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Effects of Urea, Tetramethyl Urea, and Trimethylamine *N*-Oxide on Aqueous Solution Structure and Solvation of Protein Backbones: A Molecular Dynamics Simulation Study

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The effects of urea, tetramethyl urea (TMU), and trimethylamine *N*-oxide (TMAO) on the structure and dynamics of aqueous solutions are studied using molecular dynamics simulations. It was found that urea has little effects on the water−water hydrogen-bond length and angle distributions except that it induces a slight collapse of the second shell in the hydrogen-bonding network. TMU and TMAO both strengthen the individual hydrogen bonds and significantly slow the orientational relaxation of water, but have opposite effects on the second shell structure of the hydrogen-bonding network: TMU distorts while TMAO enhances the tetrahedral water structure. Furthermore, TMAO significantly weakens the interactions between the amide carbonyl group and the water molecules, while TMU and urea both strengthen these interactions, with the effect of urea being much less significant than that of TMU. These conclusions are supported by molecular dynamics simulations of three different systems: a model amide compound $CH_3-NH-CO-CH_3$ (NMA), and two polypeptides, GB1 and ELP. Consistent with earlier studies, we also found that urea interacts strongly with the carbonyl group through direct hydrogen bonding. The simulations for the denaturation of the polypeptide GB1 in urea solutions showed that the breaking of its native hydrogen bonds follows a step-by-step process and each step is strongly coupled to the formation of water−carbonyl hydrogen bonds, and to a less extent to the urea−carbonyl hydrogen-bond formation. Our simulation results reveal the potential importance of the indirect effects of cosolvents in protein denaturation or structure protection, particularly through modifying of the water−amide interactions.

Introduction

Many cosolvents,[1−5] such as urea, tetramethyl urea (TMU), and trimethylamine *N*-oxide (TMAO), have well-documented effects on the solubility of other molecules in aqueous solutions and are especially well-known for their effects on protein structure formation and stability.[1,3,6−38] The understanding of the effects of these small molecules on protein structures plays a key role in elucidating the protein folding and unfolding mechanism.[2] In particular, two different mechanisms, direct and indirect, have been proposed for the urea-induced protein denaturing. On the basis of transfer free energy experiments, Tanford[39] postulated that urea denatures protein by an indirect change of the hydrophobic effects, through a so-called water structure breaking mechanism. In contrast, a direct interaction model[40] hypothesizes that urea changes structure and solubility of proteins in aqueous solutions by direct interacting with polar and/or apolar residues, as well as by hydrogen bonding to the backbones.[41−45] According to the examination on the roles of interactions involving protein backbone and/or side chains, both direct and indirect effects have been discussed extensively in the literature, although no decisive and indisputable conclusion has been made.[3,4,12,14,39,43,46−56]

Additionally, urea also has a well-documented effect on the solubility of simple hydrocarbons:[57,58] it decreases the solubility of methane and ethane but increases that of propane and larger hydrocarbons. At the same time, the even stronger denaturing effects of TMU,[4] which lacks the hydrogen-donating capability, also suggest that effects other than direct hydrogen bonding play crucial roles in disturbing the solubility of hydrophobes in aqueous solutions. These long-existing observations suggest that either indirect effect through changes of the water hydrogen-bonding network or direct van der Waals interactions between these denaturants and the solute is important in understanding the aqueous solutions.

At the same time, a large number of experiments have been devoted to examine the cosolvent effects on water structure and dynamics. Infrared experiments have indicated that urea has little effects on the water structure, while TMAO, on the other hand, significantly changes the water spectra.[59] Experimental dynamics studies, in particular on water orientational relaxation, have shown that TMU significantly slows the water dynamics.[60] Again, urea shows little effects, in contrast.[61] In addition, NMR experiments[37,62] on protein structures have revealed that the addition of urea to the protein solution resulted in a more flexible protein backbone, in particular the N−H bond, while the addition of TMAO reduced this flexibility and made the protein backbone more rigid. These studies suggested that direct interactions, possibly through the hydrogen bonding between urea and the protein backbone, are likely responsible for the denaturing effects of urea, due to its lacking of effects on water structure and dynamics,[41,42,44,45] while TMAO and TMU could have stronger indirect effects on proteins.

