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Gas-phase bovine ubiquitin cation conformations resolved by gas-phase hydrogen/deuterium exchange rate and extent

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Abstract

The gas-phase hydrogen/deuterium exchange of $[M + nH]^{n+}$ ($n = 5–13$) ions of bovine ubiquitin with the H/D exchange reagent D_2O are examined by electrospray ionization Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry. All of the odd or all of the even charge states were isolated by stored waveform inverse Fourier transform excitation and simultaneously reacted with D_2O leaked steadily into the ICR cell for reaction periods ranging from 1 s to 1 h. Different gas-phase protein conformations could be resolved according to difference in extent of H/D exchange. The 5+ and 6+ charge states display broad distributions of conformations ranging from 0–80% deuterium incorporation. In contrast, each of the higher charge states, 7–11+ and 13+, displays a single major isotopic distribution, whereas the 12+ charge state separates into two isotopic distributions of comparable abundance. In general, H/D exchange rates decrease with increasing charge state. External electrospray ionization source conditions (capillary current and external accumulation period) were varied while observing the conformational distribution of the 7+ charge state: increased heating in either region reduced the number of slow-exchanging conformations. At 9.4 T, it is possible to trap a large number of ions for a long reaction period (up to 1 h) at relatively high pressure (2×10^{-7} Torr). These results demonstrate the capability of FT-ICR mass analysis following gaseous H/D exchange of electrosprayed proteins to disperse different gas-phase protein conformations for subsequent isolation and characterization. (Int J Mass Spectrom 185/186/187 (1999) 565–575) © 1999 Elsevier Science B.V.

Keywords: Hydrogen–deuterium exchange; Protein; Gas phase ion–molecule reactions; Mass spectrometry; Fourier transform; Ion cyclotron resonance

1. Introduction

Hydrogen/deuterium exchange techniques have proved valuable for elucidating the solution-phase structure and conformation of biological macromolecules [1–6]. The dynamics of solution-phase H/D exchange of proteins are traditionally monitored by nuclear magnetic resonance (NMR) spectroscopy of backbone amide protons. Mass spectrometry coupled with electrospray ionization also allows the monitoring of solution phase H/D exchange [7–16]. With the introduction of electrospray ionization (ESI) mass spectrometry, a growing interest in how protein ions undergo the transition from solution to the gas phase has emerged. What happens to a protein when its

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Dedicated to Professor Michael T. Bowers on the occasion of his 60th birthday.

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solvent shell is stripped away? Does the protein maintain native solution-phase conformation(s)? These questions are currently being addressed by gas-phase experimental techniques, most notably gas-phase ion mobility [17–25] and gas-phase H/D exchange [26–29], showing that gas-phase protein ions can adopt multiple conformations that persist without interconversion for many (as many as millions) ion–neutral collisions.

Because of the relatively slow rates for gas phase H/D exchange (pseudofirst order rate constant $<10^{-11}$ cm^3 molecule^{-1} s^{-1}), it is experimentally challenging to confine ions for a long enough period to ensure complete deuterium incorporation (i.e. either short reaction period at high pressure or long reaction period at low pressure). Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) is an experimental technique that combines the ability to trap and react gaseous ions for extended periods and to perform high-resolution detection of the masses of their reaction product ions. H/D exchange reactions in a Penning trap at high magnetic field (>7 T) provides for an even longer reaction period at higher pressure and the trapping of a greater number of ions [30]. FT-ICR mass spectrometry is thus particularly well suited for gas phase H/D exchange and other ion–molecule reaction experiments. Here, we demonstrate the advantages of high field (9.4 T) FT-ICR MS for gas-phase H/D exchange of protein ions.

We chose ubiquitin as a system from which to develop methodology for performing gas phase H/D exchange of protein ions. Ubiquitin is a small protein (76 amino acids, 8565 Da for bovine ubiquitin) found in all eukaryotic cells. Ubiquitin contains 13 basic residues (4 arginines, 7 lysines, 1 histidine, and the N-terminal amino group) and a total of 144 exchangeable hydrogens for the neutral protein. The gas-phase H/D exchange reactivities for the 6+ through 11+ charge states of bovine ubiquitin have been examined by Sukau et al. by FT-ICR MS at 2.8 T [31]. It has a highly compact x-ray crystal [32,33] and native solution phase structure [34–38] consisting of a 5-stranded β-sheet, α-helix, 3_{10}-helix, and 7 reverse turns. In addition to the native state, its denatured state in alcoholic solutions has been well characterized [39–41]. Prior characterization therefore provides a firm foundation from which to elucidate the effect of desolvation on protein ion solution-phase native conformation.

2. Experimental

Bovine ubiquitin was obtained from Sigma Chemical Company (St. Louis, MO) and D_2O (99.9%D) was obtained from Cambridge Isotope Labs (Andover, MA). All reagents were used without further purification. The ubiquitin was initially dissolved in water at a concentration of 1 mM. Serial dilutions were made from this stock to a final concentration of 10 μM in approximately 50:50 (v/v) MeOH:H_2O with either 0.25% or 2.5% acetic acid.

