Gaseous Protein Cations Are Amphoteric

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Abstract: Singly- and multiply-protonated ubiquitin molecules are found to react with iodide anions, and certain other anions, by attachment of the anion, in competition with proton transfer to the anion. The resulting adduct ions are relatively weakly bound and dissociate upon collisional activation by loss of the neutral acid derived from the anion. Adduct ions that behave similarly can also be formed via ion/molecule reactions involving the neutral acid. The ion/molecule reaction phenomenology, however, stands in contrast with that expected based on the reaction site(s) being charged. Reaction rates increase inversely with charge state and the total number of neutral molecules that add to the protein cations increases inversely with cation charge. These observations are inconsistent with the formation of proton-bound clusters but are fully consistent with the formation of ion pairs or dipole/dipole bonding involving the neutral acid and neutral basic sites in the protein. The ion/ion reactions can be interpreted on the basis of conjugate acid/base chemistry in which the anion, which is a strong gaseous base, reacts with a protonated site, which is a strong gaseous acid. Adduct ions can also be formed via ion/molecule reaction which, on the basis of microscopic reversibility, implies that the neutral acid interacts with neutral basic sites on the protein cation. These results suggest that acid adduction to gaseous protein cations can be complementary in nature to chemical reactions, such as proton transfer and hydrogen/deuterium exchange, that are strongly mediated by the charge site(s).

Introduction

The advent of electrospray[1] (ES) and matrix-assisted laser desorption ionization[2] has made possible studies of the chemistry of gaseous ions derived from biopolymers. In the case of ES, ions derived from biopolymers, such as proteins, are frequently multiply charged. For multiply-charged biopolymers, most attention has been placed on unimolecular dissociation chemistry for the purpose of deriving primary sequence information.[3−5] Studies of the ion/molecule and ion/ion chemistry of multiply-charged biopolymers have also been performed, however. Examples of ion/molecule chemistry studies include exposure of gaseous protein polycations to strong gaseous bases for the study of proton transfer reactions,[6] and to D_2O for the study of hydrogen/deuterium exchange reactions.[7] Proton transfer reactions have been used to manipulate charge states, to probe higher order gas-phase structure, and to evaluate the dielectric associated with gaseous protein ions. Hydrogen/deuterium exchange reactions have been used to study higher order protein structures in the gas phase.

The reactions of isolated multiply-charged biopolymers derived from ES of proteins (cations) and oligonucleotides (anions) with singly-charged ions of opposite polarity have recently been reported.[8] Ion/ion reactions proceed over an energy hypersurface very different from those associated with ion/molecule reactions involving charge transfer. Ion/ion reactions have been shown to be more effective in manipulating biopolymer charge states as a result of the highly exothermic

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nature of the reactions.[8e] Anion attachment to gaseous multiply-charged proteins has also been noted and that the attachment reaction is highly dependent upon the identity of the anion.[8e]

This report relates both ion/ion and ion/molecule reaction studies designed to interrogate the nature of anion attachment to gaseous protein cations. This work has indicated that gaseous protein cations react differently with strong gaseous acids than they do with strong gaseous bases. Proteins, of course, have both acidic and basic functionalities and their amphoteric nature is clearly apparent in their condensed-phase behaviors. While the importance of the most acidic functionalities in gaseous protein cations, viz., protonated residues, in unimolecular and ion/molecule chemistry is recognized, the role of basic functionalities in gaseous protein cations has not be intentionally probed. The significance of the results described herein is that they lead to the expectation that use of strong gaseous acids as chemical probes of gaseous protein ions is complementary to both proton transfer and hydrogen/deuterium exchange chemistries involving gaseous bases, which, unlike the protein/acid interaction, are charge-site mediated.

Experimental Section

Bovine ubiquitin was obtained from Sigma Chemical Co., St. Louis, MO. Stock solutions for electrospray were prepared by dissolving 1 mg of ubiquitin in a 50:50 methanol−water mixture containing 1% doubly distilled acetic acid (Aldrich Chemical Co., Milwaukee, WI). Working standards at 10 μM were prepared by a 1:20 dilution of stock solution in 100% methanol containing 1% doubly distilled acetic acid. The solution was infused at rates between 1.0 and 2.0 μL/min through a 120 μm i.d. stainless steel capillary held at a potential of +3500 to +4000 V.

All experiments were carried out with a home-made electrospray source coupled with a Finnigan-MAT (San Jose, CA) Ion Trap Mass Spectrometer modified for injection of ions formed external to the ion trap through an end-cap electrode.[9] Further Improvements to the electrospray interface design and ion trap electronics have been incorporated to improve sensitivity and reproducibility associated with high mass-to-charge analysis. Details of these changes have been reported elsewhere.[8g]

The singly charged anions used in the ion/ion reaction experiments were generated by an atmospheric sampling glow discharge (ASGDI) source mounted 90° to the aforementioned electrospray source. The ion trap is situated such that there is a line of sight from the exit aperture of the ASGDI source to a 3-mm hole drilled in the ring electrode.[8e,g,10] A detailed description of the sample introduction system and control electronics for the ASGDI source have been published previously.[8g] The compounds used to generate the anionic reactants CCl_3COO^-, CF_3COO^-, I^-, and $CH_3CH_2CH_2CH_2O^-$ were trichloroacetic acid, trifluoroacetic acid, 2-iodopropane, and *n*-butyl nitrite, respectively (Chemservice Inc., West Chester, PA). The anions Cl^-, Br^-, $C_8F_{15}^-$, and $C_7F_{13}^-$ were generated from methylene chloride, 1,2-dibromotetrafluoroethane, and perfluoro 1,3-dimethylcyclohexane (PDCH), respectively (Aldrich Chemical Co., Milwaukee, WI). A nitrated dynamite sample was used as a source of NO_3^- and NO_2^- anions. Samples of C_8F_{16}, obtained from John McCall of the K-25 Site in Oak Ridge, TN, were used as a source of CF_3^- anions. The inlet pressure for the ASGDI source was set to 950 mTorr for all experiments.

For the case of ion/molecule attachment reactions involving cations of ubiquitin, the gaseous acid HI (Aldrich) was admitted into the unheated vacuum system to a pressure of 3×10^{-6} to 2.1×10^{-5} Torr. Helium bath gas was added to the vacuum system to a total pressure of 1 mTorr. These experiments consisted of a cation accumulation period, followed by a delay of 5 to 2000 ms prior to mass analysis. In the case of ion−molecule reaction kinetic measurements involving individual charge sites of ubiquitin, a mass selection step was used (see below) to select the charge state of interest. This was followed by a variable reaction period (up to 2000 ms) for the formation of ubiquitin−adduct ions. Ion−molecule adduction rates were determined by measuring the loss rate of the parent ion of the selected charge state. The rate was obtained from the slope of the plot of the negative logarithm of the I_t/I_0 intensity ratio versus time. Pseudo-first-order kinetics prevail due to the great excess of neutral gaseous acid over ubiquitin cations present in the ion trap during the reaction period. Values for the rate constant were obtained by dividing the relative rate by the calculated number density of the gaseous acid present in the vacuum system.

Ion Manipulation and Mass-to-Charge Analysis Cations were injected axially into the trap for periods ranging from 0.05 to 0.15 s. The radio frequency sine-wave amplitude applied to the ring electrode during ion injection ranged from 700 to 1200 V zero-to-peak. Anions were formed by sampling the head space vapors of the various anionic reagents in the glow discharge.

