Atomistic Mechanism of Protein Denaturation by Urea

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Effects of urea on protein stability have been studied from all-atom molecular dynamics simulations of ubiquitin, G311 protein, and immunoglobulin binding domain (B1) of streptococcal protein G (GB1) in water and 8 M aqueous urea solution. The mechanism of the change in the solvent environment and the early events in protein unfolding by urea have been identified with emphasis on the change in the interactions of hydrophilic and hydrophobic parts of the protein by calculating the potential of mean force (PMF). Urea replaces the protein−protein and protein−water contacts by forming stronger contacts with the protein, which is indicated by the longer survival times of the protein−urea hydrogen bonds.

I. Introduction

Correct folding of proteins involve (i) efficient packing of the hydrophobic side chains and (ii) saturation of the hydrogen bonding capabilities of all the polar groups of the backbone and side chains, either intramolecularly or intermolecularly with water, ligands, etc. The breaking down of this folded state by denaturants, thus, must involve the replacement of protein−protein and protein−water contacts by protein−denaturant interactions along with the loosening of the hydrophobic core. Urea has a strong effect on the folding/unfolding transitions in protein, and the effect is concentration dependent. It is known that, in addition to promoting unfolded protein states at higher concentration, urea can accumulate at the surface of folded proteins at lower concentrations without significantly altering the three-dimensional structure of the protein. Despite an enormous volume of research, there is no universal molecular mechanism that can explain the observed interaction of urea with proteins. Several mechanisms have been proposed so far. Specifically, the most popular one is that urea acts as a water structure breaker.[1] Results have been reported indicating that urea reduces hydrophobic interactions by preferentially solvating the hydrophobic residues and protein unfolds.[2–4] Contradicting the above mechanisms, Thirumalai et al. proposed a mechanism based on electrostatic effects. Preferential adsorption of urea molecules on the charged hydrophilic residues of the protein leads to repulsion between the adjacent residues on the surface of the protein, resulting in swelling out of the protein exposing the hydrophobic core.[5] Recently, the above mechanism has been supported by theoretical work.[6] It has been also proposed very recently that the polypeptide backbone is the major denaturant binding site and gives an upper limit of a few nanoseconds for residence times of denaturant molecules on the polypeptide chain.[7,8] It has also been suggested[8] that urea does not denature proteins through favorable interactions with nonpolar side chains; what drives urea-induced protein unfolding is the large favorable interaction of urea with the peptide backbone. Only ≈25% of the newly exposed surface area favorably contributes to unfolding (because of newly exposed backbone units), with ≈75% modestly opposing urea-induced denaturation (originating from side-chain exposure).

To identify the exact behavior of protein in urea, we have performed all-atom molecular dynamics (MD) simulations in 8 M urea solution on three proteins: G311 (Protein Data Bank (PDB) accession code: 1ZXH), the B1 domain of streptococcal protein G (PDB: 1GB1), and ubiquitin (PDB: 1UBQ). The objective of our work is to study the effect of urea on the protein structure at the atomic level details, thereby understanding the underlying mechanism of urea-induced protein denaturation. We have also performed MD simulations in pure water to compare the results with those obtained from aqueous urea simulations.

II. Methods

NVT simulations were performed with the CHARMM package using the CHARMM22 force field and parameters. The urea parameters were taken from ref 9. SHAKE was used to maintain the bond lengths and angles of urea and water. A nonbonded cutoff of 8 Å was used, and the nonbonded list was updated every 25 steps. Periodic boundary conditions and a minimum image were used to reduce edge effects. PME was applied to deal with the long-range electrostatic interactions with a 9 Å cutoff, and the kappa value was adjusted to 0.32. The integration time step was 2 fs and the coordinates were saved every 2 ps for analysis. Electrically neutral simulation boxes were achieved by adding two Na^+ ions in the case of G311 and four Na^+ ions in the case of GB1. The initial coordinate of ubiquitin was obtained from Protein Data Bank (PDB accession code: 1UBQ). The first model from Protein Data Bank entries 1ZXH and 1GB1 were used as the starting structures for G311 and GB1, respectively.

Boxes of aqueous urea solutions of 8 M strength were prepared by following the procedures reported in earlier theoretical studies.[9,10] The 8 M aqueous urea solution box was prepared by randomly distributing 729 urea molecules in a 51 Å × 51 Å × 51 Å box and then immersing them an equilibrated 51 Å × 51 Å × 51 Å box of TIP3P water molecules. All water molecules overlapping with the urea molecules were removed. The 8 M urea box contained 729 urea molecules and 2744 water molecules. The box was minimized with 1000 steps of steepest descent (SD) minimization followed by 1000 steps of adopted basis Newton−Raphson (ABNR) minimization. The system was heated to 325 K for 50 ps and equilibrated for 300 ps. The average density of the system was calculated to be 1.109 g/mL.[9] The proteins were then immersed in the box separately, and

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the water and urea molecules overlapping with the protein molecules were deleted in each case. For deletion, the cutoff distance between any heavy atom of protein and urea/water was set to 2.6 Å. Finally, the 8 M aqueous urea system of ubiquitin contained 438 urea molecules and 1739 water molecules. For G311, 1793 water molecules and 460 urea molecules were present in the 8 M box. In the case of GB1, 464 urea molecules and 1784 water molecules were present in the final arrangement. Each system was then minimized by 1000 cycles of SD minimization followed by 1000 cycles of ABNR minimization. The system was again equilibrated for 500 ps. For each of the three protein systems, three independent trajectories were obtained. MD simulations were truncated after 16 ns for ubiquitin and after 11 ns for G311 and GB1.

