Probing Conformational Changes of Ubiquitin by Host–Guest Chemistry Using Electrospray Ionization Mass Spectrometry

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Abstract. We report mechanistic studies of structural changes of ubiquitin (Ub) by host–guest chemistry with cucurbit[6]uril (CB[6]) using electrospray ionization mass spectrometry (ESI-MS) combined with circular dichroism spectroscopy and molecular dynamics (MD) simulation. CB[6] binds selectively to lysine (Lys) residues of proteins. Low energy collision-induced dissociation (CID) of the protein–CB[6] complex reveals CB[6] binding sites. We previously reported (*Anal. Chem.* 2011, 83, 7916–7923) shifts in major charge states along with Ub–CB[6] complexes in the ESI-MS spectrum with addition of CB[6] to Ub from water. We also reported that CB[6] is present only at Lys₆ or Lys₁₁ in high charge state (+13) complex. In this study, we provide additional information to explain unique

conformational change mechanisms of Ub by host–guest chemistry with CB[6] compared with solvent-driven conformational change of Ub. Additional CID study reveals that CB[6] is bound only to Lys₄₈ and Lys₆₃ in low charge state (+7) complex. MD simulation studies reveal that the high charge state complexes are attributed to the CB[6] bound to Lys₁₁. The complexation prohibits salt bridge formation between Lys₁₁ and Glu₃₄ and induces conformational change of Ub. This results in formation of high charge state complexes in the gas phase. Then, by utilizing stronger host–guest chemistry of CB[6] with pentamethylenediamine, refolding of Ub via detaching CB[6] from the protein is performed. Overall, this study gives an insight into the mechanism of denatured Ub ion formation via host–guest interactions with CB[6]. Furthermore, this provides a direction for designing function-controllable supramolecular system comprising proteins and synthetic host molecules. **Key words:** Ubiquitin, Conformational change, Supramolecular chemistry, Host–guest chemistry, Cucurbit[6]uril, Electrospray ionization, Collision-induced dissociation

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Introduction

Understanding structural dynamics of proteins is still a challenging field of study. Several analytical methods (e.g., single molecule spectroscopy [1], sum frequency generation spectroscopy [2], nuclear magnetic resonance spectroscopy [3], etc.) have been applied to reveal information of the dynamic states of proteins. Electrospray ioniza-

tion mass spectrometry (ESI-MS) has also been widely used for examining protein structures due to its unique advantage of isolating and characterizing particular components in the mixture [4–10].

Ubiquitin (Ub) is a highly conserved small globular protein with 76 amino acid residues. This small protein performs essential functions in eukaryotes through conjugation and ligation to the substrate protein [11, 12]. The seven lysine (Lys) residues and N-terminus of Ub are involved in polyubiquitination events [13–15], and differently linked polyubiquitin chains exhibit distinct functions [16]. The structure of Ub is well-characterized both in the solution phase and the gas phase. The native state of Ub is a tightly-folded structure containing both α -helix and β -sheet secondary structure

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elements (Supplemental Figure S1) [17–19]. This structure is very stable and is maintained over a wide range of pH values (1.2–8.5) in aqueous solution [20–22]. However, acidic condition with organic co-solvent induces conformational changes, of which an example is helix-rich A-state formed with 60 % methanol (by volume) at pH 2 [21]. Loo et al. have further studied the effects of various organic solvents in acidic condition and have reported that 20 % acetonitrile yields mainly the unfolded high charge Ub ions [22]. The gas phase Ub ions usually have charge distributions from +6 to +13 from an ESI source [5]. Charge states +11, +12, and +13 are usually from the denatured structures, and +6, +7, and +8 are from the native structure [5, 23]. The correlations between charge state signatures and protein conformations have elicited active discussions in biophysics and mass spectrometry societies [24]. However, a number of studies have reported good correlation between Ub structures and charge states [5, 22–24].

