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International Journal of Mass Spectrometry 222 (2003) 175–187

International Journal of Mass Spectrometry

www.elsevier.com/locate/ijms

Gas phase hydrogen/deuterium exchange of proteins in an ion trap mass spectrometer

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Received 23 April 2002; accepted 9 July 2002

Abstract

Electrospray ionization ion trap mass spectrometry (ESI-ITMS) coupled with gas phase hydrogen/deuterium (H/D) exchange is demonstrated to be a useful tool to investigate the gas phase conformations of proteins when coupled with a mechanistic understanding of exchange. We have investigated the H/D exchange of multiple charge states of lysozyme, cytochrome *c*, ubiquitin, insulin, thioredoxin and melittin with deuterated methanol in the ion trap. This allows a direct comparison of exchange of these well studied proteins under identical conditions. For all proteins except lysozyme, exchange results in some peak broadening but no evidence of distinct conformers is observed. Qualitatively, trends in exchange levels are consistent with prior Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR) experiments. Consistent with mechanistic studies which have shown that amine hydrogens in peptides exchange rapidly, a correlation between the number of amine hydrogens and exchange level is observed. Highest levels of exchange are observed for proteins without disulfide bonds, and for proteins which are protonated on sites other than arginine. Both of these observations are explained by the "relay" mechanism of exchange. These results indicate that a further understanding of both the dynamics of gas phase molecules and mechanisms of exchange are necessary to relate gas phase H/D exchange data more directly to protein conformation. (Int J Mass Spectrom 222 (2003) 175–187)

Keywords: Hydrogen/deuterium exchange; Ion trap mass spectrometry; Ion–molecule reactions; Proteins

1. Introduction

Physiologically, non-covalent interactions of proteins with water, specifically hydrogen bonding and electrostatic interactions, are expected to play a significant role in protein folding and structure [1]. To determine the degree to which the solvent contributes to protein structure, it is important that conformations in both an aqueous environment and in a solvent-free environment are characterized. If water does play a large role in protein folding, then one might expect the conformation of the protein would be altered when removed from its aqueous environment. Studies in non-aqueous systems have shown this to be true. The development of electrospray ionization mass spectrometry (ESI-MS) has recently made it possible to study proteins in the complete absence of solvent [2]. ESI, combined with ion mobility has been used to show that proteins in the gas phase can adopt multiple conformations, some of which are compact and similar in size to those observed in solution [3–9]. If these structures are found to be similar to those in solution,

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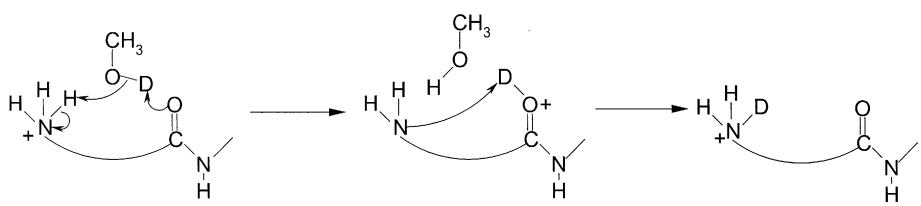
1387-3806/02/$ – see front matter
PII S1387-3806(02)00948-X

it would suggest that van der Waals interactions or intramolecular hydrogen bonding and interactions are more major contributors to protein stability than interactions with solvent [10]. Ion mobility [11–13] and mass analyzed kinetic energy spectroscopies [14,15] have been previously used to show that secondary structural motifs (α-helices and β-sheets) are stable in the gas phase.

Hydrogen/deuterium (H/D) exchange is used extensively in solution to probe protein structure. The rates and mechanisms of exchange of amide and amino acid side chains in solution are a strong function of pH and have been well characterized [16]. In a protein exhibiting secondary and tertiary structure at physiological pH, labile side chain and exposed amide hydrogens rapidly exchange. Amide hydrogens not accessible to solvent, either due to their involvement in hydrogen bonds or because they are located in the "core" of the protein exchange much more slowly. H/D solution exchange coupled with mass spectrometry has been used to observe the overall conformational changes of proteins under different solvent conditions [17,18] to count the number of surface accessible hydrogens [19,20] and to look at the dynamics of structure and folding events [21,22].

H/D exchange studies of peptides [23–30] and proteins [31–40] in the gas phase suggest this will also be a powerful technique to probe the structure of gas phase proteins. Initial H/D exchange studies of cytochrome *c* with Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR) provided evidence of multiple conformations in the gas phase. Low levels of exchange were correlated with compact structures and the higher levels observed at higher charge states with more elongated conformations [32]. H/D exchange coupled with ion mobility confirmed that the compact conformers of cytochrome *c* exchange fewer sites than elongated structures at room temperature [41] though raising the temperature results in greater maximum exchange for the lower charge (more compact) states [38]. For the protein ubiquitin, Freitas and coworkers observed that the higher charge state and presumably more elongated conformer has a lower level of exchange. These differences can be reconciled mechanistically. Wyttenbach and Bowers modeled the exchange mechanism of the protonated peptide bradykinin with D_2O and found that the collision event leading to exchange does not change the conformation of the peptide and that during the exchange event the D_2O samples the entire surface but not the interior [42]. By using a relay mechanism (Scheme 1) proposed by Beauchamp [26] and Green and Lebrilla [40] in which exchange is facilitated via interaction with a nearby basic site, they were able to model the experimental exchange behavior using an ensemble of low energy conformations. This suggests that proteins with compact structures will protect protons from exchange but that the potential inaccessibility of basic sites in elongated conformations can also result in lower exchange levels. Thus, it is important to study a series of different proteins under identical experimental conditions to further probe the intrinsic exchange behavior.

