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Water-Mediated Dimerization of Ubiquitin Ions Captured by Cryogenic Ion Mobility-Mass Spectrometry

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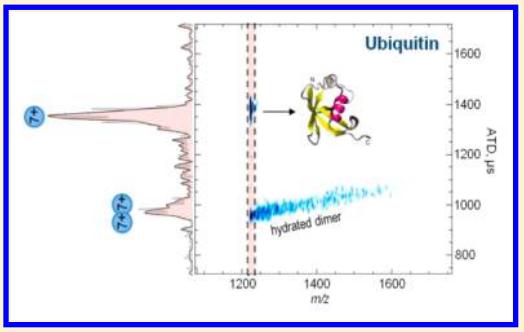
Supporting Information

ABSTRACT: The dynamics, structures, and functions of most biological molecules are strongly influenced by the nature of the peptide's or protein's interaction with water. Here, cryogenic ion mobility-mass spectrometry studies of ubiquitin have directly captured a water-mediated protein−protein binding event involving hydrated, noncovalently bound dimer ions in solution, and this interaction has potential relevance to one of the most important protein−protein interactions in nature. As solvent is removed, dimer ions, viz. $[2\ M + 14H]^{14+}$, can be stabilized by only a few attached water molecules prior to dissociation into individual monomeric ions. The hydrophobic patch of ubiquitin formed by the side chains of Leu-8, Ile-44, and Val-70 meet all the necessary conditions for a protein−protein binding "hot spot," including the requirement for occlusion of water to nearby hydrophilic sites, and it is suggested that this interaction is responsible for formation of the hydrated noncovalent ubiquitin dimer.



Water plays two important roles in determining protein structure, stability, dynamics, and function by (i) inducing the hydrophobic effect, which is reasonably well understood, and (ii) mediating hydrophilic intra- and intermolecular interactions that are important not only in structural determination but also in self-assembly and aggregation. Therefore, both long- and short-range interactions between water and biological molecules have been extensively studied both by experiment and theory.[1−6] Early studies of protein−water interactions treated water as simply an inert environment, but recently, both experimental and theoretical approaches have provided new understanding into the dynamic and active role water plays in many biological systems.[5,7] However, a detailed understanding of the effects of water on protein structures, stabilities, dynamics, and functions is largely limited to the effects of bulk solvent and specific cases where a limited number of localized water molecules strongly interact with the protein backbone and/or specific amino acid side chains.[8−12] The ability to experimentally probe the effects of solvation on peptide or protein structure will provide a better understanding of the role water plays in biological structure and functionality.

In a series of recent papers we have exploited the evaporative cooling inherent to electrospray ionization (ESI) and the low operating temperature of cryogenic ion mobility-mass spectrometry (cryo-IM-MS) as a means of directly determining changes in the conformational preferences of biomolecules as a function of the numbers of attached water molecules.[13−17] The cold drift cell (80 K) facilitates the preservation of hydrated gas-phase ions formed by ESI for structural analysis, providing a means to directly capture the evaporative dynamics of biological molecules as they transition from solution to the gas phase. For example, cryo-IM-MS studies of protonated water clusters revealed distinct changes in the size and shape of ions as the H-bond network of water molecules develops from small chainlike structures into 2D-net and stable clathrate cage structures.[13,15] Similarly, the structure(s) of alkyl diammonium cations were shown to depend on the numbers of hydrating waters, as ions undergo distinct unfolding transitions during desolvation at a critical cluster size, revealing the effects Coulombic repulsion can have on ion solvaltion.[17] For larger peptide ions, cryo-IM-MS affords the ability to relate gas-phase conformations to their solution-phase counterparts, and in certain cases, kinetically-trapped species stabilized by specific intramolecular interactions can be preserved for analysis.[14,16,18] Here, we use cryo-IM-MS to investigate the effects of hydration on ubiquitin ions formed under "native" electrospray (ESI) conditions. On the basis of prior work on lysine- and arginine-containing peptide ions, we expect cold ubiquitin ions to be hydrated by large numbers of water molecules. Previous gas-phase studies on the hydration of small peptides showed that the number of charged functional groups correlated well with the number of water adducts observed,[13,19] but as the size of the ion increases so do the number of hydrophilic sites present, increasing the capacity for hydration. The results presented herein are somewhat surprising in that we observe relatively few

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water molecules adducted to ubiquitin $[M + 7H]^{7+}$ monomer ions, but instead, we observe abundant solvated dimer ions of the type $[2\ M + 14H]^{14+}$. Additionally, dimer ions appear to undergo near-complete desolvation before dissociating into monomers, indicating the successful capture of a water-mediated binding event responsible for dictating the conformational preferences of ubiquitin ions.