Supramolecular chemistry, which examines weak intermolecular interactions, has been expanded to biological molecules [25]. Especially, well-defined host–guest systems between synthetic host molecules and proteins have been applied in various fields of applications such as protein folding and structure probing [26–30], protein immobilization and sensing [31–33], protein function control [34], and inhibition of abnormal protein self-assembly [35]. Although supramolecular synthesis of multicomponent architectures with various shapes and functions is possible in a wide range of applications, synthetic supramolecular applications with proteins are limited to small systems [25] and yet to be understood in detail at the molecular level. Cucurbit[6]uril (CB[6], Figure 1), a self-assembled neutral cyclic host molecule, is widely used in host-guest chemistry. The partially negative carbonyl-laced portals and hydrophobic interior allows specific recognition of Lys residues of proteins with moderately strong binding event in solution ($K_a \approx 10^4 \text{ M}^{-1}$) [36, 37]. Previously, we have reported that the large size (MW 996.8 Da) of CB[6] and its strong binding energy allow CB[6] to retain its binding to Lys residue of protein during the fragmentation processes in the gas phase by low energy collision-induced dissociation (CID) [37]. In addition, the doubly charged CB[6] complex of 5-iminopentylammonium (5IPA) is yielded from the formation of an internal iminium ion by multiple backbone dissociations. Then, potential utility of CB[6] to probe the surface structure

of Ub has been demonstrated using ESI-MS. A mixture of CB[6] and Ub in water shows +7 to +14 charged Ub ion peaks with multiple CB[6] in the ESI-MS spectrum. With the analysis of +13 charged Ub-1CB[6] complex ion from water, we observed that CB[6] is bound to Lys₆ or Lys₁₁ of Ub. On the other hand, an analysis of +13 charged Ub-CB[6] complex ion from acidic organic solvent shows that CB[6] can bind to all Lys residues.

In this study, we investigate the CB[6] binding site of +7 charged complex ion. Then we compare the result with that of +13 charged complex ion. A crucial difference between two charge states are observed and show that CB[6] induces different conformations depending on different binding sites. Moreover, the CID analysis of double-CB[6] binding complex ions verifies the discussions. This gives us an understanding of conformational change of Ub and high charge state formation. The mechanism at the molecular level are explained and supported by combining ESI-MSⁿ with circular dichroism (CD) spectroscopy and molecular dynamics (MD) simulation. Then, controlling folding and unfolding of Ub is demonstrated using pentamethylenediamine (PMD) as a potential supramolecular switch system for controlling protein structures.

Experimental

Chemicals and Reagents

Ub, CB[6], PMD, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents (water and acetonitrile) were of HPLC grade and purchased from J. T. Baker (Phillipsburg, NJ, USA). CB[6] stock solution (10 mM) was prepared by dissolving CB[6] in 80 % formic acid. Sample solutions were prepared in two respective solvents consisting of water or 50/50 water/acetonitrile with 0.6 % formic acid by volume (referred to as acidic acetonitrile in the paper). The final concentrations of CB[6] and Ub for ESI-MS and CD spectroscopy were adjusted to 70 μM and 10 μM , respectively. CB[6] concentration was selected to be 7-fold of Ub concentration, as seven Lys residues are present in Ub. The measured pH of the final Ub and CB[6] solution was 2.2 in water and 2.4 in acidic acetonitrile.

Figure 1. The top (left), side (middle) view, and space-filling structure (right) of cucurbit[6]uril

Electrospray Ionization Mass Spectrometry

A Thermo Scientific LTQ Velos dual ion trap mass spectrometer equipped with normal ESI source was utilized in positive ion mode for the experiments. Electrospray voltage of 3.5 kV and capillary temperature of 250 °C were set as parameters for ESI. Each spectrum was averaged from 100 scanned spectra obtained using enhanced scan mode for analysis. For the statistical analysis of mass and tandem mass spectra, at least seven spectra (each were averaged from 100 scanned spectra) obtained at different times were averaged. The nomenclature for the parent and fragment ions was adopted from Roepstorff and Fohlman [38]. The left asterisk (*) superscript for the Ub and fragment ion refers to the presence of CB[6] in the fragment. For example, a y_n^{2+} fragment ion complex with CB[6] is referred to as $*y_n^{2+}$.