In this study, we have looked at the H/D exchange of several well studied small proteins including cytochrome *c*, ubiquitin, thioredoxin, insulin and melittin using ion trap mass spectrometry (ITMS). ITMS coupled with H/D exchange has previously been used to study amino acids and small peptides [28], but has not been previously applied to larger proteins. By studying the exchange of these proteins under identical experimental conditions in the ion trap direct comparison of exchange levels of different proteins



Scheme 1.

and charge states is possible. Results are compared to exchange results observed in the FT-ICR and other drift cell techniques and discussed in terms of known mechanisms of exchange.

2. Experimental

Horse heart cytochrome *c*, bovine ubiquitin, bovine pancreas insulin, hen egg-white lysozyme, honey bee venom melittin, leucine enkephalin and triglycine were obtained from Sigma (St. Louis, MO). *E. coli* thioredoxin was obtained from Promega (Madison, WI). All proteins were used without further purification. Deuterated methanol (99.9% D) was purchased from Aldrich (Milwaukee, WI).

For mass spectrometry analysis, protein samples were prepared by dissolving ~0.1–1.0 mg of protein in 1 mL of a 49:49:1 methanol:water:acetic acid solution. Experiments were carried out in a

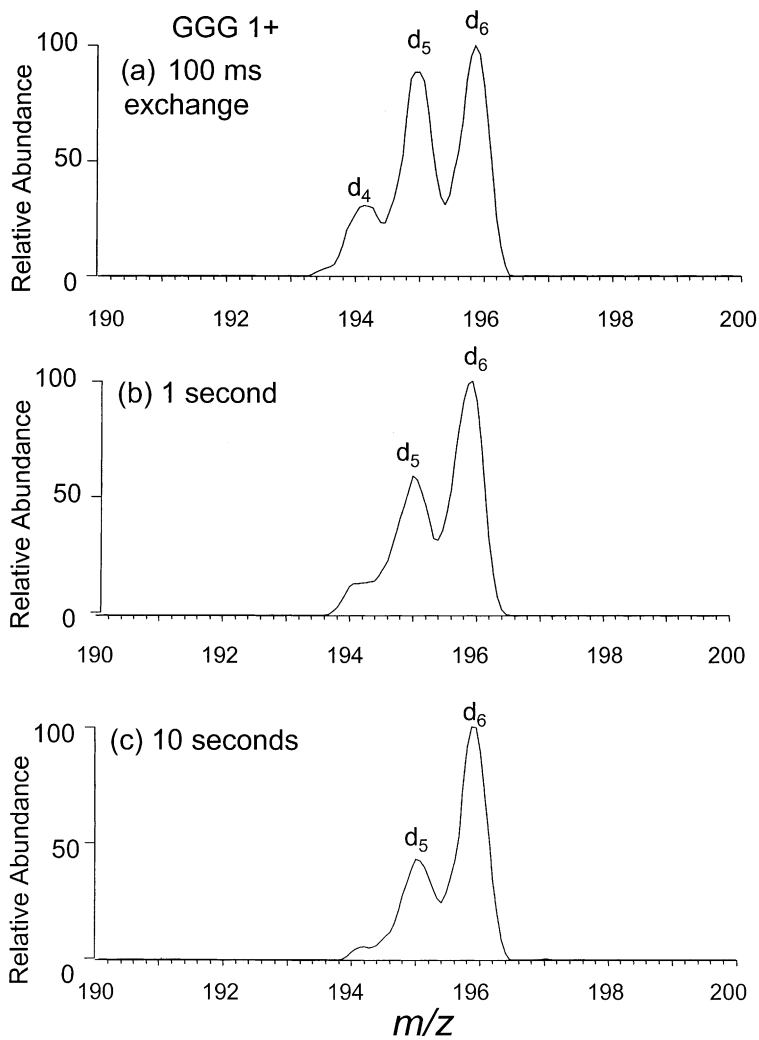


Fig. 1. Gas phase exchange of the singly protonated tripeptide, GGG (parent ion $m/z = 190.2$, six exchangeable sites) with deuterated methanol; (a) 100 ms of exchange; (b) 1 s exchange; (c) 10 s exchange.

