

Protein unfolding in freeze frames: intermediates of ubiquitin and lysozyme revealed by variable temperature ion mobility-mass spectrometry.

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Supporting Information

The gas phase is an idealized laboratory for the study of protein structure, from which it is possible to examine stable and transient forms of mass selected ions in the absence of bulk solvent. With ion mobility-mass spectrometry apparatus built to operate at both cryogenic and elevated temperatures, we have examined the conformational transitions of ubiquitin and lysozyme ions as a function of temperature. By collisionally activating the protein ions in the source, it is possible to subsequently freeze or thermally anneal the ensemble of unfolding intermediates and measure this. Data from ubiquitin suggests that the unfolding transitions proceed through diverse and highly elongated intermediate states, which then converge to more compact structures. These findings contrast with data obtained from lysozyme – a protein where (un)-folding plasticity is restricted by four disulfide linkages. We show with cold IM-MS experiments how ubiquitin can explore a wide conformational landscape due to its unrestricted nature, whereas for lysozyme the conformational restriction does not allow substantial rearrangement and facilitates enhanced resolution of the native fold as the buffer gas temperature is reduced.

Ubiquitin is a small, highly conserved protein with a native state noted for its stability under a range of solution conditions.^{1,2} Folds of ubiquitin have been comprehensively studied with a variety of experimental methods including NMR, crystallography and also computationally.^{3–6} In solution, ubiquitin exhibits two-state kinetics with a compact native N-state and a partially folded A-state, although, some studies have suggested the presence of additional on- or off-pathway intermediate states.^{7,8} Highly reversible, conformational transitions have been induced by altering solution conditions,^{9–11} pressure¹² and temperature.^{11,13} Time-resolved NMR⁷ has been used to probe transitions on a several seconds timescale, and time-resolved IR experiments have shown intermediate states occurring on a micro to millisecond timescale.⁸ The intrinsic characteristic of either spectroscopic approach is the spatially averaged nature of observables. Whilst time-resolved information is indispensable for kinetic analyses, inferring the intermediates' global geometrical structure solely from spectral data of a solvated ensemble is not straightforward. The gas-phase technique of ion mobility-mass spectrometry (IM-MS) allows measurements of a so-called collision cross section (CCS) on a millisecond timescale. In the crudest hard sphere approximation, the CCS parameter can be seen as a rotationally averaged shadow projection of a protein conformation.¹⁴ When correlated with the ion abundance, a CCS distribution yields intuitively interpretable information about the conformational preferences of a molecule. Using IM-MS, several groups have investigated conformational preferences of gaseous ubiquitin ions at ambient temperatures.^{15–19} Typically, in the absence of solution, ubiquitin ions generated from “native” solution conditions have been found to populate compact conformational states with a

^{DT}CCS_{He} of around 950 Å². This compact state has been assigned as the N-state also observed by NMR and crystallography.¹⁹ Structural changes of the compact state have been observed after prolonged residence times inside the vacuum, upon collisional activation and as a function of charge state.^{17–21} It has been observed that under such conditions, the protein adopts intermediate I-states (with ^{DT}CCS_{He} 1400–1700 Å²) which are also correlated with a solution-phase specific A-state.¹⁹ Extended structures with a ^{DT}CCS_{He} of up to 2000 Å² have also been reported and classified as gas-phase specific conformations or the unfolded U-state.^{16,17,19} Notably, Clemmer and Jarrold were the first to show that both increased temperature of the buffer gas and collisional activation can lead to elongation events,²² attributed to unfolding pathways.²³ Similarly, gas phase compaction following charge reduction has been demonstrated by Valentine *et al.*²⁴ and more recently by Laszlo *et al.*²⁵ These elongation/compaction phenomena appear substantially “step-wise”; conceptually resembling mechanically induced unfolding experiments performed by atomic force microscopy.²⁶ Breuker *et al.* investigated ubiquitin ions using ion heating followed by electron capture dissociation (ECD). The “three-state” unfolding model has been verified, however in contrast to the above studies they found an evidence for a “remarkable variety of unfolding intermediates”.²⁷