Ubiquitin is a 76 amino acid regulatory protein that is conserved in all eukaryotic cells in all species. Its function varies and is determined by a process called ubiquitination, in which the protein covalently attaches to a target protein, tagging it for a specific cellular process dependent on both the protein and the binding site.[20,21] In aqueous solution, ubiquitin occupies low charge states in the native (N-state) conformation, which has been characterized by NMR and X-ray crystallography as a compact, folded structure made up of α-helix and β-sheet structural elements.[22,23] The N-state is stable over a large pH range, from 1.2 to 8.4;[24] however, in acidic solutions (pH ~ 2) containing high concentrations (~60%) of methanol, ubiquitin unfolds to form the A-state conformation, which has substantially more α-helical character and is believed to have a more elongated conformation.[25,26] Due to the known stability of the N-state conformer, ubiquitin has been the focus of many recent studies aimed at determining the extent to which native protein structure can be retained in the gas phase.[24,27−31]

Figure 1 shows two-dimensional (2-D) plots of arrival time distribution (ATD) versus m/z for ubiquitin ions collected at two different heated capillary temperatures. Spraying from native state conditions (pure water containing 0.1% formic acid) produces primarily $[M + 7H]^{7+}$ ions of ubiquitin (m/z 1224), as shown by the mass spectrum in Figure 1b, and conformations of these ions have been previously attributed to stable, compact structures.[24,27,29] However, the plots shown in Figure 1 reveal two distinct conformer populations for $[M + 7H]^{7+}$ ions of ubiquitin, one that originates from a trendline of hydrated ions and a second observed at significantly longer arrival time with no associated hydrated ions. Upon initial observation, one might propose that the two $[M + 7H]^{7+}$ conformer distributions correspond to a compact state formed by evaporation of hydrated ions and a fully elongated state formed by either heat-induced unfolding[24] or by a different mechanism of ESI.[32] However, production of an unfolded state of ubiquitin $[M + 7H]^{7+}$ ions under cold instrument conditions is unlikely considering the broad stability of the tightly folded N-state conformer that has been observed over a wide range of experimental conditions.[27] Even under denaturing conditions, $[M + 7H]^{7+}$ ions of ubiquitin have been shown to adopt mainly compact N-state structures that resist unfolding upon collisional activation of >100 V.[24] To rule out the possibility of an elongated conformer being formed by an alternative mechanism during the desolvation process, heating studies were performed and are discussed below. Collectively, the experimental data presented herein clearly show that ions falling along the hydrated trendline in Figure 1a correspond to a noncovalent dimer of the type $[2\ M + 14H]^{14+}(H_2O)_n$ (n = 1 to ~285). The fact that these ions retain a high degree of hydration, whereas relatively few numbers of water molecules are adducted onto the $[M + 7H]^{7+}$ monomer ions, suggests that the dimer is stabilized in some way by the presence of water. Data shown in Figure 1b, collected at an increased heated capillary temperature, provide additional evidence that $[2\ M + 14H]^{14+}$ dimer ions undergo near-complete desolvation upon heating before dissociating to form monomer ions. Increasing