Finnigan LCQ (San Jose, CA) electrospray ionization ion trap mass spectrometry (ESI-ITMS) modified to allow introduction of a deuterated reagent with a gas inlet system designed by Gronert [43]. A capillary temperature of 200 °C, an ion gauge pressure of 2.0×10^{-5} Torr, and a syringe pump flow rate of 3.0 μL min^{-1} were used for all samples. For gas phase H/D exchange, deuterated methanol was introduced through a syringe pump at 300–500 μL h^{-1} into a stream of helium gas flowing at ∼1000 mL min^{-1}. The mixed gas then passed through a flow control valve and 1 mL min^{-1} was diverted directly into the ion trap. To verify reagent gas pressure day to day H/D exchange levels of cytochrome *c* were checked during every experiment period. The internal temperature of ions in the ion trap has been recently determined during over this time period to be 310 ± 20 K [43].

Mass spectra were obtained using the advanced scan menu of the Navigator software. Ions were selected at an isolation width of 10 *m/z* units, and allowed to react with the deuterated gas for periods of time ranging

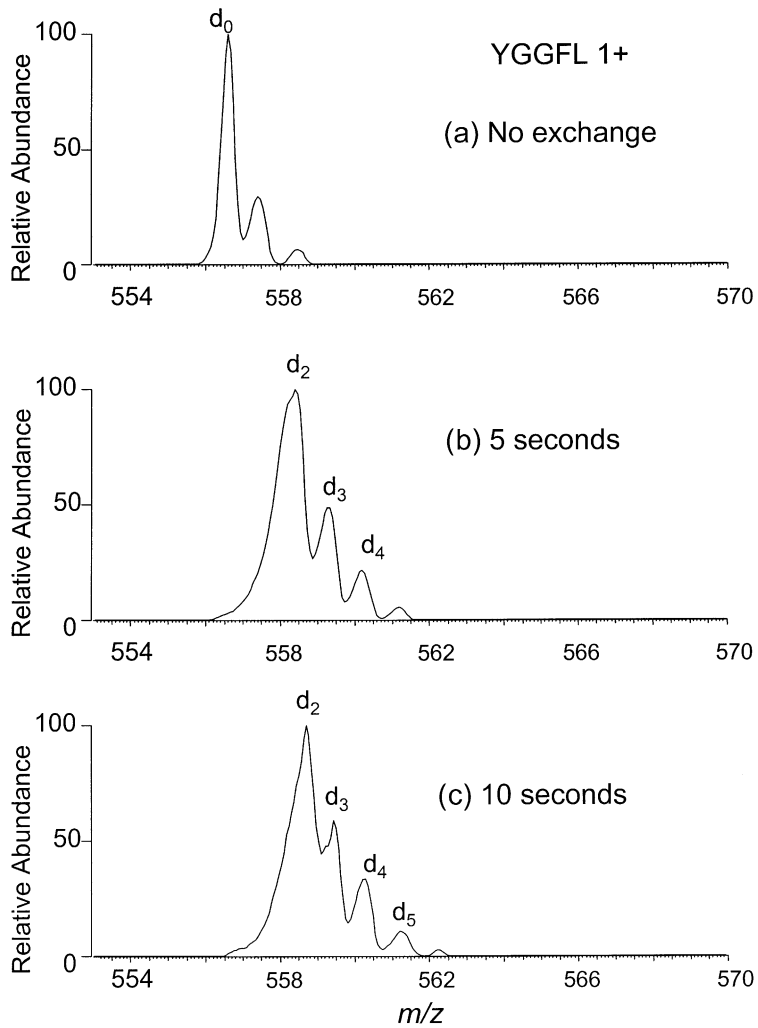


Fig. 2. Gas phase exchange of the singly protonated pentapeptide YGGFL (leucine enkephalin, parent ion $m/z = 556.6$, nine exchangeable sites) with deuterated methanol; (a) parent ion with no exchange; (b) after 5 s of exchange; (c) after 10 s of exchange.

from 1 to 10 s. Ten scans were averaged to produce all spectra.

To determine the number of hydrogens exchanged the difference between the mass (peak maximum) of the exchanged peak and the mass of the parent ion in the absence of deuterated reagent was determined. For proteins with wider peaks, indicating the presence of multiple conformations, estimates of the midpoint of the peak were used to determine number of exchanges. The percent deuterium incorporation is defined as the number of hydrogens exchanged divided by the number of exchangeable hydrogens on that fragment.

3. Results

3.1. H/D exchange of peptides

One of the limitations of the LCQ software is that ion–molecule reactions can only be studied for up to 10 s (this is a software limitation; the signal strength does not dramatically diminish over this time period). Therefore, we chose to use deuterated methanol as an exchange reagent as it has been demonstrated to undergo exchange two to four times as fast as D_2O [26,27]. To verify the utility of our deuterium inlet system and to estimate our reagent gas pressure the exchange of the peptides triglycine (6 exchangeable hydrogens) and leucine enkephalin (11 exchangeable hydrogens) was followed. Fig. 1 shows that after 100 ms of exposure to deuterated methanol, triglycine exchanges up to six sites and further lengthening the reaction time to 1 and 10 s results in more complete exchange. Fig. 2 shows leucine enkephalin exchanges up to four of nine sites within 10 s. Comparison to literature rates indicates we are seeing most fast exchanges [25,26]. We estimate our pressure is 10–50 times higher than the typical pressures of 1×10^{-7} Torr used in FT-ICR instruments (see Section 4). To determine the purity of the reagent gas we also looked at the H/D exchange of betaine, which has one exchangeable hydrogen; 80% of the parent ion is exchanged within 100 ms and complete exchange is observed after 1 s, indicating minimal back exchange under conditions employed here.