Experiments were performed with a previously described 9.4 T ESI FT-ICR mass spectrometer configured for external accumulation (Fig. 1) [42,43]. Samples (10 μM) were infused into a tapered 50 μm i.d. fused silica micro-ESI needle [44,45] at a rate of 200 nL min^{-1}. Typical ESI conditions were: needle voltage = 1.8 kV, heated capillary current = 2.5 A. Ions were accumulated in a linear octopole ion trap (operated at 1.5 MHz, 100 $V_{p\text{-}p}$) for 1.5 s and then transferred to the ICR cell through a second octopole ion guide (operated at 1.5 MHz, 100 $V_{p\text{-}p}$). The samples were prepared in either 50:50 (v/v) MeOH:H_2O with 0.25% acetic acid or 50:50 (v/v) MeOH:H_2O with 2.5% acetic acid. The 0.25% acetic acid solution was used to generate the 5+–8+ charge states whereas the 2.5% acetic acid solution was used to generate the 6+–13+ charge states. The natural isotope distributions for either all of the odd or all of the even $[M + nH]^{n+}$ ions were isolated from each solution by stored waveform inverse Fourier transform (SWIFT) excitation [46–48]. Immediately following isolation, the ions were cooled by a short pulse of He gas to 1×10^{-6} Torr. The parent ions were then allowed to react with D_2O pulsed into the vacuum system via a three-way pulse valve/leak valve combination described previously [28,49]. The partial pressure of D_2O during the pulse rose to 2×10^{-7} Torr within ~2 s and remained stable throughout the course of the H/D exchange period. Ions were allowed to react with the neutral exchange reagent for each of

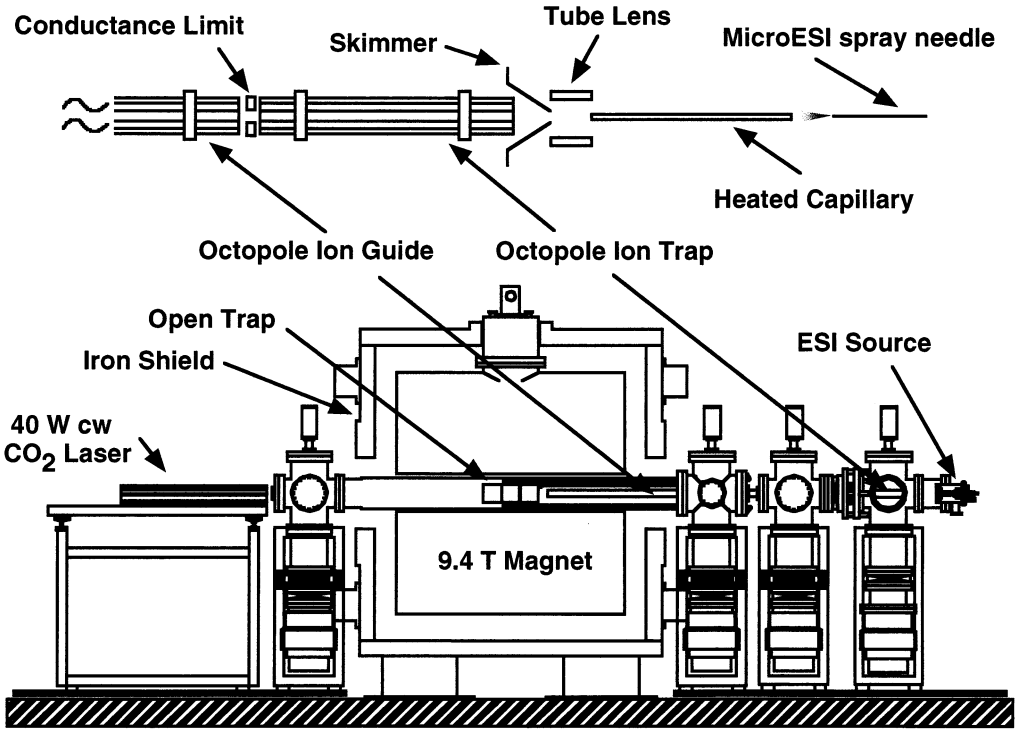


Fig. 1. Passively shielded 9.4 T ESI FT-ICR mass spectrometer configured for external accumulation. The inset at the top depicts the ESI source and first octopole in which ions are accumulated.

several varying time periods up to a maximum of 1 h. The exchange reagent pulse was followed by a 5 min pumpdown in which the pressure rapidly fell to 5×10^{-8} Torr (~10 s) and achieved a final pressure of 2×10^{-8} Torr. Neutral pressure was measured with a Granville Phillips (Boulder, CO) Model 274 ion gauge. The ions were then subjected to broadband frequency sweep excitation (50–300 kHz) and detection (300 kHz Nyquist bandwidth and 256 kWord data). Typical base pressure for the instrument was 2×10^{-9} Torr. An Odyssey™ data station (Finnigan Corp., Madison, WI) controlled all experiments.