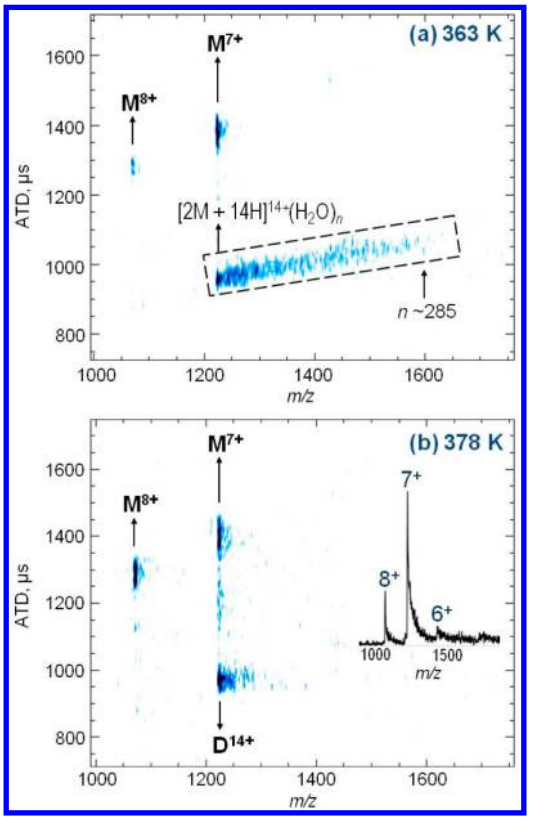

Figure 1. Two-dimensional contour plots of ATD versus m/z for ubiquitin ions collected at a heated capillary ion inlet temperature of (a) 363 K and (b) 378 K. All spectra were collected on a home-built cryogenic ion mobility-mass spectrometer that has been described in detail previously.[11] Solutions of ubiquitin were prepared at a concentration of 10 μM in pure water containing 0.1% formic acid. A representative mass spectrum is shown as an inset in panel b reflecting the typical charge state distribution produced from native state ESI conditions. Assignments of monomer and dimer ions are labeled in the spectra. Under cool inlet conditions, a trendline of hydrated ions is detected tracing to the $[2\ M + 14H]^{14+}$ dimer.

the heated capillary temperature from 363 to 378 K promotes evaporation of the hydrating waters tracing to the bare $[2\ M + 14H]^{14+}$ dimer ion. While the dimer appears to retain stability with relatively few water molecules attached, the streaking, i.e., low-abundance signals, observed in the 2-D plot (Figure 1b) between the bare dimer and monomer populations suggests that upon heating, dimer ions dissociate within the IM drift cell to form $[M + 7H]^{7+}$ monomer ions.

Further evidence supporting the conformer assignment of hydrated, noncovalently bound dimer ions is derived from data taken at different solution concentrations of ubiquitin. Assuming the population falling at shorter arrival time in the mass-selected ATDs (Figure 2A) corresponds to the $[2\ M + 14H]^{14+}$ dimer, increasing the sample concentration should result in an observed increase in the abundance of dimer ions relative to the monomer ion population. Figure 2A shows that increasing the concentration of ubiquitin from 10 to 25 and 50 μM resulted in an increase in the relative abundance of the dehydrated dimer population from 11% to 44% and 63%, respectively. The most plausible explanation for this dramatic increase is the attribution of the population to dimers, which are known to increase in solution with increasing concentration. Note that these plots do not represent the total ion abundances of the dimer population because the extracted ATDs show

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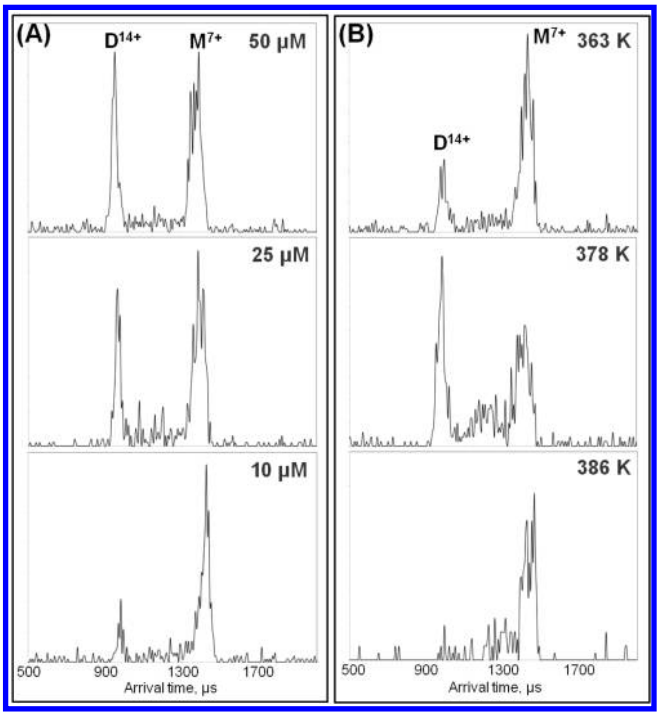

Figure 2. (A) Mass-selected arrival time distributions (ATD) of the +7 charge state of ubiquitin (m/z 1224) obtained by electrospraying 50 μM, 25 μM, and 10 μM solutions from pure water with 0.1% formic acid. All spectra were collected at a heated capillary temperature of 356 K. (B) Mass-selected ATDs of the +7 charge state of ubiquitin collected at heated capillary temperatures ranging from 363 to 386 K. Identical solution conditions were maintained for all spectra shown (10 μM from pure water with 0.1% formic acid).

signal for dehydrated ions only and do not account for hydrated ions present in the spectrum. Additional data for 75 μM samples acquired at even higher capillary temperature (366 K) provide further support for the peak assignment (see Figure S1). Finally, we note the shape of the longer ATD peak for the +7 charge state is consistent with what is expected for monomer ions. The peak for the higher mobility ions, observed at ~1000 μs, is much narrower than expected for the $[M + 7H]^{7+}$ monomer ions, even under cold conditions; the sharpening of the peak is consistent with an increase in the effective drift force associated with the more highly charged $[2 M + 14H]^{14+}$ species.