Details of ion isolation for high-mass multiply-charged ions have been given previously.[11] A single resonance ejection scan was used for isolation of parent ions. Low *m/z* ions were ejected by passing the ions through a q_z value of 0.908 by scanning the amplitude of the ring electrode radio frequency sine-wave. High *m/z* ions were ejected by a dipolar resonance ejection scan using a 8-V peak-to-peak sine-wave signal applied to the end caps at a frequency selected to eject ions at an *m/z* value slightly greater than that of the parent ion. Parent ions were isolated prior to the anion accumulation period at less than unit resolution to avoid parent ion loss, due either to dissociation or to ejection from off-resonance power absorption.[8e]

Mass-to-charge analysis was effected after the completion of all ion isolation and reaction periods using resonance ejection[12] to yield a mass/charge range (for ubiquitin) as high as 12 000 using resonance ejection amplitudes of 3.5−5.0 V_{P-P}. The mass-to-charge scale was initially calibrated using the electrospray mass spectrum of ubiquitin. In this work, the mass-to-charge ratios of the various charge states of the parent

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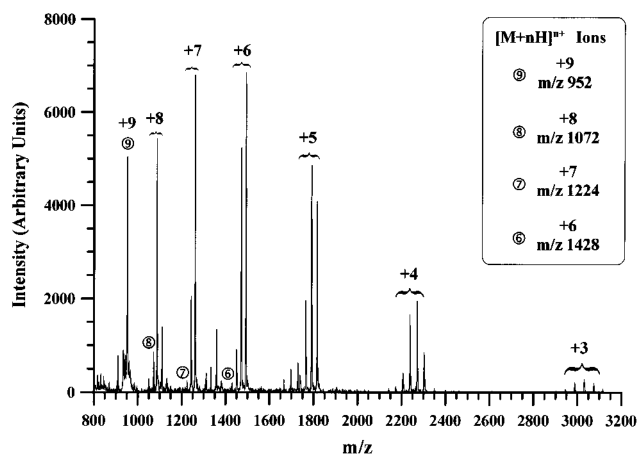
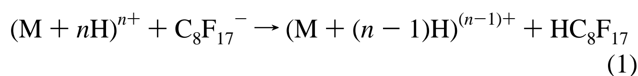


Figure 1. Product ion spectrum resulting from the interaction of the 9+ charge state of bovine ubiquitin with a population of iodide anions for 25 ms.

compound were known and could be used to determine a correction for the mass scale provided by the ion trap data system. Proton transfer reactions between multiply-charged ubiquitin cations and PDCH anions were used to calibrate the mass scale at higher mass-to-charge values. The spectra shown here were typically the average of 50−100 individual scans.[8e]

Results and Discussion

In recent gas-phase bio-ion/ion studies[8e,g,10] it has been noted that the anions $(M-F^{\bullet})^-$ and $(M-CF_3^{\bullet})^-$ derived from perfluoro-1,3-dimethylcyclohexane (PDCH) react exclusively via proton transfer with multiply-protonated peptides and proteins, e.g.:



$$(M + nH)^{n+} + C_8F_{17}^- \rightarrow (M + (n-1)H)^{(n-1)+} + HC_8F_{17} \quad (1)$$

However, we have observed that a number of even-electron anions, A^-, can, in addition to react by proton transfer, react by attachment,[8e,g] viz.,

$$(M + nH)^{n+} + A^- \rightarrow (M + nH + A^-)^{(n-1)+} \quad (2)$$

So far, the anion found to show the greatest extent of attachment to a variety of multiply-protonated peptides and proteins, relative to proton transfer, is the iodide anion, I^-. Bovine ubiquitin is used to illustrate a variety of observations made with I^-, as well as other anions, involving anion attachment to protein cations.

Figure 1 shows the results of an experiment in which the 9+ charge state of ubiquitin was isolated and allowed to react with a population of I^- anions for 25 ms. Note that the products from proton transfer, such as the $(M + 8H)^{8+}$ cation, and anion attachment, such as the $(M + 9H + I^-)^{8+}$ cation, both appear but that anion attachment dominates. For example, very little of the $(M + 6H)^{6+}$ product ion expected from three consecutive proton transfer reactions appears in Figure 1 while an abundant $(M + 9H + 3I^-)^{6+}$ product appears resulting from the consecutive attachment of three I^- anions. For multiply-protonated ubiquitin cations, proton transfer and attachment are competitive processes in reactions with the anions used in this study. In the case of I^- anions, reaction by anion attachment clearly dominates.

Figure 2 shows the results of an experiment in which the product cation population shown in Figure 1 was subjected to a rapid sweep of resonance excitation across the various charge states. This is effected by applying an oscillating dipolar electric field across the ion trap end-cap electrodes and scanning the

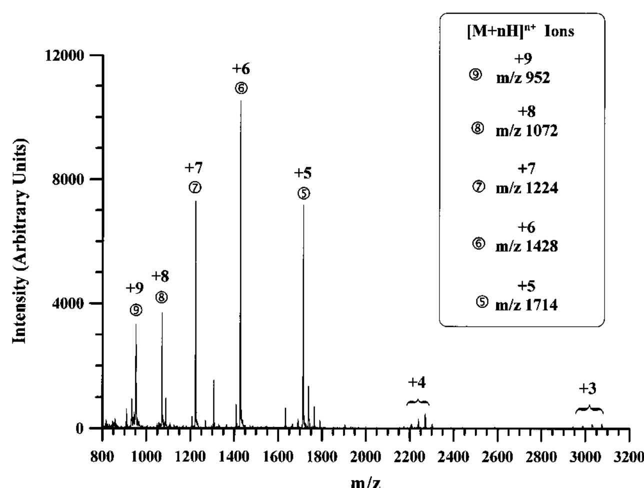
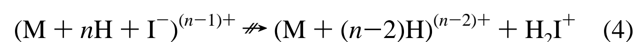


Figure 2. Product ion spectrum resulting from a rapid resonance excitation sweep across the cation population reflected in Figure 1.

amplitude of the trapping voltage applied to the ring electrode such that each ion is swept through resonance with the applied dipolar field. In effect, this procedure subjects each ion to a brief collisional activation period and is often used to desolvate ions formed via electrospray.[6h] Figure 2 shows that molecules of HI are readily removed from the $(M + nH + xI^-)^{(n-x)+}$ ions, i.e.