3. Results and Discussion

3.1. Effect of source conditions on the gas-phase ubiquitin H/D exchange

Although generally considered a "soft" ionization technique, ESI can impart considerable internal energy into ions [50]. To perform reproducible gas phase H/D exchange experiments, we therefore consider it necessary to evaluate the effect of ESI conditions on the charge state distribution and number of conformations in each charge state. Figure 1 depicts the configuration of the 9.4 T ESI FT-ICR mass spectrometer used to acquire all data reported here. The two parameters having the greatest influence on the appearance of the final FT-ICR mass spectrum were the current supplied to the heated capillary and the ion accumulation period for the octopole ion trap. It was also observed that an increase in ion accumulation period or heated capillary current (and thus increase in temperature) shifts the charge state distribution to higher mass-to-charge ratio, presumably due to charge stripping by ion–neutral collisions in the high-pressure region of the heated capillary (~2 Torr) and/or the first octopole (~10 mTorr). For cytochrome *c*, McLafferty et al. also found that experimental conditions strongly influence the rate and

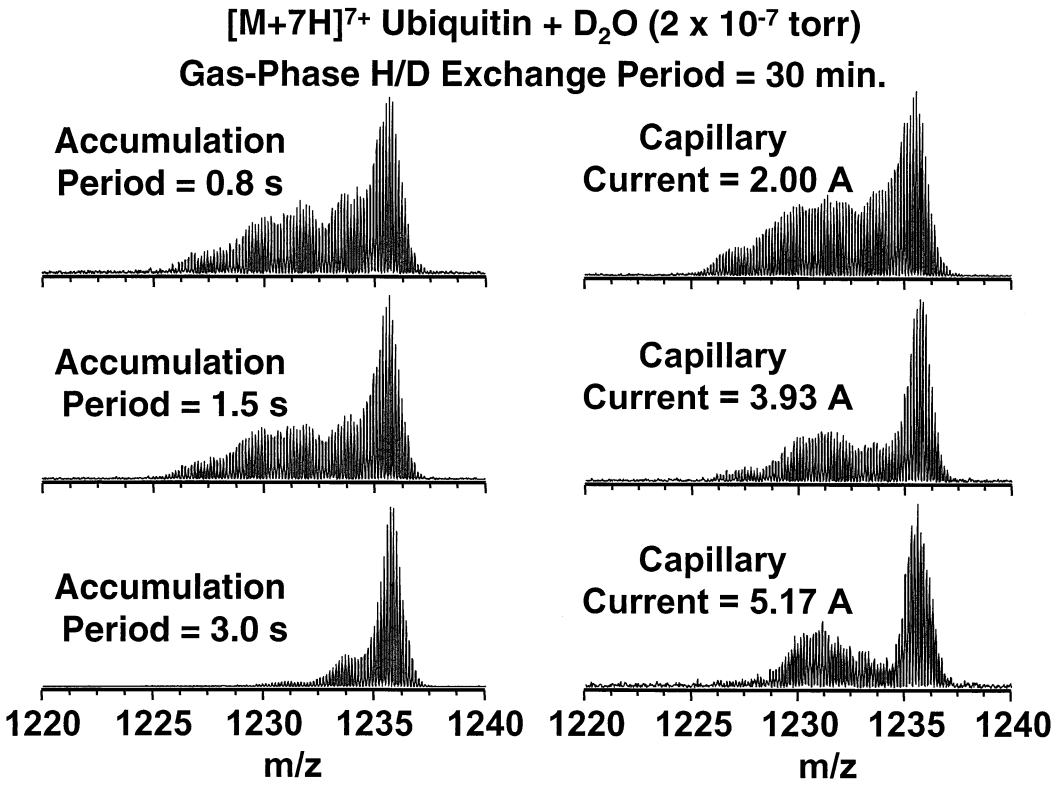


Fig. 2. Effect of the accumulation period (left) at constant capillary current (2.0 A) and the heated capillary current (right) at constant accumulation period (0.8 s) on the distribution of conformations (dispersed according to their different extent of gas-phase H/D exchange) of the 7+ charge state of electrosprayed bovine ubiquitin.

extent of gas-phase H/D exchange of cationic proteins formed by ESI [26,29]. The mechanism of formation of lower charge states of cytochrome *c* was particularly affected by experimental electrospray ionization parameters.

For gas-phase H/D exchange of the SWIFT-isolated 7+ charge state, the temperature of the heated capillary and the external ion accumulation period were found to influence the number of 7+ charge state conformations (see Fig. 2). The relative abundance of the slower-exchanging conformations clearly decreases with increasing ion accumulation period and/or heated capillary current. Thus, a consistent set of experimental conditions is necessary to yield reproducible data. We chose values of 1.5 s and 2.5 A for the external ion accumulation period and the heated capillary current for all subsequent experiments. Experimental reproducibility could then be assessed by performing the experiments under identical source conditions on three separate days. Fig. 3 illustrates an average error of ±12% for the 7+ charge state. The larger error at longer reaction periods is due to the decreased signal-to-noise ratio from trapping ions for long periods at high pressure without axialization [28,51–53].

