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Microsecond Pulsed Hydrogen/Deuterium Exchange of Electrosprayed Ubiquitin Ions Stored in a Linear Ion Trap

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Abstract

A pulse of D_2O vapour on the order of microseconds is allowed to react with the +6 to +9 charge states of ubiquitin confined in a linear ion trap (LIT). Two envelopes of peaks are detected for the ions of ubiquitin, corresponding to the ions that exchange more quickly and more slowly. The deuterium uptake of the protonated sites on ubiquitin ions accounts for the ion population with the fast exchange. The hydrogen/deuterium exchange (HDX) kinetics of ubiquitin ions trapped in the LIT for 200 ms showed comparable structural transitions to those trapped for 300 ms. When ions are trapped for longer, i.e. up to 2000 ms, mainly the slow exchanging ion population is detected. In all experiments the +7 ions exchange the most, suggesting a short distance between the surface protonated sites and nearby charged sites, and concomitantly high accessibility of surface protonated sites towards D_2O. The +6 ions are more compact than the +7 ions but have one fewer protonated site, therefore fewer surface availabilities for D_2O attack. The data suggest that the +6 ions keep most of their solution-phase contacts intact while the hydrophobic core is slightly interrupted in the +7 ions, possibly due to the exposure of charged His68 that is normally buried in the hydrophobic pocket. The +8 and +9 ions have more protonated sites but are less compact than the +7 ions because of Coulombic repulsion, resulting in a larger distance between the protonated sites and the basic sites. The data indicate that the HDX mechanism of ions with the slower exchange corresponding to the second envelope of peaks is primarily governed via a relay mechanism. The results suggest that the pulsed HDX MS method is sampling a population of ubiquitin ions with similar backbone fold as solution.

Key words: Time-resolved hydrogen/deuterium exchange mass spectrometry (HDX MS), Gas-phase HDX kinetics, Relay mechanism, Ion-molecule reactions, Time-of-flight mass spectrometry (TOF-MS), Ubiquitin, Electrospray ionization (ESI) MS, Deuterium oxide, Native MS

Introduction

The unique ability of electrospray ionization (ESI)[1] to ionize macromolecules intact has enabled the study of large biological protein complexes in the gas phase. Several mass spectrometry (MS)-based techniques have been developed to gain insight into the intramolecular interactions and the structures of biomolecules in the absence of solvent.[2-5] Studies of gas-phase protein ions potentially allow determining the effect of solvent on solution conformation. Thus complementary information for understanding the structure and dynamics of proteins in the condensed phase can be gained. Further, if conditions can be found where gas phase biomolecules retain solution structures, mass spectrometry will find widespread application in areas such as protein folding studies, enzyme binding and drug development.

Besides protein folding, understanding enzyme catalysis continues to be of interest in chemical biology.[6] Physical principles of enzymes catalysis remain a hot topic of debate as not all enzymatic rate coefficients can be described by conventional transition-state theory (TST)[7]. Enzyme catalysis[8] was initially explained with semiclassical TST in which the catalyst reduces the height of the potential energy barrier. In the case of hydrogen-atom transfer reactions in enzyme active site, the importance of quantum-mechanical hydrogen tunnelling becomes more significant.[9] For instance, the rate-determining step in soybean lipoxygenase-1 (SLO), that catalyses the oxidation of linoleic acid, is the hydrogen atom transfer from the pentadienyl moiety of linoleic acid to the active site Fe(III). It was suggested that SLO have an active site structure that is well organized to support hydrogen tunnelling.[10] Mutations in the active site of SLO were found to increase the potential energy barrier width, thus affecting the probability of hydrogen tunnelling event. In addition, temperature dependent kinetic isotope effects for

hydrogen transfer in SLO mutated in residues distant from the active site were explained with a hydrogen tunnelling model[11] that incorporated harmonic oscillators and Morse oscillators in the hydrogenic wavefunctions.[12] In another study,[13] an enhancement of catalytic activity of human purine nucleoside phosphorylase (hPNP) upon mutations, remote from the active site, was proposed to be due to dynamic coupling of the remote residues with the catalytic site. Stronger coupling of vibrations to the reaction coordinate in the mutant protein, compared to the wild-type, was proposed to increase the probability of forming the transition state and thus decrease the activation free energy. Warshel's pioneering works to study the dynamics and biological processes by MD simulations indicated the importance of electrostatic preorganization in enzyme catalysis reactions.[14] The traditional hypotheses that the compression of the donor-acceptor distance enhances tunnelling were challenged. It was found that tunnelling decreases upon compression and increasing pressure only marginally increases the activation barrier.[15] Quantifying electrostatic interactions was found to be essential for structure-function correlation in proteins.[16,17] Providing insight into the microscopic description of the atomic motion in chemical reactions,[18] experimental and computational studies in the gas phase are valuable tools to address enzyme-catalysed reactions.

Ion-molecule reactions such as hydrogen/deuterium exchange (HDX) are among gentle probes that provide information on the gaseous ions' higher order structure. HDX measurements on gas phase proteins can provide new insights into the degree to which protein conformations are retained *in vacuo*. Despite solution HDX, the mechanism of gas-phase HDX is more complex and less well understood. In addition to the instrument, the pressure and the exposure time of deuterated solvent, gas-phase HDX reactions are highly dependent on the proton affinity of the deuterated reagent. For D_2O with a relatively low proton affinity (165 kcal mol^{-1}),[19] proton transfer from the

protein and formation of a hydronium ion intermediate (i.e. the "onium ion" mechanism)[20] has a high energy barrier. Instead, exchange proceeds via a "relay" mechanism[20] in which D_2O shuttles a proton from the charged site to a nearby basic site, leaving the charge site on the protein. Thus, D_2O has to afford to form a hydrogen bond to both a protonated and a basic site. This requires the proximity of the protonated site on the surface of the protein and the basic site. Consequently, the extent of HDX does not necessarily have direct implication for gas-phase conformation. The exchange levels of a compact conformer (with less accessible protonated sites) can be higher than that for an extended conformer as a protonated and a basic site may be closer in the former.

The extent to which the gas-phase protein ions retain their solution structures is still a fundamental question in native ESI-MS. The stepwise structural evolution of a globular protein, cytochrome *c*, upon ESI was simulated by molecular dynamic (MD).[21,22] The final water molecules are removed from the protein ions in the nanosecond scale. Within ~10 picoseconds an exterior-collapsed "near-native" conformation is formed as the charges on protein side chain residues collapse onto the backbone. Subsequently, the hydrophobic interactions are lost in the millisecond time scale, followed by the loss of the electrostatic interactions. The ions then rearrange to find their most stable structure in a longer time scale, i.e., seconds to minutes. A direct correlation between the stability of proteins in solution (i.e. ΔG for global unfolding) and gas-phase unfolding is inconclusive.[23,24] Small globular proteins were found to preserve their solution-phase conformations for up to 60 ms following ESI,[22,25,26]demanding techniques to study gaseous proteins in (sub) millisecond time scale.

