Ion Spectroscopy Reveals Structural Difference for Proteins Microhydrated by Retention and by Condensation of Water

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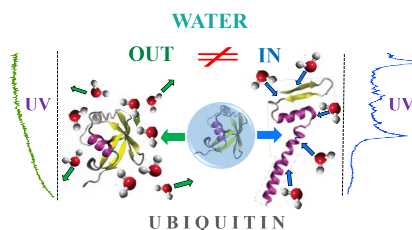
ABSTRACT

Protein ubiquitin in its +7 charge state microhydrated by 5 and 10 water molecules have been interrogated in the gas phase by cold ion UV/IR spectroscopy. The complexes were formed either by condensing water onto the unfolded bare proteins in a temperature-controlled ion trap or by incomplete dehydration of the folded proteins. In the case of cryogenic condensation, the UV spectra of the complexes exhibit a resolved vibrational structure, which looks similar to the spectrum of the bare unfolded ubiquitin. The spectra become, however, broadband with no structure, when complexes of the same size are produced by the incomplete dehydration under soft conditions of electrospray ionization. We attribute this spectroscopic dissimilarity to structural difference of the protein: condensing a few water molecules cannot refold the gas-phase structure of the bare ubiquitin, while the retained water preserves its solution-like folded motive through evaporative cooling. This assessment is firmly confirmed by IR spectroscopy, which reveals the presence of free NH and carboxylic OH stretch vibrations only in the complexes with condensed water.

TOC Graphic



Introduction

The 3D structures, properties, and functionality of many biomolecules *in vivo* are governed by a subtle balance between their noncovalent intramolecular interactions and the intermolecular couplings to the surrounding network of water molecules. The dynamical nature and, eventually, infinite size of this network in a bulk solution make a detailed study of the role of water in shaping large biomolecules difficult. To a certain extent, the first hydration shell of a biomolecule, where water molecules occupy all (most of) its hydration sites can be considered as the most important counterpart that retains the molecule in its (nearly) native conformational states. Protecting native structures of large microhydrated biomolecules is currently under extensive study and debate, for instance, in the field of cryogenic electron microscopy (cryo-EM), where soft landing of mass-selected ions is considered as a promising alternative for sample preparation.[1-3] In the gas phase, IR/UV cold ion action spectroscopy has been widely applied to smaller microhydrated biomolecules for solving a variety of problems.[4-11] In particular, vibrationally resolved IR spectra have been used for validating structures of isolated noncovalent complexes, calculated by methods of quantum chemistry. Moreover, tracking the structural changes that occur upon increasing the level of hydration may reveal, whether the geometry of the embedded biomolecule has been, indeed, already stabilized or a higher level of hydration is to be treated to reveal its native-like structure.[12-13]

One important aspect of such studies is how to generate microhydrated complexes of biomolecules. Protonated or deprotonated biomolecules are routinely produced in the gas phase directly from solution by electrospray ionization (ESI). Collisional heating on the way from atmospheric to high vacuum side of an ESI source gradually evaporates solvent molecules to generate the bare ions. By adjusting the level of this heating one can retain a desired number of

water molecules on the ions. Retaining water is, however, technically not simple, since it requires achieving and maintaining a subtle balance between the full dehydration and the formation of macro droplets.

Alternatively, one can generate fully dehydrated ions and then collect and cool them in a cryogenic ion trap, into which water vapor is injected.[8, 10, 14] Water molecules may condense onto the cold ions, thus producing a distribution of microhydrated complexes. Due to its robustness, this approach to generation of microhydrated ions is becoming widespread in structural studies of biomolecules. In this regard, the emerging question is whether the two methods of microhydration, by retention and by condensation of water molecules, produce identical structures of the embedded biomolecules and how close are these structures to the native ones. Spectroscopy may shed light on this. Indeed, if UV and IR spectra measured for the differently produced complexes are identical, this would imply identical local (around a UV chromophore) and global structures of the embedded biomolecule, respectively. Unidentical spectra of the differently generated complexes would indicate that either the biomolecule remains kinetically trapped in its solution-like geometry, or it adopts a gas-phase structure and remains kinetically trapped in this state even after rehydration. The latter implies that the structure of the embedded biomolecule is not a native-like one and has no/little relevance to biological processes in solution.

