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Evolution of Conformation and Dynamics of Solvents in Hydration Shell along the Urea-induced Unfolding of Ubiquitin

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Electronic Supplementary Information

Abstract A clear diagram for the unfolding of protein induced by denaturant is a classical but still unsolved challenge. To explore the unfolded conformations of ubiquitin under different urea concentrations, we performed hybrid Monte Carlo-molecular dynamics simulations (MC-MD) guided by small angle X-ray scattering (SAXS) structural information. Conformational ensembles sampled by the hybrid MC-MD algorithm exhibited typical 3D structures at different urea concentrations. These typical structures suggested that ubiquitin was subjected to a sequential unfolding, where the native contacts between adjacent β -sheets at first were disrupted together with the exposure of hydrophobic core, followed by the conversion of remaining β -strands and helices into random coils. Ubiquitin in 8 mol·L⁻¹ urea is almost a random coil. With the disruption of native structure, urea molecules are enriched at protein hydrated layer to stabilize newly exposed residues. Compared with water, urea molecules prefer to form hydrogen bonds with the backbone of ubiquitin, thus occupying nodes of the hydrogen bonding network that construct the secondary structure of proteins. Meanwhile, we also found that the slow dynamics of urea molecules was almost unchanged while the dynamics of water was accelerated in the hydration shell when more residues were unfolded and exposed. The former was also responsible for the stabilization of unfolded structures.

Keywords Ubiquitin; Unfolding process; Hydration behavior and dynamics; Water and urea

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INTRODUCTION

Providing a clear illustration for the unfolding of protein induced by chemical denaturants can help us understand the protein folding problem and the physicochemical mechanism of denaturing protein. Ubiquitin is a highly conserved protein in all eukaryotic organisms^[1,2] and plays a fundamental role in regulating the cellular processes of degradation.^[3] The 76-residue monomeric ubiquitin, which has a well-defined hydrophobic core comprised of five-stranded β sheets, an α helix, and a short 3_{10} helix,^[4,5] is widely used as a model in experimental and computational protein folding/unfolding studies.^[6–10] Compared with the folding/unfolding thermodynamic and kinetic properties which can be reliably obtained by fluorescence emission and circular dichroism,^[11] structural experimental techniques characterizing the short-lived transient states and highly flexible unfolded conformations still remain a great challenge.^[12,13] Till recently, fully unfolded-state ensembles of ubiquitin in 8 mol/L urea solution were built from statistical coil models and then refined by the restraints of NMR and small angle X-

ray scattering (SAXS) data.^[13,14] In addition, atomic molecular dynamics (MD) simulations are still difficult tasks for exploring the whole unfolding of globular proteins induced by chemical denaturants at room temperature^[15,16] due to the expensive computational cost. The complete unfolded state ensemble of ubiquitin in 8 mol·L⁻¹ urea solution has been described by using MD simulations, started from either the representative conformations refined by NMR and SAXS^[14] or conformations obtained from high simulation temperature (550 K).^[17] However, the intermediate states of ubiquitin unfolding which are also crucial to understand the unfolding/folding problem remain unexplored.

Urea is probably the most widely used denaturant in protein folding studies due to its non-ionic nature, the ability of denaturing protein at room temperature, and the reversibility of denaturation.^[11,18,19] The underlying molecular mechanism of how urea denatures protein has attracted a great deal of attention in the past decades.^[20,21] Two different mechanisms, *i.e.*, “indirect mechanism” and “direct mechanism”, have been proposed. “Indirect mechanism” demonstrated that urea can weaken the hydrophobic effect that is majorly responsible for the stabilization of tertiary structure by disrupting the structure of water surrounding protein.^[22] On the other hand, “direct mechanism” claimed that urea denatures protein by directly interacting with the exposed residues with

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either more favorable electrostatic or van der Waals interaction, comparing with water molecules.^[16,23–25] In recent years, “direct mechanism” has been applied more extensively to explain the role of urea in denaturing protein than the “indirect mechanism”.^[20] The “direct” interactions of urea with the residues are generally characterized in the early or terminal unfolding stage of protein.^[16,26–30] However, behavior of urea in the intermediate unfolded states between folded and random coil of proteins is awaiting for further understanding. Besides, the dynamical coupling nature of solvents in hydration shell is known to play significant roles in the protein structure and dynamics.^[31–34] The slow diffusion and long residence of urea around the hydrophobic core are favorable to the partially open structures, initialized from thermal fluctuations at the early unfolding stage.^[26] Nevertheless, dynamics of urea as well as their effects on the whole unfolding of ubiquitin have not yet been fully explored.

Herein, we performed hybrid Monte Carlo (MC)-MD simulations incorporating with the SAXS structural information^[35] to guide the unfolding of ubiquitin at different urea concentrations. The static and dynamic factors of urea/water at different stages of unfolding, as well as representative conformations at different urea concentrations, were investigated. The results provided a clear and complete picture of the unfolding of ubiquitin induced by urea and the possible static and dynamic roles of urea on denaturing ubiquitin.

METHODS

Hybrid MC-MD Simulations to Study Ubiquitin Unfolding

In the hybrid MC-MD simulations, MC simulations are used to sample the conformational space and MD simulations are used for structure relaxation to improve the MC conformational sampling efficiency.^[36,37] The hybrid MC-MD simulations are guided with the following energy function:

$$E = E_{\text{CHARMM22}} + E_{\text{Hbond}} + \Delta G_{\text{solv}} + E_{\text{SAXS_Target}} \quad (1)$$

where E_{CHARMM22} is the energy of CHARMM22^[38] force field with CMAP corrections,^[39] including stretching, bending and torsional bonded interactions, and van der Waals and electrostatic non-bonded interactions. E_{Hbond} is a native-centric hydrogen bond energy term of backbone (N–H···O–C) which is a knowledge-based potential (Fig. S1 in electronic supplementary information, ESI) from REMO.^[40] The hydrogen bond pairs of backbone were identified from the initial structure (*i.e.* the native structure) by Stride.^[41] ΔG_{solv} is the solvation free energy of protein dissolved in water solutions or urea aqueous solutions,