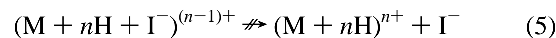
$$(M + nH + xI^-)^{(n-x)+} \rightarrow (M + (n-x)H)^{(n-x)+} + xHI \quad (3)$$

This effect is most apparent for the 9+ to 5+ charge states. The ions of the 4+ and 3+ charge states are of higher mass-to-charge and are less strongly trapped than the more highly charged ions. The latter ions therefore tend to be ejected from the ion trap under the conditions that are most effective at removing molecules of HI from the higher charge states. In separate experiments in which ions within each charge state were isolated and subsequently subjected to collisional activation via a rapid sweep of resonance excitation, as described above, reaction 3 was the exclusive reaction path. Proton transfer to HI was not observed, e.g.,



$$(M + nH + I^-)^{(n-1)+} \not\rightarrow (M + (n-2)H)^{(n-2)+} + H_2I^+ \quad (4)$$

and loss of I^- was not observed, e.g.,



$$(M + nH + I^-)^{(n-1)+} \not\rightarrow (M + nH)^{n+} + I^- \quad (5)$$

Fragmentation of protein covalent bonds was not observed, e.g.,

$$(M + nH + I^-)^{(n-1)+} \not\rightarrow m_aH_{(n-x)}{}^{(n-x)+} + m_bH_xI^{x+} \quad (6)$$

where m_a and m_b represent protein fragments. This set of observations suggests that the attached species are loosely bound (probably not covalently attached). Moreover, the anion attachment product is probably an intermediate in the proton transfer reaction since collisional activation of the adduct species leads to the expected proton transfer products.

Figure 3 shows a general energy diagram consistent with these observations indicating the relevant reaction thermochemistry. The enthalpy of the proton transfer reaction is simply the difference between the proton affinities of I^- and $(M + (n-1)H)^{(n-1)+}$, i.e.,

$$\Delta H_{rxn}(H^+ \text{ transfer}) = PA((M + (n-1)H)^{(n-1)+}) - PA(I^-) \quad (7)$$

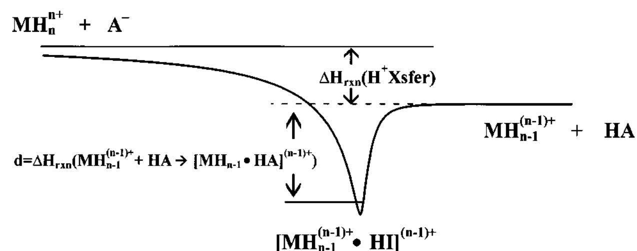
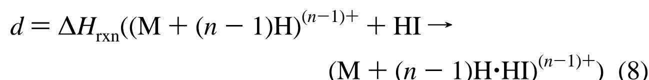


Figure 3. Energy diagram associated with ion/ion proton transfer from a multiply-protonated protein, $(M + nH)^{n+}$, to I^-.

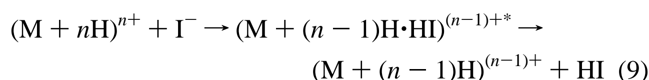
The well-depth, *d*, associated with the adduct ion is the enthalpy associated with the attachment of HI to $(M + (n - 1)H)^{(n-1)+}$. That is,



$$d = \Delta H_{rxn}((M + (n - 1)H)^{(n-1)+} + HI \rightarrow (M + (n - 1)H{\cdot}HI)^{(n-1)+}) \quad (8)$$

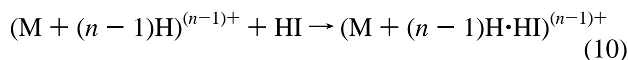
The likelihood that the initially formed $(M + (n - 1)H{\cdot}HI)^{(n-1)+*}$ species survives in the ion trap environment is a function of $\Delta H_{rxn}(H^+ \text{ transfer})$, *d*, the number of degrees of freedom in the adduct ion, and the rate at which the initial ion/ion condensation energy can be removed from the adduct.[13]

If the hypothesis that the anion attachment product is an intermediate in the proton transfer reaction



$$(M + nH)^{n+} + I^- \rightarrow (M + (n - 1)H{\cdot}HI)^{(n-1)+*} \rightarrow (M + (n - 1)H)^{(n-1)+} + HI \quad (9)$$

is correct, the adduct should also be formed via the reverse reaction, i.e.

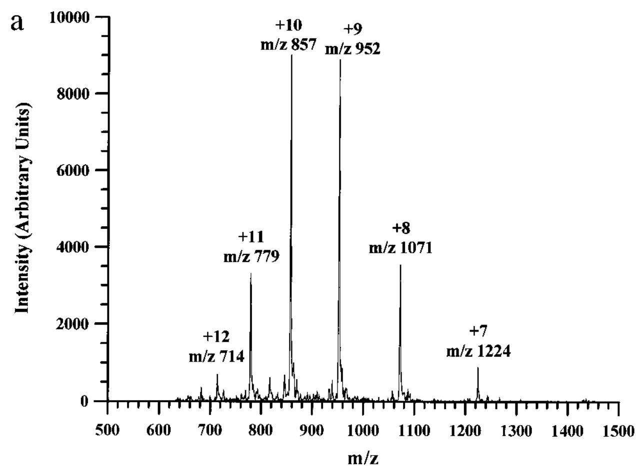


$$(M + (n - 1)H)^{(n-1)+} + HI \rightarrow (M + (n - 1)H{\cdot}HI)^{(n-1)+} \quad (10)$$

Figure 4b shows the results obtained by exposing the ubiquitin cation population shown in Figure 4a to HI at an estimated pressure of 3.7×10^{-6} Torr for 250 ms. Indeed, adduct ions are readily formed. These adduct ions exhibit behavior identical to those formed via ion/ion reactions when subjected to collisional activation in that they react exclusively by loss of one or more molecules of HI. While these results do not prove that the structures of $(M + (n - 1)H{\cdot}HI)^{(n-1)+}$ ions formed via ion/molecule reactions are equivalent to those formed via ion/ion reactions, they cannot be distinguished on the basis of their behavior under collisional activation conditions. Furthermore, it seems reasonable that once proton transfer to I^- occurs in the ion/ion reactions with $(M + nH)^{n+}$, an ion/neutral complex is formed that can rearrange to form whatever complex is formed from the ion/molecule collision involving $(M + (n - 1)H)^{(n-1)+}$ and HI.

The results of Figure 4b, which show extensive clustering of HI to ubiquitin cations, are a suprising result when viewed from the context of protein ion solvation in the gas phase. Adduct ions are commonly observed when gaseous protein ions are exposed to strong bases.[6] Highly charged proteins react largely by proton transfer, whereas clustering becomes important as the charge state of the protein decreases.[6] These observations have been interpreted on the basis of the formation of proton-bound clusters involving the charge sites on the protein and the neutral bases.[6m] The relative degrees to which products from proton transfer versus adduct formation are observed are strong

(13) Under the conditions used here, in which a helium bath gas pressure of 1 mtorr is employed, modeling studies have suggested that large bio-ions can be internally thermalized in on the order of a few hundred microseconds. See ref 8d.