In evaluation of similar effects but with a different means for assigning conformers, Li et al.'s ion mobility experiments showed that solvent conditions and capillary temperature affect the distribution of gas-phase protein ion conformations [21]. Specifically, an increase in their capillary temperature resulted in increased collision cross section of ubiquitin cations. Unfortunately, their ESI source was of a different configuration than ours, thereby allowing for only qualitative comparisons between their ion mobility and our H/D exchange experiments.

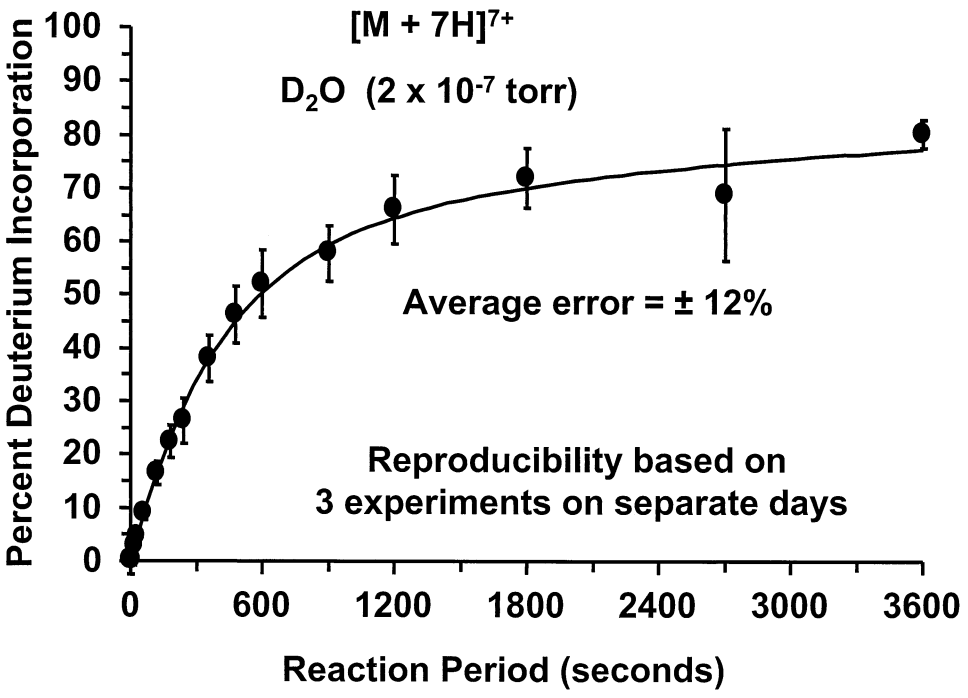


Fig. 3. Experimental reproducibility of gas-phase H/D exchange of the ubiquitin 7+ charge state. Experiments were repeated on three separate days with identical source conditions (1.8 kV ESI needle, 2.5 A capillary current, 1.5 s accumulation period). Each error bar represents one standard deviation above and below the averaged experimental value.

3.2. H/D exchange of multiply charged ubiquitin ions

After appropriate ion source conditions had been established, gas phase H/D exchange of the 5–13+ charge states of ubiquitin were carried out. To reduce the time required to perform the H/D exchange experiments on all of the charge states, either all of the odd or all of the even charge states (from solutions at either 0.25% or 2.5% acetic acid) were SWIFT isolated and allowed to undergo simultaneous H/D exchange in separate experiments [29]. The percentage of deuterium incorporation was calculated as the ratio of the observed mass shift for the zero-charge species to the total number of exchangeable hydrogen atoms.

3.2.1. Charge states 5+ and 6+

Gas-phase H/D exchange of the 5+ and 6+ charge states reveals that these ions adopt a multitude of gas-phase conformations. The conformational distributions for the 5+ and 6+ charge states after an exchange period of 1 h with D_2O (2×10^{-7} Torr) are shown in Fig. 4. These conformations most likely arise from different combinations of the (5 or 6) charges among 13 basic sites. If all sites had an equal probability of being occupied then there could be 13!/(5!8!) = 1287 possible combinations for the 5+ charge state or 13!/(6!7!) = 1716 for the 6+ charge state. However, with the reasonable assumption that all four arginine residues are protonated so that the remaining charge(s) are limited to the 7 lysine residues, the number of possibilities drops to 7 and 21 for the 5+ and 6+ charge states. Those values could account for the experimentally observed broad distribution of conformations for the 5+ and 6+ charge states.