$$\begin{aligned} \Delta G_{\text{solv}} &= \Delta G_{\text{water}} + \Delta G_{\text{tr}}([c_{\text{urea}}]) \\ \Delta G_{\text{water}} &= \Delta G_{\text{elec}} + \Delta G_{\text{np}} \\ &= -\frac{1}{2} \left(\frac{1}{\epsilon_{\text{p}}} - \frac{1}{\epsilon_{\text{s}}} \right) \sum_{ij} \frac{Q_i Q_j}{\sqrt{r_{ij}^2 + R_i R_j \exp(-r_{ij}^2/4R_i R_j)}} \\ &\quad + \gamma \sum_{i=1}^N \text{SASA}_i \\ \Delta G_{\text{tr}}([c_{\text{urea}}]) &= \omega \sum_{i=1}^N \alpha_i [c_{\text{urea}}] \text{SASA}_i \end{aligned} \quad (2)$$

where ΔG_{elec} can be estimated by Generalized Born with a simple switching (GBSW) solvation energy model,^[42] ϵ_{p} and ϵ_{s} are the dielectric constants assigned to the solute molecule and solvent, respectively. Q_i and R_i are the charge and the effective Born radius of atom i , respectively,^[42] γ is the phenomenological surface tension coefficient which is set to 2.093 kJ·mol^{−1}·nm^{−2}, N is the total number of atoms in protein, and SASA_i is the solvent-accessible surface area of atom i .

$\Delta G_{\text{tr}}([c_{\text{urea}}])$ is an empirical model which accounts for the free energy of transferring protein from water to an osmolyte solution according to the Tanford's transfer model (TM).^[19] ω is the phenomenological scaling factor for α_i which is set to be 125, α_i (in units of kJ·mol^{−1}·L·mol^{−1}·nm^{−2}, listed in Table S1 in ESI) is the surface interaction potential of atom type i of per unit area and urea concentration. The surface interaction potentials α_i of different atom types were taken from Guinn's report.^[21] We re-scaled α_i by re-calculated the SASA of these structural models used by Guinn *et al.* with LCPO approach^[43] with less computational cost, comparing with Surface Racer method^[44] in Guinn's report. Atom types include aliphatic carbon, aromatic carbon, hydroxyl oxygen, amide oxygen, anionic carboxylate oxygen, amide nitrogen, and cationic nitrogen.

$E_{\text{SAXS_Target}}$ represents the discrepancy between the SAXS intensity profile of present conformation calculated by FoXS^[45] and the SAXS intensity profile of target state determined by experiment. $E_{\text{SAXS_Target}}$ is used to guide the sampling toward the experimental target state and can be estimated as follows:

$$E_{\text{SAXS_Target}} = A \times \chi = A \times \sqrt{\frac{1}{N} \sum_{i=1}^N \left(\frac{I_{\text{exp}}(q_i) - cI(q_i)}{\sigma(q_i)} \right)^2} \quad (3)$$

where A is an empirical regulator of χ which is taken as 4186 kJ·mol^{−1} in MC simulations and 0 kJ·mol^{−1} in MD simulations (MD simulations are used for structure relaxation and thus do not include the $E_{\text{SAXS_Target}}$ energy term); N and q represent the number of data points and scattering vector in the scattering intensity profiles, respectively; c is the scaling factor to minimize χ and $\sigma(q)$ is the experimental error at q . Experimental scattering profiles for ubiquitin in water solutions and in urea aqueous solutions were taken from Small Angle Scattering Biological Data Bank (code: SASDAQ2)^[46] and the study reported by Huang *et al.*,^[47] respectively. The boundaries of q in the experiment are 0.64 nm^{−1} (0.86 nm^{−1} for $[c] = 1$ mol·L^{−1} for excluding the significant interparticle interactions at low scattering angles^[47]) and 3.32 nm^{−1}, respectively.

Unfolded conformational ensembles of ubiquitin were sampled by the hybrid MC-MD method^[35] at different urea concentrations with implicit solvent. The hybrid MC-MD method is found to significantly accelerate the conformational sampling compared with single MC or MD method does.^[35,36,48] Protonation states of residues in ubiquitin were titrated to pH 2.0 (Fig. 1). 60-Cycle MC-MD simulations were performed, with 500 steps of MC simulations for sampling conformations, followed by 100 steps of steepest descent, 100 steps of BFGS^[49,50] energy minimization, and

least 3 residues and within a distance of 0.45 nm in native conformation; N_{ij} is the total number of native contact pairs in native conformation; the number of native contact pairs for evolving conformations in MC simulations is the sum of Heaviside function $\theta(x)$; d_{ij} and d_{ij}^0 are the distances between native contact pair (i, j) in evolving and native conformation, respectively; λ is set to be 1.8 by considering the fluctuations within the native conformation.

Hydrogen bonds (D—H···A) formed by solvents and protein were defined according to the criteria of $d_{\text{H-A}} < 0.3$ nm and angle $\text{D-H-A} > 120^\circ$ (Fig. 2). The partition coefficient K_p of urea is expressed approximately by the ratio of Ratio_{UW} (the urea/water ratio) in the first solvent shell (FSS) to R_{UW} in bulk water. FSS (Fig. 2) and bulk solvents were defined as solvent molecules within 0.5 nm of any atom of protein and 0.6 nm beyond any atom of protein, respectively.

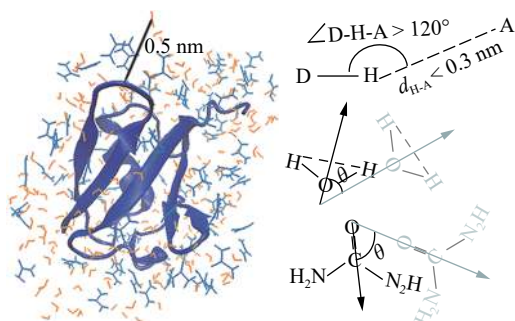


Fig. 2 Schematic illustration of the first solvent shell (FSS) of ubiquitin, hydrogen bond, and the dipole angles of water and urea molecules. The dark and grey forms stand for the orientation of the same molecule at time of 0 and t , respectively.