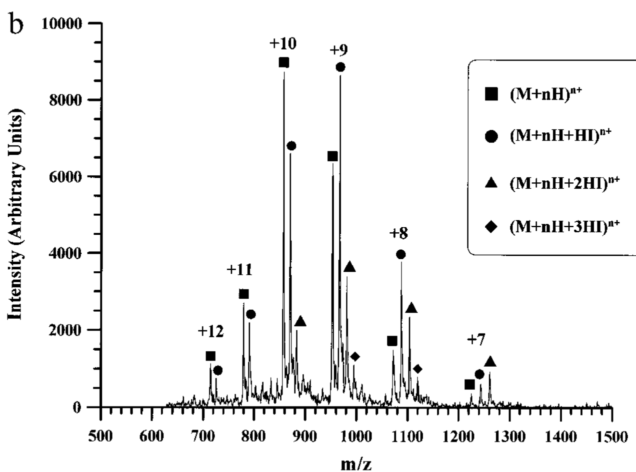


Figure 4. (a) ES mass spectrum of ubiquitin. (b) Product ion spectrum resulting from the exposure of the cation population shown in part a to HI at an estimated pressure of 3.7×10^{-6} Torr for 250 ms.

functions of the proton affinity of the base. Weak bases, such as water (PA = 166.5 kcal·mol^{-1})[14] and methanol (PA = 181.9 kcal·mol^{-1}),[14] are not observed to undergo either proton transfer or clustering with the protein ions in the dilute gas phase.[15] The proton affinity of HI (PA = 147 kcal·mol^{-1})[14] is much lower than that of water and is therefore not expected to solvate a protonated site as well as water. In the ion/ion reaction case, it is clear that I^- must attack a protonated site initially forming either an ion pair of the form $R{-}NH_3^+{\cdot}I^-$ or a dipole/dipole complex written nominally as $R{-}NH_2{\cdot}HI$. For this species to form an ion/dipole interaction (i.e., a proton-bound dimer of the form $R{-}NH_3^+{\cdot}HI$), the HI molecule must transfer to a charged residue elsewhere on the protein.

The observations just discussed suggest that the adduct ions formed either by ion/ion or ion/molecule reaction involving I^- or HI, respectively, may not be charge-centered clusters like those formed with strong gaseous neutral bases. Rather, they

(14) Lias S. G.; Bartmess, J. E.; Liebman, J. F.; Holmes, J. L.; Levin R. D., W. G. Mallard *J. Phys. Chem. Ref. Data* **1988**, *17*, Suppl. 1

(15) There is evidence that suggests that the solvent molecules in ES can strip protons from multiply-protonated molecules in the desolvation process. See, for example, (a) Chait, B. T.; Chowdhury, S. K.; Katta, V. *Proceedings of the 39th Conference on Mass Spectrometry and Allied Topics*, Nashville, TN, 1991; p 447. (b) Schnier, P. D.; Gross, D. S.; Williams, E. R. *J. Am. Soc. Mass Spectrom.* **1995**, *6*, 1086. (c) Winger, B. E.; Light-Wahl, K. J.; Smith, R. D. *J. Am. Soc. Mass Spectrom.* **1992**, *3*, 624. (d) Chillier, X. F. D.; Monnier, A.; Bill, H.; Gulacar, F. O.; Buchs, A.; McLuckey, S. A.; Van Berkel, G. J. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 299. However, protein ions that have survived the drying process do not undergo proton transfer or clustering with either water or methanol under the conditions in which HI has been observed to attach to ubiquitin cations.

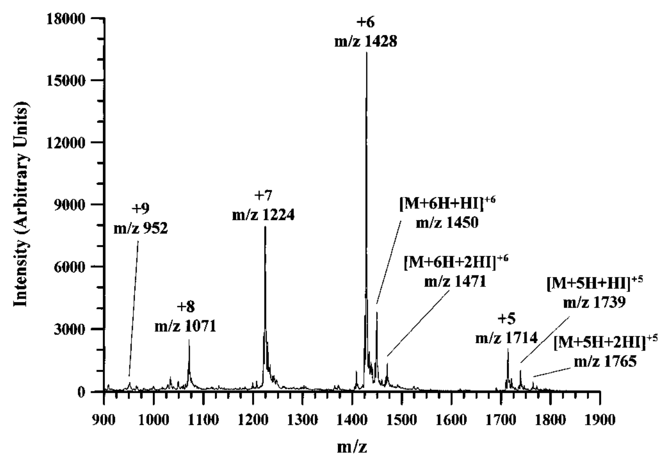


Figure 5. ES mass spectrum of a bovine ubiquitin solution containing 10 pmol/μL protein in 50/50 methanol−water and 3 mM potassium iodide.

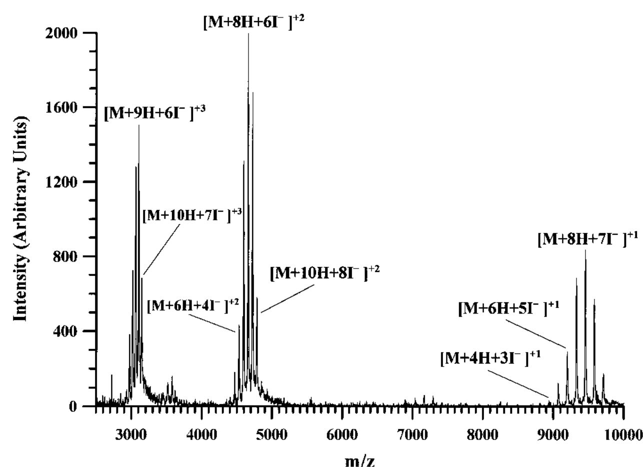


Figure 6. Product ion spectrum resulting from the reaction of all ES charge states derived from bovine ubiquitin with a population of I^- for 125 ms.

may involve either ion pair or dipole/dipole formation. Ion pair formation in solution involving protein cations and anions of strong acids has been proposed as leading to reduction of charge states in electrospray mass spectra[16] and for the observation of relatively weakly-bound adduct ions in ES mass spectra involving acidic species, such as sulfuric acid.[17] Chait et al. have proposed a mechanism in which gas-phase dissociation of adduct species formed initially in solution can lead to charge state reduction in ES mass spectra of proteins.[16] The ion/ion reaction experiment leading to the results of Figure 2 is a purely gas phase analog in which the adduct ions are both formed and dissociated in the gas phase. While the nature of the binding in the gas phase of the clusters reported by Chait et al. was not discussed,[16,17] it was shown that significantly higher temperatures were necessary to remove the acid adducts than was necessary for removal of the electrospray solvent. This indicates that the adducts formed in solution were more strongly bound in the gas phase than the electrospray solvent, which is consistent with the gas-phase ion/molecule reaction results described here. That is, HI is observed to attach to the ubiquitin cations when water and/or methanol under similar conditions do not. If the parallels between the condensed-phase and the gas-phase ion/ion reactions just discussed are consistent, adducts comprised of HI should also be formed in solution. Figure 5 shows the mass spectrum derived from a 10 μM solution of ubiquitin containing 3 mM KI. Indeed, adducts of HI are observed for the 6+ and 5+ charge states. While the relative abundances of the adduct species formed in the gas phase versus the condensed phase cannot be compared directly,[18] the experiment leading to Figure 5 provides further support for the similarity of the clusters formed with strong acids via gas phase and solution chemistries.

The formation of ion pairs involving protein cations and anions in solution, and the observation of adduct ions in the gas phase consistent in mass with the ion pairs, does not necessarily imply that the structures and nature of binding are preserved in the gas phase. Rearrangement to proton-bound species might occur upon removal of solvent. Furthermore, cooperative binding involving a protonated site and a neutral basic site might add to the stability of the adduct. For example, the iodide anion of HI might interact with a protonated site while the proton of HI interacts with a nearby neutral amine group in a "salt bridge" type of interaction.[19] Such an interaction might account for the unexpected stability of the HI adducts. However, both the "solvated proton" and "salt bridge" pictures require the presence of one or more charge sites while the ion pair (or dipole/dipole) picture does not. Several experiments have therefore been performed to probe the behavior of ubiquitin ions of low charge state. Figure 6, for example, shows the results of an experiment in which all of the charge states of ubiquitin were exposed to I^- anions for a sufficient period (125 ms) to observe the 3+, 2+, and 1+ charge states. A broad distribution of adduct ions is observed for each charge state due both to the initial distribution of parent ion charge states and the competition between proton transfer and attachment. Perhaps the most noteworthy observation with respect to the question of the nature of binding in the adducts is that there are as many as nine molecules of HI (or nine I^- anions) attached to the 1+ charge state of ubiquitin. It seems unlikely that all nine molecules of HI can solvate the charge site in the room temperature helium bath gas storage conditions of the ion trap. Furthermore, it seems unlikely that nine "salt bridges" to a single proton could account for this observation. Such a situation would be entropically disfavored and would afford decreasing energetic benefit with each additional HI molecule.