The denaturation of a number of proteins and polypeptides by urea studied using molecular dynamics simulations[1,6,24,63−66] has not been conclusive on whether direct or indirect effect is the main driving force for protein denaturation. In the indirect mechanism, urea was observed to "free" water molecules from its hydrogen-bonding network, which then interacts more strongly with the protein backbone,[42] while the direct interacting mechanism involves the interaction of urea with both the

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backbone and the polar residues and/or the apolar side chains.[66,67] Similarly, both the direct and the indirect mechanisms have also been considered and tested for TMAO. In particular, the simulations by Zou et al. are consistent with the transfer free energy studies,[48] which show that the main effect on the relative stability between the native and unfolded states arises from interactions with the protein backbone, with TMAO disfavoring the unfolded state. This study also showed that TMAO significantly changes water structure and hydrogen bonding, consistent with an indirect effect. However, other simulation studies questioned the existence of the indirect effect of TMAO on water structure.[11,12] A more complete analysis of the effects of TMAO on water hydrogen-bonding interactions and a better characterization of the indirect effects of TMAO are needed.

Another important question in understanding the effects of cosolvents on protein structures has been on the side-chain hydrophobic interactions. Probably partly due to the cancellation of hydrophobic and hydrophilic effects[12] and due to the weakness of each individual factors, the effects of TMAO on the hydrophobic interactions have been found small by simulations,[11,12] in agreement with experiments.[14,48] The contribution of side chains in transfer free energy was also found to be small[14,54,68,69] in the case of urea solution. In contrast, the transfer energy of an amide group from water to TMAO (as discussed earlier) or urea solution was found to be large, which is about 1 order of magnitude larger than that for the side chains, with the former being less and the latter more favored for the unfolded states.[14,48,70] It is interesting to note that a recent simulation showed that the side chains could play more important roles if they possess strong van der Waals interactions,[42,71] based on which protein denaturation mechanism driven by direct dispersion interactions between urea and the proteins was proposed. Their results on the van der Waals interaction between urea and other molecules are consistent with earlier simulation studies of Ikeguchi et al.[72] and the theoretical analysis of Graziano.[73] The importance of the direct van der Waals interaction between urea and protein side chains was also shown in an impressive 1 μs MD simulation for denaturing of lysozyme in 8 M urea solution.[14,64]

As discussed above, the concept of "direct" and "indirect" effects on the protein stability in aqueous solutions with cosolvents added has been widely used. Either or both effects might play essential roles in denaturing or renaturing of proteins. Because of the complexity in the interactions between water, cosolvent, and solute, a molecular-detailed characterization of the "indirect" effects on protein structure is unclear and questionable, yet. Apparently, a unified atomic-detailed picture of denaturant/renaturant on their indirect effects on protein hydration is still missing. For example, the mile-stone simulation for the lysozyme denaturation described in great detail the direct van der Waals interactions between urea and side chains and further suggested these interactions as the driving force for protein denaturation,[14,64] but the roles that protein backbone plays in this process were not addressed. In contrast, it was experimentally found that the transfer free energy of protein backbone from water to urea is much greater than that of amino acid side chains.[14,48,54,68−70] Furthermore, the molecular mechanism of native hydrogen bonds breaking has not been investigated in these earlier studies, although the contact of urea and water to protein has been analyzed in detail in these simulations.