Circular Dichroism Spectroscopy

A Jasco J-815 spectropolarimeter equipped with peltier cell holder and 0.1 cm quartz cuvette was used to obtain CD spectra under constant nitrogen flow. All spectra are recorded in a wavelength interval of 190–260 nm, 0.1 nm data pitch by scanning at the rate of 20 nm/min at 20 °C. The spectra used in the present study were an average of three scans and smoothed using a FFT filter.

Computational Modeling

MD simulations were performed using the large scale atomic/molecular massively parallel simulator (LAMMPS) code [39]. The inter-atomic interactions were described by using the all-atom CHARMM PARAM27 force field (FF) [40], and the partial charge distribution of CB[6] was determined using Mulliken charge population analysis of the quantum mechanical (QM) wave function from density functional theory (DFT) calculation (B3LYP/6-31 G(d,p) using Gaussian 09) [41–43]. Table S1 shows the partial charge distribution of CB[6] from QM and FF. We prepared four different complex structures of Ub and CB[6] depending on the interacting residue of CB[6] (Lys₆, Lys₁₁, Lys₄₈, and Lys₆₃). Ub structure was from PDB entry 1D3Z, which was charge-neutral with all lysine and arginine protonated, and all glutamic acid and aspartic acid deprotonated. Each of these was solvated with 8134–8159 water molecules. After the solvated complex was minimized, we elevated the simulation temperature from 0 to 300 K for 50 ps to properly distribute the momentum to each atom. We then carried out 50 ps canonical ensemble MD simulations (NVT) to equilibrate the system at 300 K, and then performed ~10 ns isobaric-isothermal ensemble MD simulations (NPT). We

note that a Nosé-Hoover thermostat and barostat were employed for the temperature and pressure control.

Results and Discussion

ESI-MS of Ub with CB[6]

Previously, we have reported that addition of CB[6] to Ub solution results in charge shift into high charge state [37]. The major charge states of Ub ions in aqueous solution range from +5 to +8, and the ions are regarded as native-like structures. In acidic solution with high concentration of acetonitrile (50 % by volume), an additional charge distribution ranging from +9 to +13 is observed [37]. These additional charge states are typically regarded as denatured structures [5, 23]. When CB[6] is added to the solutions, dramatic changes occur. The abundance of low charge state ions are lowered and charge state distribution shifts into high charge state, inducing +12 and +13 charge state ions to be the most abundant.

To examine the phenomena quantitatively and confirm the relevance of charge shifts of Ub with CB[6], we have statistically investigated the relative abundances of Ub, Ub-1CB[6], and Ub-2CB[6] complexes of each charge state. It is found that in water, the relative abundance of the uncomplexed Ub ion in high charge states is significantly low (Figure 2a). On the other hand, in the +7 charge state, the relative abundance of Ub ion without CB[6] is the greatest. This shows that in water, denatured high charge state is formed by host-guest interaction of CB[6] with Ub. A

Figure 2. Relative abundances of Ub, Ub + 1CB, Ub + 2CB ions with +7 to +13 charge states from (a) water and from (b) acidic acetonitrile. The relative abundance of Ub + 1CB is arbitrarily assigned to be hundred. Data from seven independent spectra were averaged

difference is observed with a denaturing condition of acidic acetonitrile (Figure 2b). In this condition, the relative abundance of uncomplexed Ub ion in high charge states is comparable, or even exceeds the abundances of complexed ions. The result is in accordance with the denaturing solvent condition, which provides a distinct pathway of Ub denaturation. As formation of high charge state is possible by solvent-induced denaturation, the existence of high charge state ions without CB[6] is not only possible, but is also very probable, as the data suggest.