Recently, a pulsed HDX MS method allowed studying the HDX reactions of protein ions immediately after ESI with a 1 ms pulse of D_2O.[27] D_2O with a low proton affinity and small

degree of charge stripping was shown to be a very sensitive reagent to probe millisecond time-resolved HDX reactions. This work has now been extended to demonstrate the ability of pulsed HDX MS to study trapped protein ions with a much shorter pulse of D_2O (100-500 μs) and shorter trapping times (i.e. 200 ms). The newly-developed technique is applied to investigate an already well-studied protein, ubiquitin. Ubiquitin is the major protein in ubiquitination, a fundamental regulatory post-translational modification controlling a wide range of intracellular signalling processes. Here, the HDX reactions of trapped ubiquitin ions with minimal interruption of folding transitions are studied. Two envelopes of peaks are observed for the ions of ubiquitin over the trapping time, corresponding to slower- and faster-reacting ion populations. The deuterium uptake of the protonated sites on ubiquitin ions accounts for the ion population with the fast exchange. Based on current data, the exchange for ions corresponding to the second envelope of peaks mainly occurs via a relay mechanism. The sub-millisecond time scale of current technique enables capturing gaseous protein ions with implications for the preservation of their solution-phase conformation.

Experimental

Chemicals

Ubiquitin from bovine red blood cells (≥ 98%, U6253) and caesium iodide (99.99%) were from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. The protein was 5 μM in 10:90 methanol/water (pH 6.5). Deionized 18 MΩ water was obtained from a Millipore Q-grade 1 purification system (Etobicoke, ON, Canada). Methanol (HPLC grade) was from Fisher Scientific (Fair Lawn, NJ). Deuterium oxide (99.9%) was from Cambridge Isotope

Laboratories Inc. (Andover, MA). All gases, nitrogen, Ar (UHP 5.0 grade) and compressed air (breathing grade), were from Praxair (Mississauga, ON, Canada).

ESI Linear Ion Trap Time-Of-Flight Mass Spectrometry

Solutions of ubiquitin were infused at 1.0 μL min^{-1} into a pneumatically-assisted electrospray ion source of a home-built linear quadrupole ion trap reflectron time-of-flight (LIT-TOF) MS.[28,29] D_2O, stored in a stainless steel sample cylinder (Swagelok, Solon, OH), was immersed in a heated water bath (80 °C) to maintain 354.8 Torr vapour pressure backing the valve. D_2O vapour was pulsed into the LIT chamber through a solenoid pulse valve with an orifice diameter of 0.039" (Series 9, Parker Hannifin Corp., Pine Brook, NJ) as described previously.[27] Protonated ions formed by ESI (+4.3 kV) at room temperature pass through an aperture in a curtain plate (1000 V), a dry nitrogen curtain gas (2 L min^{-1}), an orifice (220 V), and a skimmer (30 V), and enter a chamber containing two linear radio frequency (rf)-only quadrupole ion guides, Q_0 (length 5 cm, DC offset 14 V) and Q_1 (length 20 cm, DC offset 10 V). The quadrupole region was pumped to a base pressure of ~2 mTorr, measured with a precision capacitance manometer (Baratron model 120 AA, MKS Instruments, Andover, MA). Ions reach the source region of the reflectron-TOF (SCIEX, Concord, ON, Canada) after passing through a stack of four focusing lenses (L1 = L2 = -20 V, L3 = L4 = 0.0 V). Ions were extracted into the flight tube (SCIEX, Concord, ON, Canada) with a series of acceleration pulses and detected by a dual microchannel plate (3400 V). The pressure in the TOF chamber was 1.2×10^{-6} Torr (360 $L.s^{-1}$ turbomolecular pumps backed by mechanical pumps). Mass calibration was performed with a CsI solution (1 mM in 30% MeOH).

Trap Timing Sequence

The timing parameters are given in Fig. 1a with each trapping cycle containing four segments: ion drain, ion injection, trapping, and ion detection. In the drain phase of each cycle, both the LIT entrance (Q_0/Q_1) and exit (L1) plates were kept at low voltages to eliminate the ions from the trap. For ion injection, the entrance voltage remained low (+15 V) while a high stopping potential of +40 V was applied to the exit plate. Ions were confined in Q_1 by increasing the LIT entrance plate voltage to +40 V during trapping and detection.

Pulse Valve Timing and LIT Pressure vs. Time Profile

The pulse valve was triggered by the LIT entrance plate (Q_0/Q_1). Several sets of experiments were designed to collect time-resolved data, including varying D_2O pulse duration (denoted in Fig. 1a by "On"), D_2O pulse position (controlled by changing "Off time" in Fig. 1a), and trapping time ("Trap" in Fig. 1a). For each set of experiments the total cycle time (Fig. 1a) was kept the same by adjusting the timing sequence. The "Off time" was adjusted to probe ions HDX at several time intervals during the trap such that the sum of Injection and Trap is equal to the sum of Off time, On, and Δt.

The profile of pressure in the LIT chamber (5445 cm^3) vs. time under current experimental condition is simulated[30,31] and is shown in Fig. 1b.

Results and Discussion

HDX of Ubiquitin Ions in the LIT with 10, 100 and 500 μs D_2O Pulse

Under the experimental conditions applied, the +6 to +9 charge states of ubiquitin ions were produced. Ubiquitin ions were injected into the LIT for 1 ms, trapped for 200 ms, detected for 20

ms, and drained for 4 ms (total cycle time = 225 ms). During ions storage in the trap, a pulse of D_2O was introduced after a delay time of 1 ms (Off time = 1 ms in Fig. 1a). Pulse lengths as short as 10 to 100 μs did not result in any detectable HDX products. With increasing the pulse duration up to 500 μs, a small shift toward higher mass-to-charge ratio (*m/z*) with slight broadening of the isotopic envelopes was detected (shown in Fig. S1 for the +8 ions). This indicates that at the very beginning of the reaction, a population of ions with fast exchange is dominant. The broadening of the isotopic envelope indicates another population of ions with slower exchange. Thus a 500 μs pulse of D_2O was applied to explore the details of HDX reactions of ubiquitin ions over the trapping time. To characterize the reactions occurring between the multiply charged ubiquitin ions and D_2O, several sets of experiments were conducted, A4, C4, C'4 and D4, that are summarized in Table 1. Repeat experiments produced very similar results.