Heating studies were also performed by varying the temperature of the heated capillary ion inlet, as shown in Figure 2B, in order to elucidate the role water plays in stabilizing the interactions at the binding interface. Upon heating, the dimer first loses virtually all of its hydrating water, as evidenced by the truncation of the hydration trendline in Figure 1b, before dissociating to form monomer ions, as evidenced by the streaking observed between the dehydrated dimer and monomer populations (across the mobility axis). It appears from the mass-selected ATDs shown in Figure 2B that the relative abundance of the dimer population increases initially upon heating before dissociating completely to form monomer ions. This can be explained by the desolvation pathway undergone by ubiquitin ions during ESI. After minimal heating, hydrated dimer ions undergo stepwise desolvation to lose the weakly bound H-bond network of water molecules solvating and stabilizing the ion. Because it is likely that less energy will be required to drive off these hydrating waters than the amount required to induce dissociation, upon initial heating, the dehydrated state of the dimer will appear to increase in abundance relative to the monomer, as observed in the mass-selected ATDs shown in Figure 2B. Upon additional heating, the dehydrated dimer ions begin to dissociate in the drift cell forming monomers, as indicated by the streaking observed between the two conformer distributions as the ion inlet temperature is increased. Eventually, the $[2 M + 14H]^{14+}$ dimer population disappears completely leaving a single conformer distribution for $[M + 7H]^{7+}$ ions that we attribute to the stable, compact N-state monomer. Increasing the effective ion temperature of the dimer to induce dissociation during the IM measurement can also be achieved by operating at high field strengths in the drift tube (Figure S2). Additional evidence supporting the peak assignments of dimer and monomer ions is included in the Supporting Information (Figure S3).

Collectively, the cryo-IM-MS results indicate that the presence of water mediates a protein−protein binding event resulting in formation of hydrated dimer ions that remain stable until near-complete desolvation. Because the dimer ions retain stability throughout the desolvation process, this suggests that while water may drive association, it is not essential to the preservation of the noncovalently bound species. Therefore, additional interactions must serve to stabilize the binding interface in the absence of water. Evidence for such interactions was recently reported by Liu et al. in which NMR data showed that free ubiquitin dimerizes noncovalently in solution at high concentrations and upon doing so forms a hydrophobic interface between portions of the β-sheet regions of two ubiquitin molecules.[33] While such an interface may be weakly interacting in the absence of water, buried hydrophobic surfaces have been shown to contribute significantly to the stability of protein−protein interfaces. Brunori and co-workers estimated the free-energy gain associated with burying hydrophobic residues on the surface of a protein through protein−protein binding to be ~15 cal/mol per square angstrom of surface area buried.[34] Simulated annealing calculations performed by Liu et al. suggest that noncovalent dimerization of ubiquitin buries an area of ~600 Å on average.[33] Therefore, formation of noncovalent dimers of ubiquitin may be favored in solution, where the hydrophobic effect drives the binding of two interfaces, and then the noncovalently bound species is able to survive transfer to the gas phase under gentle instrument conditions.

Further evidence supporting the formation of a hydrophobic interface between ubiquitin ions comes from recent studies revealing the hydrophobic region of the native-state structure of ubiquitin to be the most common interaction site in ubiquitination events.[21] While it is reasonable to presume that the presence of water would facilitate the binding of these regions, when water mediates this interaction during desolvation, it is likely that "hot spots" form within the interface.[2] Such regions have been shown to contribute significantly to the stability of the bound species, in part by occluding water from the hydrophobic interface. In the case of ubiquitin, the side chains of Leu-8, Ile-44, and Val-70 have been shown to form a hydrophobic patch in the β-sheet region of the protein that participates strongly in protein-binding events.[10,33,35] Wand and co-workers have shown that the water molecules solvating these hydrophobic sites exhibit restricted hydration dynamics, similar to what is expected for protein binding sites.[10] Additionally, reports by Liu et al. on noncovalent dimerization of ubiquitin in

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solution pointed to residues surrounding Leu-8, Ile-44, and Val-70 as the primary sites of binding within the hydrophobic interface.[33] Therefore, we propose that the hydrated ubiquitin dimer observed here results from the water-mediated binding of two hydrophobic interfaces encompassing the β-sheet region of the protein, specifically the hot spots formed from the side chains of Leu-8, Ile-44, and Val-70.