Solution and gas-phase behavior of lysozyme contrasts that of ubiquitin. It is approximately twice the size of ubiquitin with 129 amino acids and due to its four intramolecular disulfide bonds²⁸, it often exists in highly stable folds both in solution and in the gas-phase.^{29,30} In solution, the folding pathway of lysozyme is typically reported as a two-state transition between the native N-state and the denatured helical H-state without intermediate structures. However, kinetically trapped, partially folded transient intermediates may be possible.³¹ In the gas-phase, at ambient temperatures, the lysozyme 8+ ion presents with a ^{DT}CCS_{He} of ~1350 Å² (N-state) whilst the unfolded H-state has a ^{DT}CCS_{He} of ~1750 Å².³²

Here we employed a variable temperature ion mobility-mass spectrometer (VT IM-MS)³³ in an attempt to record ion mobility spectra of the “unfolding intermediates”, advocated by Breuker *et al.* For ubiquitin, our results indicate that the intermediates are initially highly elongated and then rapidly converge to compacted structures. We note that only the latter species are observed using ambient temperature IM experiments. The gas-phase folding pathway of non-reduced lysozyme is expectedly more restrained, resembling the two-state solution model. However, cryogenic IM-MS still captures some, partially folded intermediates between the N- and H-states.

Observation of these folding transitions and transient intermediates in well-studied proteins illustrates the potential of VT-IM-MS methodology in studying fast structural transitions, thus delineating their gas-phase un-folding pathways. The key advantage here is the ability to freeze the metastable transition states, assess their size and compare it to that of the “native” and “unfolded” forms.

Experimental

Samples. Ubiquitin (from bovine erythrocytes) and lysozyme (from chicken egg white) were purchased from Sigma Aldrich, UK. Ammonium acetate was purchased from Fisher Scientific, UK. Final protein concentrations were prepared to 50 μ M ubiquitin and 30 μ M lysozyme, both in 50 mM ammonium acetate, pH 6.8.

VT IM-MS. Our experimental arrangement and measurement principles have been described in detail elsewhere.³³ Briefly, the ions are created in a nano-ESI source (capillary 1-1.4 kV), then transferred *via* two ion guides into the variable temperature (VT) IM cell. The temperature of the ion source and the first two guides is held close to 300 K, whereas the temperature of the drift gas can be varied between 150 and 500 K. Ion activation is induced by increasing the voltage offset between the first two ion guides, both held close to ambient temperature. In-source activated ions then enter the VT compartment. Inside the VT chamber, the ions acquire the temperature of the helium gas while being accumulated for 16 and 22 ms for ubiquitin and lysozyme, respectively. Upon release as 20 μ s packets, the ions travel through the drift region (50.5 cm) where the mobility separation takes place. Compact structures drift faster than the extended forms of the same charge state. After IM separation, the m/z of ions are measured in a time-of-flight mass analyzer. In such IM-MS experiments, m/z data obtained for each ion has an associated arrive time distribution (ATD), which can be then converted to a collision cross section distribution.^{33,34}

Ubiquitin

We have obtained VT IM-MS data for the 5+ and +6 charge states of ubiquitin with and without in-source activation. Figure 1 shows the collision cross section distributions obtained for 6+ ubiquitin ions between 150 and 500 K. The $^{DT}CCS_{He}$ distribution for the 5+ and 7+ charge states and the relevant mass spectra are presented in Figures S1–S3. Ion activation, prior to transfer into the variable temperature cell, occurs between the first and the second ion guides by increasing the voltage offset from 0 to 70 V (non-activated (black) versus activated (dashed gray) traces in Figure 1). When the VT compartment is held at sub-ambient temperatures (lower 2 traces of Figure 1), the internal temperature of the collisionally activated ions is rapidly reduced; this leads to kinetic trapping of energetic conformational states. Data obtained at sub-ambient temperatures indicates that these forms can be, to a large extent, preserved on the measurement timescale. When the IM cell is held at ambient or elevated temperatures, collisionally activated ions may re-fold during accumulation and, to a lesser extent during separation in the drift region. The typical drift times are between 4 and 12 ms and can be altered by changing the applied electric field. No apparent change is seen in these $^{DT}CCS_{He}$ distributions as a function of drift time, which implies that structural rearrangements occur primarily during accumulation.