We have also performed simulations of the three protein systems in pure water at 325 K following the above-mentioned procedure. Each of the proteins was solvated separately in a cubic water box of 48 Å lengths filled with TIP3P water molecules. Water molecules overlapping with the protein were deleted. Resulting water boxes had 2265, 2348, and 2321 water molecules for ubiquitin, G311, and GB1, respectively, giving rise to density in the range 0.945−0.952 g/mL in these three systems. A set of three independent MD simulation trajectories were obtained for each of the three proteins in water following the same procedure as that of the urea-containing systems. Each trajectory was truncated after 15 ns.

Potential of Mean Force (PMF). We have calculated the potential of mean force between the solutes using

$$W_{\alpha,\beta}(r) = -k_B T \ln(g(r)) \quad (1)$$

where $W_{\alpha,\beta}(r)$ ($\alpha = \beta = C_\beta$ of hydrophobic residue, or $\alpha = C_\beta$ of positively charged hydrophilic residue and $\beta = C_\beta$ of negatively charged hydrophilic residue) is the PMF, k_B is the Boltzmann constant, T is the simulation temperature, and $g(r)$ is the radial distribution function between the solutes.

Layer Survival Time Correlation Function. The layer survival time correlation function can be defined as

$$C_R(t) = \frac{1}{N_W} \sum_{j=1}^{N_W} \frac{\langle P_{R,J}(0)\, P_{R,J}(t)\rangle}{\langle P_{R,J}(0)^2\rangle} \quad (2)$$

where $P_{R,J}(t)$ is the binary function that takes the value of 1 if the jth water/urea molecule stays in the layer of thickness R, for a time t without getting out in the interim of this interval, and 0 otherwise. The quantity $C_R(t)$ measures the probability that a water/urea molecule remains in a given layer at a certain time t without getting exchanged with bulk water/urea before.

III. Results and Discussion

The analyses were done from all three independent trajectories in each protein system. The trends and averages of the data obtained were very similar (Supporting Information). The reported data contain analysis from one of the three trajectories for each of the three proteins. We have found no unfolding in any of the nine trajectories obtained for the three proteins in water. Hence, the unfolding that is observed for the proteins in 8 M aqueous urea solutions at 325 K is solely due to the denaturing power of urea and is not due to the effect of temperature. The urea-induced denaturation can hypothetically involve several steps: (1) breaking of the water structure by urea molecules; (2) removal of water solvation shell from protein surface; (3) formation of strong hydrogen bonds with polar groups of proteins; (4) potential ability of urea to form hydrogen bonds simultaneously with multiple amino acid residues, which in turn can readjust the local structures. To identify the structural features of the water−urea mixed system, various radial distribution functions are plotted in Figure 1 (for the ubiquitin system). The different classes of hydrogen bonds were observed between water and urea, namely water−water, water−urea, and urea−urea. The first peaks in the pair distribution functions located within 1.3−1.6 Å are indicative of the formation of the usual hydrogen bond network in the mixed system as in that of pure water. Thus the oxygen atoms in urea are distributed locally in a pattern similar to the water oxygen in bulk water. These observations are consistent in all three protein systems. The ability of large amounts of urea to dissolve in water is a consequence of the minimum disruption of the overall hydrogen bonding in aqueous solution. Contrary to previous postulations, the role of urea as structure breaker may not be the most crucial part of the mechanism in protein denaturation. The water−water radial distribution function remains almost identical in aqueous (0 M urea) and 8 M urea solutions (Figure 1), indicating strongly that urea does not alter the hydrogen bonding network of the water molecules.

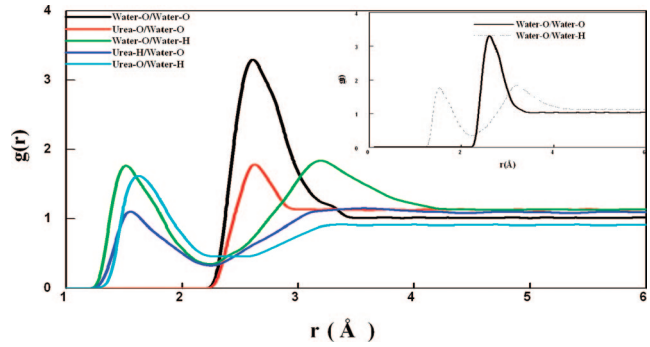

Figure 1. Urea−water and water−water radial distribution functions derived from 8 M aqueous urea solution of ubiquitin (indicated by five different colored solid lines, namely, black, red, green, blue, and cyan, for five different distributions for easy understanding). From the distributions, it is evident that aqueous urea solution can mimic the liquid water system as there is a nice positional overlap of the first peak in oxygen−oxygen distribution between urea−water and water−water. Inset: distributions for the hydrogen bonds between water molecules derived from the water simulation of ubiquitin (indicated by one solid line (—) and one dotted line (•••) for two different distributions).