Despite the importance for the field, there are only a few studies that allow comparison of the structures for hydrated biomolecules generated by the two methods. For instance, the structure of the smallest amino acid glycine hydrated by single water, likely, remains the same for both methods of hydration.[15-16] Our recent study provides direct evidence of this for protonated amino acid tryptophane hydrated by 1 to 6 water molecules.[12] To our knowledge, there are no reported cases, where the two structures (spectra) of such differently microhydrated biomolecules would

appear to be unidentical, however. While adiabatic relaxation to the same common conformational states might be an often case for small hydrated biomolecules, one may expect kinetic trapping to be predominant for large species or to the species with high energy barriers of inter-conformational transitions. Here we explore the approach of the "dual" hydration and push it to small protonated protein ubiquitin in the +7 charge state. The IR and UV spectra of this ion hydrated by retaining and by condensing 5 and 10 water molecules have been measured, compared and linked to the global conformational states of ubiquitin. It is of crucial importance for comparing the spectra that the differently hydrated biomolecules have been interrogated with the same spectroscopic method and on the same instrument under identical conditions.

Ubiquitin is a well-studied benchmark protein. Its gas-phase structure was extensively studied by H/D exchange,[17-20] proton transfer reactions[21-23] and ion mobility mass spectrometry methods.[24-29] Recently we showed the capability of cold ion UV and IR spectroscopy to track structural changes in ubiquitin.[11] To sum up, depending on the charge state, solution composition, and collisional heating, the global gas-phase structure of ubiquitin varies from folded to partially and fully unfolded states. Compare with other charged states, ubiquitin in +7 one is a particularly convenient object for this study, since, depending on these conditions, it can be readily found either in the folded or partially unfolded states. This structural instability reflects the subtle balance between the Coulomb repulsion of the seven charges and the intramolecular non-covalent interactions that keep the protein folded. Whether this balance can be shifted toward refolding by condensing a few water molecules onto the bare "chilly" protein is the main question that we address herein.

Experimental methods

Ubiquitin from bovine erythrocytes (≥98% purity) was purchased and used without further purification. Water solution with 50 μM ubiquitin concentration with the addition of 1% of acetic acid was used for electrospray ionization. Water and acetic acid are of LC-MS grade. Bare and microhydrated ions with retained water molecules are generated by nano-electrospray ionization (nano-ESI) and transferred through a metal capillary (0.5 mm I.D., 10 cm long) and three consecutive inline molecular skimmers. The diameter of the first, second, and third skimmer is 1, 2, and 3 mm respectively. The differential pumping sections are organized between each molecular skimmer. The distribution of microhydrated ions is mostly controlled by capillary temperature and voltage. After the third skimmer, the ions pass an octupole ion guide and enter a linear octupole ion trap (pre-trap). This pre-trap operates at a constant pressure controlled by two independent gas dosing valves. One valve is to supply pure nitrogen; another valve is to supply pure water vapor. The pre-trap is cooled by a pulse tube cryocooler (TC4189, Lihan) and can operate in the range of 60-320K. The cold head of the cryocooler and pre-trap are thermally connected by ~15 cm long braided copper cable in our setup. The ions are accumulated in the temperature-controlled pre-trap. To condensate the water molecules on the bare ions a small amount of water vapor was seeded into the pre-trap. After accumulation, thermalization, and condensation the ions are released, mass-selected by a quadrupole mass filter, and guided into a cold octupole ion trap, which is kept at 6 K. Once the ions are trapped and cooled down to ~10 K in collisions with He buffer gas, they are interrogated by UV or IR laser pulses. UV photofragmentation spectroscopy is performed using the 2±0.3 mJ output of a frequency-doubled dye laser pumped by 7 ns pulses of a Nd:YAG laser at 355 nm. Absorption of UV photon produces the fragments with 45-100 Da loss by the bare protein and the loss of water molecules for microhydrated complexes. IR light (15±3 mJ/pulse, 1 cm^{-1} spectral linewidth), is produced by an optical parametric oscillator (OPO, Laser Vision),

which is pumped by 8 ns pulses of a Nd:YAG laser. The absorption of IR photons by microhydrated complexes results in the loss of water molecules. The remaining parent and fragment ions are released from the cold trap and detected by a quadrupole mass spectrometer. We average 10 measurements at each UV and IR wavelength at a repetition rate of 10 Hz. Each spectrum was recorded at least 3 times to ensure its reproducibility. A wavelength meter measures the wavenumber of the dye laser before a frequency doubling and the Nd:YAG laser and of the signal wave of the IR OPO, pumped by this laser, thus providing the wavenumber of the generated by the difference frequency mixing IR light with ±0.2 cm^{-1} accuracy.