At ambient temperature (300 K), non-activated ions present as a compact population around 950 \AA^2 , consistent with the reported literature data.^{19,35} Upon reducing the temperature, the $^{DT}CCS_{He}$ of the compact population shifts to $\sim 1050 \text{ \AA}^2$ at 150 K.

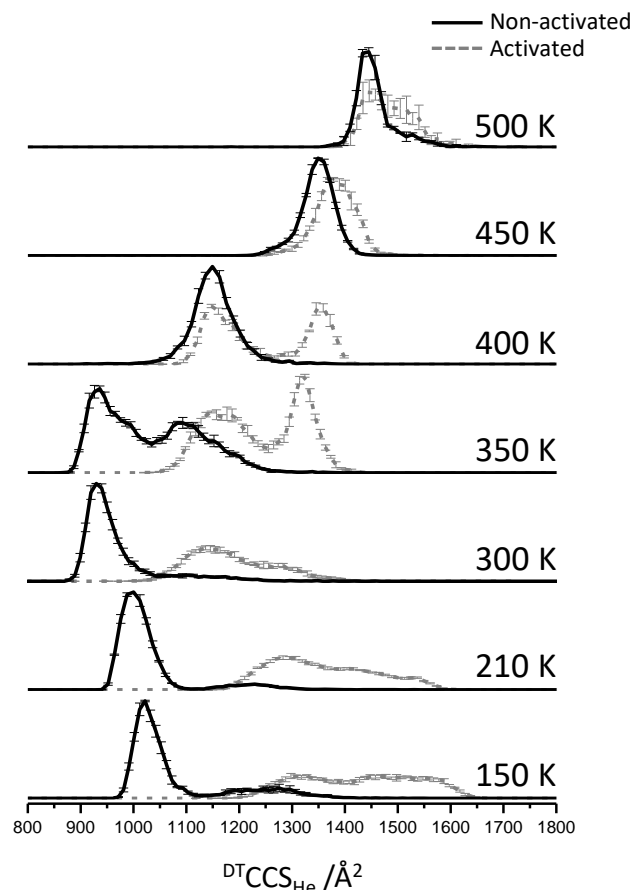


Figure 1. Collision Cross Section distributions of ubiquitin 6+ sprayed from 50 μ M solution in 50 mM ammonium acetate, pH 6.8. Solid lines represent data obtained with no in-source activation at a range of temperatures. Dashed lines represent the respective data recorded with the in-source activation on. Error bars correspond to standard deviation from three, 1 minute long acquisitions. Activated data has been normalized to the area of the non-activated data.

We attribute this increase to the expected dependence of ion-molecule interactions rather than a conformational transition.³⁶ At 350 K and above, several conformational transitions are observed for non-activated ions. At room temperature, the $^{DT}CCS_{He}$ profiles of the activated ions appear between 1100 and 1400 \AA^2 . However, at 150 K source activation produces significantly more extended forms with ($^{DT}CCS_{He}$ 1250 - 1650 \AA^2). Tuning the collisional activation conditions allows refolding intermediates to be captured in the low temperature drift tube (Figure S4). The most extended state achieved at 150 K (1650 \AA^2) aligns with the expected 150 K $^{DT}CCS_{He}$ of the most extended state at 500 K, suggesting this is a highly extended form; numerous other lower CCS intermediate states are also observed. Ion-molecule interactions may be different for elongated ions than for the compact forms, nevertheless the magnitude of the change and difference in the shape of the distribution cannot be explained solely by ion-buffer gas interaction potentials.³⁷

Lysozyme

The above findings are in contrast with the data obtained from lysozyme. Figure 2 shows the $^{DT}CCS_{He}$ distributions for the 8+ ions of lysozyme between 160 and 295 K; the accompanying mass spectra and non-activated and activated data for 7+ ions can be found in the Figures S5 and S6. For the +8 ions, the non-activated species