PMFs were calculated by following the same procedure as done by Thirumalai.[6] In pure water the PMFs of the C_β's of hydrophobic and hydrophilic pairs are similar; i.e., both figures show a clearly defined contact minimum (CM), a barrier, and a solvent-separated minimum (SSM). In both solutions the positions of the minima (4 and 7 Å) remain the same; however, the depth of the minima increases at the CM by 0.2 kcal/mol in 8 M urea solution (Figure 2). This indicates a stabilization of both the hydrophobic and hydrophilic side chains in urea compared to that in aqueous solution. Thirumalai and co-workers[6] reported a shift by ∼2 Å in the locations of CM and SSM for the $M^+$and M^- pairs compared to the methane pairs, but they did not observe any changes in the depth of the minima, concluding that there is no significant difference in stabilization of the charged species in urea solution compared to that in the aqueous solution. This difference might arise due to the fact that we have used a full protein with all atoms explicitly defined whereas Thirumalai and co-workers used a pair of small solutes.

We next tried to explore whether urea alters the dynamics of the solvation shell of water, thereby disrupting the solvation pattern of the protein. The dynamic behavior of interfacial water can be described by evaluating the water molecule residence

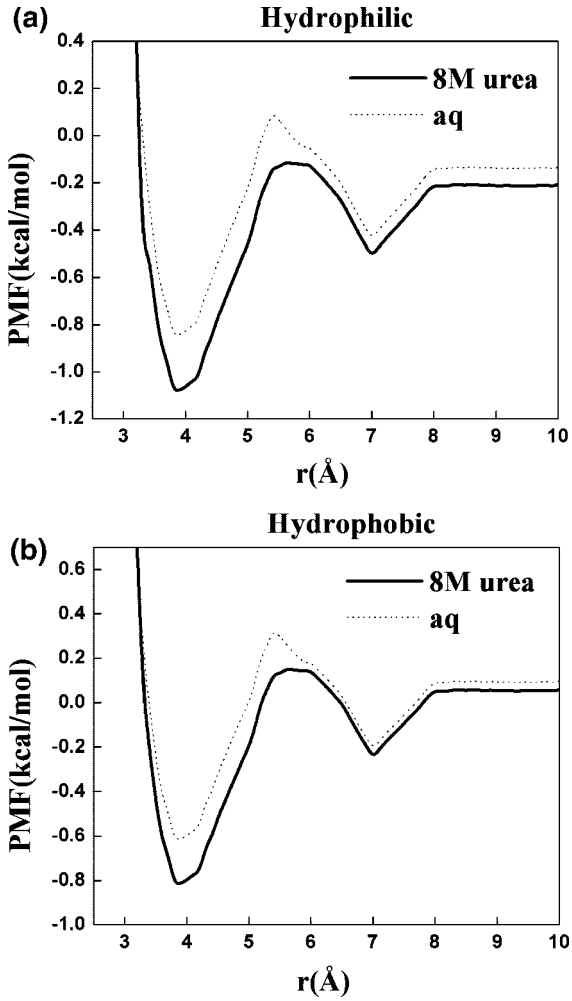

Figure 2. Potentials of mean force (PMFs) between the hydrophilic residues (a) and the hydrophobic residues (b) obtained from the 8 M aqueous urea (indicated by solid line, —) and water simulations (indicated by dotted line, •••) of ubiquitin. Only the C_β atoms of the corresponding residues are considered. A considerable change in the desolvation barrier was observed for each of the two categories.

