+ and 7+ charge states of the protein are observed. Ubiquitin was then subjected to ETD (Figures 1a) and backbone dissociations at the two termini but not in the middle of the protein were observed (Figure 1b). Based on the NMR structure of ubiquitin [25], salt bridges exist in the solution phase between E18 and K29, D21 and K29, K27 and D52, E51 and R54, and R54 and D58. Interestingly, no product ions corresponding to cleavages between residues 18 and 58 are observed in the ETD spectrum; this is consistent with previous ECD studies on this protein [14, 26, 27]. We measure a series of c ions from c_6^+ to c_{17}^+ , but the next c ion in the series is c_{74}^{4+} . Similarly, z_{15}^{2+} , z_{17}^{2+} and z_{18}^{2+} are observed, but the next z ion is z_{59}^{3+} . The lack of product ions in the region spanned by the salt bridges suggests that these electrostatic interactions might still exist in the gas phase, thereby stabilizing the protein's structure in this region and inhibiting the generation of product ions from this region. Indeed, Skinner et al. recently argued that the regions spanned by the salt bridges of ubiquitin were the slowest to become destabilized in the gas phase [26].

To further test the idea that native salt bridges influence the ETD pattern of ubiquitin, the charge-reduced ion $(M+6H)^{5+\bullet}$ of intact ubiquitin, which is generated during ETD, was subjected to CID in an MS³ experiment. As described in the experimental section, the CID voltage was chosen to be low enough to prevent the dissociation of covalent bonds. As Figure 1c indicates, several new product ions are formed. Many of these new product ions are formed by cleavage of backbone bonds at locations between the native salt bridges, e.g. E51-R54, R54-D58 and D21-K29. Presumably these dissociations are observed after CID because initial ETD is able to cleave N-C _{α} bonds but electrostatic interactions (e.g. salt bridges) that still exist in the charge reduced ions prevent the protein ions from fully

dissociating [27]. Subsequent collisional activation by the low amplitude CID voltage results in the disruption of these interactions and the observance of numerous new product ions. Figure 2 provides a comparison of the results from ETD and ETD followed by CID (i.e. ETD/CID). From this figure, it is clear that product ions in the region spanned by the salt bridges are only observed in the ETD/CID experiment. Comparable results are also obtained for the +5 charge state of ubiquitin, and its charged-reduced $(M+5H)^{4+}$, although the poorer signal for the +5 ion lead to fewer measurable product ions (Figure S2).

We also collisionally activated (but not dissociated) the 6+ ion of ubiquitin before ETD and before ETD/CID. When doing this on a quadrupole ion trap, the process is analogous to activated ion ECD [27], but a key difference is that the ions are allowed to cool for approximately 5 msec before subsequent dissociation by ETD. The purpose of this experiment was to determine if ETD and/or ETD/CID data could provide evidence for collisionally activated shuffling or disruption of the salt bridges that were initially maintained upon transfer into the gas phase. The resulting data, as illustrated in the green and blue lines in Figure 2, shows that collisional activation (CA) prior to ETD results in almost no change to the ETD pattern (green lines) but substantial changes to the ETD/CID pattern (blue lines). In the CA/ETD/CID data, numerous new product ions are observed in the regions spanned by the native salt bridges. These results indicate that CA has disrupted the protein's structure in some way but the salt bridge pattern may have not been substantially changed. It is possible that CA disrupts some intramolecular interactions but during the 5 msec cooling time, the protein re-forms its initial salt bridges. In the meantime, however, how the protein is structured between the salt bridges and/or the location of the protonation sites have changed. Consequently, during CID cleavages occur at new sites but the same parts of the protein are still held together by electrostatic interactions.