(solid black line) predominantly present as the compact N-state ($\sim 1360 \text{ \AA}^2$ at 295 K). As for ubiquitin, the $^{DT}CCS_{He}$ increases with decreasing buffer gas temperature (250 K, 1370 \AA^2 ; 210 K, 1400 \AA^2 ; 160 K, 1460 \AA^2) which again we attribute to the expected temperature dependence of the ion-molecule interaction.³⁸ Theoretical $^{DT}CCS_{He}$ for 160 - 295 K were calculated using the projected superposition approximation (PSA) method.³⁹ The change in theoretical $^{DT}CCS_{He}$ across the presented temperature range is predicted to be $\sim 6\%$, in good agreement with that measured experimentally ($\sim 7\%$). Following collisional activation, we see an increase in the population of the extended state ($\sim 1750 \text{ \AA}^2$ at 295 K) and the retention of some N-state population. Following in-source activation at temperatures 295 K – 250 K, we observe partial refolding back to the N-state (Figures 2 and S6). As we decrease the temperature of the drift cell further, we observe that population H shifts away from the un-activated N-state, the effect being most pronounced at 160 K. This indicates that as for ubiquitin, we are able to ‘freeze’ some of the transient intermediates within the timescale of the experiment. Similar behavior is found for the 7+ charge state (Figure S6), although the available activation energy is not sufficient to substantially populate the H-state.

Discussion

For ubiquitin, we conclude that at low temperatures we are able to kinetically trap metastable intermediate/transition states not experimentally observable during the timescales of ambient temperature measurements. This hypothesis is consistent with data obtained at elevated temperatures. At 350 K and above, the conformational profiles of the activated ions ubiquitin are significantly different to the low T profiles. Our data suggests that at elevated temperatures (above 150 K), extended intermediates ‘frozen out’ at 150 K (1300-1600 \AA^2) can refold to adopt temperature specific conformations (e.g. 1150 \AA^2 at 300 K). In particular, at 400 K we observe two distinct populations, of apparently equal stability, both of which may be due to different annealing pathways from the extended forms. At 450 and 500 K, more extended forms dominate the $^{DT}CCS_{He}$ distributions which are also narrower, suggestive of a reduction in the number of conformations present. We can consider whether conformational transitions observed here bear any resemblance to the reversible conformational transitions, exhibited by ubiquitin in solution.¹³ Changes in $^{DT}CCS_{He}$ are to a large extent “reversible” for conformations between 1100 and 1650 \AA^2 while this is not the case for the structures with $^{DT}CCS_{He}$ below 1000 \AA^2 . We do not observe any “compact intermediates” which suggests that once activated, they cannot find the initial state in the timescale available. As an additional check, we performed in-source activation at a range of collision energies (Figure S4 and S7); yet again no “compact intermediates” are observed. Therefore, the initial compact conformers of ubiquitin ($\sim 950 \text{ \AA}^2$) are likely contain solution-phase specific secondary structural which can be preserved in the gas phase with very gentle ESI conditions.¹⁹ Ubiquitin data shows that all transitions from such solution-specific folds appear “irreversible” on the timescale of our experiment. This is in accord with original idea of Smith and Light-Wahl: “Once the structure of the molecular ion has been lost, however, long-range coulombic forces should effectively preclude the reverse process.”⁴⁰

A contrasting behavior is observed for lysozyme where, even at 160 K, there is a significant overlap of activated and non-activated arrival time distributions. This suggests that the structural motifs in activated ion populations are to a large extent similar to those in non-activated ions. This correlates with the known, high stability of lysozyme both in solution and in the gas-phase.