The data of Figure 6 cannot be used to determine the number of HI molecules that might have been lost as a result of charge state reduction. An ion/ion reaction experiment employing two different anion reagents used in turn was performed to determine the extent to which removal of protons affects the binding of HI molecules in the adduct ions. Figure 7a shows the 5+ charge state products formed from the ubiquitin parent ion charge state distribution following reaction with a population of I^- for 50 ms. Figure 7b shows the results of reactions of the ion population of Figure 7a with the $C_8F_{17}^-$ and $C_7F_{15}^-$ anions derived from PDCH, which are known to react exclusively by proton transfer with ubiquitin cations.[8e] If protons are necessary for binding, the loss of HI molecules in going from the 5+ charge state to the 1+ charge state might be expected to occur. Within experimental uncertainly, however, the relative abundances of the $(M + nH)^{n+}$ and adduct ions $(M + nH{\cdot}(HI)_x)^{n+}$ do not change as the charge state is reduced. The stability of the 1+ charge state adducts would be significantly reduced relative to the 5+ charge state adducts for either the proton-

(16) Mirza, U. A.; Chait, B. T. *Anal. Chem.* **1994**, *66*, 2898.

(17) Chowdhury, S. K.; Katta, V.; Beavis, R. C.; Chait, B. T. *J. Am. Soc. Mass Spectrom.* **1990**, *1*, 382.

(18) It is not possible to create equivalent reaction conditions in solution and in the ion trap. Furthermore, the cluster ions formed in solution are highly dependent upon the electric fields present in the atmosphere/vacuum interface.

(19) Campbell, S.; Rodgers, M. T.; Marzluff, E. M.; Beauchamp. J. L. *J. Am. Chem. Soc.* **1995**, *117*, 12840.

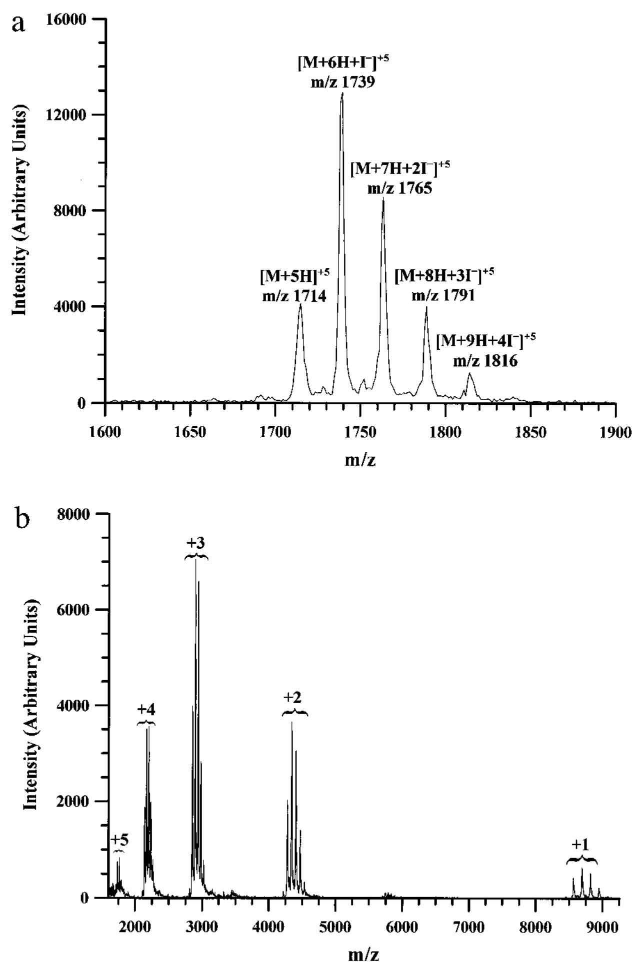


Figure 7. (a) Product ion distribution of the isolated 5+ charge state resulting from the reaction of the ES charge state distribution with a population of I^- for 50 ms. (b) Product ion distribution resulting from the reaction of $C_8F_{17}^-$ and $C_7F_{15}^-$ anions derived from PDCH with the cation population shown in part a.

bound or salt bridge structures but such a loss in stability is not reflected in the data of Figure 7b. The formation of ion pairs or dipole/dipole interactions is more consistent with these results because such interactions are independent of the locations and numbers of charges elsewhere on the protein. The data suggest that the adducts are merely spectators in the proton transfer reactions involving the $C_8F_{17}^-$ and $C_7F_{15}^-$ anions.

The ion/ion reactions can be viewed within the context of conjugate acid−base chemistry. The iodide anion is a gaseous base whereas a protonated residue of the protein is an acid. Upon proton transfer, the neutral protein residue is a neutral base whereas HI is an acid. By extension, the ion/molecule reaction can be viewed within this context if the acid, HI, interacts with the basic moieties of the protein, the neutral basic residues, rather than with the protonated residues. Such a scenario runs counter to the usual picture of ion/molecule chemistry whereby the neutral interacts with the charge site. We have therefore examined the kinetics associated with the ion/molecule reactions of some of the ubiquitin cations with HI. Figure 8 shows a plot of the measured rate constants for HI attachment for each of the ubiquitin charge states from 6+ to 12+.[20] The ion/molecule collision rate, based on polarization, should increase linearly with charge state.[21] Experimentally measured proton transfer reaction rates involving strong gaseous bases have consistently been observed to increase with charge state, although a linear dependence is frequently not observed due to a dependence of reaction efficiency upon charge.[6a,e,f,k,l] The

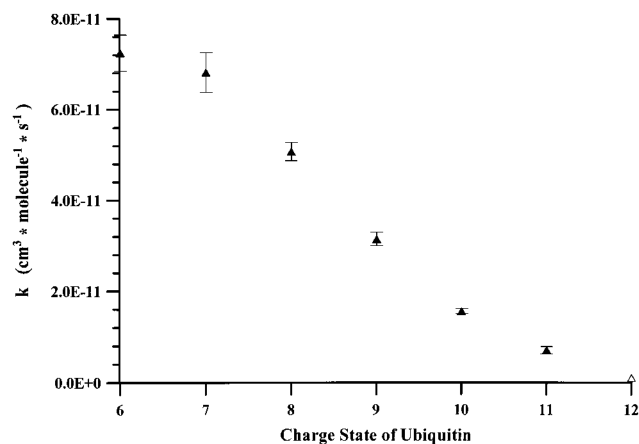


Figure 8. Plot of the rate constants for HI attachment for the 6−12+ charge states of ubiquitin. An open triangle is used to represent the rate for the 12+ parent ion and is represented thusly due to the fact that the rate of proton transfer to background gases was significantly greater than that of HI attachment such that a precise rate measurement could not be made. The rate constant for this charge state must be less than 4×10^{-12} $cm^3 \cdot molecule^{-1} \cdot s^{-1}$.