For a more quantitative evaluation of the influence of the salt bridges on protein dissociation, the weighted average numbers of salt bridges and ionic hydrogen bonds that were disrupted during ETD and ETD/CID were calculated using equation 1. In equation 1, the number of salt bridges or ionic hydrogen bonds broken refers to the number of these interactions that must be broken to observe the experimentally measure product ions. Table 1 shows for the most abundant charge state of ubiquitin that the weighted average numbers of broken salt bridges and ionic hydrogen bonds broken during ETD are 0 and 0.00153, respectively, whereas the values for ETD/CID are 0.179 and 0.231, respectively. The +5 charge state of ubiquitin gave comparable results (Table S1 in the Supporting Information). The lower values for the ETD experiment are consistent with the ability of ETD to break covalent bonds without disrupting non-covalent interactions, suggesting that the protein's salt bridges and some ionic hydrogen bonds remain intact after transfer into the gas phase. The higher numbers for ETD/CID are consistent with the idea that the charge reduced ions are those protein ions that have already undergone backbone cleavages during ETD but fail to liberate product ions because of the salt bridges holding the ion together.

$$\text{Weight Average} = \frac{\sum (\text{Intensity of product ion}) \times (\text{number of Salt Bridge/Ionic H.B. Broken})}{\sum \text{Intensity of product ion}} \quad (1)$$

We also considered the values for the product ions that are not observed. For ubiquitin, the average numbers of salt bridges and ionic hydrogen bonds that would have to be broken in the product ions that are not observed are shown in table 2. The higher values, especially for the salt bridges, for the product ions that are not observed are again consistent with the idea that electrostatic interactions remain intact to some degree after transfer of ubiquitin into the gas phase. It should be noted that our data does not directly confirm that salt bridges still exist in the gas phase ions, but certainly interactions that exist between the above-mentioned

residues add substantially to the gas-phase stability of the protein. Moreover, given the high number of basic residues and carbonyl oxygens that can stabilize salt bridges in the gas phase [23] along with the compelling results above, it is reasonable to speculate that many of the electrostatic interactions persist once the ions are transferred into the gas phase.

CRABP I

Several other proteins were also electrosprayed under native-like conditions and dissociated by ETD and ETD/CID to monitor the extent to which the native salt bridges remained intact. ETD of the 10+ charge state of the His-tagged R131Q mutant of CRABP I resulted in the formation of mostly c type ions that are formed by dissociations near the N-terminus of the protein (Figure 3a). This observation is consistent with the unstructured N-terminal region that has a His tag and salt bridges that span most of the rest of the protein, starting from R31. Despite the His tag and Arg to Gln mutation at position 131, this form of the protein is known to maintain its native fold in solution [29]. The salt bridge pattern and resulting cleavages during ETD are shown in Figure 3a. Even though the formation of a few product ions (i.e. c_{31}^{3+} , c_{33}^{3+} , c_{34}^{3+} , c_{41}^{3+} , c_{43}^{3+}) requires the breakage of a salt bridge, the vast majority of the product ions are formed without disruption of these electrostatic interactions. Unfortunately, ETD of less abundant charge states of CRABP I (i.e. +9 and +8) did not give sufficient signal to obtain similar data from another charge state.

The importance of the salt bridges in maintaining the gas-phase structure of CRABP I is further indicated when ETD/CID is performed on the charge-reduced ion produced during ETD. Just like with ubiquitin, in this experiment the charged-reduced ion $(M+10H)^{9+}$, which did not dissociate during ETD, was subjected to low-amplitude CID. New product ions are formed during this experiment, including z-type product ions near the C-terminus of the protein (Figure 3a). When the weighted average number of salt bridges broken is calculated using equation 1, we find that 0.1494 salt bridges on average are broken during ETD, while 0.8009 are broken during the ETD/CID experiment (Table 1). The same calculation for the ionic hydrogen bonds results in values of 0.6497 for ETD and 2.043 for ETD/CID. As with ubiquitin, these results are consistent with a native-like pattern of salt bridges being maintained in the gas phase. Moreover, the average numbers of salt bridges and ionic hydrogen bonds that would need to be broken in the unobserved product ions are 1.161 and 3.866, respectively (Table 2).

Azurin

Comparable ETD and ETD/CID results are also obtained with the protein azurin. Upon ETD of the 8+ charge state of this protein, many fragment ions arise from regions that are not spanned by salt bridges (Figure 3b); although, based on several cleavages between positions 84 and 104 it appears that the native salt bridge between K24 and E104 may not be present in many of the protein ions or it exerts a lesser effect. A disulfide bond between Cys3 and Cys26 also strongly influences the dissociation pattern based on the relatively few cleavages near the N-terminus. Interestingly, the interactions between Cu and its binding residues, G45, H46, C112, H117, and M121, are either disrupted upon transfer into the gas phase or they exert a lesser effect on the ETD pattern than the salt bridges, as indicated by the numerous cleavages near the C-terminus. This fact could be caused by the relatively weaker ion-dipole interactions between Cu(II) and the carbonyl oxygen of G45 and the neutral His and Met residues.