Results and discussion

Figure 1 shows the UV photofragmentation spectra of the +7 charge state of cold ubiquitin and its microhydrated complexes with 5 and 10 water molecules in the spectral range of electronic band origin of Tyr aromatic residue. The conditions in the ESI source were tuned to optimize the signal of the studied ions. For generating the bare Ubi^{+7} and its rehydrated complexes the conditions in the ESI source were the same mildly “harsh”, tuned to optimize the signal of the studied ions. Under these conditions no water molecules have been left on the ions prior entering to the cryogenic pretrap. This ensures that the protein unfolds and the water in the interrogated complexes is, indeed, condensed in the pretrap, but not retained after ESI. To produce the protein with retained water molecules, the conditions of ESI source had to be tuned to be “soft” for minimizing the collisional heating before the pretrap. The spectrum of Ubi^{+7} (Figure 1a) is vibrationally resolved with the first absorption band centered at 35470 cm^{-1}. The onset of this band is very close to the band origin in neutral Tyr.[30-31] As we suggested earlier,[11] this observation implies that in the gas phase the side chain of the Tyr-59 residue of the protein does not experience any noncovalent interactions and the protein resides in its unfolded or partially unfolded states. In

contrast, the spectra of the $(Ubi–n\cdot H_2O)^{+7}$ (n = 5, 10) complexes with retained water molecules (Figures 1b,c; green traces) appear smooth and do not contain any vibrationally resolved transitions. Our previous study[11] demonstrated that these spectra resemble the spectrum of the fully dehydrated Ubi^{+7} produced under gentle conditions of ESI that, in main, preserve unfolding of the protein. The same study[11] also suggests that the microhydrated protein remains in a folded, native-like state. The large spectral broadening may arise from variation of the length of the hydrogen bond between the hydroxyl of Tyr-59 and the amide group of Glu-51.[11, 32] An alternative explanation involves interaction of the proton of Arg-74, which resides in the intrinsically unstructured in the gas phase C-terminus of the protein.[33]

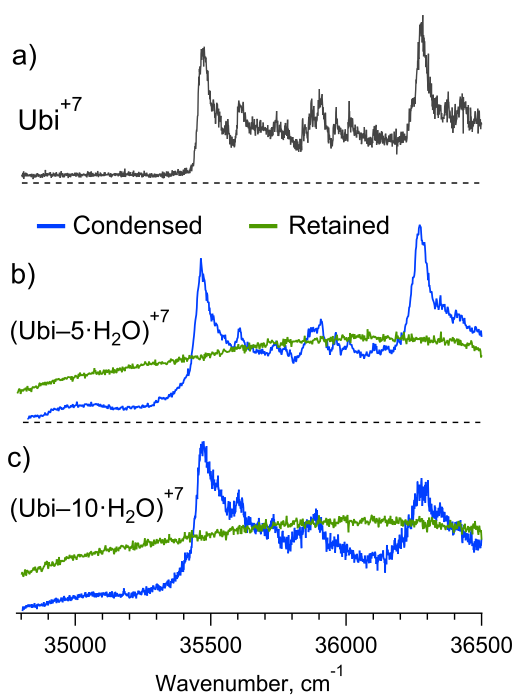


Figure 1. UV photofragmentation spectra of (a) bare Ubi^{+7} ion and of its microhydrated complexes with (b) five and (c) ten water molecules produced by condensation (blue traces) and by retention (green traces) of water molecules. In all the cases the ions were prestored for 40 ms in the ion pretrap maintained at T= 200 K.

The spectra of the same $(Ubi–n\cdot H_2O)^{+7}$ (n= 5, 10) complexes change drastically however, when they are produced by condensation of water molecules onto the bare protein. Regardless of the number of these molecules, the spectra (Figures 1b,c; blue traces) appear vibrationally resolved and, apart from a slight broadening of the vibrational bands, look very similar to the spectrum of the bare Ubi^{+7} (Figure 1a). This observation clearly indicates that the 3D structures of the same but differently produced microhydrated complexes must be different, at least, in the vicinity of the Tyr chromophore. We may suggest that the unfolded in the ESI process bare protein cannot anymore refold back even with the assistance of the rehydration, at least on the 50-ms scale of our experiment. The retained and condensed water molecules may occupy different hydration sites among many of those available in the protein.[32] Most likely, the few water molecules will, first, bind to the charges, which themselves can occupy different available protonation sites of the protein in different folding states.[34] The proposed inhomogeneous condensation is consistent with the observed (Figure 1; black and blue traces) increase of inhomogeneous spectral broadening upon the increase of the number of the condensed water molecules from n = 0 to 5 and further to n = 10.