Circular Dichroism Studies of Ub-CB[6] Complex in the Solution Phase

In order to confirm the correlation between the observed charge distribution changes in the ESI-MS spectra and structural changes of Ub in the solution phase by addition of CB[6], CD spectroscopy is used to examine the secondary structures of Ub in the solution phase. Characteristic CD ellipticity for each secondary structural component (α -helix, β -sheet, and random structures) is listed in Supplemental Table S2. The CD spectrum of Ub in water shows a good agreement with the previously reported CD spectrum of the native state Ub (Supplemental Figure S2a), which comprises a mixed structure of α -helix (positive ellipticity at 190 nm and negative ellipticity at 208 and 222 nm) and β -sheet (negative ellipticity at 215 nm) elements [19]. The CD spectrum of Ub in water with formic acid shows that the portion of random structure slightly increases (Supplemental Figure S2a). Addition of CB[6] exhibits significant increase of random structure of the protein (Supplemental Figure S2a). This indicates that the complexation with CB[6] via host-guest interaction induces major conformational change of Ub from its native state to a denatured state in the solution phase. This supports that the observed shift in the charge states upon addition of CB[6] in ESI-MS spectra is caused by the conformational change of Ub following the complexation.

The CD spectrum of Ub and its mixture with CB[6] in acidic acetonitrile shows a clear difference compared with the structural change of Ub in water (Supplemental Figure S2b). The helical secondary structure component of Ub increases due to denaturation following the addition of acid to water/acetonitrile. The CD spectrum of Ub in acidic acetonitrile shows high similarity to the previously reported CD spectrum of the A-state structure of Ub [44]. No significant secondary structure change is observed by addition of CB[6]. This is due to the relatively weak correlation between the denaturation of Ub and CB[6] complexation in acidic acetonitrile. The denatured conformation is already formed by the solvent effect. Therefore, the role of CB[6] in acidic acetonitrile is limited to the conformation change of the native-like structured Ub. Thus

the conformational change induced by CB[6] is minor so that the CD spectrum does not show much difference.

ESI-MS² of Ub-1CB[6] Complex Ions

It is interesting that in both solvent conditions, CB[6] binding does not necessarily induce high charge state formation. +7 charge state ions with CB[6] are present in significant quantity. In other words, some conformations with CB[6] lead to the denaturation of Ub, whereas some do not. We hypothesized that different binding site of CB[6] in Ub may be the cause. Our previous study shows that CB[6] binding site could be deduced with CID and that CB[6] is bound to Lys₆ or Lys₁₁ for +13 charge state ions from water, and to any Lys for +13 charge state ions from acidic acetonitrile [37].

In the present study, we have carried out multiple CID investigation of +7 charged Ub-1CB[6] complex ions both from water and acidic acetonitrile. A recent study by Oldham and co-workers discusses the relationship between the stability of noncovalent interactions between Ub-modified substrates and Ub-binding domains along with their charge states [45]. The study has reported that highly charged complex ion has higher tendency to lose its noncovalent interactions because of the repulsion between charged binding partners and relatively high kinetic energy gained during ESI process [45]. However, this is not applicable to our case as CB[6]-Lys interaction is an ion-dipole interaction, and also is strong enough to be retained during CID processes [37].

CID spectra of +7 charged Ub with a single CB[6] from both water and acidic acetonitrile show identical fragments (Supplemental Figures S3a and S3b). Both spectra show that CB[6] prefers to bind to y₅₈₋₆₃ fragments, and MS³ analysis reveals that CB[6] is found at Lys₄₈ and Lys₆₃ (Supplemental Figures S3c and S3d). As discussed earlier, +7 charge state of Ub represents its native folding state in the solution

Scheme 1. Dissociation pathways of +7 charged Ub-1CB[6] complexes from both water and acidic acetonitrile. Both solvent condition exhibit identical dissociation pathways. Indicated products are further probed by MS³. Observation of only fragment-CB[6] complexes in MSⁿ spectra is denoted as an empty circle. Solid circle denotes the presence of both fragments and fragment-CB[6] complexes

Figure 3. Relative abundances of b_{16} , b_{17} , b_{18} fragments with and without CB[6], and y_{58} , y_{59} , y_{60} , y_{61} fragments with and without CB[6], in the CID spectra of Ub-1CB[6] complex ions with +13 charge state (a) from water and (b) from acidic acetonitrile, and with +7 charge state (c) from water and (d) from acidic acetonitrile. The Lys₆ and Lys₁₁ are included in b-fragments, and five other Lys (Lys₂₇, Lys₂₉, Lys₃₃, Lys₄₈, Lys₆₃) are included in the y-fragments, and CB[6] existence on each fragment means CB[6] binding to the respective Lys. The gray and slashed bars represent the relative abundance of fragments without and with CB[6], respectively. Data from six independent spectra were averaged

phase [5, 23]. Thus, the observed charge state and binding location of CB[6] indicate that no significant change in conformation of Ub occurs with the attachment of CB[6] to Lys₄₈ and Lys₆₃ residues. The CID processes of +7 charged Ub-1CB[6] complex ions are summarized in Scheme 1.