ETD/CID on the 8+ charge state of azurin results in several new product ions in regions spanned by the salt bridges. An obvious point of comparison with the ETD data is a new series of product ions between D98 and K101, once again illustrating the effect of salt bridges on dissociation during ETD. Likewise, several new product ions appear in other

regions spanned by salt bridges. The quantitative evaluation of product ion formation is very consistent with the qualitative evaluation (Table 1). The weighted average number of salt bridges broken during ETD is 0.384 as compared to 1.175 for ETD/CID. Very similar values are obtained when considering the ionic hydrogen bonds. If the salt bridge between K24 and E104 is ignored, the weighted average number of salt bridges broken during ETD and ETD/CID are 0 and 0.182 respectively. The lower weighted average values when leaving out the K24-E104 salt bridge suggest that this salt bridge may not exist in a substantial portion of the protein ions in the gas phase. If we consider the weighted average number of Cu amino acid interactions that are broken during ETD and ETD/CID, the relatively high values of 2.701 for ETD and 2.842 for ETD/CID causes us to conclude that these interactions are not playing much of a role in maintaining the structure of the protein in the gas phase. Again, this may mean that some of the metal amino acid interactions do not exist after the protein is electrosprayed or they are less important than the salt bridges in maintaining protein structure in the gas phase.

β2m

β2m was also investigated to observe the relative effects of salt bridges and a disulfide bond on the ETD patterns. As is clear in Figure 3c for the +7 charge state and Figure S3 for the less abundant +8 charge state, most of the backbone cleavages are near the N-terminus of the protein. This pattern is presumably due to both the salt bridges near the C-terminus and the disulfide bond between Cys25 and Cys80 that spans most of the protein. ETD/CID experiments on β2m indicate that a few new product ions are observed within regions spanned by the salt bridges (i.e. between residues 81 and 87); however, the overall dissociation pattern appears to be dominated by the location of the disulfide bond.

A more quantitative look at the dissociation patterns again reveals the importance of the native salt bridges in influencing dissociating patterns. For β2m, the weighted average number of salt bridges and ionic hydrogen bonds broken during ETD is 0.008 and 0.799, respectively, while during ETD/CID the numbers are 0.342 and 0.895 (Table 1). The +8 ion, which is the second most abundant charge state, gave comparable results (Table S1 in Supporting Information). These results are consistent with the protein's salt bridges remaining intact after transfer into the gas phase. Furthermore, the average number of interactions in the bonds that are not broken in the product ions that are not observed is very high for β2m (Table 2).

Cytochrome C

Cytochrome c was the final protein chosen as its gas phase structure has been studied extensively [11, 12, 15]. Unlike the previous proteins, ETD of cytochrome c results in product ions spread across much of the protein, including regions spanned by the native salt bridges (Figure 3d for the +7 charge state; Figure S4 for the +8 charge state). The ETD/CID experiments do give rise to new product ions, but the cleavages mostly occur in the same regions as observed in the ETD experiments. The weighted average numbers of salt bridges and ionic hydrogen bonds that are broken during ETD and ETD/CID is consistent with the qualitative observations. On average 1.31 salt bridges and 2 ionic hydrogen bonds are broken in the formation of the product ions in ETD, whereas fewer, 0.8 and 1.94, salt bridges and ionic hydrogen bonds are broken during the ETD/CID experiments. Data for the +8 ion, which is the second most abundant charge state, show comparable results (Table S1 in Supporting Information). Moreover, the average number of salt bridges and ionic hydrogen bonds that need to be broken in the product ions that are not observed are lower than for the ions that are observed (Table 2). These more quantitative results strongly suggest that the native salt bridges and ionic hydrogen bonds are not maintained in the gas phase ions of cytochrome c. This observation is consistent with previous gas-phase

experiments with cytochrome c that indicate this protein has a very different structure in the gas phase than in solution [11, 12]. Overall, these data suggest that unlike the previous four proteins the electrostatic interactions of cytochrome c in the gas phase may be quite different than in solution. This observation could be related to the fact that cytochrome c has less secondary structure than the four other proteins in this study, thereby providing fewer overall intramolecular interactions to maintain the native fold in the gas phase.