"hard-sphere" collision rate, based on protein size, is larger for most proteins than the point-charge/(induced) dipole associated with a single charge and is not expected to change with charge state unless the higher order structure of the protein is charge dependent.[22] Remarkably, the rate constants for HI attachment shown in Figure 8 increase inversely with ubiquitin charge state. Such behavior is inconsistent with that expected for charge-site mediated reactions but is at least qualitatively consistent with the picture that the HI molecule is reacting with a neutral basic site, insofar as the number of neutral basic sites is inversely related to charge state. The rate constant behavior of Figure 8 does not strictly follow an inverse linear relationship, as would be expected if each neutral basic site reacted with the same rate constant. This observation might arise from possible differences in three-dimensional protein structure associated with ions of different charge and/or the degree of intramolecular proton

(20) Rate constants were determined by measuring the rate of loss of the $(M + nH)^{n+}$ ions as a function of time. The number density of HI was determined from an ionization gauge measurement corrected for the differential sensitivity of the gauge according to Bartmess and Georgiadis (Bartmess, J. E.; Georgiadis, R. M. *Vacuum* **1983**, *33*, 149). For each charge state, linear pseudo-first-order kinetics were observed over the entire reaction time range. Corrections were made for significant parent ion loss at the highest charge states due to proton transfer to residual pyridine vapor from previous experiments. These corrections were made by determining the kinetics for proton transfer to residual pyridine prior to admitting HI vapors and subtracting the rate associated with proton transfer from the total rate measured when HI was admitted. As expected, the rate for proton transfer increased with charge state. The correction was most important, therefore, for the 10+ through 12+ charge states whereas the rate of proton transfer was less than a few percent of the rates for HI attachment at the lower charge states. No pyridine adducts were detectable in these studies.

(21) (a) Langevin, P. M. *Ann. Chim. Phys.* **1905**, *5*, 245. (b) Eyring, H.; Hirschfelder, J. O.; Taylor, H. S. *J. Chem. Phys.* **1936**, *4*, 479. (c) Gioumousis, G.; Stevenson, D. P. *J. Chem. Phys.* **1958**, *29*, 294. (d) Su, T.; Bowers, M. T. In *Gas Phase Ion Chemistry*; Bowers, M. T., Ed.; Academic Press: New York, 1979; Vol. 1, Chapter 3. (e) Su, T.; Bowers, M. T. *Int. J. Mass Spectrom. Ion Phys.* **1973**, *12*, 347. (f) Su, T.; Bowers, M. T. *Int. J. Mass Spectrom. Ion Phys.* **1975**, *17*, 211. (g) Chesnavich, W. J.; Su, T.; Bowers, M. T. *J. Chem. Phys.* **1980**, *72*, 2641. (h) Su, T.; Bowers, M. T. *J. Chem. Phys.* **1982**, *76*, 5183.

(22) (a) Covey, T.; Douglas, D. J. *J. Am. Soc. Mass Spectrom.* **1993**, *4*, 616. (b) Cox, K. A.; Julian, R. K.; Cooks, R. G.; Kaiser, Jr., R. E. *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 127. (c) von Helden, G.; Wyttenbach, T.; Bowers, M. T. *Science* **1995**, *267*, 1483. (d) von Helden, G.; Wyttenbach, T.; Bowers, M. T. *Int. J. Mass Spectrom. Ion Processes* **1995**, *146*, 349. (e) Clemmer, D. E.; Hudgins, R. R.; Jarrold, M. F. *J. Am. Chem. Soc.* **1995**, *117*, 10141. (f) Collings, B. A.; Douglas, D. J. *J. Am. Chem. Soc.* **1996**, *118*, 4488.

bonding. We are currently examining this behavior further with a variety of multiply-charged proteins.

It is also worthy of note that the values of the measured rate constants, which are all less than 10^{-10} cm^3·molecule^{-1}·s^{-1}, are significantly lower than rate constants associated with efficient ion/molecule reactions (i.e., on the order of 10^{-9} cm^3·molecule^{-1}·s^{-1})[21,23] and low relative to the value predicted for a point charge of mass 8500 Da colliding with HI at room temperature (i.e., 5.7×10^{-10} cm^3·molecule^{-1}·s^{-1}).[23] Indeed, these values are one-to-two orders of magnitude lower than those measured for highly exoergic proton transfer reactions. It could be concluded either that the reactions are inefficient or that the reaction rates are dependent upon the collision rate between HI and the neutral basic sites of the protein, or both. In any case, a decrease in reaction rate with increasing charge state is difficult to rationalize based on the interaction of HI with protonated residues, whereas an explanation based on the interaction of HI with neutral basic residues, at least in a qualitative sense, is straightforward.

The rates of loss of the various $(M + nH)^{n+}$ ions of ubiquitin reflect the reactivity of the various charge states for the addition of a single molecule of HI. These rates, however, do not reflect differences in the numbers of HI molecules that can attach to the various charge states under a fixed set of conditions. We have observed that ubiquitin ions of lower charge states not only add the first HI molecule at higher rates than ions of higher charge states but also add more HI molecules. Figure 9, for example, compares the results obtained for the 12+ (Figure 9a) and 1+ (Figure 9b) charge states of ubiquitin over comparable periods of time (roughly 500 ms) and in the presence of the same number density of HI (estimated to be 2.5×10^{11} cm^{-3}). Small peaks are evident in between those associated with successive additions of HI. The peaks correspond to the addition of a molecule of nitric acid and presumably result from the residual nitric acid present in the vacuum system from a previous ion/molecule reaction experiment. Nitric acid and the NO_3^- anion have both been observed to attach to protein cations including those of ubiquitin (data not shown). The 12+ charge state clearly adds a single molecule of HI, but not significant numbers in excess of one over this reaction time period. The 1+ charge state, on the other hand, shows the addition of as many as eight molecules of HI over this time period. In fact, the $(M + H)^+$ ion population is almost completely depleted as a result of consecutive additions of HI, in contrast with the 12+ charge state where most of the ubiquitin signal remains associated with the $(M + 12H)^{12+}$ parent ion after this reaction period. This dramatic contrast is consistent with the argument that more neutral basic sites are available for ion pair or dipole/dipole formation in the low charge state ion than in the high charge state ion. Such behavior is inconsistent with the formation of proton-bound adducts whereby greater numbers of HI molecules would be expected to bind with the higher charge state ion.