The dynamics of water and urea in contact with the protein (*i.e.* within 0.35 nm of any protein atoms) can be characterized by the survival time correlation function $S(t)$ and dipole-dipole time correlation function (TCF), $C_\mu(t)$:

$$S(t) = \langle \delta(0, t) \rangle \quad (5)$$

$$C_\mu(t) = \langle \cos \theta(0, t) \rangle \quad (6)$$

where $\delta(0, t) = 1$ for solvents continuously in contact with protein lasting time of t , and otherwise, $\delta(0, t) = 0$. $\theta(0, t)$ is the dipole angle for a molecule at time of 0 and t (Fig. 2). The angular brackets $\langle \dots \rangle$ stand for ensemble average. The dipole vectors of water and urea are defined as the oxygen atom to the center of two hydrogen atoms and the oxygen atom to the carbon atom, respectively.

RESULTS AND DISCUSSION

Evolution of Ubiquitin Conformations during Unfolding

To explore the unfolding of ubiquitin induced by urea, we carried out hybrid MC-MD simulations incorporating the SAXS structural information for ubiquitin under different urea concentrations. Evolutions of backbone RMSDs of ubiquitin in reference to the initial structures are plotted in Fig. 3(a). It shows that the systems get equilibrium after 10000 MC steps. In order to demonstrate conformational ensembles sampled by the hybrid MC-MD algorithm that can enrich sampling of the realistic conformational space

for ubiquitin at different urea concentrations, we compared the calculated SAXS profiles with the corresponding experimental curves^[47] (Fig. 3b). The calculated SAXS profiles were obtained by averaging over 900 equilibrated conformations of ubiquitin (300 conformations $\times$ 3 parallel trajectories). Fig. 3(b) demonstrates that the calculated SAXS profile for each urea concentration matches very well with the experimental one. Mean differences (χ) for all urea concentrations (listed in Table 1) have prominently decreased relative to the initial values. The largest χ between the calculated and experimental SAXS profiles is only 1.12 at 8 mol·L⁻¹ urea concentration, which is more conserved than MD-results reported by Orozco *et al.* with $\chi^2 = 1.4$ ^[60] and the conformational ensemble refined by NMR and SAXS with $\chi^2 = 2.1$.^[14] The good superimpositions in these SAXS profiles demonstrated that the hybrid MC-MD simulations could effectively sample the conformational ensembles which reliably represent the conformations of ubiquitin in real solutions. In addition, the integration of SAXS structural information also well confined the sampling in 0 mol·L⁻¹ urea near the crystal structure (Fig. 3b). The representative structure at 0 mol·L⁻¹ urea concentration overlaps very well with the crystal structure (RMSD of 0.13 nm), and its calculated SAXS profile has a lower χ (0.18) than that of the

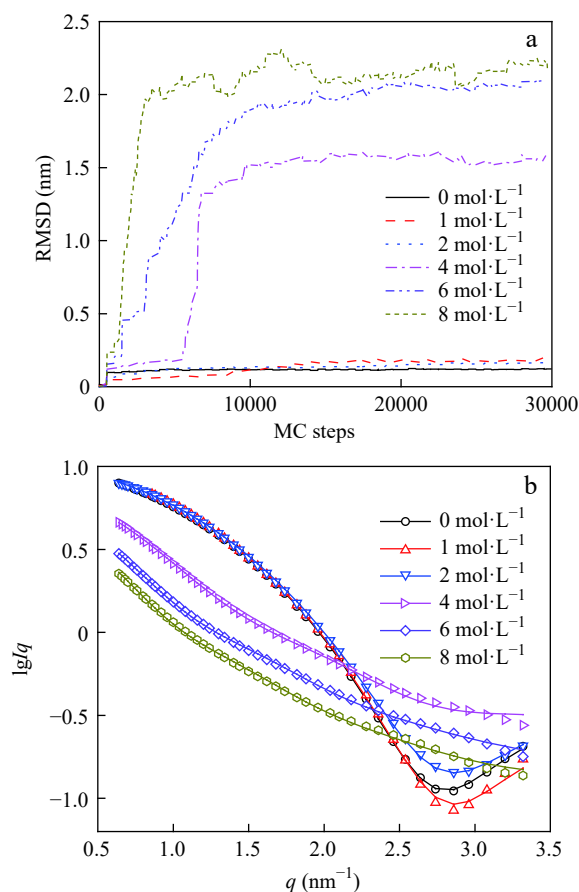


Fig. 3 (a) MC time evolution of backbone RMSD and (b) comparison of experimental and calculated SAXS profiles for equilibrated conformations averaged of 3 parallel trajectories from the MC-MD simulations including SAXS restraint. The experimental and calculated SAXS profiles are depicted with symbols and lines with identical colors.

crystal structure (0.52), as depicted in Fig. S2 (in ESI). This result confirms again that the present hybrid MC-MD method could effectively reproduce the ubiquitin conformations in water solutions.

Fig. 4 illustrates the unfolding of ubiquitin at different urea concentrations. The representative conformations were obtained by clustering analysis of 900 conformations. To make the top 3 clusters cover more than 90% decoys, cutoffs of 0.2, 0.2, 0.2, 0.5, 0.5, and 1 nm were used for 0, 1, 2, 4, 6, and 8 mol·L⁻¹ urea, respectively. For 0, 1, and 2 mol·L⁻¹ urea, all ubiquitin conformations were conserved in only 1 cluster, whereas 3 clusters were produced for 4, 6, and 8 mol·L⁻¹ urea. The central structure of each cluster was selected as the representative conformation. As seen from Fig. 4, ubiquitin gradually deviates from its native state, and its secondary and tertiary characteristics disappear sequentially as the urea concentration increasing. For 1 and 2 mol·L⁻¹ urea, ubiquitin has minor deviations from its native conformation with averaged RMSDs lower than 0.2 nm (Table 1). However, they are far away from the native conformation for 4, 6, and 8 mol·L⁻¹ urea with averaged RMSD of 1.55, 2.06, and 2.17 nm, respectively. Meanwhile, ubiquitin swells gradually with the increasing urea concentration. R_g can be steadily deduced from SAXS profiles or calculated from the coordinates of the structure. Table 1 shows that R_g of ubiquitin increases from 1.16 nm (in water) to ~2.51 nm (8 mol·L⁻¹ urea), indicating that ubiquitin stretched apparently, as observed in Fig. 4. Furthermore, the calculated R_g (2.51 nm) of