HDX of Ubiquitin Ions Trapped in the LIT for 300 ms with 500 μs of D_2O

In C4, ubiquitin ions were trapped for 300 ms while D_2O was pulsed for 500 μs. The total cycle time for C4 was kept equal at 325 ms. During ion storage in the trap, a pulse of D_2O was introduced after a delay time of 1 to 300 ms (Off time = 1 to 300 ms, Δt = 299.5 - 0.5 ms in Fig. 1a). The pulse of D_2O enables systematic probing of the HDX at time intervals from the beginning to the end of Trap fill time by changing the delay time (i.e. Off time in Fig. 1a). Bimodal distributions were observed for all charge states, indicating that hydrogen exchange has been very fast in some populations of ubiquitin ions and very slow in others. The two envelopes of peaks are denoted as "first peak" and "second peak", corresponding to the ions that exchange more quickly and more slowly, respectively. Each envelope of peaks can correspond to either the

presence of an ensemble of conformers with similar exchange rates or fast interconversion of several conformers with different exchange rates. Plots of intensity vs. *m/z* at different delay times (Off time in Fig. 1a) for C4 are shown in Fig. 2a. While the bimodal distribution is fully resolved for the +6 and +7 ions, the first and the second peaks overlap somewhat for the +8 ions.

The fast-reacting ions corresponding to the first peaks (Fig. 2b) exchange only a few hydrogens (~max 11). As previously reported,[27,32] the deuterium uptake of the first peak possibly accounts for the number of protonated sites on ubiquitin ions (i.e. 6, 7 and 8), within the uncertainties arising from the reading *m/z* values of the deuterated peaks. In current experiments, for the +8 ions, slightly higher deuterium uptake (~11) than expected (8) is observed. This could simply result from *m/z*-reading uncertainties due to broadening of the +8 ions (Fig. 2a). An alternative explanation is that the charged sites on the +8 ions might be located on basic sites that are involved in salt bridging to acidic residue(s). If the exchange proceeds through other mechanisms such as "flip-flop",[20] then the number of hydrogens exchanged depends on the number of residues that salt-bridge to the basic sites. This also explains the higher HDX uptake of the +8 ions corresponding to the second peak when trapped for 300 ms (C4) than 200 ms (C'4, discussed below).

For the +6 and +7, the slow exchanging ions corresponding to the second peak reach a plateau near the end of trapping time (i.e. Off time > 150 ms), reflecting the completion of HDX reactions just after the midpoint of trapping time. The +7 ions exchange the most (33 hydrogens), while the +6 ions exchange the least (~18 hydrogens). The +8 ions exchange about the same as the +7 ions. The time at which the relative intensities of the first and second peaks are equal, referred to as $t_{midpoint}$, is calculated from the plots in Fig. 2c (Table 1). For the +8 ions, at the

beginning of the experiment (i.e. Off time values of 1 and 10 ms), slight shift to higher *m/z* values and significant broadening are detected. The second peak for the +8 ions becomes more resolved when the D_2O probe is introduced at longer delays (i.e. Off time ≥ 50 ms) as more deuterated ions corresponding to the second peak are formed. This possibly indicates a more heterogeneous population of ions for the +8 ions, either because of a richer ensemble of conformers or that the ions fluctuate between different conformations on a time scale considerably longer than the exchange time. Also, it is possible that several reaction channels are present owing partly to the variations of the location of protons on the gas-phase ions.

In previous experiments with a 1 ms D_2O pulse,[27] the deuterium uptake at the end of trapping time (i.e. $\Delta t = 0$) was significantly lower than that seen for the second set of peaks. This was expected and explained from the pressure profile of D_2O inside the LIT chamber as these ions are given less time (only 1 ms) to react with D_2O. For current experiments with a pulse of 500 μs, D_2O reaches a steady state pressure of ~6 mTorr in 0.5 ms and takes ~70 ms to be completely pumped away (Fig. 1b). Thus, ions exposed to D_2O at Off time = 300 ms ($\Delta t = 0.5$ ms) have been given less time for exchange to occur compared to the rest of ion with Off time < 300 ms.

HDX of Ubiquitin Ions Trapped in the LIT for 200 ms with 500 μs of D_2O

Similar to C4, 500 μs of D_2O (i.e. On = 500 μs in Fig. 1a) was pulsed in C'4, while ions were trapped for a shorter time, 200 ms (a total cycle time of 225 ms). The +7 to +9 ions are studied (Fig. 3). In agreement with C4, the +7 ions exchange the most (33 hydrogens). The +8 and +9 ions exchange about the same (Fig. 3b). All ions studied have small $t_{midpoint}$ values (Fig. 3c and Table 1). The second peak for the +8 and +9 is already present at the beginning of the experiment (i.e. Off time = 1 ms).

To examine whether the broadening of the isotopic envelope of the +8 ions reported in C4 is because of its smaller *m/z* value (thus smaller spacing between the two envelopes of peaks for a given number of hydrogens exchanged) or due to a greater heterogeneity in its gas-phase reaction, the +9 ions of ubiquitin were also studied in C'4 experiments. The peaks corresponding to the +9 ions are not fully resolved, however; they are better resolved than the peaks corresponding to the +8 ions (Fig. 3a). This suggests less heterogeneity in the ensemble of conformers/reaction channels in the +9 ions compared to the +8 ions. As the charge density increases, the gas-phase reactivity of ions depends more strongly on Coulombic contributions rather than the intrinsic reactivity of a particular charge state.

During HDX reactions of ubiquitin ions with D_2O, the *m/z* value of deuterated peaks and the spacing between the two envelopes of each charge state remains approximately constant in both C4 and C'4. The HDX level of the slow-exchanging ion population is about the same for the +7 ions in C4 and C'4 (Figs. 2b and 3b). However, the +8 exchanges slightly more in C4 than in C'4. The fast-exchanging ion population of the +8 also exchanges more in C4 than in C'4. As discussed above, this might be due to the differences in the location of charged sites for the +8 ions.

Effect of Pulsing D_2O 100 ms to the End of Trapping Period

The effect of trapping time on HDX behaviour of +6 to +8 ions of ubiquitin was investigated in A4 experiments (Fig. 4). Solution of ubiquitin was injected into the trap for 1 ms, trapped for 100 to 2000 ms, detected for 20 ms, and drained from 4 to 1904 ms to maintain a total trapping cycle of 2025 ms. One ms D_2O was pulsed at 100 ms before the end of Trap (i.e. On = 1 ms and Δt = 100 ms in Fig. 1a). Results are summarized in Fig. 4. Bimodal distributions were resolved for the

+7 and +8 ions (Fig. 4a). The +7 ions exchange the most with a maximum of 53 hydrogen uptakes and have smaller $t_{midpoint}$ than the +8 ions. Ions corresponding to the first peak are completely depleted when trapped for longer than ~350 ms (Fig. 4c).