3.2. H/D exchange of proteins

Table 1 summarizes the extent of exchange observed for a variety of proteins with different charge states

Table 1
Observed percentage of exchange for proteins as a function of charge state of labile hydrogens with under similar conditions with deuterated methanol after 8 s

Protein	Charge	Labile H's[a,b]	Exchange (%)	Arginines[b]	Amine (%)
Lysozyme	+11	266	14.3	11	5.6
Lysozyme	+10	265	13.1	11	5.7
Lysozyme	+9	264	13.3	11	5.7
Lysozyme	+8	263	9.2	11	5.7
Cytochrome *c*[b]	+15	213	57.0	2	18.3
Cytochrome *c*[b]	+8	206	46.5	2	18.9
Thioredoxin	+12	185	11.3	1	11.9
Thioredoxin	+7	180	28.2	1	12.2
Ubiquitin	+10	154	32.6	4	10.4
Ubiquitin	+7	151	40.3	4	10.6
Insulin	+4	90	11.8	1	6.7
Insulin	+3	89	6.5	1	6.7
Insulin B-chain	+3	52	1.5	1	7.7
Melittin	+3	53	10.8	2	15.1
Melittin	+2	52	1.4	2	15.4

The number of arginines and percent of sites located on amines is also given.

[a] Number of labile H's = total for neutral protein + nH$^+$, where n = charge on protein.

[b] The total number of labile H's is 198 for neutral cytochrome *c*, see text for details.

after 8 s. To facilitate comparison of different proteins, the exchange level is expressed as a percentage of exchangeable sites. The number of exchangeable sites on the neutral protein was determined by looking at the known protein structure, and counting the labile hydrogens. For cytochrome *c*, the 198 exchangeable sites reported in prior studies includes the C–H hydrogens on the three histidine side chains, which exchange slowly in solution [33,34,38]. There is also some evidence the two carboxyl groups on the heme group are deprotonated, which would result in 196 exchangeable sites [34]. In this case, a protein with a net charge of +8 and +2 deprotonated sites requires the addition of 10 (exchangeable) protons, still resulting in 198 exchangeable hydrogens. For consistency with other mass spectrometry work, with the other proteins, the C–H exchangeable sites on the histidine side chains were not counted. However, no other

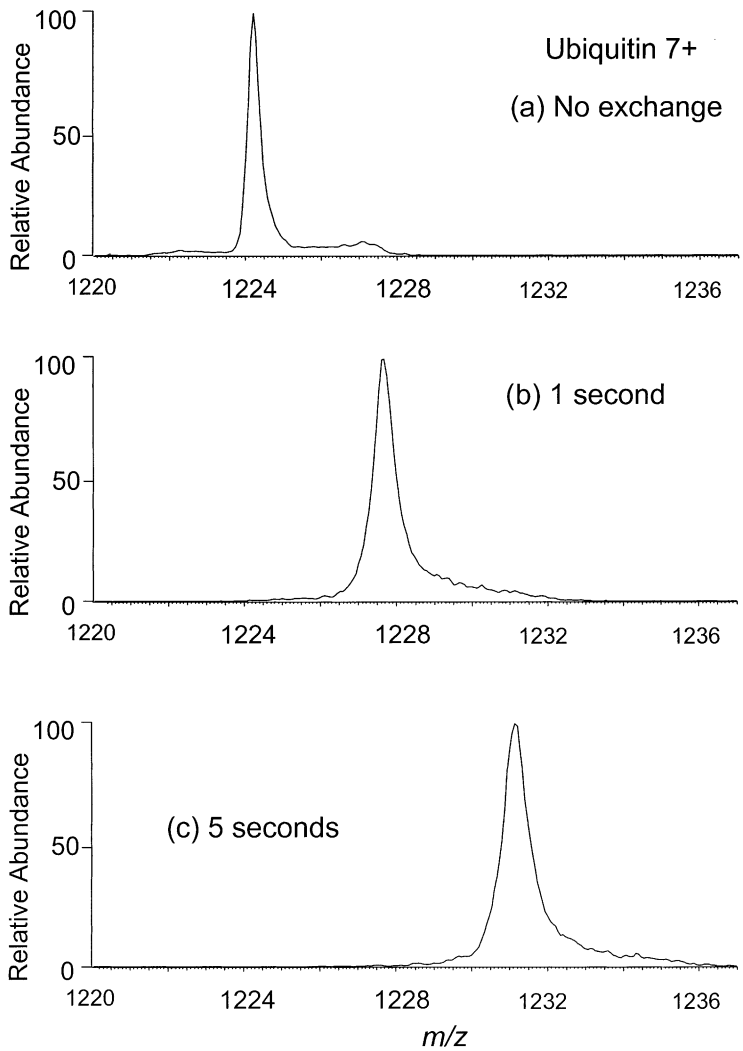


Fig. 3. Gas phase exchange of the +7 charge state of ubiquitin (76 amino acids, 151 exchangeable sites, calculated parent ion $m/z = 1224.5$) with deuterated methanol; (a) parent ion with no exchange; (b) 1 s of exchange. Peak-width at half height is twice the parent ion; (c) 5 s of exchange. Peak-width at half height is ~1.75 times the parent ion.

protein has more than one histidine so this does not introduce a significant error when comparing exchange levels.