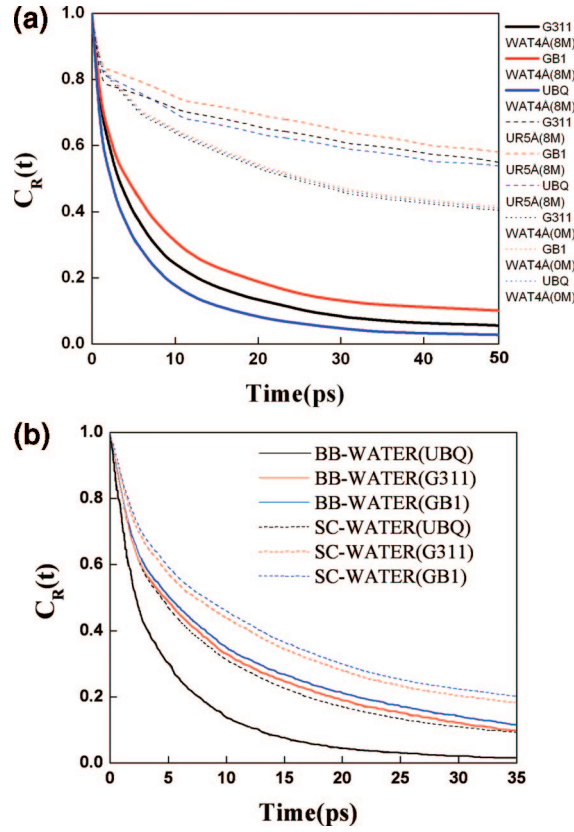

Figure 3. (a) Survival time correlation functions for all three protein systems (black for G311, red for GB1, and blue for ubiquitin). Solid lines (—) indicate protein−water hydrogen bond survival time obtained from the simulations in 8 M aqueous urea solution; dashed lines (---) demonstrate protein−urea hydrogen bond survival time obtained from the same solution, and dotted lines (•••) illustrate protein−water hydrogen bond survival time obtained from water simulations of the three proteins. For protein−water hydrogen bond survival time calculation, water molecules within 4 Å from the protein (i.e., within the first hydration shell; for both 8 M aqueous urea and water simulations) are considered, and for protein−urea hydrogen bond survival time calculation, urea molecules within 5 Å from the protein (8 M aqueous urea simulations) are considered. Protein−water hydrogen bonds in the first hydration shell are less stable in 8 M urea solution than in pure water, which is indicated by their faster decay, whereas protein−urea hydrogen bonds have considerably higher lifetimes. The parameters extracted by a second-order exponential fit are reported in Table 1. (b) Survival time correlation functions for all three protein systems (black for ubiquitin, red for G311, and blue for GB1). Solid lines (—) indicate backbone−water and dotted lines (•••) illustrate side chain−water hydrogen bond survival time correlation functions obtained from the simulations in 8 M aqueous urea solution of the three proteins.

time in the first hydration shell of protein atoms exposed to the solvent from an appropriately defined "layer survival time correlation function",[11] describing the probability of a water molecule remaining in a given layer at a certain time *t*, without getting exchanged with bulk water (Figure 3a). All the curves show a usual fast initial decay, on a time scale of a picosecond or even less, followed by a slower decay.[11,12] Comparison of these data shows that the mobility of the water molecules is much more enhanced in 8 M aqueous urea solutions than the observed trend in pure water. This high mobility of the water molecules, which are very close to the protein surface, is due to the penetration of urea molecules to the hydration shell of the protein, and this is probably the first step of the mechanism of protein denaturation by urea. The fact that urea can bind protein surfaces has long been reported.[13–16] We have also assessed the survival time correlation function for the protein−urea hydrogen bonds (Figure 3a). The fitting parameters of the decay curves for the protein−water and protein−urea survival times are shown in Table 1. It is evident for all three protein systems that the lifetimes of the protein−urea hydrogen bonds are significantly greater than those of the protein−water hydrogen bonds. Thus urea not only replaces the solvation shell water molecules, but also stays in the vicinity of the protein for a longer period of time. Moreover, urea preferentially disturbs the conformational pattern of the protein backbone rather than the side chain. This is validated by the survival time correlation functions which were calculated separately for water molecules attached to the backbones and side chains of the protein (Figure 3b). From the figure it is evident that the backbone bound water molecules show a faster decay than the side chain bound water molecules. This implies that, as simulation progresses, urea preferentially displaces the backbone bound water and makes contacts with it. Our observation is in good agreement with that proposed by earlier experiments.[7,8]

As urea molecules attach themselves successfully to the protein, the protein−protein and protein−water hydrogen bonds get replaced by protein−urea hydrogen bonds. Time evolution of the number of hydrogen bonds between protein−water and protein−urea are calculated separately for both the hydrophobic and charged hydrophilic residues of the three protein systems

TABLE 1: Fitting Parameters for the Survival Time Correlation Functions Shown in Figure 3a for All Three Proteins from both 8 M Aqueous Urea and Water Simulations [a]

protein	A	τ_s (ps)	B	τ_l (ps)
G311 (8 M) (water 4 Å)	0.4	1.36 ± 0.1	0.54	9.88 ± 0.36
GB1 (8 M) (water 4 Å)	0.3	0.87 ± 0.06	0.6	9.85 ± 0.19
UBQ (8 M) (water 4 Å)	0.4	1.7 ± 0.13	0.6	13.8 ± 0.71
G311 (0 M) (water 4 Å)	0.16	0.54 ± 0.066	0.46	18.54 ± 0.454
GB1 (0 M) (water 4 Å)	0.15	0.58 ± 0.07	0.46	18.60 ± 0.46
UBQ (0 M) (water 4 Å)	0.16	0.57 ± 0.069	0.46	18.57 ± 0.457
G311 (8 M) (urea 5 Å)	0.20	0.14 ± 0.03	0.32	34.54 ± 1.19
GB1 (8 M) (urea 5 Å)	0.15	0.29 ± 0.08	0.34	35.11 ± 1.93
UBQ (8 M) (urea 5 Å)	0.18	0.24 ± 0.15	0.32	21.18 ± 1.00

[a] Parameters are obtained by fitting the graphs by a second-order exponential. τ_s and τ_l are short- and long-time decay constants (in picoseconds), respectively, and A and B are the exponents.