The ions corresponding to the second peak are present at the beginning of the experiment with Trap times as short as 100 ms. Note that for ions trapped for 100 ms, the D_2O pulse is introduced right after injecting the ions into the LIT, giving the shortest elapsed time between ESI and HDX reactions. Ions might be vibrationally and translationally hot at the very beginning of trap, acting differently from the thermalized ions. At Trap = 100 ms, the +6 ions show a significant broadening of the isotopic envelope rather than a bimodal distribution that observed for the +7 and +8 ions. The higher velocities of ions with lower *m/z* might account for this observation. The first peak for the +6 ions was only detected for Trap = 100 ms. The second peak for the +7 ions when trapped for 100 ms has a small shoulder that is not fully resolved, possibly because of contributions from at least one more reaction. Both +7 and +8 ions have a rather small $t_{midpoint}$, 129 and 195 ms, respectively (Fig. 4c and Table 1). When ions are trapped for 500 ms or longer, ions corresponding to the first peak are completely depleted. Broadening of the isotopic envelope, most prominently for the +7 ions (Fig. 4a), was observed for ions trapped for longer (i.e. Trap ≥ 750 ms). The deuterium was introduced at Δt = 100 ms, providing identical reaction time in all experiments in A4 (i.e. Trap = 100 to 2000 ms). Overall, the HDX level gradually increases with longer trapping time.

Several factors can contribute to the behaviour of ions at longer trapping times. Mass discrimination effects are among those factors.[33] Ions were injected into the LIT for only 1 ms to keep the charge density low in the LIT and eliminate mass discrimination effects triggered by

space charge. The slight increase in the pressure of the LIT upon introducing D_2O helps to further minimize mass discrimination, possibly because collisional cooling of ions prevents large amplitudes of radial motion which can cause ion ejection or fragmentation.[34] Charge stripping of highly charged ions and formation of a *new* population of low-charged ions upon increasing the trapping time can account for mass discrimination. The broadening of the +7 and +8 ions is noted in Fig. 4b by considering HDX uptake for a possible third peak in the mass spectra. Structural changes from keeping ions longer in the trap[25] and the occurrence of more than one reaction channel might account for the differences observed here. Also, the longer trapping time could alter the competition between various ion-molecule reactions.

Effect of Pulsing D_2O at the End of Trapping Period

The effect of trapping time on HDX reactions of +6 to +8 ions of ubiquitin was investigated in D4 experiments (Fig. 5). The trapping cycle was identical to A4 (i.e. ions were injected into the trap for 1 ms, trapped for 100 to 2000 ms, detected for 20 ms, and drained from 4 to 1904 ms for a total trapping cycle of 2025 ms). The position of pulse was further delayed such that 1 ms D_2O was pulsed just before the end of Trap (i.e. On = 1 ms and $\Delta t = 0$ in Fig. 1a). Results are summarized in Fig. 5. Note that in addition to the position of D_2O pulse, D4 differs from A4 in the amount of D_2O that is admitted into the LIT. Under the conditions used to collect the data, a 1 ms D_2O pulse in the LIT reaches a steady state pressure of ~12 mTorr in 1 ms and takes ~80 ms to be completely pumped away (Fig. 1b). Ions exposed to D_2O in D4 ($\Delta t = 0$) have only 1 ms to react with D_2O, whereas in A4 ($\Delta t = 100$ ms) the ions are exposed to D_2O for the whole 81 ms. Thus, the exchange level is about half of that observed in A4 (max 24 hydrogens).

The fast exchanging ions were only observed when ions were trapped for 100 ms. In agreement with A4, the broadening of the isotopic envelope of the second peak becomes more pronounced for ions trapped in the LIT for longer. Again, the +7 ions exchange the most.

Comparison of the Pulsed HDX Reactions of Ubiquitin Ions

The pulsed HDX results of ubiquitin, conducted under several conditions, show a somewhat similar trend. In all experiments discussed above (i.e. A4, C4, C'4 and D4 and those discussed previously[27]), the +7 ions consistently show the highest HDX level. Numerous experiments suggest that the +7 ions of ubiquitin have the most native fold and require up to 0.1 s after ESI to unfold.[35-36] Native electron capture dissociation (ECD) of +7 ions of ubiquitin 0.2 s after ESI showed that ubiquitin's solution fold is overall unstable in the gas phase, but unfolding caused by desolvation is slowest in regions stabilized by salt bridges.[37] HDX reactions are very sensitive to the location of charged sites. The higher HDX level of the +7 ions suggests that a protonated site is not only accessible on the surface of the protein, but also near a charged site. The suitability of the +7 ions for a relay mechanism (i.e. compactness) can be attributed to the additional stabilization of the gaseous ions by salt bridges. Out of 12 basic residues (i.e. seven K, four R, and one H residues), five are stabilized by forming more than one salt bridge with acidic residues (D and E). If the protons in the +7 ions of ubiquitin are located on basic residues that are not heavily stabilized by salt bridges (i.e. K6, K11, K63, R72, R74, K48, and H68), then this would result in a more stable structure in the gas-phase. H68 is normally buried in the hydrophobic pocket. Slight disruption of the hydrophobic core in the gas phase exposes H68 accessible for HDX. The +6 ions are more compact than the +7 ions in the gas phase, and possibly more stable because of retaining the hydrophobic core. However, the +6 ions have one fewer protonated site,

therefore less surface availabilities for D_2O attack compared to the +7 ions. The data suggest that the +6 ions keep most of their solution-phase contacts intact while the hydrophobic core is more interrupted in the +7 ions. Although the higher charge state ions (i.e. +8 and +9) have more protonated sites for D_2O attack, they are less compact than the +7 ions due to Coulombic repulsions. The distance between the protonated sites and the basic sites is larger in +8 and +9 ions, resulting in less efficient HDX compared to the +7 ions. Therefore, the HDX mechanism of ions with the slower exchange corresponding to the second envelope of peaks is primarily governed via a relay mechanism.

The maximum HDX uptake is detected for ions trapped for longer. A maximum exchange of 37% was observed for ubiquitin in experiment A4. Although the trapping time in D4 was the same as that for A4, D_2O exposure time was shorter in D4, resulting in lower HDX uptake. No mass shifts indicative of the formation of proton-bound adducts with D_2O were observed.

Energetics of Pulsed HDX Reactions of Protonated Ubiquitin

The HDX reaction of ubiquitin ions in gas phase has significantly changed potential energy surface compared to that of solution. Overall, aqueous solvent facilitates charge transfer and results in more stabilisation of ions by gaining favourable free energy of solvation compared to that of bare gaseous ions. The species with greater electrostatic character should become more stable in the presence of an aqueous solvent. Adoption of multiple conformers adds to the complexity of reaction paths and energy profile in gas phase. It has been proposed that under gentle ESI conditions, ions that are in a global minimum on the solution-phase potential energy surface may be trapped in a local minimum on the gas-phase (unsolvated) potential energy surface.[38] This local minimum (not necessarily the most stable structure) may be one in which

the ion retains elements of the solution-phase structure. The extent of conformational changes of gaseous ions after ESI is highly time-dependent.