ubiquitin at 8 mol·L⁻¹ urea is rather close to the theoretical value (2.69 nm) estimated by Flory scaling law ($R_g = a_D N^{0.6}$, where $a_D \approx 0.20$ nm, $N = 76$ ^[61]) for a random coil in good solvent. This suggests that most of ubiquitin conformations behave as random coils in 8 mol·L⁻¹ urea, and 8 mol·L⁻¹ urea solution is a good solvent for ubiquitin. The backbone dihedral angles also confirm that the ubiquitin conformations in 8 mol·L⁻¹ urea are more outstretched and flexible. As shown in Fig. S3 (in ESI), the distribution of dihedral angles becomes more and more disperse at higher urea concentration and increases in corner regions of Ramachandran plots and “polyproline II” (PPII) region (centered at -75°, 145°).^[62] Both the extension and PPII conformations of ubiquitin in 8 mol·L⁻¹ urea are more favorable for urea interaction with residues due to the greater surface available for binding. To further evaluate the conservation of native folds for conformations at different urea concentrations, the fraction of native contact (Q) was calculated (Table 1). Q decreases as the urea concentration increases, and only 3.0% of native contacts are held in 8 mol·L⁻¹ urea. In 1 and 2 mol·L⁻¹ urea, there are still ~90% native contacts in the conformations of ubiquitin, while about 60% native contacts have been lost in 4 mol·L⁻¹ urea. It could be deduced that the denaturation midpoint c_m (the urea concentration when Q equals 50%) should be located between 2 and 4 mol·L⁻¹. CD experiment^[8] measured that c_m was ~3.2 mol·L⁻¹. The evaluation for c_m by MC-MD simulations reasonably agrees with the experimental measurement.

Table 1 Averaged RMSD, R_g , fraction of native contact (Q), number of backbone hydrogen bond (N_{HB}), and the deviation χ between the calculated and experimental SAXS profiles over 900 decoys from the MC-MD simulations including SAXS restraint

c_{urea} (mol·L ⁻¹)	RMSD (nm)	R_g (nm)	Q	N_{HB}	χ (Initial χ)
0	0	1.16	1	44	0.18 (0.52)
1	0.18 ± 0.02	1.20 ± 0.01	0.90 ± 0.02	40.36 ± 0.02	0.73 ± 0.11 (0.92)
2	0.16 ± 0.01	1.21 ± 0.01	0.91 ± 0.01	42.81 ± 1.77	0.52 ± 0.10 (1.09)
4	1.55 ± 0.10	1.85 ± 0.06	0.41 ± 0.05	24.01 ± 3.27	0.89 ± 0.21 (8.31)
6	2.06 ± 0.06	2.28 ± 0.07	0.27 ± 0.02	11.33 ± 1.90	0.61 ± 0.29 (9.19)
8	2.17 ± 0.12	2.51 ± 0.12	0.03 ± 0.01	4.30 ± 1.55	1.12 ± 0.67 (9.10)

The initial χ values are also listed in parentheses. Values in 0 mol·L⁻¹ urea are calculated from the representative conformation selected from conformational ensembles in 0 mol·L⁻¹. This representative conformation is the reference structure for other urea concentrations.

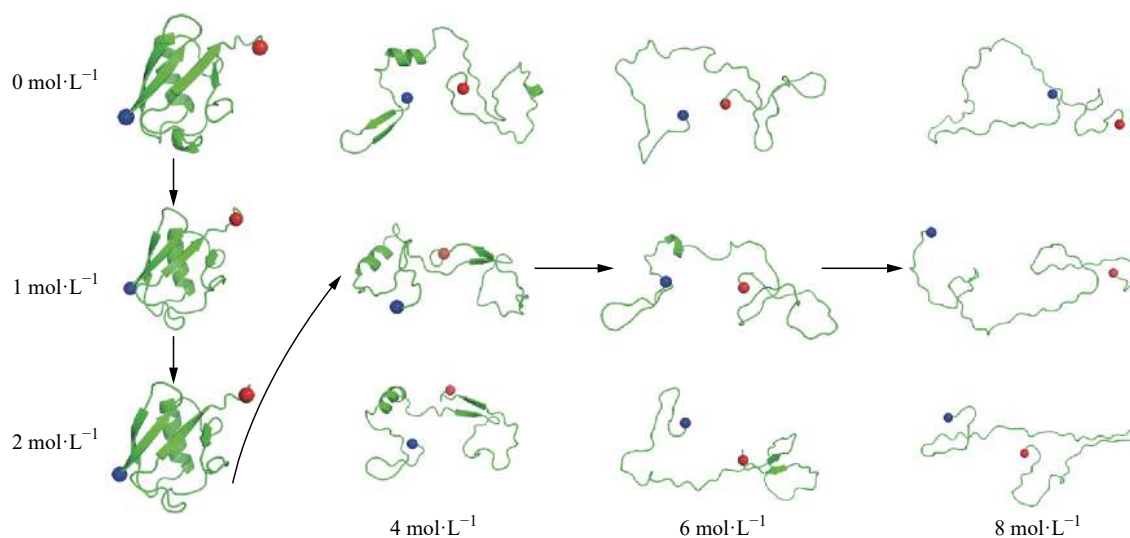


Fig. 4 Representative conformations of unfolded ubiquitin at different urea concentrations from the MC-MD simulations including SAXS restraint. The blue and red spheres represent N-term and C-term of ubiquitin, respectively.