It is interesting to consider possible structures for these conformations. From solution-phase NMR of ubiquitin under alcoholic solvent conditions, ubiquitin is proposed to exist in a mixture of two states, native and denatured [39–41]. The tightly folded native

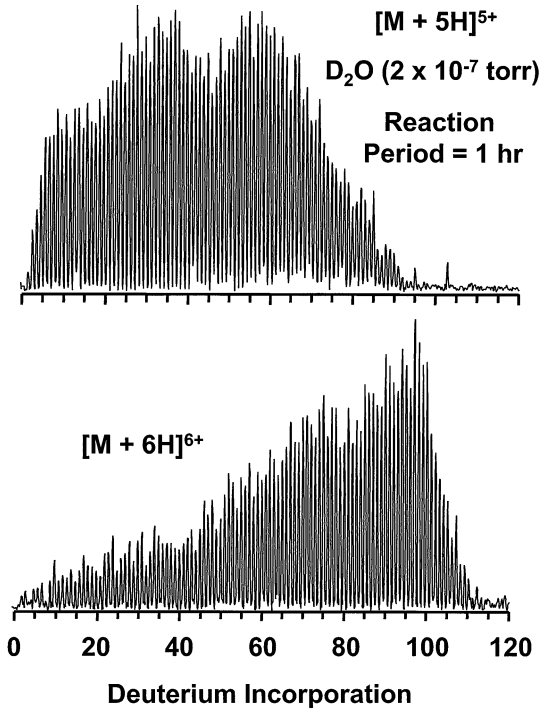


Fig. 4. FT-ICR mass spectra following gas-phase H/D exchange for 1 h at 2×10^{-7} Torr D_2O for the 5+ (top) and 6+ (bottom) charge states of bovine ubiquitin. The abscissa represents a convolution of the deuterium incorporation distribution after 1 h and the initial natural-abundance isotopic distribution of the fully protonated ion. Note the wide dispersion in deuterium uptake, corresponding to multiple slowly interconverting gas-phase ubiquitin conformations.

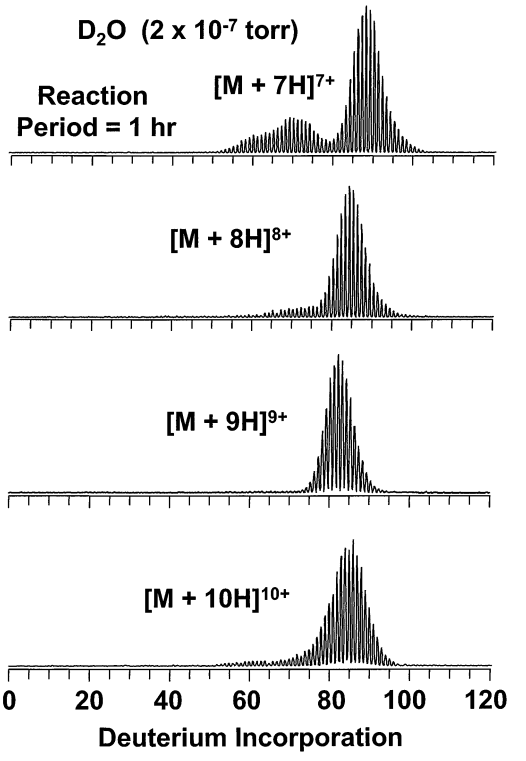


Fig. 5. FT-ICR mass spectra (abscissa as in Fig. 4) following gas-phase H/D exchange for 1 h at 2×10^{-7} Torr D_2O for the 7–10+ charge states of bovine ubiquitin. For these charge states, either one conformation predominates, or several conformations interconvert in much less than 1 h.

state, also called the *N* state, has been characterized in solution by NMR [36,37] and agrees well with the x-ray crystal structure [32,33]. The denatured form, also called the *A* state, are observed in 60% CD_3OD/40% D_2O at a pH of 2 (i.e. conditions very similar to those for our ESI solutions [40]. In the *A* state, Harding et al. found that the 3_{10} helix and residues 37–62 become random coil whereas three strands of the five-strand β sheet are retained. It therefore seems plausible that the observed mixture of gas-phase conformations could arise from protonation of different sites of the *A* state. Although gas-phase H/D exchange data do not directly report three-dimensional (3D) structure, the H/D exchange data do offer a valuable tool in understanding the dynamic process by which macromolecular ions unfold in the gas phase.

3.2.2. Charge states 7–10+

The H/D exchange deuterium uptake patterns for the 7+ to 10+ charge states after an exchange period of 1 h with D_2O (2×10^{-7} Torr) (Fig. 5) suggest multiple gas-phase conformations but not the broad distributions observed for the 5–6+ charge states. The corresponding reaction progress curves are shown in Fig. 6. In contrast to the 5+ and 6+ charge states, these higher charge states exhibit fewer conformations and all exchange to virtually the same final extent. Each of these charge states is characterized by a single isotopic distribution at an exchange level of ~75% along with a broad isotopic distribution at lower exchange level. Of these charge states, the 7+ charge state had the highest relative population of conformations at lower exchange level. After a shorter reaction period, the slower-exchanging con-