Fig. 3 shows the mass spectra as a function of exchange time for the +7 charge state of ubiquitin. This figure is representative of the appearance and relative width of the peaks observed for the majority of the proteins studied. The peak-width at half height broadens by a factor of 2 after 1 s of exchange, and slightly narrows at longer times. The presence of one peak is proposed to be indicative of one or a set of similar conformers or an ensemble of rapidly interchanging conformers. The tailing observed at higher masses is probably not significant, but is due to sodium adducts that are not fully ejected from the trap prior to exchange. Fig. 4 shows the mass spectra as a function

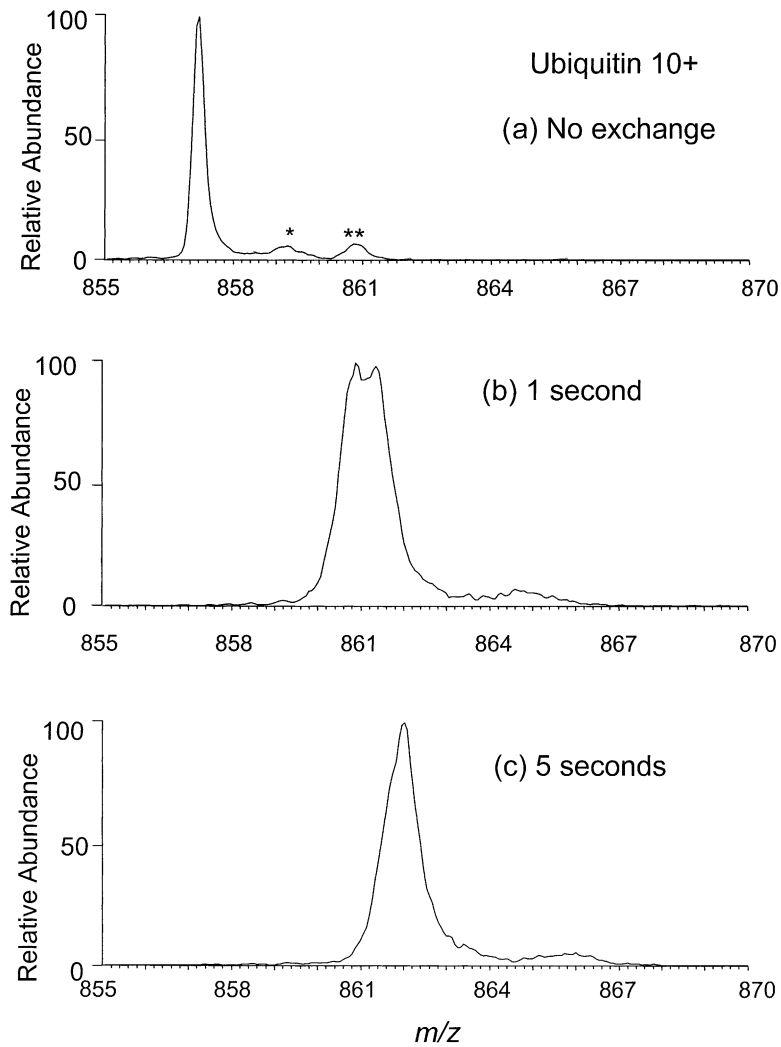


Fig. 4. Gas phase exchange of the +10 charge state of ubiquitin (76 amino acids, 154 exchangeable sites, calculated parent ion $m/z = 854.5$) with deuterated methanol; (a) parent ion with no exchange. Single asterisk (∗) corresponds to parent ion plus 1 sodium and double asterisks (∗∗) correspond to parent ion plus 2 sodiums; (b) 1 s of exchange. Sodium peaks not present because they were not selected for exchange. Peak-width at half height is 3.7 times the parent ion (c) 5 s of exchange. Peak-width at half height is 2.7 times the parent ion.

of exchange time for the +10 charge state of ubiquitin, where the wider peak observed after 1 s is again attributable to similarly exchanging or interconverting conformers, though we do not resolve distinct conformers. After 5 s, the peak becomes narrower, perhaps indicating completion of the interconversion, and stays that width for the remainder of exchange.

Fig. 5 shows the exchange of the +8 peak of lysozyme where a very small amount of exchange is observed, but significant peak broadening is seen. To estimate the extent of exchange for this protein, the peak midpoint was used. Lysozyme is the only protein for which significant broadening is seen, and it is observed for all charge states studied.