The relative abundances between CID fragments of Ub with and without CB[6] provide strong correlation between charge state of Ub and CB[6] binding sites (Figure 3). The abundances of the sum of b_{16} , b_{17} , b_{18} fragments are compared with the abundances of corresponding CB[6] complex fragments ($*b_{16}$, $*b_{17}$, $*b_{18}$). The relative abundances of the sum of y_{58} , y_{59} ,

y_{60} , y_{61} , and the corresponding CB[6] complex fragments ($*y_{58}$, $*y_{59}$, $*y_{60}$, $*y_{61}$) are also compared. Figure 3a shows that the +13 charged Ub-1CB[6] ion from water has its interacting CB[6] exceptionally at b fragments: complexation at Lys₆ or Lys₁₁. In contrast, +7 charged Ub-1CB[6] ion has its interacting CB[6] exceptionally at y fragments: complexation at Lys₂₇, Lys₂₉, Lys₃₃, Lys₄₈, or Lys₆₃ (Figure 3b). Further CID (MS³) investigation reveals the binding sites to be Lys₄₈ and Lys₆₃. Thus, it can be concluded that a binding of CB[6] to Lys₄₈ and Lys₆₃ does not induce denaturation, whereas a binding of CB[6] to Lys₆ or Lys₁₁ induces denaturation.

Scheme 2. Dissociation pathways of +12 charged ubiquitin with two CB[6] (a) from water and (b) from acidic acetonitrile inferred from CID. Indicated products with two CB[6] are further probed by MS³. Observation of only fragment-CB[6] complexes in MSⁿ spectra is denoted as an empty circle. Solid circle denotes the presence of both fragments and fragment-CB[6] complexes

Figure 4. (a) Native structure of Ub (PDB entry 1D3Z). (b) Final snapshot after 10.0 ns of MD simulations of Ub complex of CB[6] to Lys₁₁

The +13 charged Ub-1CB[6] ion from acidic acetonitrile shows a different fragmentation pattern (Figure 3c). Both b fragments (b₁₆, b₁₇, b₁₈) and y fragments (y₅₈, y₅₉, y₆₀, y₆₁) with CB[6] are observed. MS³ investigation shows that CB[6] binding occurs at even Lys₂₇, Lys₂₉, and Lys₃₃, which are buried inside of the protein when it is in the native state. The denaturing solvent condition leads to the exposure of inner Lys and allows CB[6] to bind. Figure 3d, the CID data of +7 charged Ub-1CB[6] ion from acidic acetonitrile, is almost identical to that of the ion from water. This further confirms that binding CB[6] to Lys₄₈ and Lys₆₃ preserves the native state of Ub and retains its low charge state.

ESI-MS² of Ub-2CB[6] Complex Ions

To verify the observed correlation between the complexation of Ub with CB[6] and its structure, the CID analysis of Ub

complex ions with two CB[6] (Ub-2CB[6]) is further performed. The CID of the +12 charged Ub-2CB[6] complex ion from water yields *y₅₈, *b₁₇, and *b₁₈ fragments (Supplemental Figure S4a). This indicates that a CB[6] is attached at Lys₆ or Lys₁₁ and the other CB[6] is attached to any one of other Lys residues from 27 to 63. We infer that once the protein structure is changed to the unfolded structure by complexation of CB[6] to Lys₆ or Lys₁₁, all the rest of Lys residues are accessible to CB[6].