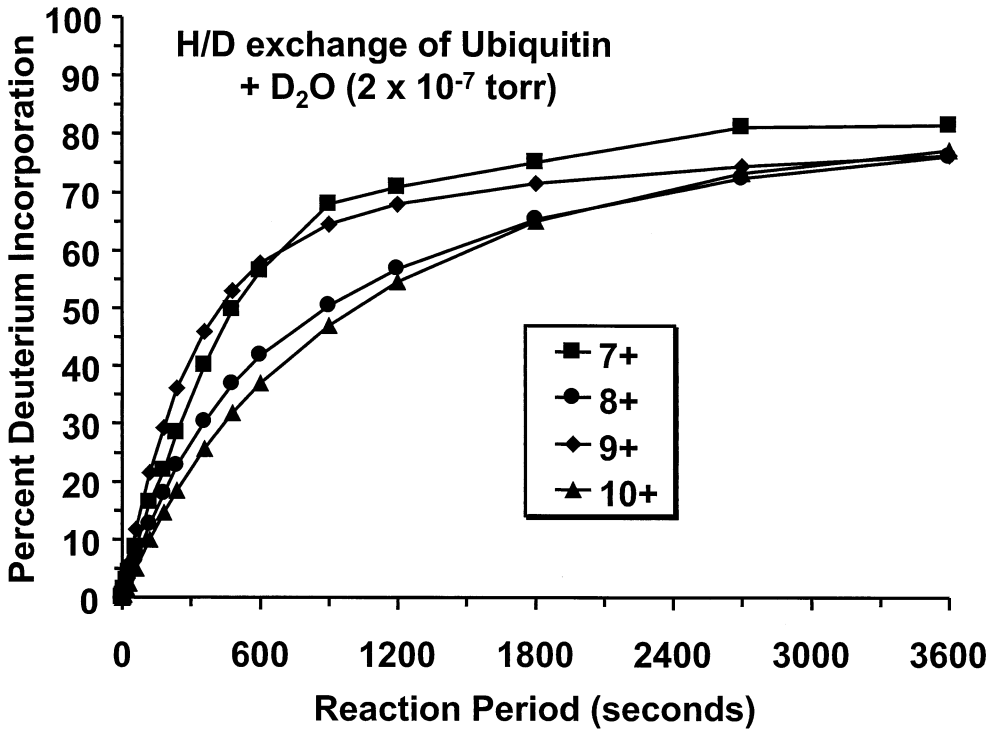


Fig. 6. Reaction progress curves for gas-phase H/D exchange of the 7–10+ charge states with D_2O (2×10^{-7} Torr) at 9.4 T.

formations of the 7+ charge state are readily apparent (reaction period = 30 min for spectra in Fig. 2). For a reaction period of 1 h, the slower-exchanging conformations tend to overlap and form the major isotope distribution along with some minor distributions. The 8–10+ charge states contain primarily a single isotopic distribution, suggesting a single conformation. However, it should be noted that several different conformations could undergo exchange with similar exchange rates and could thus make up the same isotope distribution. Alternatively, several conformations with different H/D exchange rate could interconvert rapidly during the H/D exchange period, to yield a single average rate of deuterium uptake. Finally, the presence of multiple conformations with overlapping deuterium uptake patterns could explain why the isotopic distributions for the 8+ and 10+ charge state are wider than the 9+ charge state (which possesses the lowest abundance of slower exchanging species).

3.2.3. Charge states 11–13+

The conformational distributions for the 11–13+ charge states after an exchange period of 1 h with D_2O (2×10^{-7} Torr) appear in Fig. 7. From the H/D exchange patterns of the 11+ and 12+ charge states, it is clear that at least two conformations exist for each of these charge states. The 13+ charge state, however, exhibits only a single isotopic distribution. Because all 13 basic sites are protonated in that case, the protein must either adopt a single conformation or multiple conformations with similar exchange rates or multiple rapidly interconverting conformations. The 12+ charge state is particularly interesting due to the complete separation of its two isotopic distributions. In prior gas-phase H/D exchange and deprotonation experiments on the two isotopic distributions of the 12+ charge state [27], Cassady and Carr found that the faster-exchanging species was also the more easily deprotonated species. In any case, one of the most striking aspects of the H/D exchange of ubiquitin's