"Mobile proton"[39,40] may be considered as gas-phase analogues of Grotthuss mechanism.[41] Rapid proton exchange along the protein backbone can generate a heterogeneous ensemble of protonated species. In gas phase the hydrogen tunnelling does not significantly contribute to transfer reactions at room temperature. The internal energy required for the proton mobilization event depends on the difference between the energy of the most stable form and the protonated form generated after proton mobilization. Indeed, the presence of D_2O molecule can facilitate the proton migration in isolated cations via "transport" catalysis.[42,43] The presence of water molecule dramatically drops the calculated height of the energy barrier for proton migration in small systems (e.g.[43]).

In the fast exchange, exchange of protonated side chains, the intramolecular hydrogen bonding should be the same for reactants (ubiquitin ions) and intermediate product (deuterated ubiquitin ions corresponding to the first peak in the mass spectra). Thus, one can assume the entropy change of the fast exchange reaction is near zero. The free energy change is therefore independent of temperature (i.e. $\Delta G \approx \Delta H$). During the slow exchange reaction, however, the gaseous ions may rearrange to find their most stable conformation. This might result in new intramolecular hydrogen bonding and subsequently loss of rotational modes in the final deuterated product (corresponding to the second peak in the mass spectra). Thus kinetically the rate of exchange should be higher for the intermediate product than for the final deuterated product (as seen in current experiments for lower charge states). One can conclude that the potential energy surface of ubiquitin ions reactions with D_2O in gas phase consists of at least

eight critical points. The overall reaction is exothermic as the deuterated products that have somewhat rearranged to find their most stable conformations in the reaction timescale are observed. The fast exchange proceeds via a minimum three-step process:[44] the formation of a loose hydrogen-bonded complex (2), intramolecular proton transfer within this complex (3) and hydrogen scrambling, and lastly dissociation of the complex (4) to yield the deuterated protein species (5) (shown below).

$$(M+nH)^{n+} + nD_2O \leftrightarrow (M+nH)^{n+}\ldots D_{2n}O_n \leftrightarrow M\ldots D_{2n}O_nH_n^{n+} \leftrightarrow (M+nD)^{n+}\ldots D_nO_nH_n$$

(1) (2) (3) (4)

$$\leftrightarrow (M+nD)^{n+} + nDOH \leftrightarrow \ldots. \leftrightarrow (M+(n+m)D)^{n+} + (n+m)\ DOH$$

(5) (6)

Within an ion-neutral complex, energy randomization is often fast. Thermodynamically, the energy released from the exothermicity of complex formation is sufficient to overcome the barrier to internal proton transfer. Ion-neutral complexes are comparable to ion pairs in solution. Overall, the ion-neutral complex intermediate with protonated carbonyl group (Scheme I) has ~120 kJ/mol higher energy than N-protonated forms. The second reaction also proceeds via at least a three-step process. The final deuterated product (6) is the most stable species. The potential energy surface cross section for proton transfer and isotope exchange reactions of ubiquitin ions with D_2O is oversimplified in Scheme I. The first exchange product (5), corresponding to the fast exchange, possibly forms via an energy-neutral reaction (isotope effects are ignored). The electrostatic effects have been shown to play a central role in determining the

ion-molecule well depths.[45] The multiple exchanges observed for (5) correlates to protonated sites.

Clemmer et al.[46] proposed unfolding of gas-phase conformers of ubiquitin ions in the 35 ms to 30 s time scale. Measuring collision cross sections of ubiquitin ions electrosprayed from different solutions, several groups have proposed native state and A state in the gas phase.[36,47,48] However, the time scale of ion mobility experiments is not comparable to current experiments.

Thermochemistry and chemical kinetics are both essential to fully understand gas-phase ion chemistry. Dynamics of molecular collisions in gas-phase HDX reactions might be better understood by undertaking MD simulations. MD simulations of the evaporation of water molecules from partially solvated ubiquitin at 300 K show that ubiquitin shrinks with decreasing hydration shell and exposes more of its hydrophilic surface area to the vacuum.[49] For current experiments one has to consider several additional factors in MD simulations as follows. Ion motion and trajectory in the quadrupole field of the LIT must be considered. The collisional cooling effect of the pulse of D_2O and subsequent increase in the pressure of the LIT must also be taken into consideration (amongst other factors). The MD simulation studies of current data are beyond the scope of this work.

Comparison of Current Results with Measurements in Solution

Several biophysical techniques have been applied to fully characterize ubiquitin (reviewed by Jackson[50]). Early 2D NMR studies of HDX labelling of ubiquitin[51] indicated that ubiquitin folds very rapidly and cooperatively. Amide protons in the β-sheet, the α-helix, and their interface are 80% protected against labelling in an 8-ms time scale. Slower protection rates for residues Tyr-59, Ile-61, and Leu-69 with irregular structure were found. Minor slow-folding species due to

unfolded molecules containing one or more proline residues with cis peptide bonds were also found. Overall, the initial formation of hydrogen-bonded structure in ubiquitin was concluded to occur at a more rapid rate with slow folding species being less prominent. However, a direct comparison of the current gas-phase HDX results with those obtained in solution is not feasible. Simulations of solvated proteins suggested that the dielectric constant of proteins is not constant;[16] larger for the active site residues and ionisable groups than that for other nonpolar regions. In addition to the differences in the dielectric constant in vacuum and solution, the mechanism of gas-phase HDX is not well-understood. The presence of a bimodal peak not only can correspond to two conformers but also the presence of two (or more) reaction channels. Note that one of the aspects of the current experiments is to find the conditions that gas-phase protein ions properties can better resemble those of solution. The fast exchange of the protonated sites on ubiquitin ions implies that the near-native[22] conformation of ubiquitin (i.e., intact hydrophobic core and electrostatic interactions) is captured by the μs-pulsed HDX method. Note that the fast exchange here is due to the exchange of protonated side chains whereas in solution backbone amide hydrogens exchange. Thus pulsed HDX MS experiments suggest that gas-phase measurements immediately after trapping protein ions in the LIT more directly correlate to solution properties.

Conclusions

A time-resolved HDX with 500 μs pulse of D_2O is applied to characterize the rapid conformational changes and short-lived intermediates of ubiquitin ions immediately after they are stored in the trap. The low proton affinity and small degree of charge stripping marks D_2O a very sensitive reagent for HDX reactions.

Two approaches were considered for experimental design. First, a D_2O probe was introduced into the trap by varying the delay time over a constant trapping time, thereby providing the snapshots for gradual evolution of deuterated peaks. Second, the Trap time was systematically increased to monitor the effects of trapping time on the HDX reaction of ions.

Fast intermediates and presumably the least energetically stable isomers not detected by continuous HDX were identified. The fast-exchanging ion population is attributed to the exchange of protonated sites located on the exterior-collapsed "near native" protein.[21,22,26] The HDX trend correlates with solution behaviour, i.e. ions with higher charge states exchange more than those with lower charge states. This possibly indicates the population of ions that keep their solution compactness intact. A second population of ions with slower exchange was also detected, possibly corresponding to transient structure with interrupted hydrophobic interactions. In all experiments the +7 ions exchange the most. The current experiments suggest that gas-phase measurements immediately after trapping protein ions in the LIT more directly relate to solution properties and thus can possibly be of biological interest.