Distinct CB[6] binding sites are observed from the CID of Ub-2CB[6] complex ions from acidic acetonitrile (Supplemental Figure S4b). The CID analysis of +12 charged Ub-2CB[6] complex ion additionally yields **y₅₈, whose two CB[6] interact with two Lys residues from 27 to 63. Since the protein is already at a denatured state, of which all Lys residues are exposed outside, two CB[6] can bind to any two Lys residues from 27 to 63. In this case, binding of CB[6] to Lys₆ or Lys₁₁ residue is not a necessity for highly charged complex formation from acidic acetonitrile. The CID processes of +12 charged Ub-2CB[6] complex ions are summarized in Scheme 2.

Conformational Changes of Ub by Host–Guest Chemistry with CB[6]

The present study demonstrates that binding of CB[6] to Lys₆ or Lys₁₁ induces conformational change of Ub in water differently from the change induced by organic solvent. MD simulations provide further insight into structural changes of Ub by complexation with CB[6] in an aqueous environment. We use the all-atom CHARMM PARAM27 force field and TIP3P potential to mimic a water environment [40]. These simulations exhibit no critical changes in intramolecular interactions of Ub by complexation of Lys₄₈ and Lys₆₃ with

Figure 5. Proposed mechanistic diagrams for structural changes and charge states of Ub by host–guest chemistry with CB[6]. The figure is for illustrative purposes only

a CB[6] (Supplemental Figures S5a and S5b, respectively), which is consistent with experimental observation. Significant changes in intramolecular interactions of Ub are observed when CB[6] is attached to Lys₁₁. The host–guest interaction between CB[6] and Lys₁₁ prohibits Lys₁₁ from forming a salt bridge with Glu₃₄, which links between β -sheet in region I (residues 1–18) and α -helix in region II (residues 19–35, Figure 4a). As a result, an intra-regional salt bridge is formed between Glu₃₄ and Lys₃₃, while an inter-regional H-bond between Lys₃₃ and Thr₁₄ is broken (Figure 4b). Two important inter-regional interactions between region I and region II are broken by host–guest interaction between Lys₁₁ and CB[6]. This results in loosening of the tightly folded structure of Ub, and eventually induces the conformational change of Ub in water. This theoretical investigation supports the experimental observations that conformational change of Ub occurs by specific host–guest interaction between Lys₁₁ and CB[6] in the solution phase. No significant change in inter-regional interaction is observed when CB[6] is attached to Lys₆ (Supplemental Figure S5c). We infer that CB[6], which binds to Lys₁₁, interacts with adjacent Lys₆ [46] simultaneously during the conformational changes of Ub to induce further changes in protein conformation.

The interaction between Lys residues and CB[6] is known to be moderately strong ($K_a \approx 10^4 \text{ M}^{-1}$) in the solution phase [36], so the specific interaction of CB[6] is expected to be limited to Lys residues which do not have strong salt bridge interactions. Thus, it is hard to expect that CB[6] alone can defy the salt bridge between Lys₁₁ and Glu₃₄ and induce conformational change of Ub. The mixture solution of 10 μM Ub and 70 μM CB[6] contains approximately 0.6 % of formic acid since CB[6] is dissolved in acidic solution [47, 48]. The native structure of Ub is very stable and is maintained upon addition of acid in aqueous solution [20, 21]. We have tested ESI-MS of Ub in water with 0.6 % formic acid to understand the effect of the acid alone to the charge distribution in the MS spectrum. The ESI-MS spectrum of Ub from water with formic acid (Supplemental Figure S6a) shows very similar charge distribution of Ub from water without formic acid (Supplemental Figure S6b). This indicates that the native structure of Ub is not directly influenced by acidic solution condition. We infer that the acidic condition of the solution may weaken the salt bridge between Lys₁₁ and Glu₃₄ to allow CB[6] to bind to Lys₁₁. Once the salt bridge is weakened by acidic condition, the attachment of CB[6] to Lys₁₁ is considered to induce further denaturation of Ub. Additional expansion of Ub structure would be expected in the charged droplet from ESI which results in the observed charge shift in the ESI-MS spectrum (Figure 5). Previous study of structures of Ub using selective noncovalent adduct protein probing (SNAPP) by Julian and co-workers has reported weak salt bridge between Lys₁₁ and Glu₃₄ along with dynamic structural

motions of the protein involved with Lys₁₁ [27]. A recent report by Breuker and co-workers has also addressed the importance of salt bridges in stabilizing gas phase structures [49].