In this study, to investigate whether cosolvents affect water/water or water/protein interactions, we first perform detailed analyses and comparison of the effects of urea, TMAO, and TMU on the bulk water properties, in particular on its hydrogen-bonding network. It was found that urea has very weak effects on the water−water hydrogen-bond length and angle distributions, consistent with earlier studies.[74] TMU and TMAO both strengthen the individual hydrogen bonds and significantly slow the orientational relaxation of water, but have opposite effects on the second shell structure of the hydrogen-bonding network: TMU distorts while TMAO enhances the tetrahedral water structure. These results suggest the possibility of indirect effects of the cosolvents, in particular TMU and TMAO, on protein structures. We then examine whether urea, TMAO, or TMU have any significant effect on the interactions between water and proteins using MD simulations. In particular, motivated by the experimental observation that amide group contribution dominates the transfer free energy, we focus on the interaction between the solvent/cosolvent and the protein amide group. Two protein systems were simulated in the present study, a short peptide from the B-domain of the G-protein (hereinafter called GB1) and a 90-residue elastin-like protein (ELP). Simulations were performed with the folded GB1 structure in pure water and in urea solutions, to study the unfolding process. It was found that urea easily denatures GB1 in two independent 100 ns trajectories. Simulations were also performed for the unfolded GB1 in different solutions and conditions, to analyze in detail the interactions between different components of the polypeptide and various solvent/cosolvent molecules. By examination of the effects of cosolvents on the interaction between water and the polypeptide, it was found that, consistent with their effects on the tetrahedral water hydrogen-bonding structure, TMAO weakens the hydration of the carbonyl group on polypeptide backbone and TMU strengthens the water−carbonyl interactions. Urea also strengthens the interactions between water and the amide carbonyl group, but to a less extent as compared to TMU. On the other hand, significant hydrogen-bonding interactions were also identified between urea and the amide groups, suggesting that both direct and indirect mechanisms are involved in protein denaturation in urea solution. Besides the hydrogen bonds to the backbone, interactions between the solvent/cosolvent molecules and the side chains of polypeptide were also analyzed, and the results suggest that the extent of accumulation around the side chains heavily depends on cosolvents as well as the properties of the side chains.

To further evaluate how cosolvents affect water−protein interactions and protein conformations, similar simulations and analysis were also performed for ELP in TMAO and TMU solution, indicating that the two molecules strongly affect water structures and the interactions between water and the GB1 amide groups. It was found that ELP takes an extended structure in the TMU solution and, in contrast, a compact structure in the TMAO solution, consistent with the improved hydration of the amide carbonyl in the presence of TMU and a depletion of interacting water in the presence of TMAO, as evident from their effects on the radial distribution function of water surrounding the carbonyl group.

Simulation Details

For all simulations presented here, the SPC/E[75] water model was used. All MD simulations were performed using the AMBER 9 suite of programs.[76] The force field for urea was developed by Duffy et al.,[77,78] and that for TMAO was developed by Kast et al.[79] The geometry and intramolecular terms of the force field for TMU were kept the same as those for urea, while the AMBER99 force field was utilized for the methyl groups. The charge distributions on all atoms in TMU

were obtained using the RESP-fit method[80,81] based on B3LYP/cc-pVTZ density functional theory (DFT) calculations.[82−85]

Cuboid boxes were created with water and solute molecules evenly distributed in the initial configuration. The systems of water, urea, TMAO, and TMU solutions contain 8049, 6898, 6115, and 4359 waters, respectively. All solutions contain 800 solute molecules, resulting in ∼5 M urea and TMAO solutions, as well as a ∼4 M TMU solution.

To obtain a reasonable initial structure, an NPT (number, pressure, temperature) ensemble calculation was performed first. In this process, a 20 ps MD simulation was first conducted to heat the system from 0 to 360 K, followed by a 200 ps equilibration at 360 K and 1 atm. Next, the system was cooled to 300 K by another 20 ps MD simulation followed by a 1 ns equilibration at 300 K and 1 atm. The resulting final cuboid boxes for pure water, urea, TMAO, TMAO/urea, and TMU are approximately 40 × 40 × 155, 37 × 37 × 200, 40 × 40 × 190, 35 × 35 × 210, and 40 × 40 × 210 Å^3, respectively.