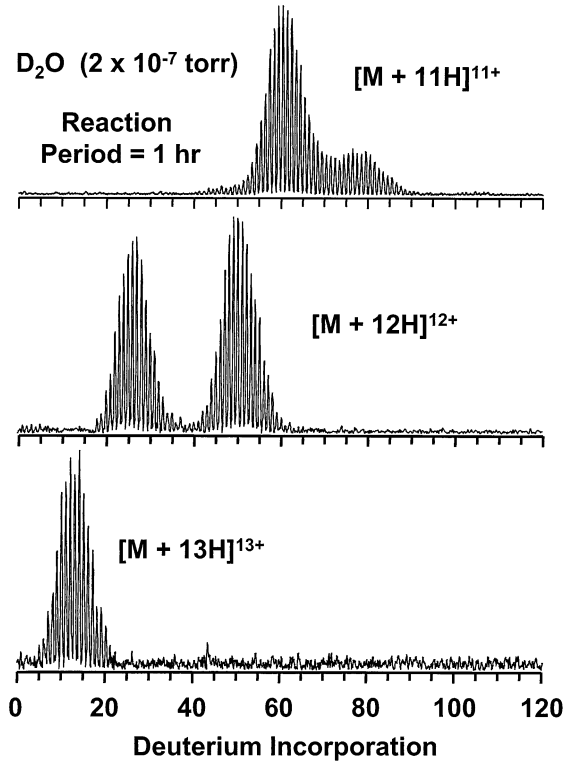


Fig. 7. FT-ICR mass spectra (abscissa as in Fig. 4) following gas-phase H/D exchange for 1 h at 2×10^{-7} Torr D_2O for the 11–13+ charge states of bovine ubiquitin. Note the presence of two comparably abundant gas-phase conformers of the 12+ charge state, baseline-resolved by extent of deuterium incorporation.

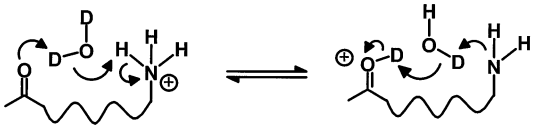
higher charge states is the dramatic decrease in exchange level as the protein becomes more protonated.

3.3. Gas-phase ion mobility versus gas-phase H/D exchange

To date, the most extensive comparison of gas-phase ion mobility and gas-phase H/D exchange has been provided by the McLafferty group on cytochrome *c* [26]. Although the results differ in detail, both techniques reveal multiple gas-phase conformations for low charge states, and fewer and/or unresolved conformations for high charge states. The collision cross sections for the 6–13+ charge states of bovine ubiquitin have been measured by Valentine et al. [25]. Gas-phase ion mobility experiments reveal trends that correlate well with those from our H/D exchange experiments. However, differences in both the nature of the experimental techniques and the solvent conditions allow for only qualitative comparisons. In the ion mobility experiments, the spectra for the 6−8+ charge states contained broad distributions of collision cross sections, suggesting several gas-phase conformations. As the number of charges on the protein increases, the collision cross section also increases. From molecular modeling of the shape (and thus cross section) of ubiquitin conformations, the distributions of collision cross sections for the 6+ and 7+ charge states were interpreted as mixtures of compact, partially folded and elongated conformations. The 8+ charge state thus exhibited partially folded and elongated conformations. Finally, the 9–13+ charge states were assigned to elongated conformations in which the collision cross section increases as the charge state increases.

We also observe mixtures of conformations for the 5–7+ charge states. As the charge state increases we observe isotope distributions that suggest a predominately single conformation. However, H/D exchange can apparently resolve conformations having similar collision cross sections. For example, the 12+ charge state exhibits a single collision cross section in ion mobility experiments, whereas our present data (and that of Cassady and Carr [27]) clearly expose the presence of at least two distinct species. A similar difference between gas-phase ion mobility and gas-phase H/D exchange results has been observed for cytochrome *c* [22,26]. We infer that subtle differences in the location of the charge do not significantly change the collision cross section but can lead to large differences in exchange rate.

H/D exchange suggests that the elongated conformations inferred from ion mobility experiments are also the slowest-exchanging species. This effect of decreased gas phase exchange with increased elongation of the protein is counterintuitive to what one would expect in solution. However, the mechanism by which H/D exchange occurs in solution phase versus the gas phase is different. In solution, the protein's exposed hydrogens are exchanged rapidly with the exchange solvent. Upon unfolding, the solvent can



Scheme 1.

undergo additional exchange with labile hydrogens in the protein core. Conversely, in the gas phase, exchange occurs only by ion-molecule reactions. Bowers and Beauchamp have investigated the mechanism by which gas phase H/D exchange occurs with peptide ions [54,55], including recent extensive modeling of the H/D exchange of the peptide, bradykinin [56]. Their calculations show that a D_2O molecule collides with a peptide cation and may be deflected or form a long-lived collision complex. During the lifetime of the complex, the D_2O molecule samples many different exchange sites on the peptide's surface. Exchange occurs by means of a "relay mechanism" (Scheme 1) in which the D_2O molecule complexes with both the charge site and a neighboring basic site [56]. Although compact conformations possess fewer exposed hydrogens, the density of neighboring basic sites is greater. The situation is reversed for the elongated conformations. For the lower-charge states we observe a broad distribution of conformations by H/D exchange. It is plausible to assume the slower exchanging species could arise from many conformations with differing numbers of exposed hydrogens. But as the charge increases, so does Coulomb repulsion forcing the conformation to become elongated. The elongated conformations are at a disadvantage: almost all of their exchangeable hydrogens are exposed to the exchange reagent, but those hydrogens cannot exchange unless they can adopt the right conformation. Thus the "relay mechanism" model fits our experimental observations in that we observe decreasing levels of exchange for the higher charge states (Fig. 8).