Ubiquitin secondary and tertiary structures were investigated to characterize its spatial order at different urea concentrations. The number of residues composed of different secondary structural elements under all urea concentrations is displayed in Fig. 5. The cross correlation maps determining the tertiary structure of ubiquitin for all urea concentrations are depicted in Fig. 6. Secondary structural elements have minor changes in 1 and 2 mol·L⁻¹ urea. Quantitative similarities are reflected in the few reductions of backbone hydrogen-bond numbers, which is the main force in stabilizing the secondary structure (Table 1). Similar observations in cross correlation maps show little loss of contact information in 1 and 2 mol·L⁻¹ urea compared with 0 mol·L⁻¹ state. These indicate that ubiquitin conformations in 1 and 2 mol·L⁻¹ urea are more native-like, which is in good agreement with observations from NMR experiments.^[13,47] By further calculating the mean distances between adjacent β -sheets, it was found that β 3- β 5 sheets expanded 0.08 nm, and β 3- β 4 sheets shrank 0.06 nm in 2 mol·L⁻¹ urea (Table 2). These subtle changes are similar to the early thermal denaturation process of ubiquitin detected by 2D IR experiment^[63] which revealed that ubiquitin unfolding may begin with the change of β 3- β 4 and β 3- β 5 sheets. As the urea concentration increased to 4 mol·L⁻¹, contacts between β 1- β 5 completely disappeared, resulting in the exposure of hydrophobic core and an extended conformation with $R_g \approx 1.8$ nm, which was much larger than $R_g \approx 1.2$ nm in 0, 1, and 2 mol·L⁻¹ urea. At the same time, contacts between β 3- β 5 were more broken than β 1- β 2 (Fig. 6d). This agrees with the experiment evidences that N-terminal (β 1, β 2, and α helix) was more stable than C-terminal (β 3, β 4, and β 5).^[12,63,64] At 6 mol·L⁻¹ urea concentration, bits of orderly secondary structural elements

(α -helix and β -sheet) could be detected, while there were not any orderly secondary structural elements (helices or sheets) in 8 mol·L⁻¹ urea (Fig. 5 and Table 1). Although most of orderly secondary structural elements vanished in 6 mol·L⁻¹ urea, some contacts between β 1- β 2 and β 3- β 5 sheets still remained (Fig. 6e). Those contacts completely disappeared as the urea was increased to 8 mol·L⁻¹ (Fig. 6f).

To examine the effect of incorporating SAXS structural information into MC-MD simulations on guiding the unfolding of ubiquitin, we also carried out the same simulation process without SAXS structural information in the MC-MD simulations. The corresponding characteristic parameters are listed in Tables S2 and S3 (in ESI), and the structur-

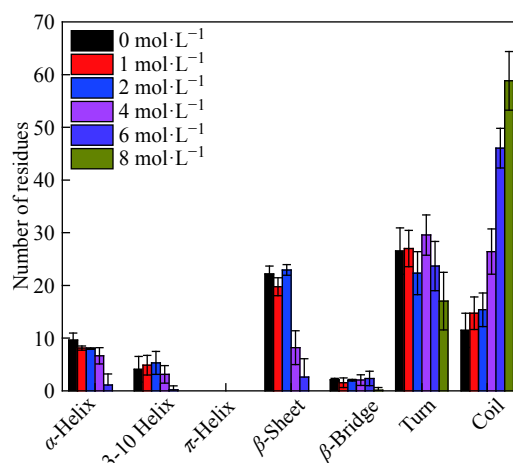


Fig. 5 The number of residues consisting of the corresponding secondary structural element of ubiquitin under different urea concentration from the MC-MD simulations including SAXS restraint

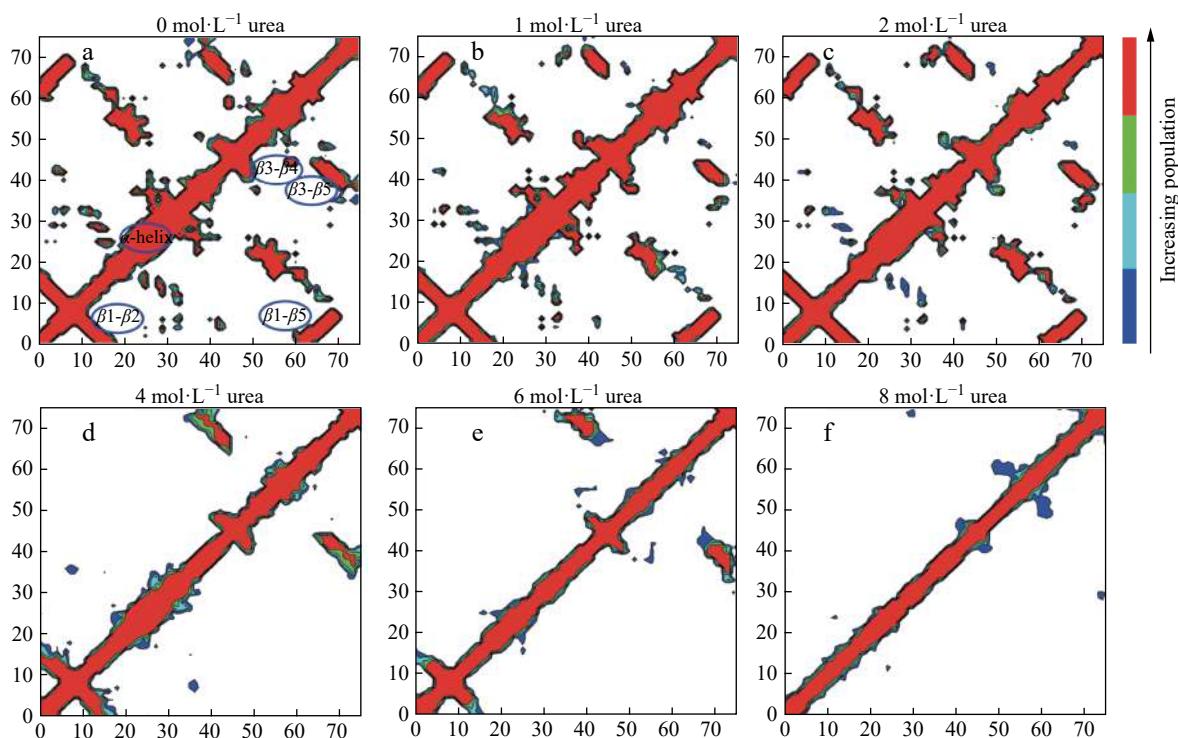


Fig. 6 Cross correlation maps of backbone $C\alpha$ atoms of ubiquitin conformations from the MC-MD simulations including SAXS restraint in (a) 0, (b) 1, (c) 2, (d) 4, (e) 6, and (f) 8 mol·L⁻¹ urea, respectively