It is reasonable to examine if the heme interactions with Cys14 and Cys17 influence the dissociation patterns of cytochrome c. Our data indicate that the heme contacts are probably not significantly influencing the dissociation patterns of the protein. The weighted average numbers of heme contacts broken during ETD/CID are slightly higher than in ETD (Table 1), but the average numbers of heme interactions that need to be broken in the unobserved ions are slightly lower (Table 2). At most the heme-protein interactions might have a marginal effect.

Computational Analysis

The ETD and ETD/CID results for ubiquitin, CRABP I, azurin, and β 2m suggest that the native salt bridge patterns remain largely intact after transfer into the gas phase. It is important to ask, though, could any other salt bridge pattern in these proteins explain the data equally well or better? To answer this question, we considered all the possible salt bridge patterns for each protein by assuming that any of the acidic (i.e. Glu and Asp) residues could interact with any basic (Lys and Arg) residue to form a salt bridge. For each salt bridge pattern the weighted average number of salt bridges that would need to be broken to give rise to the experimentally measured ETD spectrum was then calculated. The algorithm used for this calculation is illustrated in Figure 4a. To constrain the very large number of possible salt bridge patterns for any given protein, we limited the total number of salt bridges for each protein to the number that is present in the known NMR or X-ray crystal structure of the protein. For example, ubiquitin has five salt bridges in its native structure, so when considering all the salt bridge permutations for this protein, we limited the total number of salt bridges for this protein to five. Even with this constraint, there are still 25,613,280 possible patterns given all the acidic and basic residues in this protein.

After generating all the possible permutations for each protein, we then identified the salt bridge patterns that gave the lowest weighted average number of broken salt bridges in the product ions that arose during ETD. For each protein except CRABP I, the native salt bridge pattern was amongst the top 5% of salt bridge patterns that gave the lowest number of broken salt bridges (Figure 4b). For example, for ubiquitin only 0.88% of all the possible salt bridge combinations can lead to the experimental dissociation pattern without disrupting any salt bridges, and the native salt bridge pattern is among this 0.88%. The fact that >99% of possible salt bridge patterns cannot explain the data as well as the native salt bridge pattern supports the idea that these salt bridges remain largely intact in gas phase ubiquitin. Amongst the 0.88% of salt bridge patterns that have the same score as the native salt bridge pattern, the vast majority still involve the exact same residues with just different pairings of residues. For example, the native structure of ubiquitin has a salt bridge between E18 and K29. Amongst the other salt bridge patterns that score equally with the native structure, there commonly exists a salt bridge between E18 and K27 instead.

For β 2m, azurin and CRABP I, the native salt bridge pattern is among the top 0.62%, 5.3%, and 10.2% of all scores. There is a small percentage of salt bridge patterns for each of these proteins that could give rise to the experimental data without disruption of any salt bridges. Indeed, 0.083%, 0.067% and 1.26% of the salt bridge patterns for β 2m, azurin, and CRABP I, respectively, could lead to the observed ETD spectra without disrupting any salt bridges. In these cases, though, the salt bridge patterns that give a slightly better match to the

experimental data would require major structural changes to achieve the necessary electrostatic interactions. Interestingly, for cytochrome c the native salt bridge pattern still remains in the top 4.8% of scores, even though the experimental data suggests that its gas-phase structure is quite different to solution structure. However, only 0.000121% of cytochrome c salt bridge patterns could result in the experimental spectrum with no salt bridges broken.