(Figure 4) throughout the total simulation time. In doing so, a hydrogen bond is considered if the distance between the donor D and the acceptor A is ≤3.6 Å and the angle D—H---A is ≥120°. The number of hydrogen bonds of the hydrophilic residues with urea is found to increase more rapidly than the corresponding decrease in the number of hydrogen bonds with water. For the hydrophobic residues, the number of hydrogen bonds with water remains almost constant, but that with urea increases only slightly with time. This correlates well with the results obtained by Thirumalai et al.[6]

Interaction of the hydrophilic and hydrophobic residues with urea can also be inferred from the radial distribution functions involving urea and proteins (Figure 5; calculated for all three systems, shown for ubiquitin). The interaction between urea and charged side chains is illustrated in the pair function between the oxygen atoms on the side chains and hydrogen, nitrogen, and carbon on urea. Solvation of the charged side chains by urea is supported by the first and second peaks (Figure 5a). In addition to direct electrostatically dominated interactions with the charged side chains, urea forms hydrogen bonds with the carbonyl group of the protein backbone (Figure 5b). Urea interacts substantially also with the hydrophobic residues of the protein as indicated by the pairwise radial distribution plot (Figure 5c). Hydrogen bonding is the likely cause of the experimentally observed favorable free energy change upon transferring a peptide unit from water to aqueous denaturant solution.[21,22] This finding supports the direct binding mechanism of denaturation, where the hydrogen bonds between denaturant molecules and protein are thought to stabilize the denatured state and lead to protein denaturation.

The change in the number of protein−protein and protein−solvent contacts as a function of time was calculated. A contact is defined if the distance between two heavy atoms lies within 4 Å. For all three protein systems, the loss in number of contacts between protein−protein and protein−water is strongly correlated with the formation of protein−urea contacts (Figure 6a). Therefore, urea binds to the protein and consequently the protein releases a large number of water molecules to the bulk solvent. This phenomenon indicates that the denaturation process is entropically favorable. This is further validated in Figure 6b, where we have plotted the time evolution of the number of contacts between protein−urea and protein−water. We have found that the decrease in the number of protein−water contacts is many-fold higher than the increase in the number of protein−urea contacts. Thus, release of a large number of water molecules (favorable entropic factor) outweighs the effect that arises due to the binding of urea to protein (unfavorable entropic

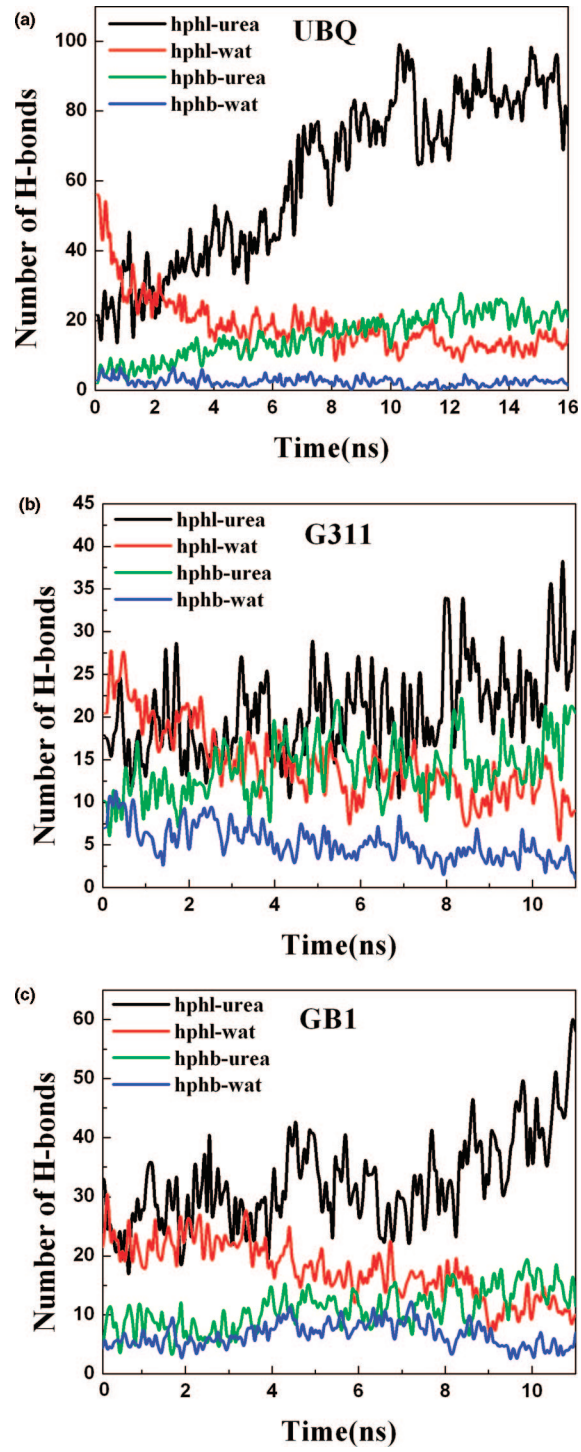

Figure 4. Time evolution of the number of hydrogen bonds with urea and water for the hydrophilic and the hydrophobic residues of (a) ubiquitin, (b) G311, and (c) protein GB1 obtained from 8 M aqueous urea simulation.