Figure 9c shows the results obtained when the singly charged ubiquitin ions were stored in the presence of the same number density of HI used to collect the data of Figures 9a and 9b for roughly 2 s, rather than 500 ms. This spectrum shows the depletion of all of the signal associated with the $(M + H)^+$ ion as well as with the $(M + H + nHI)^+$ ions, where $n \leq 4$, and also shows as many as twelve molecules of HI attached to the singly charged ion. It was noted that the appearance of the spectrum changed very little beyond a reaction period of about 1 s, indicating that the ions with large numbers of HI molecules already attached were reacting very slowly. We have not observed the attachment of more than twelve molecules of HI. It is interesting to note that there are four arginine residues,

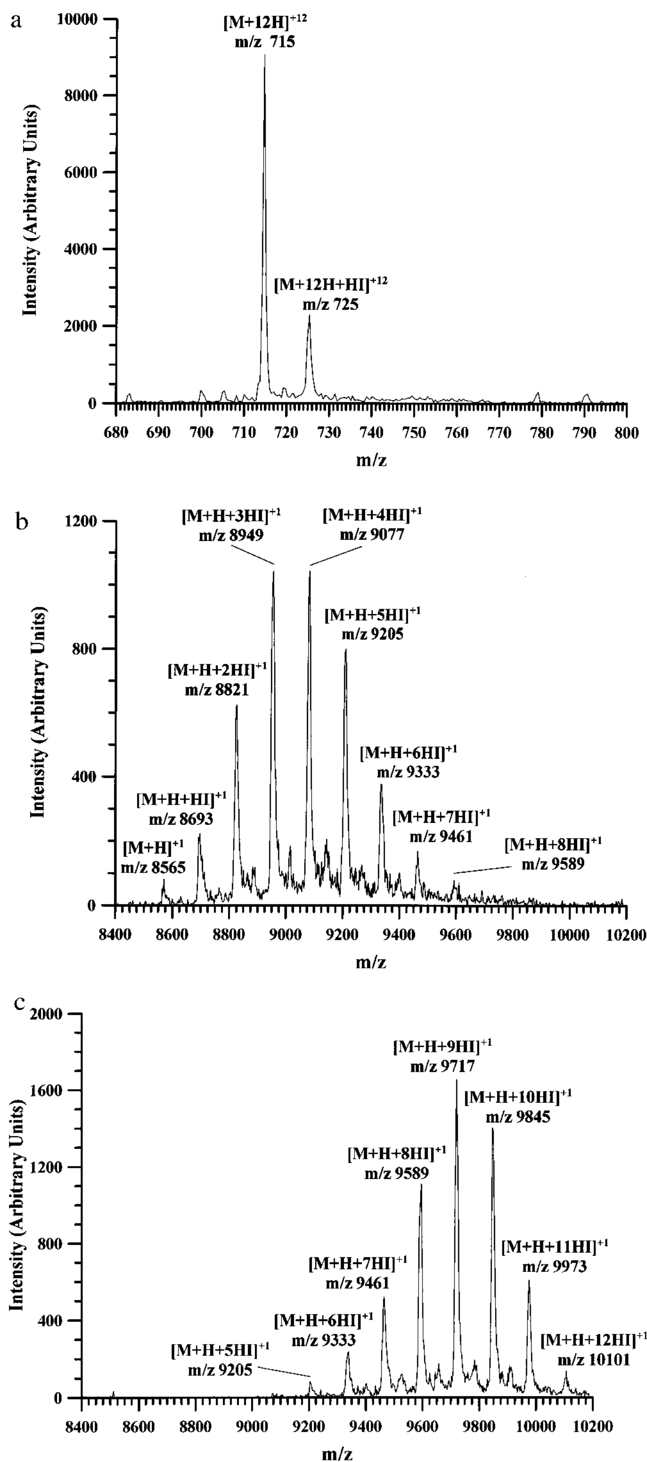


Figure 9. (a) Product ion distribution following the isolation of the $(M + 12H)^{12+}$ cation and reaction with neutral HI ($\sim 2.5 \times 10^{11}$ cm^{-3}) for 500 ms. (b) Product ion distribution following the formation of the $(M + H)^+$ ion via ion/ion reactions and reaction with neutral HI ($\sim 2.5 \times 10^{11}$ cm^{-3}) involving a total time available for ion/molecule reactions of roughly 500 ms. (c) Product ion distribution following the formation of the $(M + H)^+$ ion via ion/ion reactions and reaction with neutral HI ($\sim 2.5 \times 10^{11}$ cm^{-3}) involving a total time available for ion/molecule reactions of roughly 2000 ms.

(23) For a compendium of a wide variety of ion/molecule reaction rate constants for both efficient and inefficient reactions see: Ikezoe, Y.; Matsuoka, S.; Takebe, M.; Viggiano, A. *Gas Phase Ion−Molecule Reaction Rate Constants Through 1986*; Maruzen Company: Tokyo, 1987. For a point charge of mass 8500 reacting with HI, the collision rate constant is predicted to be 5.7×10^{-10} cm^{-3}·molecule^{-1}·s^{-1} using the rate theory described in ref 21h.