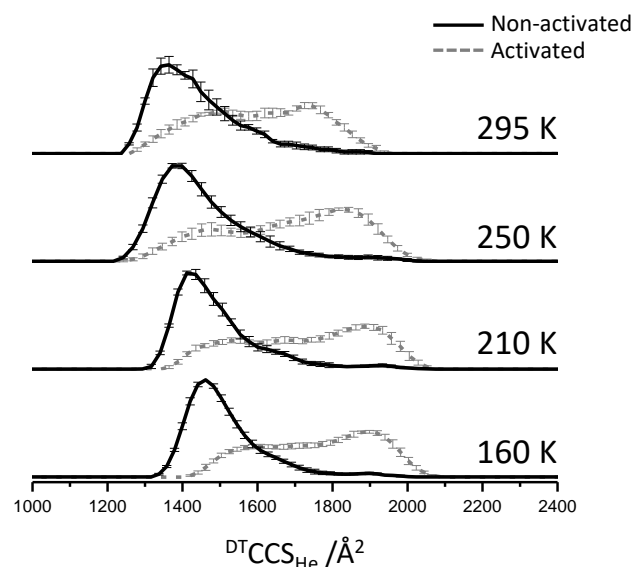


Figure 2. Collision cross section distributions of lysozyme 8+ sprayed from 30 μM solution in 50 mM ammonium acetate, pH 6.8. Solid lines represent data obtained with no in-source activation at a range of temperatures (160 to 295 K). Dashed lines represent the respective data recorded with in-source activation (voltage offset of 90 V). Error bars correspond to standard deviation from six, 30 second long acquisitions. Activated data has been normalized to the area of the non-activated data.

The results obtained for both 5+ charge state ubiquitin and 7+ charge state lysozyme (Figures S1 and S5, respectively) show effects considerably less pronounced than for the respective more highly charged species (Figures 1 and 2, respectively). The energy gained during the activation step is less for the lower charge states; moreover the drift time is longer and therefore the likelihood of rearrangement during IM separation is higher (quantitatively, the difference of either effect is approximately 17%). Nevertheless, the magnitude of difference between +5 and +6 charge states for ubiquitin and +7 and +8 charge states for lysozyme suggests that the presence of one additional proton (or a lack of thereof) plays a key role during gas-phase unfolding and re-folding of the protein. It has been shown previously that the coordination (self-solvation) plays an important role in this process.^{24,25} To rule out the possibility of charge stripping during activation, mass selected IM-MS of ubiquitin 6+ and 7+ and lysozyme 8+ and 9+ were recorded at increasing activation energies (see Figures S8A-B and S9A-B, respectively).

The above findings can be summarized with a toy picture of the gas-phase folding free energy landscapes compared to that in solution (Figure 3). Considering the data obtained for ubiquitin, a structurally flexible protein, we conclude that the transition from the initial “compact” ions (denoted N^{6+} , 950 \AA^2) is irreversible on our experimental timescales – thus either the energy of this state is higher in the gas phase (solid line) or that the barrier between this and the intermediates and extended states is elevated in the absence of solution (dotted line). Following activation at room temperature, unfolding proceeds through a variety of intermediates and transition states (denoted TS) which at room temperature converge to structures at 1100-1400 \AA^2 . At cryogenic temperatures (blue shaded area), we can slow down the interconversion process and perhaps kinetically trap those metastable species, which results in broad CCS profile (Figure 1, 210-150K). We hypothesize that protein undergoes an inside-out transition,⁴¹ which may involve both self-solvating of “native protonation sites” and accommodating for

the coulombic repulsions. In high temperature environment (500K), the system presents as a single, substantially unfolded population at $\sim 1450 \text{ \AA}^2$. Presence of a shoulder at $\sim 1530 \text{ \AA}^2$ following activation, suggests presence of an additional high energy state.

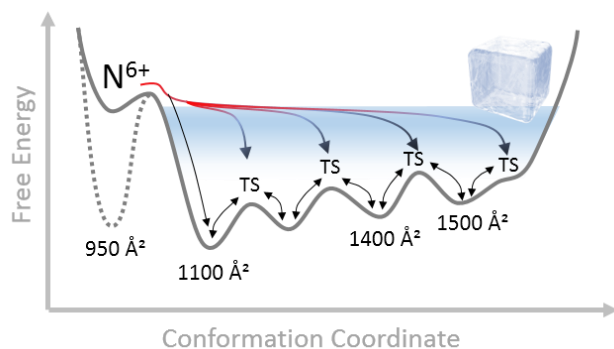


Figure 3. Hypothetical 1D gas-phase folding free energy surfaces of ubiquitin in the gas phase. The initial compact state obtained from solution via ESI process is denoted N^{6+} . Upon activation ubiquitin can no longer access the native state, and samples a plethora of unfolded states via multiple transition states (denoted TS). Most of these transitions appear to be reversible. Shaded blue area depicts the principle of performing the measurements at low temperature interconverting and transition states can be preserved ('frozen') on the time scale of experiment. Corresponding sketches contrasting solution and gas phase behavior of ubiquitin and lysozyme are presented in Figure S10.