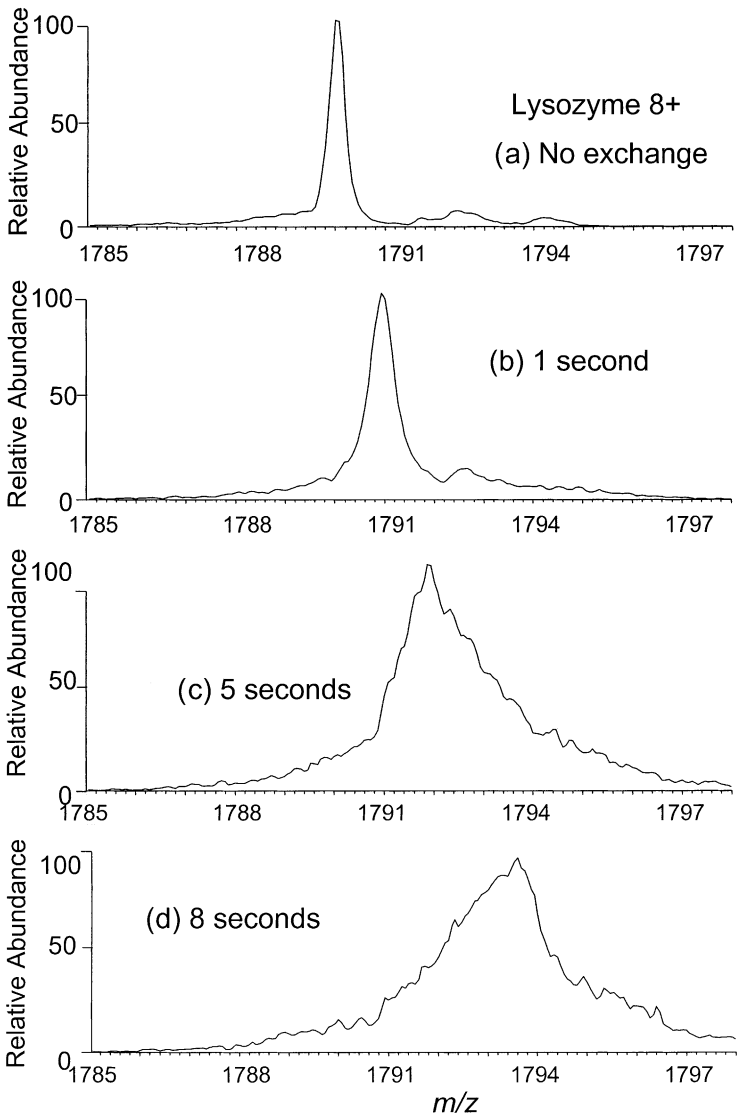


Fig. 5. Gas phase exchange of the +8 charge state of lysozyme (129 amino acids, 271 exchangeable sites, calculated parent ion $m/z = 1790.1$) with deuterated methanol; (a) parent ion with no exchange; (b) 1 s of exchange. Peak-width at half height is 1.5 times the parent ion; (c) 5 s of exchange; (d) 10 s of exchange. The wide peak at longer times is attributed to multiply co-existing conformers.

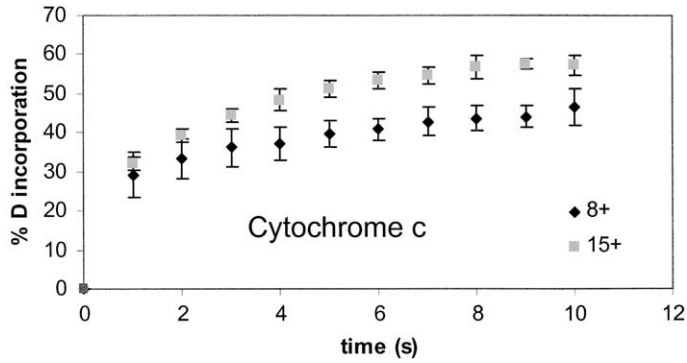


Fig. 6. Percent deuterium incorporation as a function of time for cytochrome *c* for the +8 and +15 charge states (104 amino acids, $198 + nH^+$ exchangeable sites, where n = charge). The error bars indicate the standard deviation for three separate trials.

Fig. 6 shows the extent of exchange as a function of time for cytrochrome *c* for the +8 and +15 charge states. The +15 exchanges 57% of its total labile hydrogens fairly rapidly, and the +8 exchanges 47%. Between 8 and 10 s there is no significant increase, although we are not able to probe longer times and so it would not be correct to characterize these as maximum exchange levels. The error bars show the standard deviation of three trials. The deviation is primarily due to reproducibility of establishing identical gas pressures and leads to an estimated error of 2–4%. It is likely that the deviation is greater for the +8 state than for the +15 primarily because the peak is broader (data not shown) for the +8 indicating again the presence of multiple conformers, and also making the estimate of the number of exchanges more difficult.