Table 2 Mean distances between adjacent β -sheets in 0, 1, and 2 mol·L⁻¹ urea from the MC-MD simulations including SAXS restraint

Urea (mol·L ⁻¹)	$d(\beta1\beta2)$ (nm)	$d(\beta1\beta5)$ (nm)	$d(\beta3\beta5)$ (nm)	$d(\beta3\beta4)$ (nm)
0	0.44 ± 0.01	0.81 ± 0.01	0.50 ± 0.02	0.59 ± 0.03
1	0.48 ± 0.04	0.82 ± 0.03	0.59 ± 0.01	0.52 ± 0.01
2	0.45 ± 0.01	0.83 ± 0.02	0.58 ± 0.01	0.53 ± 0.02

al evolutions are depicted in Fig. S4 (in ESI). The results show that incorporating SAXS structural information into MC-MD simulations could not bring significant improvement on biasing the sampling space compared with the case without SAXS structural information at urea concentrations of 0, 1, and 2 mol·L⁻¹, whereas it could significantly guide the unfolding of ubiquitin into the targeted conformational space at high urea concentration (*i.e.* 4, 6, and 8 mol·L⁻¹).

Deciphering conformational states of proteins along the unfolding pathway is highly useful to help us understand the mechanism of protein folding because the order of native structure formation is correlated with relative structural stability in the unfolded state.^[7,9,65] The denatured states are paid with much attention because they are thought to serve as a starting point for protein folding. Our simulation results revealed that the conformational states of ubiquitin in 8 mol·L⁻¹ urea followed Flory's scaling law for a random coil in a good solvent. Almost no existing local residual structures and random coil conformations were in accordance with the NMR experiments^[10] and the MD simulations^[60] started from conformations refined by NMR and SAXS.^[14] In 4 mol·L⁻¹ urea, there are about 71.5% helices and 42.1% sheets maintained but with exposure of hydrophobic core. This illustrates that the denaturation of ubiquitin by urea goes through a sequential unfolding with a commensurate level of hydrogen-bond disruption and surface exposure. This sequential unfolding is in accordance with the reverse folding of ubiquitin, where the experimental observations of no early collapse phase occurred after denaturant dilution^[9] and the majority of structure and topology formed before the transition state.^[66] The sequential folding is expected to reduce the sampling search space and stabilize the formed intermediate structure which can reasonably explain the Levinthal's paradox.^[67,68]

Distributions and Dynamics of Urea/Water in the Hydration Shell

It is well known that distributions and dynamics of solvents in the hydration shell play significant roles in mediating the stability and functions of protein.^[31] To explore the effects of distributions and dynamics of urea/water on the unfolding of ubiquitin, all atomic MD simulations of 12 representative conformations spanning the whole unfolding (Fig. 4) were performed in the explicit 8 mol·L⁻¹ urea solution to eliminate the influence of urea concentrations on the absolute number of urea in FSS. Urea partial coefficient K_p is used for characterizing the relative distribution of urea in FSS and bulk. As listed in Table 3, K_p increased from 1.10 in the native state to 1.24 in the complete unfolded state induced by 8 mol·L⁻¹ urea solution. The growing number of urea molecules with the increasing extent of ubiquitin unfolding suggests that the newly exposed residues are more prone to interact with urea than with water. The invariant number of urea molecules in unit SASA (as displayed in Fig. S5 in ESI) also shows that the newly exposed residues are immediately wrapped by urea. The direct interaction of urea with the newly exposed residues should be beneficial to stabilizing the unfolded ubiquitin conformations and preventing ubiquitin from refolding,^[26,27] indicating that ubiquitin should be denatured by urea with the "direct mechanism".

The organization of urea and water in FSS was characterized by the distribution of their oxygen atoms (O) which depended on the distance away from any atom on the surface of ubiquitin, and is displayed in Fig. 7. Similar number and position of peaks can be observed for whether urea or water. Ratio_{UW} (the ratio of urea and water) within 0.16 nm distance of ubiquitin surface larger than 1.0 (inset in Fig. 7) suggests that urea penetrates into protein interior more deeply than water, leading to a larger effect on ubiquitin unfolding. Hydrogen bond (HB) analysis reveals that the first peaks of both urea and water should be the characteristic peaks of HB. Within 0.25 nm of protein surface, more than 60% of urea and water form HBs with ubiquitin, and as-formed HBs account for more than 90% of the total HBs between urea or water and ubiquitin. The number ratio between per urea and water formed HBs with ubiquitin is 1.89 in the native conformation and increases along the ubiquitin unfolding (Table 3), indicating that urea might possess much stronger

Table 3 Comparison of the solvent accessible surface area (SASA) of ubiquitin, the partition coefficient of urea in FSS and bulk (K_p), the ratio of hydrogen bonds (HBs) of urea and water formed with ubiquitin, and the HBs ratio of urea or water formed with backbone and side chain of ubiquitin in urea solutions of different concentrations