The spacing between the two populations of ions remained constant for constant trapping times while it gradually increased for ions trapped for varied time. More stable gas-phase structures via the formation of new noncovalent bonds require much longer time (i.e. seconds to minutes). In addition to time-dependent changes of ions structure, shorter exchange reaction time resulted in lower HDX uptake.

HDX reactions are more sensitive to the location of charged sites than collision cross sections.[52] The +8 ions show slightly higher HDX when trapped for about the same length of time (i.e. 300 and 200 ms). This is attributed to the differences in the location of charged site, and possibly the

role of a “flip-flop” mechanism for HDX reactions. Therefore, ions with close cross sections can show different HDX behaviour.

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Table 1. Values of $t_{midpoint}$ (ms) for the +6 to +9 ubiquitin ions calculated from the plots in Figs. 2c-4c.

				$t_{midpoint}$			
Exp. code	**Trap/ms**	**On/µs**	**Δt/ms**	**+6**	**+7**	**+8**	**+9**
A4	100 to 2000	1000	100	-	129	195	-
C4	300	500	299.5 to 0.5	118	82	112	-
C'4	200	500	199.5 to 0.5	-	16	38	37
D4	100 to 2000	1000	0	-	-	-	-

Scheme I

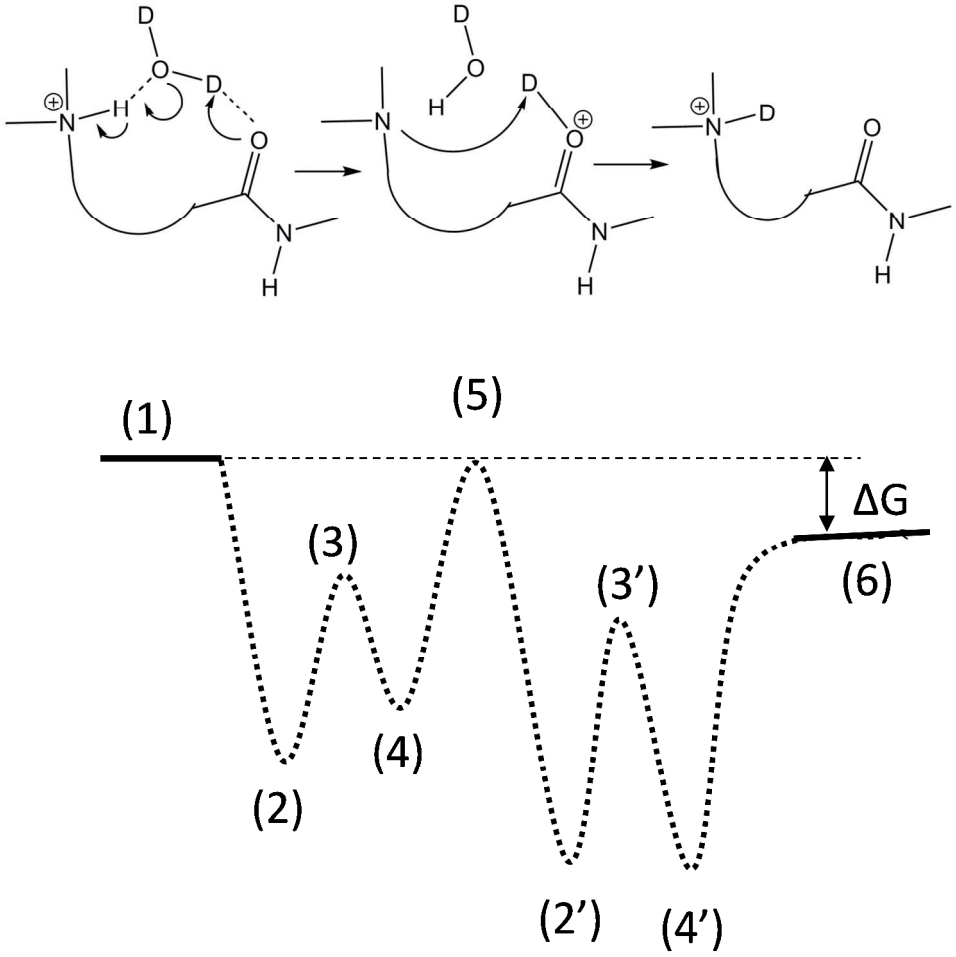


Figure Captions:

Figure 1. (a): timing sequence for the LIT and pulse valve in a trapping cycle. Q0/Q1 and L1 are entrance and exit plates of the LIT, respectively. D_2O represents the pulse valve. (b): calculated change in the LIT chamber pressure when D_2O is pulsed for 500 μs and 1 ms into the trap chamber with a base pressure of 2 mTorr.

Figure 2. Representative plots of (a) Intensity vs. *m/z* at different D_2O pulse delay times (Off time) for experiment C4 (D_2O pulse time of On = 500 μs and Off time = 1 - 300 ms). (b) HDX levels. (c) Normalized intensity vs. Off time (ms) for the +6 to +8 ubiquitin ions. Color codes for (b) and (c): +6 (black), +7 (red), +8 (blue), the first peak (dash line), and the second peak (solid line).

Figure 3. Representative plots of (a) Intensity vs. *m/z* at different D_2O pulse delay times (Off time) for experiment C'4 (D_2O pulse time of On = 500 μs and Off time = 1-200 ms). (b) HDX levels. (c) Normalized intensity vs. Off time (ms) for the +7 to +9 ubiquitin ions. Color codes for (b) and (c): +6 (black), +7 (red), +8 (blue), the first peak (dash line), and the second peak (solid line).

Figure 4. Representative plots of (a) Intensity vs. *m/z* at different Trap times for experiment A4 (D_2O pulse time of On = 1 ms and Δt = 100 ms). (b) HDX levels. (c) Normalized intensity vs. Trap time (ms) for the +6 to +8 ubiquitin ions. Color codes for (b) and (c): +6 (black), +7 (red), +8 (blue), the first peak (dash line), and the second peak (solid line).

Figure 5. Representative plots of (a) Intensity vs. *m/z* at different Trap times for experiment D4 (D_2O pulse time of On = 1 ms and $\Delta t = 0$). (b) HDX levels for the +6 to +8 ubiquitin ions. Color codes for (a) and (b): +6 (black), +7 (red), and +8 (blue).

Figure 1.