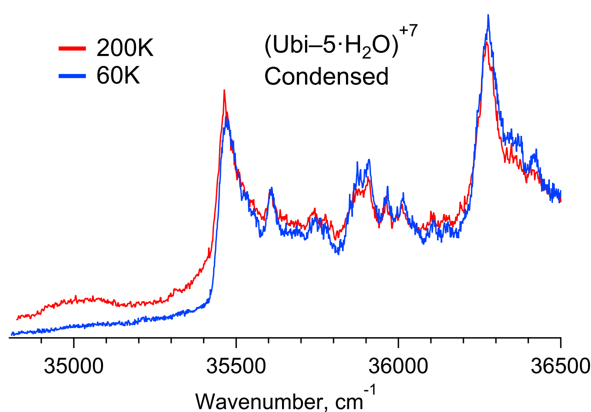


Figure 2. UV photofragmentation spectra of $(Ubi–5\cdot H_2O)^{+7}$ complexes produced by condensation of water during 40 ms in the cryogenic ion pretrap cooled to 200 K and 60 K.

The spectra of the bare protein and its re-hydrated by condensation complexes with five water molecules locally differ around 35100 cm^{-1} (Figures 1a,b), where a low-intensity broad peak appears in the spectra of the complexes. The band becomes more pronounced and less resolved for the complexes with n = 10 (Figure 1c). It significantly reduces however upon cooling of the $(Ubi–5{\cdot}H_2O)^{+7}$ complex in the pretrap from 200 K to 60 K (Figure 2). The position, width and intensity of this band are very similar to that of the redshifted band earlier observed in the spectrum of bare ubiquitin in the +10 charged state.[11] The band has been attributed to the conformers of Ubi^{+10} with one of the protons residing close (through space) to Tyr chromophore. Regarding these three observations, we may speculate that the condensed water assists in proton migration across the protein, such that in some of the conformers a charge appears close to the chromophore. The increased level of hydration facilitates the charge transfer through the network of H-bonds, while lowering the temperature slows the migration of water molecules and protons. It is worth noting that, the same lowering of temperature does not change the UV spectra measured for both the bare protein and the complexes with five retained water molecules (Figure 3). The implication of the latter observation is that, regardless of the used pre-storage temperature, the few retained water molecules are able to protect the folded, native-like, structure of the protein.

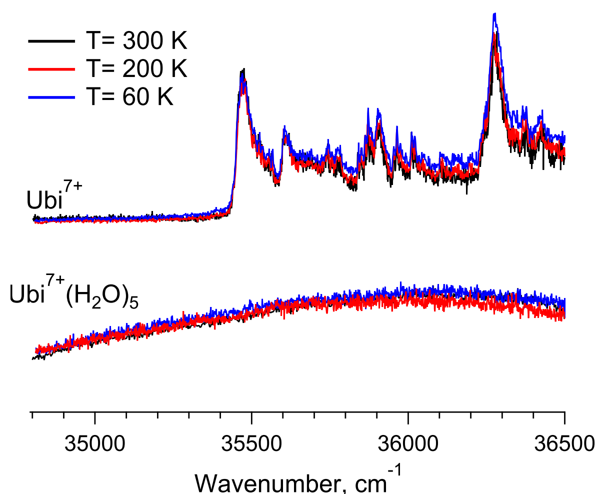


Figure 3. UV photofragmentation spectra of Ubi^{7+} and its complexes with 5 retained water molecules measured after thermalization in the pretrap at 300 K, 200 K, and 60 K.

While UV spectra are sensitive only to the local 3D structure of the protein around the chromophores, IR spectra reflect its global geometry. Figure 4 shows the IRMPD spectra of the same $(Ubi–5\cdot H_2O)^{+7}$ complexes produced by retaining and by condensing water molecules. A striking difference between the two identically measured spectra is observed in the region of 3420-3580 cm^{-1}. Despite the "spectroscopically" very large size of the protein, the spectrum of the complexes with condense water exhibits a number of sharp resolved peaks in this region. These peaks can be readily assigned to the free stretch vibrations of the NH and carboxylic OH groups that are not involved in any noncovalent interactions, which would redshift and broaden the transitions.[35] This observation provides a direct evidence of that, that the protein is, at least, partially unfolded.