Ubiquitin contains 12 basic residues, most of which are likely protonated in acidic solutions and located near the surface of the native state structure, isolated from the hydrophobic region of the molecule (see Figure 3).[11,33] Interestingly, a number of

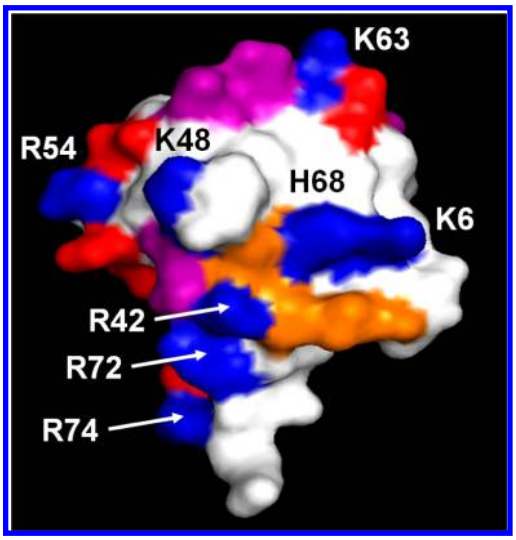

Figure 3. Structure of the N-state conformer of ubiquitin illustrating the hydrophobic region (orange) and surrounding basic residues (blue). The acidic residues (red) and glutamines (purple) are also shown. Expected sites of hydration include the protonated side chains of the exposed basic residues. The location of K6, K11 (not visible), R42, K48, H68, R72, and R74 around the hydrophobic binding region suggests that these hydrophilic sites may serve as initiators of dimer formation.

these hydrophilic sites are located in close proximity to the β-sheet region of the molecule, surrounding the hydrophobic core. These hydrophilic sites are expected to be solvated and may serve as initiators of dimer formation. Recent studies of diammonium cations have shown experimental evidence for the structural implications of forming stable water bridges between ammonium ions where the stability provided by solvation offsets the effects of Coulombic repulsion and induced folding of the ions within a water droplet.[17,36] In addition to the energy gain associated with the buried hydrophobic surface area, water bridging between protonated lysine and/or arginine side chains of ubiquitin ions could be aiding in the stabilization of noncovalently bound dimers. Because it is likely that water would be excluded from a hydrophobic dimer interface, the most favorable hydration sites on the protein surface are assumed to be the exposed charged groups.[19] The location of many of these charged groups, at the periphery of the proposed binding interface, suggests that water molecules could potentially form a ringlike orientation around the hydrophobic interface. Similar hydration behavior has been observed for protein–protein interfaces,[9] and the stabilization of these sites through the formation of "water bridges" between hydrophilic sites would assist in the facilitation of the water-mediated binding event responsible for formation of the ubiquitin dimer.

Probing the effects of solvation on the conformer preferences of ubiquitin under native state conditions identified a water-mediated protein–protein binding event leading to the formation of noncovalently bound dimers in solution. Interestingly, the absence of extensively hydrated N-state $[M + 7H]^{7+}$ ions suggests that monomeric ubiquitin ions are formed by dehydration of the dimer and subsequent dissociation. This observation is not totally unexpected considering the recent report by Liu et al. on the formation of noncovalently bound dimers of ubiquitin in solution,[33] which, under gentle instrument conditions afforded by cryo-IM-MS, can be preserved upon transfer to the gas phase. Water plays a key role in determining structural preferences of proteins as well as structure–function relationships, and there is growing evidence indicating that the formation of protein–protein hot spots amidst hydrophobic binding regions is largely dependent on the initial presence and ultimate occlusion of water. Although insights about protein hydration and water-mediated processes have been gleaned from molecular dynamics simulations, there are few experimental tools for understanding the effects of water on protein structure and protein–protein interactions. The cryo-IM-MS approach adds a new dimension to studies of hydrated protein ions and may ultimately shed light on many water-mediated processes that dictate the structure and function of proteins.

EXPERIMENTAL SECTION

The home-built cryo-IM-MS instrumentation has been described in detail previously.[13−15] Briefly, hydrated ions are generated via electrospray ionization and guided into the variable temperature ion mobility drift tube, which is cooled to 80 K via liquid nitrogen cooling. Once inside the drift tube, ions are separated on the basis of size and shape and detected using an orthogonal time-of-flight mass spectrometer. The extent of hydration observed for ions is controlled by the temperature of the heated capillary ion inlet. Ubiquitin (bovine erythrocytes, >90% purity) was purchased from Sigma-Aldrich, diluted in deionized water (18 MΩ), and stored at −20 °C before specific solutions were prepared. All solutions were prepared at 10 μM in pure water containing 0.1% formic acid, unless otherwise noted.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpclett.5b02382.

Additional evidence supporting the peak assignments for monomer and dimer ions of ubiquitin (PDF)

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Notes

The authors declare no competing financial interest.

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