Model	SASA (nm ²)		K_p	Protein-solvent HB			
	Total	$R_{\text{hydrophobic}}$		R_{UW}	R_{UW}^*	$U_{\text{BB/SC}}$	$W_{\text{BB/SC}}$
0 mol·L ⁻¹	45.97	0.26	1.10 ± 0.18	0.49 ± 0.10	1.89 ± 0.40	0.81 ± 0.21	0.70 ± 0.09
1 mol·L ⁻¹	49.29	0.28	1.16 ± 0.13	0.51 ± 0.10	1.98 ± 0.40	0.96 ± 0.24	0.74 ± 0.12
2 mol·L ⁻¹	52.70	0.30	1.24 ± 0.13	0.55 ± 0.10	2.10 ± 0.38	0.96 ± 0.23	0.77 ± 0.11
4 mol·L ⁻¹ #	78.35	0.39	1.21 ± 0.13	0.58 ± 0.09	2.12 ± 0.34	1.34 ± 0.28	1.11 ± 0.14
6 mol·L ⁻¹ #	89.99	0.41	1.20 ± 0.11	0.62 ± 0.10	2.24 ± 0.35	1.61 ± 0.32	1.19 ± 0.15
8 mol·L ⁻¹ #	103.77	0.39	1.24 ± 0.10	0.66 ± 0.09	2.36 ± 0.32	1.80 ± 0.34	1.37 ± 0.16

#All results were obtained by averaging over 3 representative conformations (Fig. 4); $R_{\text{hydrophobic}}$ represents the proportion of SASA of hydrophobic residues in total SASA of ubiquitin; R_{UW} and R_{UW}^* are the ratios of protein-urea (U) HBs and protein-water (W) HBs before and after normalizing with the number of urea and water molecules in FSS, respectively; $U_{\text{BB/SC}}$ and $W_{\text{BB/SC}}$ are the ratios of HBs of urea and water formed with backbone (BB) and side chain (SC) of ubiquitin, respectively.

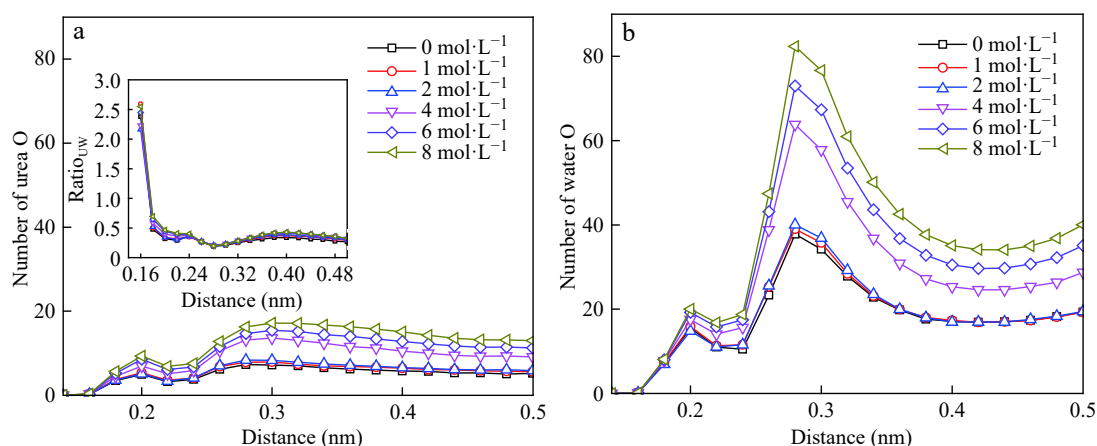


Fig. 7 Distributions of oxygen atoms of (a) urea and (b) water depending on the distance away from any atom of ubiquitin surface. The inset shows the distribution of ratio of oxygen atoms of urea and water in (a) and (b).

interaction with ubiquitin comparing with that of water. Furthermore, it could be found from Table 3 that the proportion of HBs of urea and water formed between the backbone and side chain of ubiquitin ($U_{BB/SC}$ and $W_{BB/SC}$) increases significantly as the ubiquitin becomes more outstretched. At the early unfolding states (1 and 2 mol·L⁻¹ urea concentrations), the increase of $U_{BB/SC}$ (from 0.81 to 0.96) is more evident than $W_{BB/SC}$ (from 0.70 to 0.77), which has also been found at the early unfolding of lysozyme using MD simulations.^[16] Considering that backbone HBs of ubiquitin play an import-

ant role in maintaining its secondary structure, the disruption or displacement of urea for the intra-backbone HBs of ubiquitin should have pivotal effects on denaturing the ubiquitin.

The microscopic dynamical properties of water and urea in contact with ubiquitin were investigated by survival time correlation function $S(t)$ and dipole-dipole time correlation function (TCF) $C_{\mu}(t)$, which characterize the translational and rotational dynamics, respectively. The corresponding decay curves are plotted in Fig. 8. The attenuations of urea are