factor). It is seen that the loss in interaction energy of the protein with water is only partially compensated by the protein−urea interaction energy (figure not shown). Thus protein−urea interaction is enthalpically unfavorable. This is in nice agreement with the earlier findings, obtained from some model systems, that the entropic effect outweighs the opposing enthalpic effect in protein−urea interaction.[17−20] All these observations collectively suggest a possible mechanism of a urea-mediated denaturation pathway as follows: Urea becomes solvated in water without disrupting the water structure (due to its ability

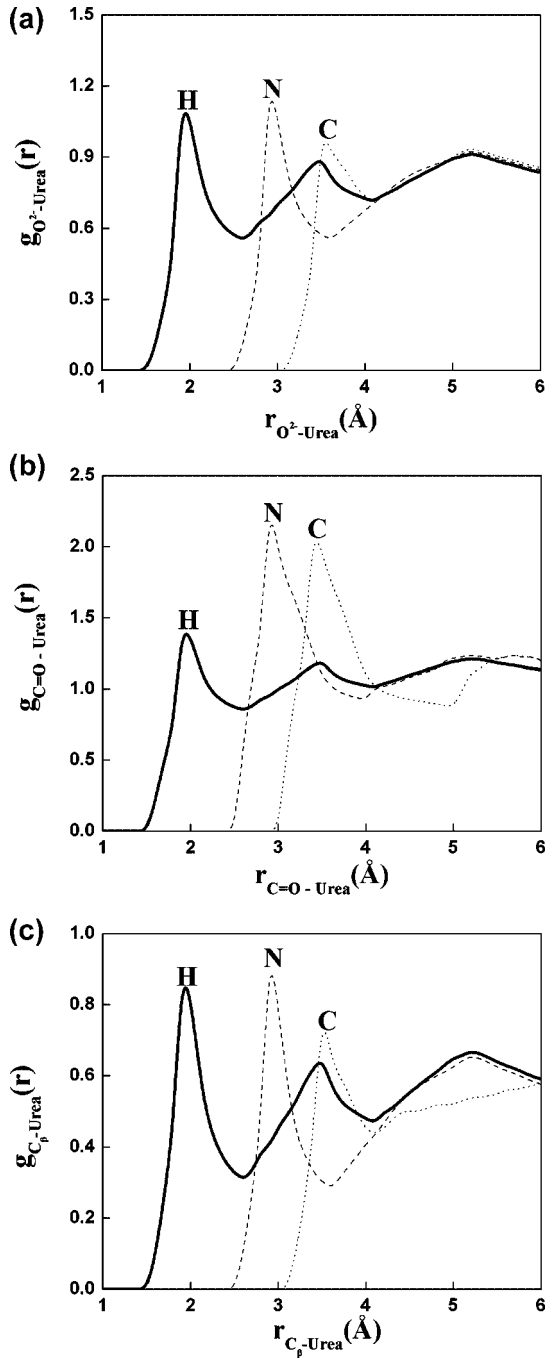

Figure 5. Radial distribution functions (a) between urea molecules and oxygen on the negatively charged side chains of ubiquitin at 8 M urea. Pair functions between hydrogen, nitrogen, and carbon on urea and oxygen on the negatively charged side chains are shown in solid (—), dashed (---), and dotted (•••) lines. (b) Radial distribution functions between urea molecules and oxygen that are part of backbone carbonyl groups of ubiquitin at 8 M urea. Pair functions between hydrogen, nitrogen, and carbon on urea and oxygen on the backbone carbonyl are shown in solid (—), dashed (---), and dotted (•••) lines. (c) Radial distribution functions between urea molecules and C_β atoms that are part of the hydrophobic residues of ubiquitin at 8 M urea. Pair functions between hydrogen, nitrogen, and carbon on urea and C_β atoms of the hydrophobic residues are shown in solid (—), dashed (---), and dotted (•••) lines. For the other two protein systems similar results are obtained.