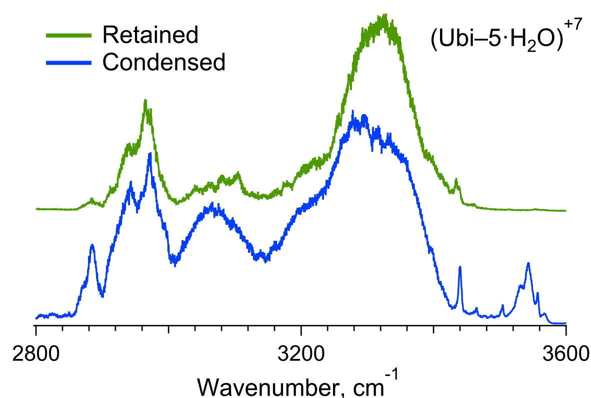


Figure 4. IRMPD spectra of $(Ubi–5\cdot(H_2O)^{+7}$ complexes with retained and condensed water molecules.

In contrast, no transitions above 3470 cm^{-1} have been detected in the spectrum of the complexes with retained water. We must admit, however, that for such large molecule as Ubi, breaking even a relatively weak non-covalent bond requires absorption of several IR photons. As

a result of the non-linearity of IRMPD, isolated transitions of OH/NH stretches, potentially, could be missed because of their low absorption intensity. When numerous, these transitions may overlap giving rise to the high collective absorption intensities, which are picked up by IRMPD. The lack of these transitions in the spectrum of ubiquitin with retained water molecules implies a lack or a low number of free NH and carboxylic OH stretches in these complexes. The involvement of these functional groups to intramolecular couplings suggest that the protein remains folded, held by the numerous H-bonds.[36] It is worth noting that, apart from the small differences in relative intensities of some peaks, the IR spectra of the complexes formed by water condensation at 200 K and 60 K look nearly identical (Figure 5). This indicates that the structures of the complexes formed by condensation at different temperatures, globally, remain similar; due to the substantial spectral congestion, no specific spectral features could be attributed to the minor conformers that may give rise to the broad peak around 35000 cm^{-1} in the UV spectra of this complex (Figure 2).

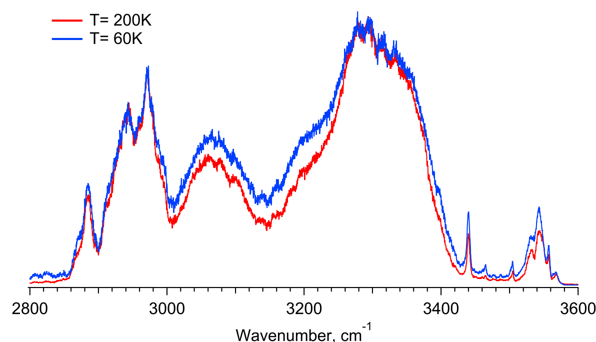


Figure 5. IRMPD photofragmentation spectra of Ubi^{7+} with 5 condensed water molecules measured after thermalization in the pretrap at 200 K and 60 K.

Conclusions

In conclusion, our study provides firm spectroscopic evidence that the same but differently formed microhydrated complexes of electrosprayed biomolecules may have significantly different 3D structures. Both UV and IR spectroscopy indicate that in the microhydrated complexes that are

generated directly from aqueous solution by soft ESI, the native-like folded structure of the embedded ubiquitin is preserved. In the gas phase, the folded motive is protected by continuous evaporative cooling, but not by the hydrogen bonds that involve a few retained water molecules. Condensing a few water molecules onto the already unfolded (during ESI) protein does not refold it on the 50-ms timescale of our experiments. We believe that a significant structural difference (or a significant difference in conformational distributions) may appear not only for the differently hydrated proteins, but also for much smaller biomolecules that have sufficiently high energy barriers of conformational rearrangements – a subject of our further study.

One practical implication of our study is that solving the native structure of biomolecules by optical spectroscopy or, for instance, by cryo-EM with soft landing of microhydrated complexes may require their hydration by retention rather than by condensation of water molecules. We also may propose that for the best protection of the electrosprayed native structures mass spectrometry should, perhaps, balance the desire of a high mass resolution with an incomplete dehydration of biomolecules, which can serve as an indicator of softness of ESI.

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Notes

The authors declare no competing financial interests.

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