Fig. 7 shows the extent of exchange as a function of time for ubiquitin. In contrast to cytochrome *c*, in ubiquitin the lower charge state exchanges more rapidly than the higher charge state. Also, in ubiquitin the more rapidly exchanging lower charge state is associated with the narrower peak-width during exchange, while in cytochrome *c* the more rapidly exchanging higher charge state is associated with the narrower peak. As Table 1 shows, thioredoxin behaves similarly to ubiquitin (the lower charge state exchanges a greater percentage within 8 s), and insulin and lysozyme behave similar to cytochrome *c*.

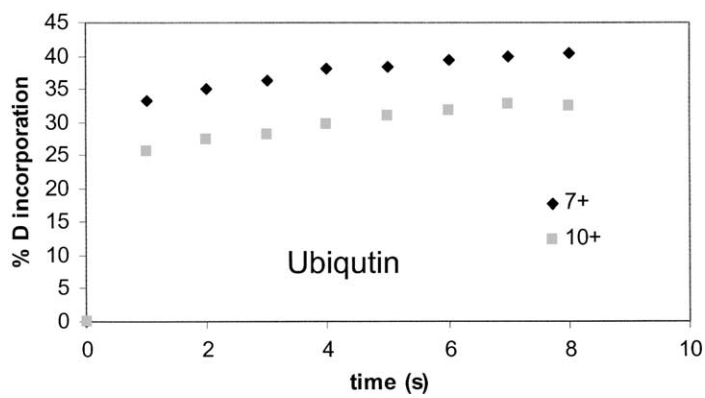


Fig. 7. Percent deuterium incorporation as a function of time of ubiquitin for the +7 and +10 charge states (76 amino acids, $144 + nH^+$ exchangeable sites, where n = charge).

4. Discussion

While we cannot directly measure the rate of pressure inside the ion trap, we estimate the pressure of deuterated methanol to be $\sim(1–5) \times 10^{-6}$ Torr through comparison to prior literature studies of H/D exchange of protein and peptides. The typical ion trap operating pressure is 2×10^{-3} Torr and Gronert reported a mixing ratio of reagent gas to helium as $\sim 10^3$ in an inlet system similar to ours [43]. Comparison of the observed rate of exchange of betaine to the literature rates with methanol (1.0×10^{-10} cm^3 molecule^{-1} s^{-1} [26]) yields an estimated pressure of $\sim 5 \times 10^{-6}$ Torr. Finally, our observed rates of exchange for proteins are about 10–50 times faster than typical FT-ICR experiments with deuterated methanol at $\sim 10^{-7}$ Torr [40] and similar to those obtained in an ion guide [44] with D_2O at a pressure of $\sim(1–3) \times 10^{-6}$ Torr.

4.1. Comparison to other studies

The general trends observed in FT-ICR studies of proteins are consistent with our work. The +10 state of ubiquitin was observed to exchange fewer hydrogens (with D_2O) than the +7 [37] and with both charge states primarily one conformation was observed at long times. For cytochrome *c* (also with D_2O) the +15 state exchanged more than the +8, though unlike in our work, more than one conformation was observed for the +8 state [32–34]. Low levels of exchange (with MeOD) for lysozyme compared to cytochrome *c* were previously observed by Green and Lebrilla [40].

For cytochrome *c* we observe exchange of 121 (57%) sites in 8 s for the +15 charge state and 96 (47%) sites for the +8 charge state. In FT-ICR studies with D_2O one conformation (consistent with our narrow peak) was observed for the +15 state which exchanged 125 sites in 30 min [34]. Multiple conformers were observed for the +8 state, and our level of exchange falls in the middle of the two primary conformations (exchanging 82 and 113, respectively) consistent with our seeing an interconversion of structures and hence a wider peak in the ion trap [32]. Recent experiments combining ion mobility and ITMS of cytochrome *c* demonstrate that the +7 to +10 states are reasonably compact when injected into the trap and then rapidly (within 70–100 ms) adopt more diffuse conformations [45]. For the +8 charge state two species with different mobilities persisted up to 200 ms (the longest time studied). It is possible our broad peak is indicative of multiple states slowly interconverting and the narrowing at longer times is due to full interconversion. Our results are not comparable to the 64 exchanges observed for the +8 state by Valentine and Clemmer in the ion mobility drift cell for the more diffuse conformer with D_2O even though the pressures and temperatures are comparable between the drift cell and ion trap [41]. In later work, Valentine and Clemmer were able to increase exchange by raising the temperature, resulting in almost complete exchange and greater maximum exchange for higher charge states [38].

For ubiquitin we observe that 49 (31%) sites exchange for the +10 charge state and 58 (39.4%) for the +7 charge state. Prior studies in the FT-ICR with D_2O observed that the +7 also exchanges more than the +8, +9 and +10 states. Significantly more exchange was observed after 1 h than we observe (83% for the +7 and 78% for the +10) [37]. Both in this study and the FT-ICR work, primarily one isotopic distribution was observed for the +7 and +10 charge states, and at long times the +10 is wider, though at shorter times multiple conformations are seen for the +7 charge state. In contrast to our work the +10 peak is more significantly broadened than the +7, perhaps indicating we are sampling structures similar to those seen in longer time scales in the FT-ICR.