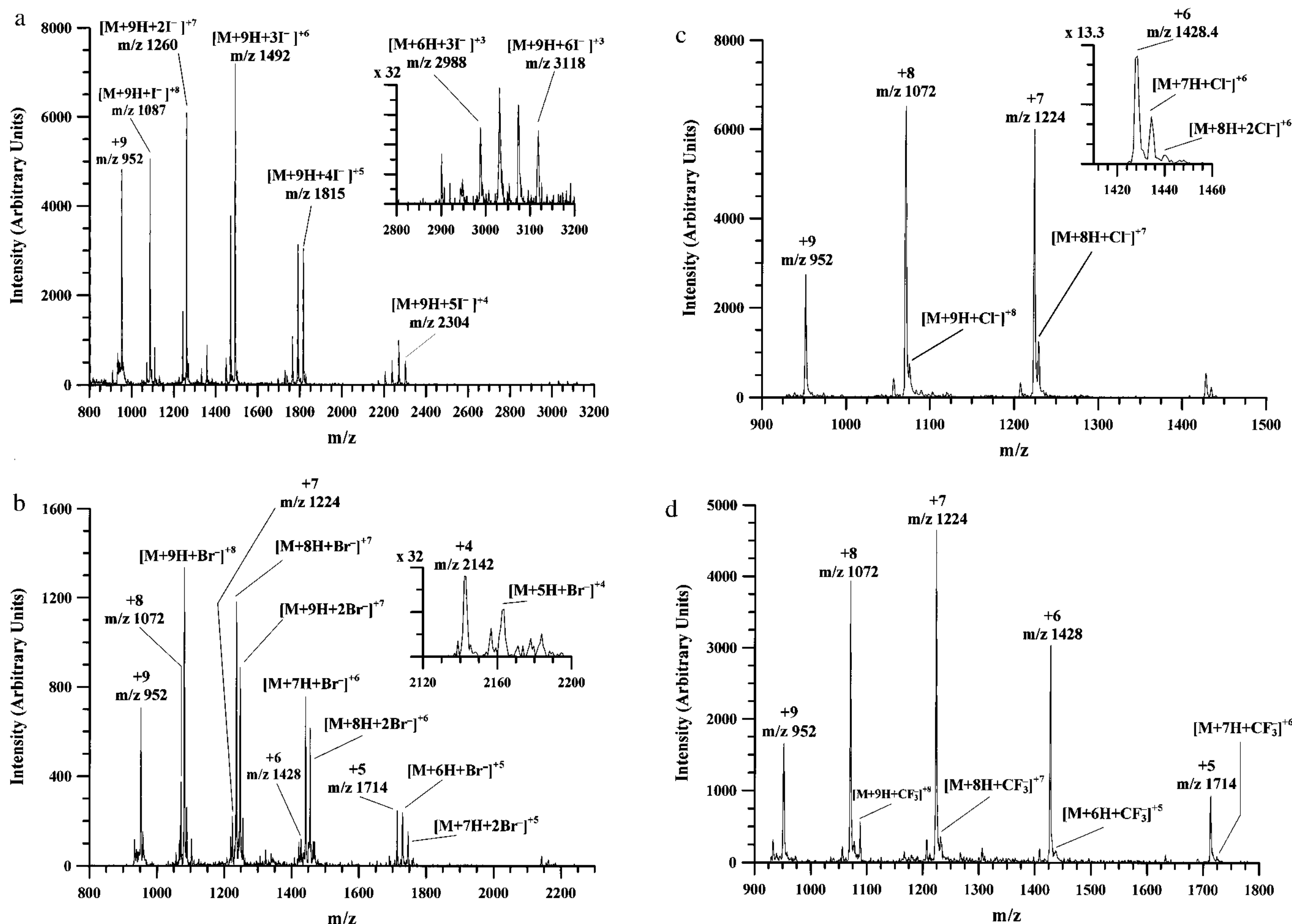


Figure 10. (a) Product ion spectrum resulting the reaction of the $(M + 9H)^{9+}$ cation population derived from ES of bovine ubiquitin with a population of I^- for 25 ms. (b) Product ion spectrum resulting from the same experiment as in part a except that a population of Br^- was used as the anionic reactant. (c) Product ion spectrum resulting from the same experiment as in a) except that part a population of Cl^- was used as the anionic reactant. (d) Product ion spectrum resulting from the same experiment as in part a except that a population of CF_3^- was used as the anionic reactant.

seven lysine residues, one histidine residue, and one N-terminus in ubiquitin. There are, therefore, nominally thirteen highly basic sites in the neutral protein. The number of basic sites in the ions is $13 - n$ or 12, in the case of $(M + H)^+$, and 1, in the case of $(M + 12H)^{12+}$. While it cannot be concluded from these results alone that the maximum number of acid molecules that can attach to a singly-protonated protein is equal to the number of exposed basic residues, these results are consistent with such a conclusion and further support the notion that the ion/molecule chemistry associated with strong acids interacting with the protein cations may very well be charge remote. That is, the protonated site does not play a major role in the protein cation/acid chemistry.

The data acquired for the ion/ion reactions involving ubiquitin cations and I^- and ion/molecule reactions involving ubiquitin cations and HI are more consistent with the formation of ion pairs or dipole/dipole bonding than with binding around a charge site. Results with other anions are also consistent with this observation. Figure 10 compares the results obtained from the reactions of I^-, Br^-, Cl^-, and CF_3^- with the 9+ charge state of ubiquitin under a common set of reaction conditions. While the extent of charge state reduction cannot be compared directly, due to difficulty in forming equal numbers of anions, the extent of adduct formation relative to proton transfer can be seen for each anion type. The I^- and Br^- anions clearly show the greatest degree of adduct formation whereas Cl^- shows substantially less and CF_3^- shows almost none. The I^- versus CF_3^- comparison is particularly interesting in that the proton affinities of HI and HCF_3 are essentially equal, whereas the gas-phase acidities are 314.3 and 376.9 kcal·mol^{-1}, respectively.[14] The reaction involving CF_3^-, therefore, is significantly more exothermic than the reaction involving I^-. In general, we have not observed adduct formation for any anion with a proton affinity greater than about 330 kcal·mol^{-1}. The proton affinity of the anion (or, equivalently, the gas-phase acidity of the conjugate acid), however, is not the sole parameter determining whether or not adduct formation competes with proton transfer. Table 1 lists the conjugate acids of the anions thus far studied with ubiquitin cations along with their proton affinities,[14] gas-phase acidities,[14] qualitative degree of observed adduct formation via ion/ion reactions, and, where available, dipole moments[24] and polarizabilities.[25] Note that while the gas-phase acidity of trifluoroacetic acid is less than that of HBr, Br^- shows a far greater tendency for adduct formation. Perhaps a better correlation for adduct survival could be found with *d*, the well-depth associated with the adduct (see Figure 4), or, more likely, with some combination of gas-phase acidity and *d*. Values for *d* have not been measured but are expected to

(24) (a) *CRC Handbook of Chemistry and Physics*, 69th ed.; Weast, R. C., Ed.; CRC Press: Boca Raton, 1988−89. (b) McClellan, A. L. *Tables of Experimental Dipole Moments*, 2nd ed.; W. H. Freeman: San Francisco, 1963.

(25) Molecular polarizabilities were calculated according to the method of Miller and Savchik (Miller, K. J.; Savchik, J. A. *J. Am. Chem. Soc.* **1979**, *101*, 7206).

Table 1. Proton Affinities (PA), Polarizabilities (α), Dipole Moments (μ) and Gas Phase Acidities (ΔH_{acid}) of the Conjugate Acids of Anions Subjected to Ion/Ion Reactions with Multiply-Protonated Ubiquitin Cations and the Relative Tendencies for Adduct Formations

acid	PA (kcal/mol)[14]	α ($Å^3$)[25]	μ (debye)[24]	ΔH_{acid} (kcal/mol)[14]	adduct tendency
HCl	128.6	2.64	1.08	333.4	small
HBr	139	3.86	0.82	323.6	large
HI	147	6.18	0.44	314.3	large
HCF_3	147	2.65	1.65	376.9	very small
n-HOC_4H_9	191	1.66	375		very small
HNO_3	178	3.55	2.17	324.6	moderate
HNO_2	187	2.72		339.6	very small
HO_2CCH_3	190.7	5.15	1.74	348.7	none
HO_2CCF_3	176			322	small
HC_8F_{15}		15.41		>314.3[26]	none
HC_7F_{13}		13.5		>314.3[26]	none

reflect the avidity of the acid/base interaction. Therefore, concepts associated with hard−soft acid−base chemistry may prove to be particularly relevant in this situation.

Conclusions

Iodide anions and hydroiodic acid both attach to cations derived from bovine ubiquitin cations in the gas phase. The resulting adduct ions dissociate upon collisional activation via loss of neutral molecule(s) of HI. Greater numbers of adducted species are observed with lower charge states than with higher charge states and ion/molecule reaction rates are inversely related to charge state, in contrast with rates of reaction for ubiquitin cations with strong gaseous bases. No change in the relative abundances of adduct species in an intermediate charge state are observed in reducing the charge state to 1+ via ion/ion proton transfer chemistry, an unexpected result if the adduct species are proton bound to the protein. All of the experimental observations are consistent with adduct species being bound via either ion pair or dipole/dipole interactions, as opposed to charge-centered clustering. These results indicate that strong gaseous acids interact with protein cations differently than do strong gaseous bases. The results are consistent with interaction of the acids with basic sites of the protein (i.e., neutral basic residues) rather than with protonated sites. If so, the interaction of gaseous neutral acids with gas-phase protein cations may be a chemical probe complementary to other chemical probes, such as interactions with strong gaseous bases and hydrogen/deuterium exchange, the latter reactions being charge-site mediated.

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(26) The major high mass anions derived from PDCH were found to undergo rapid proton transfer with HI in the vacuum system indicating that the gas phase acidities of the relevant acids (empirical formula HC_8F_{15} and HC_7F_{13}) are greater than that of HI.