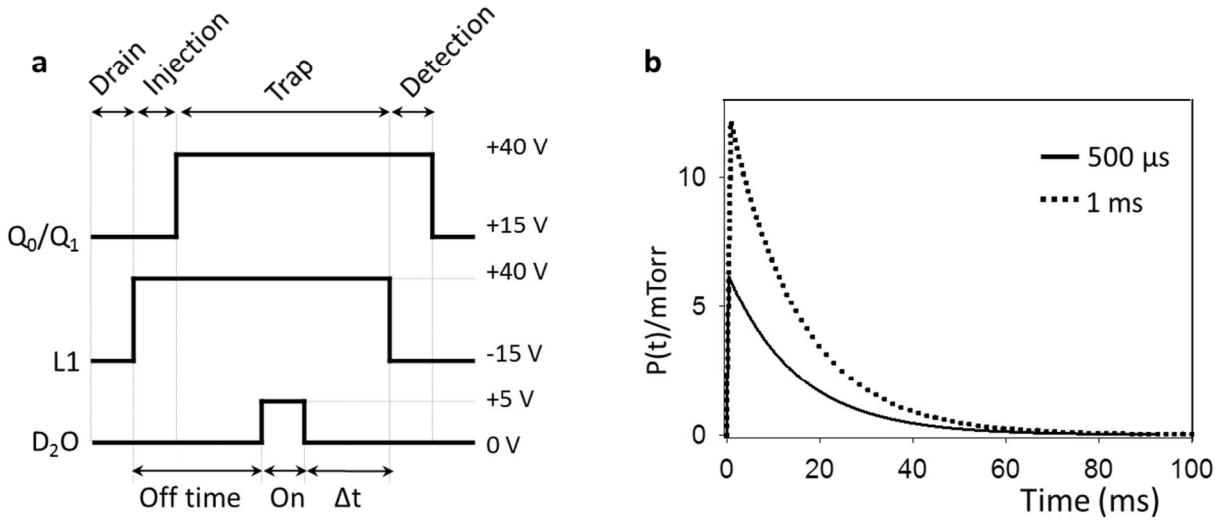


Figure 2.

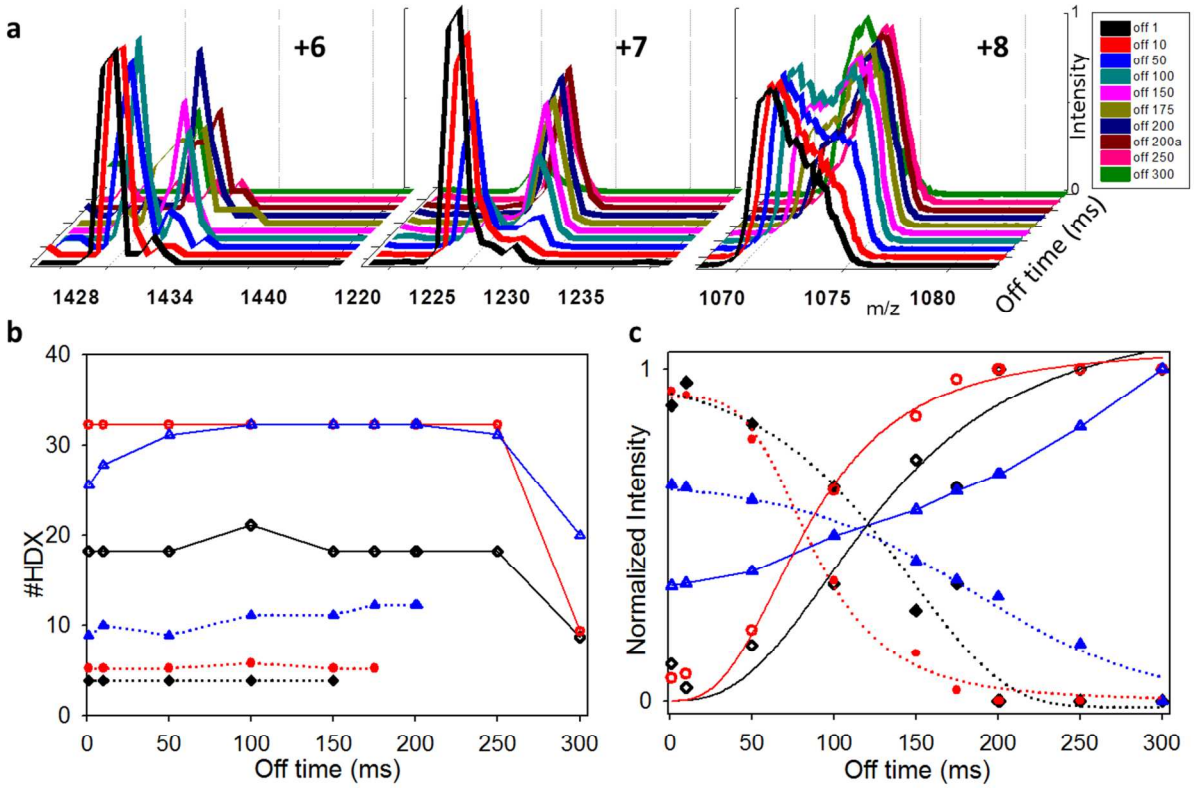


Figure 3.

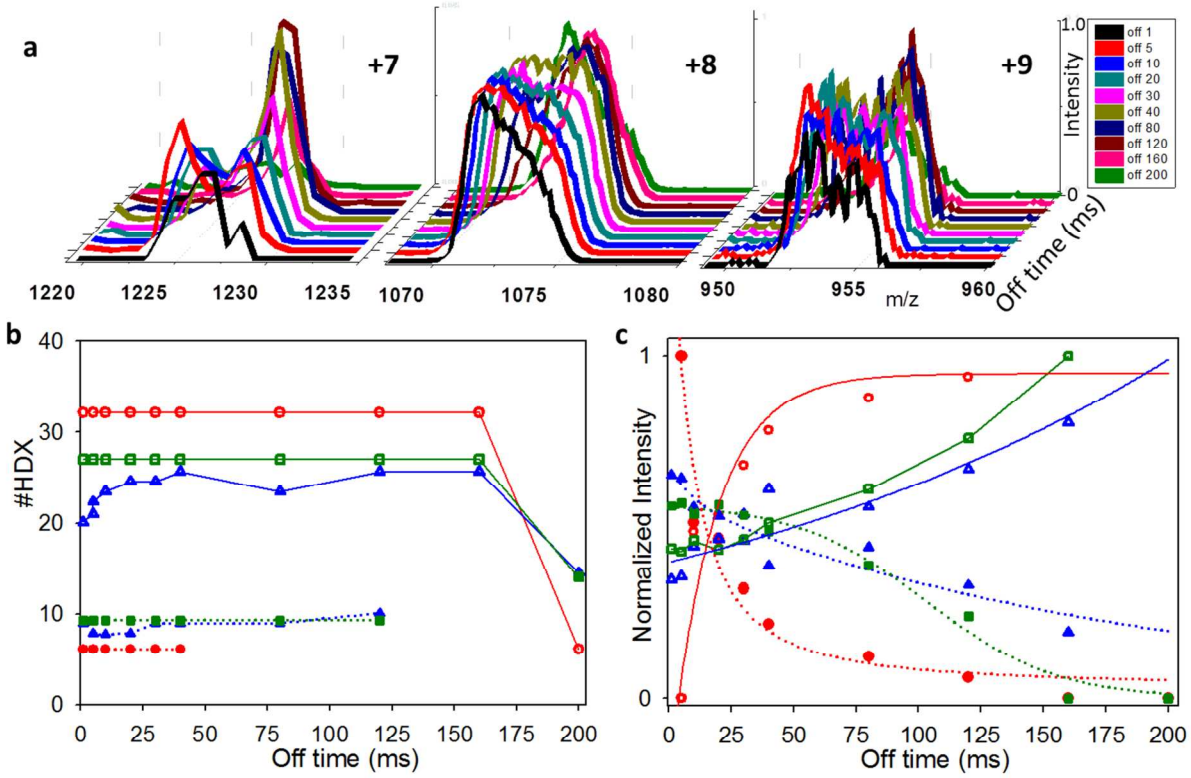


Figure 4.