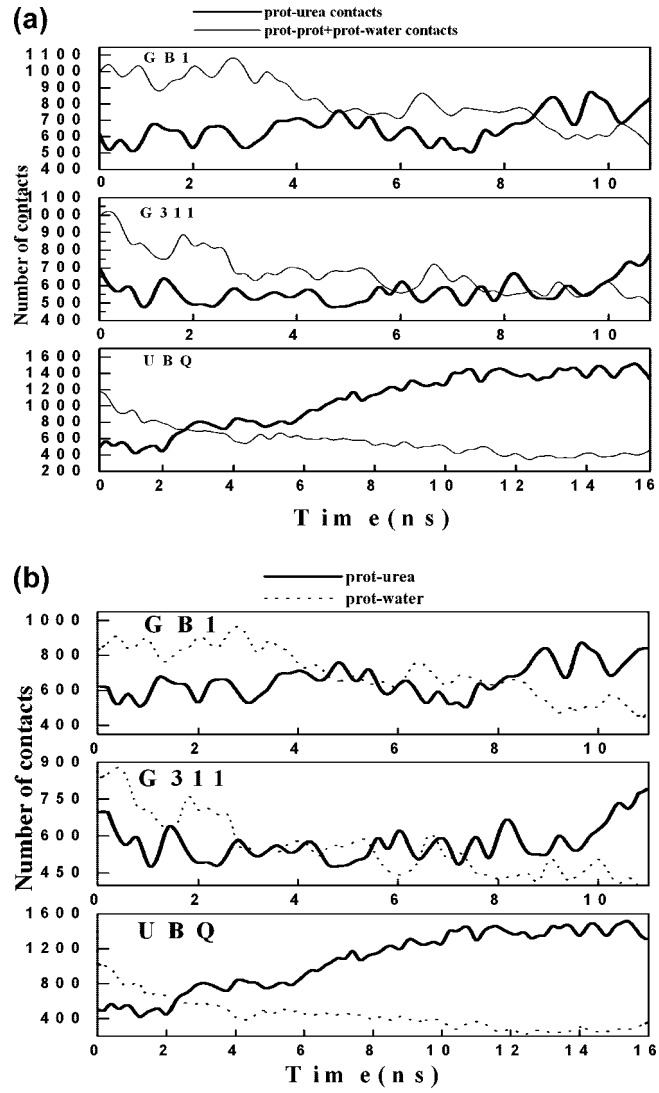

Figure 6. (a) Time evolution of the number of contacts of ubiquitin, G311, and protein GB1 obtained from 8 M aqueous urea simulations. Number of contacts within the protein, between protein and water, and between protein and urea are calculated as a function of simulation time. Then the sum of protein−protein and protein−water contacts is calculated and compared with the protein−urea contacts for all three protein systems. A nice correlation is obtained between these two types (protein−protein + protein−water and protein−urea) of contacts throughout the simulation time for all of the protein systems. This clarifies that urea breaks both the protein−protein and protein−water contacts. A contact is defined if the distance between two heavy atoms is within 4 Å. (b) Time evolution of the number of contacts of ubiquitin, G311, and protein GB1 obtained from 8 M aqueous urea simulations. Number of contacts between protein and water and also between protein and urea are plotted as a function of simulation time.

to form hydrogen bonds not only with itself but also with the protein and cosolvent). Urea then preferentially binds to the side chain atoms of charged hydrophilic residues exposed to the surface of the protein which triggers the loss of tertiary contacts. The long-lasting hydrogen bonds of protein−urea help the protein to experience an external drag that makes the core hydrophobic residues more exposed to the solvent (indicated by the increase in the solvent accessible surface area (SASA) in Figure 7), and consequently the protein starts to lose its tertiary contacts, giving more available sites for urea to bind.

IV. Conclusions

Motivated by the need to understand the structural basis of urea-induced destabilization of proteins, we have investigated the alterations in the hydrophobic and ionic interactions in protein in aqueous urea solution. Urea, a polar nonelectrolyte,

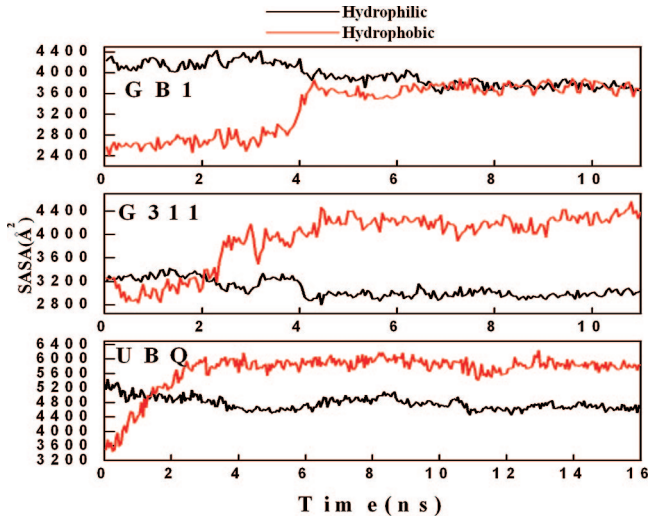

Figure 7. Change in solvent accessible surface area (SASA) with simulation time for the hydrophilic (black lines) and hydrophobic residues (red lines) of the three proteins. With progress of simulation time, SASA increases rapidly for the hydrophobic residues, but for the hydrophilic residues, a slow decrease is observed. This indicates gradual opening of the hydrophobic core of the protein.