Controlling Ub Conformations by Host–Guest Chemistry of CB[6] with PMD

The observed conformational changes of Ub is primarily induced by moderately strong ($K_a \approx 10^4 \text{ M}^{-1}$) interactions between CB[6] and Lys₁₁ in the solution phase [36]. CB[6] exhibits strong binding properties to α,ω -alkyldiammonium cations with alkyl chain, which comprises four to six carbons [50]. Refolding to the native state of the Ub unfolded by CB[6] beforehand, is examined using pentamethylenediamine (PMD, Supplemental Figure S7) as a potential supramolecular switch for controlling protein structures. PMD binds to CB[6] strongly ($K_a \geq 10^5 \text{ M}^{-1}$) in

Figure 6. ESI-MS spectra of (a) Ub (10 μM), (b) Ub (10 μM) with CB[6] (70 μM), (c) a mixture of Ub (10 μM) and CB[6] (70 μM) followed by an addition of PMD (70 μM), and (d) a mixture of Ub (10 μM), CB[6] (70 μM), and PMD (70 μM) followed by an addition of CB[6] (70 μM) from water

the solution because of its suitable alkyl chain length (~ 8 Å) to the cavity depth (~ 9 Å) and two ammonium groups, which interact with two carbonyl-laced portals [50].

ESI-MS spectrum of Ub in water shows a dominating charge distribution in the range of +5 to +8, which represents the native structure of Ub in the solution phase (Figure 6a) [5, 23]. The complexations and conformational changes of Ub (10 μ M) by CB[6] (70 μ M) is confirmed by ESI-MS (Figure 6b). The ESI-MS of a mixture of Ub and CB[6] from water shows a significant shift in charge states of Ub up to +14 with multiple (up to four) CB[6] attachments as reported from our previous study [37]. Then, 70 μ M PMD is added to a mixture of Ub and CB[6] (10 μ M and 70 μ M, respectively) in water to refold Ub to its native state. The ESI-MS spectrum of a mixture of Ub and CB[6], after the addition of PMD, shows major peak at m/z 550.2, which corresponds to doubly charged CB[6]-PMD complex ion (Figure 6c). The addition of PMD to the Ub-CB[6] mixture detaches CB[6] from Ub by forming CB[6]-PMD complex in the solution. Ub ions exhibit a major charge distribution in the range of +5 to +8, which represents the native structure of Ub in the solution phase. A minor charge distribution in the range of +9 to +13 is additionally observed from the spectrum. This minor charge distribution results from a relatively low abundance of permanently denatured Ub that has been formed by host-guest interactions with CB[6]. No significant Ub-CB[6] complex ion is observed in the spectrum. Finally, 70 μ M of CB[6] is, again, added to the solution which formerly comprises 10 μ M Ub, 70 μ M CB[6], and 70 μ M PMD. Addition of extra amount (70 μ M) of CB[6] to the final solution, again, results in charge shift, and hence, conformational changes of Ub (Figure 6d).

Conclusions

We have probed distinct conformational change mechanisms and dynamics of Ub by host-guest chemistry with CB[6] at the molecular level compared with solvent driven conformational change using ESI-MS combined with CD spectroscopy and MD simulations. Under acidic condition, CB[6] causes dramatic conformational change of the protein and this results in shifts in major charge states up to +14 with multiple CB[6] attachments in the ESI-MS spectrum. Tandem MS analysis indicates that binding of CB[6] to Lys₆ and Lys₁₁ causes conformational change of the protein. Then, controlling folding and unfolding of Ub is demonstrated by addition of a series of stoichiometric amount of CB[6] and PMD by attaching and detaching CB[6] from Ub. Our study provides mechanistic details about the conformational changes of Ub by host-guest interactions with CB[6]. Small residual changes by host-guest interactions can induce dramatic structural change of the protein, which is an important factor for designing building blocks for biologically functional supramolecular architectures using proteins and synthetic host molecules. Understanding supramolecular

interactions of the system in detail at the molecular level provides us a direction to control protein structures, which is directly related to controlling protein functionality.

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