3.4. From solution to the gas phase

Little is known about how proteins react to desolvation during the process of electrospray ionization. It is clear from the present H/D exchange results that rapid desolvation results in formation of many different conformations. However, it is unclear if any of those conformations is the same as in solution. It is conceivable that upon desolvation, the protein ion unfolds rapidly due to the absence of the hydrophobic interactions that induce folding. The electrostatic interaction between each charge site and the solvent is no longer present, and the charge site must now be stabilized by intramolecular interactions. Two factors come into play in determining the gas phase conformation that the protein ion adopts. The first factor, Coulomb repulsion, arises from the absence of the high dielectric constant of solvent molecules to stabilize the charge [22,57–59]. As the number of charges on the protein increases, an elongated conformation will minimize the Coulomb repulsion. The second factor is self-solvation of the charge site. Based on ion mobility data and molecular dynamics calculations, Bowers has proposed that the peptide, bradykinin, undergoes self-solvation [56]. That interaction would also change the conformation of the protein dramatically from that in solution. Both effects reduce the likelihood that the gas-phase conformation will maintain all conformational elements found in solution. What is most likely is that the protein can adopt a multitude of configurations once the driving force to minimize the hydrophobic interactions is eliminated. However, some elements of secondary structure may be retained. Although the degree to which gas-phase techniques can distinguish between such elements remains debatable, gas-phase ion mobility and gas-phase H/D exchange provide the most direct methods now available for analysis of gas-phase protein ion conformations.

4. Conclusion

This work describes the behavior of gas phase proteins by means of gas phase H/D exchange experiments. Our goal in performing these studies was to evaluate the effectiveness of gas phase ion–molecule reactions, in particular H/D exchange, to lend insights into the mechanism by which proteins undergo the

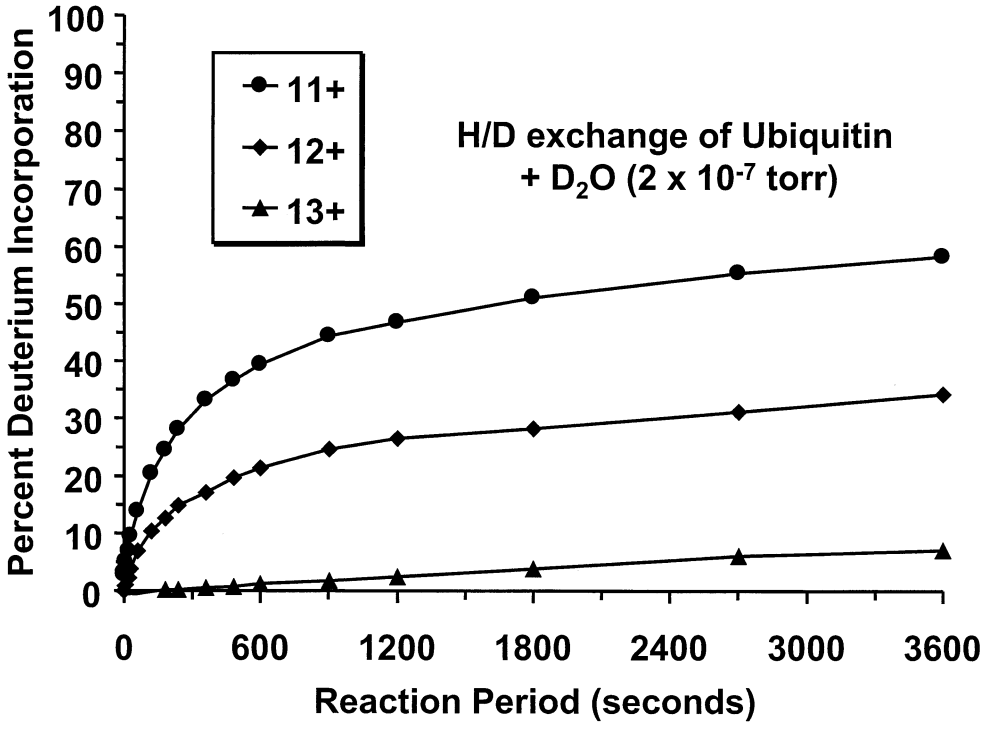


Fig. 8. Reaction progress curves for gas-phase H/D exchange of the 11–13+ charge states with D_2O (2×10^{-7} Torr) at 9.4 T. The data for the 12+ charge state represent an average of the two conformations to provide a consistent basis for comparison to the exchange levels of other charge states.

transformation from solution to the gas phase. We find that H/D exchange experiments, especially when combined with ion mobility data, provide a powerful probe into the conformational heterogeneity and conformational stability of gas-phase protein ions. H/D exchange is particularly sensitive for distinguishing between multiple conformations of gas-phase protein ions. High field (9.4 T) FT-ICR MS greatly facilitates the acquisition of data following the long ion storage periods needed for completion of ion–molecule reactions. We shall next employ H/D exchange to mass-disperse various gas-phase protein conformations, and then isolate an individual conformer by mass-selective SWIFT ejection of other conformers. FT-ICR MS and MS/MS allow investigation of the reactivity and stability of individual gas-phase protein ion conformers. It will also be necessary to carry out extensive modeling of the conformations by means of force-field and semiempirical calculations.

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