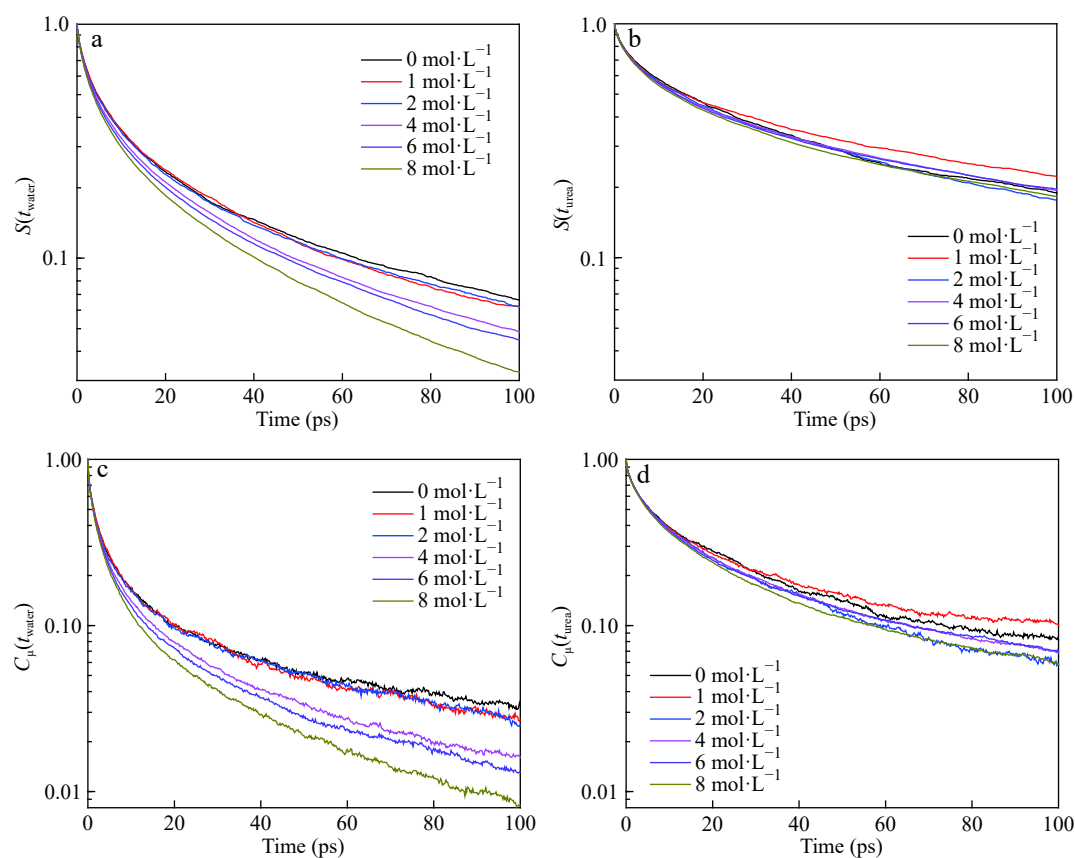


Fig. 8 (a, b) Survival time correlation function $S(t)$, and (c, d) rotational time correlation function (TCF) $C_{\mu}(t)$ of (a, c) water and (b, d) urea within 0.35 nm of any ubiquitin surface atoms. Results for 4, 6, and 8 mol·L⁻¹ urea were the average of 3 conformations.

apparently slower than that of water. To quantitatively describe and compare dynamics of urea and water, we fitted each of the decay curves with triple exponential function:

$$f(t) = \sum_{i=1}^3 A_i e^{-t/\tau_i} \quad (7)$$

where A_i and τ_i are the corresponding amplitudes and time constants, respectively. R^2 of all fitting curves are above 0.99. The amplitude-weighted average times ($\langle\tau\rangle = A_1\tau_1 + A_2\tau_2 + A_3\tau_3$) were calculated and are listed in Table 4. It can be found that the translational and rotational dynamics of urea are 2–3 times slower than that of water. The slower dynamical properties of urea might be attributed to its higher mass and stronger interactions with protein (about ~1.8 times stronger than that of water, Table 4). The average survival time and rotational relaxation time of water molecules in the hydration shell for native ubiquitin (0 mol·L⁻¹) are about 1.5 and 1.9 times longer than those for the fully unfolded ubiquitin (8 mol·L⁻¹), respectively. The 1.9 times faster rotational dynamics of water is in accordance with the experimental observations, where the rotational dynamics of water molecules in the hydration shell of unfolded protein L was twice faster than that in the folded state.^[69] However, the unfolding extent of ubiquitin has a marginal influence on the translational and rotational dynamics of urea molecules located in the hydration shell. The persistently slower dynamics of urea are favorable to stabilize the newly exposed residues during ubiquitin unfolding. In brief, the closer contact and long residence of urea around ubiquitin should be crucial for denaturing the ubiquitin.

Table 4 Amplitude-weighted average survival time ($\langle\tau_S\rangle$) and rotational time ($\langle\tau_C\rangle$) of water (W) and urea (U) molecules within 0.35 nm of any ubiquitin atoms and average interaction strength between them and protein

Model	Survival time (ps)		Rotational time (ps)		Interaction energy (kJ·mol ⁻¹)	
	$\langle\tau_S^W\rangle$	$\langle\tau_S^U\rangle$	$\langle\tau_C^W\rangle$	$\langle\tau_C^U\rangle$	$\langle E_{PW}\rangle$	$\langle E_{PU}\rangle$
0 mol·L ⁻¹	23.39	57.06	10.69	26.54	-21.31	-38.22
1 mol·L ⁻¹	21.92	66.10	10.05	30.39	-21.35	-36.92
2 mol·L ⁻¹	21.95	52.09	9.63	22.48	-20.97	-34.66
4 mol·L ⁻¹	18.21	57.77	7.09	22.77	-16.24	-28.84
6 mol·L ⁻¹	17.22	59.92	6.46	24.01	-15.20	-29.01
8 mol·L ⁻¹	14.84	55.17	5.53	21.17	-14.19	-26.79

CONCLUSIONS

In this investigation, we illustrated the urea-induced unfolding of ubiquitin using the hybrid MC-MD simulations integrated with SAXS structural information. In 1 and 2 mol·L⁻¹ urea, the majority of secondary structures in ubiquitin still remained and only $\beta 3$ - $\beta 5$ sheets started to be opened. Increasing urea to 4 and 6 mol·L⁻¹, ubiquitin unfolding reached intermediate states that contained a few secondary structures, till 8 mol·L⁻¹ it was totally unfolded into random coils. We found that urea molecules were enriched in the first hydration shell, and the degree of enrichment was more pronounced at the unfolded state than the native state. Urea has much stronger interaction with

ubiquitin comparing with water. As the ubiquitin became more unfolded, hydrogen bonds between urea/water and the backbone of ubiquitin were more preferentially formed, contributing to the disruption of secondary structure. The translational and rotational dynamics of urea molecules in the hydration shell were much slower than that of water molecules. Besides, the unfolding extent of ubiquitin conformations had a major influence on these dynamics of water molecules but hardly any effect on urea molecules. Persistent slow dynamics of urea on the surface of ubiquitin is responsible for the stabilization of the unfolded conformations.

Electronic Supplementary Information

Electronic supplementary information (ESI) is available free of charge in the online version of this article at <http://dx.doi.org/10.1007/s10118-019-2238-8>.

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