For lysozyme, our results are consistent with an FT-ICR study by Green and Lebrilla in which only very low levels of exchange were observed [40]. An increase in exchange was observed between the +9 and the +10 charge states whereas we observe one between the +8 and +9 charge states. The low level of exchange was attributed to the high number of arginines localizing the charge and significantly slowing exchange [40]. The increase in exchange between the +8 and +9 is consistent with prior work by Gross et al. looking at the gas phase ion–molecule reactivity

of lysozyme [46]. Modeling of the reactivity of these ions indicated that the charge states of +8 and below are fairly compact while the higher charge states have significant fractions of unfolded conformers. Ion mobility of lysozyme provides evidence for two primary conformers, one folded and one elongated, with relative intensity a strong function of injection voltage. The ion mobility studies do not show a dramatic change in conformation going from the +8 to +9 state, though at high injection voltages the +9 opens up almost completely, while the +8 has a significant amount still folded [4]. An interpretation that unfolding at higher charge states leads to increased exchange is not in conflict with the interpretation of argnine playing a major role in controlling the exchange rate. Protonation of non-arginine residues at higher charge states (lysozyme has 11 arginines, but several are in close proximity and it is probable at higher charge states it may be more energetically favorable to protonate a lysine) might also be accompanied by a change in structure.

4.2. Mechanistic considerations

As discussed in the introduction, the effect of charge state on exchange level in a protein can be influenced by two competing factors. Lower charge states associated with more compact conformations can protects hydrogens from exchange with deuterated reagent gas and would be expected to therefore exchange fewer hydrogens. However, the potential inaccessibility of basic sites (Scheme 1) in the highly charged elongated conformations can also result in lower exchange levels. Separating these two effects is complicated by influence of individual amino acid side chains on exchange, which is not yet fully understood.

Site directed studies of the exchange of the dipeptide gly–gly indicates that the amine hydrogens exchange fastest, followed by the amide and then the carboxylic acid hydrogens [27]. The percent of amine hydrogens (primarily on lysines) in each protein is also tabulated in Table 1 and there is a correlation between the number of total amines in a protein and how much exchange is observed, which is consistent with fast exchange of amine hydrogens followed by additional exchange by other groups. The "relay" mechanism resulting in rapid exchange of amine hydrogens is shown in Scheme 1 [26,40]. A similar mechanism would also be possible if the amides were protonated. It is clear the presence of basic residues is essential for exchange, and recent work on a series of protonated peptides with deuterated methanol showed that peptides with no basic groups (modified N-terminus removing that amine) undergo little to no exchange in the same time period that peptides with arginine and lysine undergo exchange of 60–80% of their labile hydrogens [30].

Arginine is the most basic site in all these proteins and is preferentially protonated. As already discussed with lysozyme, lower levels of exchange have been attributed to protonation on arginine [40]. We also observe that the exchange level for the peptide melittin experiences a jump when the charge exceeds the number of arginines (Table 1). With the exception of the insulin B-chain (see below) all the proteins whose charges greatly exceed the number of arginines exchange at much higher levels.

There is also a trend relating number of disulfide bonds with exchange levels. Lysozyme has four disulfide bonds, potentially holding it in a rigid conformation that makes it difficult for basic sites to facilitate exchange throughout the protein. The insulin B-chain exhibits less exchange than melittin despite being of similar size and it is also held together by a disulfide bond which, given its small size, would take away flexibility. The other proteins in this study have at most one disulfide bond, and ubiquitin and cytochrome *c*, which have the highest levels of exchange do not have any. McLafferty and coworkers examined disulfide intact and reduced Rnase S and observed significantly increased exchange levels in the reduced form [32]. The reactivity [45] and ion mobility [4] of both intact and reduced lysozyme have been investigated, and both studies indicate the disulfide reduced structure adopts a more open form than the intact protein. Future work will look at the difference in exchange levels in intact and reduced proteins, to continue to try to understand the

relation of exchange levels to conformation and mechanism.

5. Conclusions

ITMS can be coupled with H/D exchange to investigate the gas phase conformations of proteins. While software limitations on the commercial instrument limit the timescale of exchange available for study, overall results are in good agreement with exchange observed with other methods, particularly FT-ICR studies. The levels of exchange observed are correlated with the number of amine groups, which indicates that the protein adopts a conformation in which the amines (primarily lysine side chains) are on the surface. While, it is tempting to attribute the higher exchange levels to more open conformations it is difficult to separate that effect from mechanistic data that predicts slower exchange for elongated conformations. A more full understanding of the mechanisms for exchange of proteins are necessary to more fully related exchange levels to protein conformation.

Acknowledgements

Funding for instrumentation was provided by Grinnell College. E.M.M. gratefully acknowledges funding from the Dreyfus Foundation (Faculty Start-Up Grant) and an American Society for Mass Spectrometry (ASMS) Research Award. Some stipend support for S.E.E. and N.L. was provided by the Grinnell College Fund for Excellence.

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