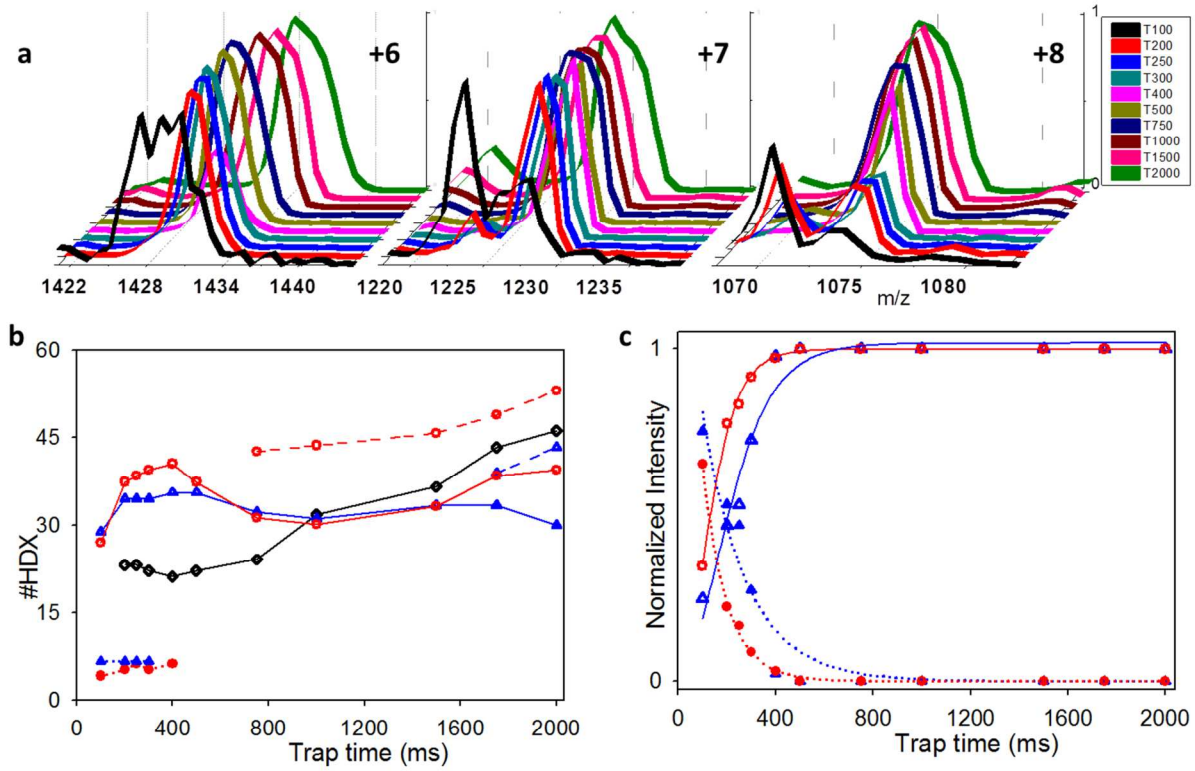
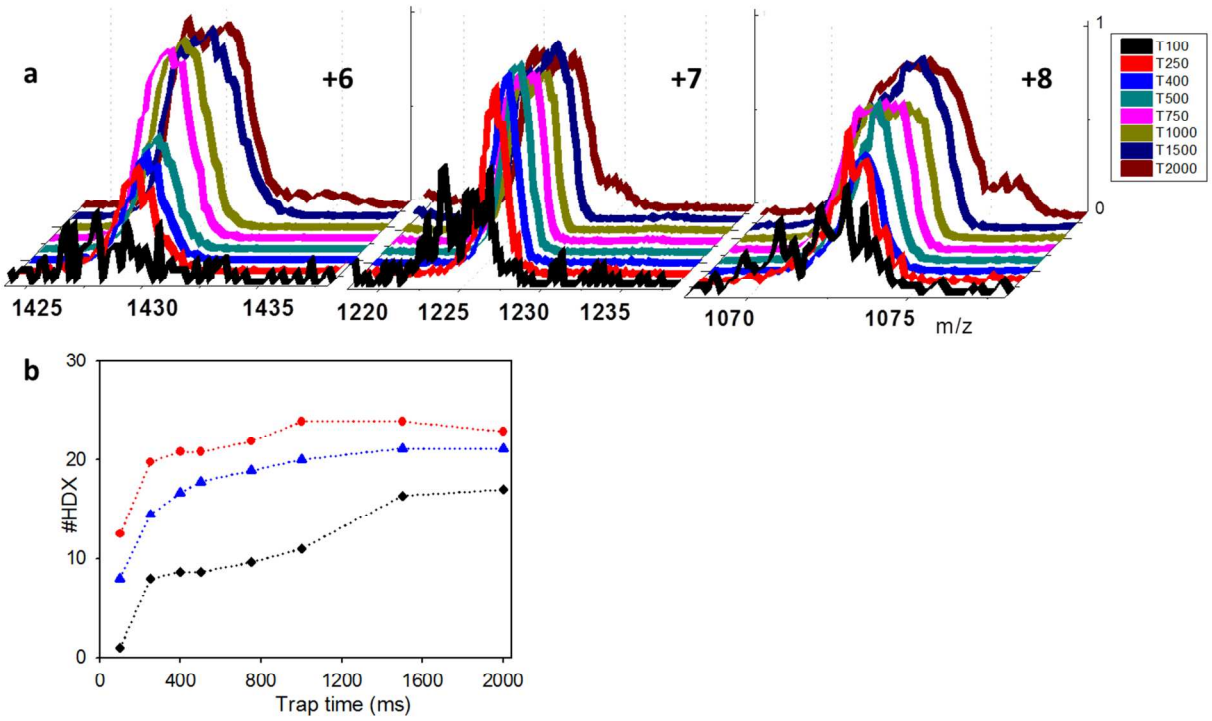


Figure 5.



Graphical abstract

Pulsed HDX MS method is sampling a population of ubiquitin ions with similar backbone fold as solution.