Conclusions

Results from ETD and ETD/CID experiments on five proteins electrosprayed from native-like solutions suggest that some proteins can maintain their native salt bridge pattern in the gas phase. For four of the five proteins studied, the formation of product ions during ETD is limited to the regions not spanned by salt bridges, confirming previous observations that ETD can dissociate proteins along the backbone without breaking non-covalent interactions such as salt bridges. ETD followed by CID on the charge-reduced ions is consistent with this observation as CID disrupts the non-covalent interactions to generate new product ions in the regions spanned by salt bridges. Interestingly, for cytochrome c, which is the one protein that behaves differently than the rest, there are previous studies [11, 12, 14] that suggest this protein undergoes substantial structural changes upon transitioning into the gas phase. After considering all possible salt bridge patterns for each protein, we further find that the native salt bridge pattern in most cases explains the experimental ETD data better than nearly all other possible salt bridge patterns. Overall, we conclude that native salt bridge patterns are one aspect of solution phase structure that is likely to be maintained in gas phase protein ions electrosprayed under native-like conditions. Moreover, we predict that ETD and ETD followed by CID might be valuable means of identifying the approximate locations of salt bridges in other proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Example ETD and ETD/CID spectra of the $(M+6H)^{6+}$ ion of ubiquitin and product ion summaries for this ion. (a) ETD spectrum; (b) a summary of the abundances of the observed ETD product ions plotted against their sequence number. Blue and green bars indicate c and z ions, respectively; (c) ETD/CID spectrum; (d) a summary of the abundances of the observed ETD/CID product ions plotted against their sequence number. Orange and red bars indicate c and z ions, respectively.

Figure 2.

Summary of the cleavage sites from the ETD, ETD/CID, CA/ETD and CA/ETD/CID experiments on the 6+ ion of ubiquitin. The amino acid sequence of the protein is shown at the top of the figure. The black bars on this sequence indicate the locations of the salt bridges.

Figure 3.

Summary of cleavage sites from the ETD and ETD/CID experiments on the (a) 10+ ion of CRABP I, (b) 8+ ion of azurin, (c) 8+ ion of $\beta 2m$, and (d) 7+ ion of cytochrome c. The black lines indicate the product ions that are observed during ETD. The red lines indicate the product ions that are observed upon dissociating the charge reduced state (e.g. $[M + nH]^{(n-1)+}$) by a low amplitude CID voltage. The amino acid sequence of each protein is shown at the top of the each figure. The black bars on the sequence indicate the locations of the salt bridges. The orange bars indicate the disulfide bonds. In (b), red letters indicate Cu binding amino acids, and the gray bar indicates a likely salt bridge involving K101, which has an unresolved amino group on its side chain (see SI for details). In (d), the green bars show the amino acids that are linked through the heme group in cytochrome c.

Figure 4.

(a) Flow chart illustrating the algorithm used to compare all possible salt bridge patterns with the experimental ETD data. (b) The percentage of all possible salt bridge patterns that have higher or lower weighted average numbers of broken salt bridges (equation 1) than the native solution salt bridge pattern. The red represents the percentage of patterns that result in the same or fewer numbers of salt bridges broken than the native salt bridge pattern. The blue represents the percentage of patterns that result in a greater number of salt bridges broken than the native salt bridge pattern.

Table 1

Weighted average numbers of salt bridges, ionic hydrogen bonds, metal-amino-acids bonds and heme interactions that are disrupted during ETD and ETD/CID for all proteins in this study.

Protein, charge state	dissociation method	salt bridges broken	ionic hydrogen bonds broken	heme/metal interactions broken	overall weighted average
ubiquitin, 6+	ETD	0	0.0153	-	0.0153
	ETD/CID	0.179	0.231	-	0.410
CRABP I, 10+	ETD	0.0701	0.480	-	0.550
	ETD/CID	0.801	2.043	-	2.844
azurin, 8+	ETD	0.384	0.384	1.102	1.411
	ETD/CID	1.175	1.070	2.013	3.576
β 2m, 7+	ETD	0.008	0.799	-	0.808
	ETD/CID	0.342	0.895	-	1.23
cytochrome c, 7+	ETD	1.31	2	1.44	4.76
	ETD/CID	0.8	1.94	2.59	5.35

Table 2

Average numbers of salt bridges, ionic hydrogen bonds, metal-amino-acids bonds and heme interactions that need to be broken in the product ions that are not observed by ETD and ETD/CID for all the proteins in this study.

Protein, charge state	salt bridges not broken	ionic hydrogen bonds not broken	heme/metal interactions not broken	overall
ubiquitin, 6+	1.12	0.225	-	1.345
CRABP I, 10+	1.129	3.761	-	4.89
azurin, 8+	0.827	1.160	2.429	4.404
β2m, 7+	2.01	1.33	-	3.35
cytochrome c, 7+	0.24	1.68	1.44	3.37