can efficiently form hydrogen bonds with water molecules as well as with other species such as the protein backbone or charged species provided there are no restrictions due to excluded volume interactions. The ability of urea, which is twice as large as a water molecule, to efficiently form a hydrogen bond is the primary reason that the water structure is unperturbed even in high denaturant concentration (Figure 1). Urea forms hydrogen bonds with the protein backbone or with the charged hydrophilic residues in the same way as it does with water. This high ability of hydrogen bond formation of urea is because urea mimics the protein backbone. The hydrogen bonds formed by urea with protein have longer lifetimes (Figure 3a). From our simulations, we see that urea preferentially binds to the protein backbone (Figure 5b). However, probabilities of binding with the hydrophilic and the hydrophobic residues are comparable (Figure 5a,c). These observations lead us to the direct interaction mechanism in urea-mediated protein denaturation, that urea directly binds to the protein backbone, which is in good agreement with that reported by Thirumalai.[6] However, our results deviate from the above-mentioned work of Thirumalai[6] in the case of PMFs between the hydrophobic residues. The PMFs between the C_β atoms of the hydrophobic residues show that urea has a considerable effect on the depth of the contact minimum and also on the solvent-separated minimum (Figure 2b). However, this effect is also observed for the PMFs of the $C_\beta$atoms of the charged hydrophilic residues, but for the later one the effect is larger (Figure 2a). Thus, urea has effects on both the hydrophilic and hydrophobic residues.

In summary, we can say that the mechanism of urea-mediated protein denaturation consists of a series of events occurring one after another. At first, urea gets solvated in water by forming a large number of hydrogen bonds. Then it approaches the surface of the protein without disrupting the water structure. After penetrating the first hydration shell of protein, it binds to its surface by forming hydrogen bonds with the backbone and the charged hydrophilic residues exposed to the surface. It stays there for a considerable time. Then, due to electrostatic repulsions, the protein starts to unfold, providing a passageway to urea to the core hydrophobic residues. Thus more and more sites become available to urea, where it can form hydrogen bonds. This makes the unfolding process easier. As urea forms hydrogen bonds with the protein, a large amount of water molecules get released from the protein surface, which makes the unfolding process entropically favorable. With time the core hydrophobic residues get more and more exposed to the denaturant solution, which results in the increase in the solvent accessible surface area of the hydrophobic residues. Consequently, we have also seen a decrease in the SASA of the hydrophilic residues. These events collectively explain the swelling out mechanism of the core hydrophobic residues and also the protein denaturation.

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Supporting Information Available: Time evolution of radius of gyration (R_g) of all three proteins in all three independent trajectories. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Frank, H. S.; Franks, F. *J. Chem. Phys.* **1968**, *48*, 4746–4757.
(2) Tanford, C. *Adv. Protein Chem.* **1970**, *24*, 1–95.
(3) Roseman, M.; Jencks, W. P. *J. Am. Chem. Soc.* **1975**, *97*, 631–640.
(4) Alonso, D. O. V.; Dill, K. A. *Biochemistry* **1991**, *30*, 5974–5985.
(5) Wallqvist, A.; Covell, D. G.; Thirumalai, D. *J. Am. Chem. Soc.* **1998**, *120*, 427–428.
(6) O'Brien, E. P.; Dima, R. I.; Brooks, B.; Thirumalai, D. *J. Am. Chem. Soc.* **2007**, *129*, 7346–7353.
(7) Möglich, A.; Krieger, F.; Kiefhaber, T. *J. Mol. Biol.* **2005**, *345*, 153–162.
(8) Auton, M.; Holthauzen, L. M. F.; Bolen, D. W. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 15317–15322.
(9) Caflisch, A.; Karplus, M. *Structure (Folding Des.)* **1999**, *7*, 477–488.
(10) Smith, L. J.; Berendsen, H. J. C.; van Gunsteren, W. F. *J. Phys. Chem. B* **2004**, *108*, 1065–1071.
(11) Rocchi, C.; Bizzarri, A. R.; Cannistraro, S. *Phys. Rev. E* **1998**, *57*, 3315–3325.
(12) Dastidar, S. G.; Mukhopadhyay, C. *Phys. Rev. E* **2003**, *68*, 021921.
(13) Hibbard, L. S.; Tulinsky, A. *Biochemistry* **1978**, *17*, 5460–5468.
(14) Liepinsh, E.; Otting, G. *J. Am. Chem. Soc.* **1994**, *116*, 9670–9674.
(15) Lin, T.; Timasheff, S. N. *Biochemistry* **1994**, *33*, 12695–12701.
(16) Bennion, B. J.; Daggett, V. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 5142–5147.
(17) Wetlaufer, D. B.; Malik, S. K.; Stoller, L.; Coffin, R. L. *J. Am. Chem. Soc.* **1963**, *86*, 508–514.
(18) Brandts, J. F. *J. Am. Chem. Soc.* **1964**, *86*, 4291–4301.
(19) Creighton, T. E. *Biochem. J.* **1990**, *270*, 1–16.
(20) Zou, Q.; Habermann-Rottinghaus, S. M.; Murphy, K. P. *Proteins: Struct. Funct. Genet.* **1998**, *31*, 107–115.
(21) Nozaki, Y.; Tanford, C. *J. Biol. Chem.* **1970**, *245*, 1648–1652.
(22) Pace, C. N. *Methods Enzymol.* **1986**, *131*, 266–280.

JP800370E