For Lysozyme, the conformational restriction induced by the disulfide bonding, prevents the protein from full departure from the native like fold. The structural diversity of the transient folding intermediates is, again, much decreased compared to that in the unrestricted protein. This suggests that solution and gas phase folding free energy surfaces for this conformationally restricted protein are somewhat similar (Figure S10).

For both charge states of lysozyme (Figures 2 and S6) the width of the un-activated $^{DTCCSD}_{He}$ at 250 K is greater than at 210 K and the data at 250 and 160 K indicates more resolved conformers at $\sim 1650 \text{ \AA}^2$ as well as some N-state. The enhanced resolution is expected - it should scale with \sqrt{T} ; ⁴² the fact that we do not see this for ubiquitin is more evidence for the disruption of the solution N-state into many closely related conformers that are readily disrupted by activation. The increased width at 250 K for un-activated lysozyme 8+ has been reported by us previously ³² and may indicate a temperature specific transition at around 0 °C, which does not happen at lower temperatures.

Summary and Outlook

By manipulating solution and experimental conditions, it has been shown that gas-phase conformations of proteins can in turn be manipulated and transitions from compact to extended forms have been reported. Data and interpretation presented here attempts to expand the current understanding of gaseous, "native" conformations of proteins. Collectively, our results show that gas-phase unfolding can proceed through highly extended, intermediate states which then converge to "gas-phase compacted" structures. With our newly developed instrument and VT-IM-MS methodology, we can directly visualize the remarkable structural diversity of those states, first detected by ECD.²⁷ By affecting the temperature at which the refolding occurs, we can direct the folding process of a protein. This highlights the potential of this technique to map free

energy surfaces for proteins. In this work, we transpose our experimental findings to hypothetical free energy surfaces for the two contrasting cases.

Considering that a "compact" state of protein in the gas phase is itself a kinetically trapped form, for small and flexible proteins these measurements show that we still need to better understand the bias given to solvated ensembles by the electrospray and desolvation process and by the dominance of electrostatics in gas-phase structures. In solution, a thermally induced, reversible conformational transition for ubiquitin has been reported to occur between 330 and 370 K. Moreover, an irreversible transition was found to occur above 400 K.¹³ Although it is difficult to compare the mechanistic details of the thermally induced unfolding in solution with that which occurs in the absence of solvent, our high temperature data is consistent with these solution-phase findings; however, it remains to be seen whether this is a general effect.

Finally, we note that the experiment presented here is analogous to "simulated annealing", an approach employed frequently in computational simulations of protein structure and where ubiquitin is often used as a benchmark.³ In the future we intend extend the VT-IM-MS method to allow for measurement of timescales of the folding events presented here. We envisage that such time-resolved data could constitute a unique tool for assessing protein dynamics in the gas phase.

ASSOCIATED CONTENT

Supporting Information

$^{DTCCSD}_{He}$ profiles of 5+ and 7+ ubiquitin and 7+ lysozyme charge states and accompanying n-ESI mass spectra. Details of experimental setup and data processing. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interests.

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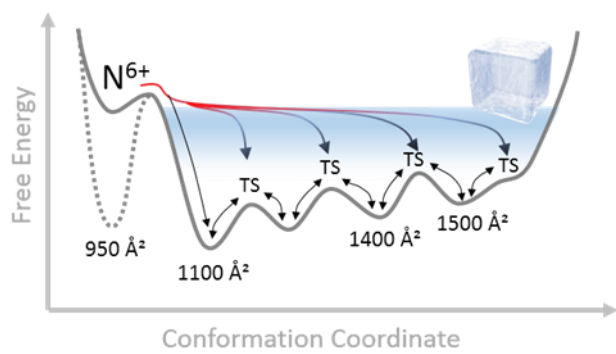
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