The simulation and analysis of trajectories of urea denaturation of the β-hairpin structure formed by the 16 C-terminal residues (GEWTYDDATKTFTVTE) of protein GB1 were also performed using the AMBER 9.0 program. The initial structure was taken from the model conformation of the full Protein G (PDB code: 1GB1). The terminals of GB1 peptide are oppositely charged. Boxes of aqueous urea solution of 5 M strength were prepared by immersing 16-residue peptide of GB1 into a cubic box containing 720 urea molecules and 6180 SPC/E water molecules (5 M, in a separate simulation an 8 M urea solution was also used), and three Na^+ ions were added to neutralize the charge of the system.

The system was minimized with a total of 2500 steps: 1000 of steepest descent with the peptide being fixed by harmonic restraints of a force constant of 500.0 kcal mol^{-1} Å^{-2} applied to the backbone atoms, followed by 1500 steps of conjugate gradient minimization. After that, the system was heated to 360 K for 20 ps and equilibrated for 1 ns at 360 K, followed by a 200 ps of cooling from 360 to 300 K performed with harmonic restraints (force constant = 10.0 kcal mol^{-1} Å^{-2}) applied to the backbone. The production run of 100 ns was used for the data analysis, with the data being collected every 0.5 ps.

We have also performed three simulations on the unfolded β-hairpin peptide obtained from the urea denaturation of peptide in the pure water, TMU, and TMAO system at 300 K following the above-described procedure. The water system contains 7766 SPC/E water molecules, and the TMU system contains 752 TMU and 5532 SPC/E waters, corresponding to a TMU concentration of ∼4 M. The TMAO system contains 720 TMAO molecules and 5571 SPC/E waters, corresponding to a TMAO concentration of ∼5 M. For each system, a 20 ns trajectory was obtained following the same procedure as described above for the urea system.

To more clearly analyze the interactions between amide groups and solvent molecules, and to avoid the complication brought in by protein side chains, a model compound, $CH_3-NH-CO-CH_3$ (NMA), in different solvent systems, pure water, 5 M urea, 4 M TMU, and 5 M TMAO, was simulated using the same molecular dynamics procedure described above. The initial configuration of the pure water system was generated with 923 water molecules; the 5 M urea system contains 78 urea molecules and 670 waters; the 4 M TMU system contains 63 TMU molecules and 470 waters; and the 5 M TMAO system contains 78 TMAO molecules and 596 waters.

Also, simulations of the elastin-like polypentapeptide, $(VPGVG)_{18}$ in water, TMU, and TMAO solutions, were performed at 300 K following the same procedure as described above. The β-spiral model of the polypeptide was taken from ref 86. Besides the polypeptide, the TMU system contains 1008 TMUs and 7455 SPC/E waters with a TMU concentration of ∼4 M, and the TMAO system contains 1008 TMAO molecules and 7779 SPC/E waters with a TMAO concentration of ∼5 M.

All of these systems were further extended by using the periodic boundary condition. The SHAKE algorithm[87] was used to constrain all bonds involving hydrogens. A cutoff of 10.0 Å was applied for nonbonding interactions. The Particle Mesh Ewald method was applied to treat long-range electrostatic interactions.[88]

Hydrogen Bonds Definition

The hydrogen bond, analyzed in the simulations, is defined as a geometry with a cutoff length of 3.2 Å between the two heavy atoms of the hydrogen donor and acceptor and an X−H···Y (X and Y stand for heavy atoms) angle cutoff of 135°. A hydrogen bond is counted when the distance between X and Y is less than 3.2 Å and the X−H···Y angle is greater than 135°.

Results

1. Effects of Cosolvent on Water Hydrogen Bonding and Dynamics. Various structural and dynamical properties of water, especially of the hydrogen-bond network, in pure water and the aqueous solutions of urea, TMAO, and TMU were investigated first. In Figure 1a−c, two-body distribution functions, the radial distribution function of water oxygen around a central water oxygen atom and the water hydrogen-bond length and angle distribution functions, are shown for these systems. Figure 1d shows the O−O−O angle distribution for three water molecules that are connected by hydrogen bonds.

An inspection of Figure 1a−c reveals that the various cosolvents do affect the water interactions in very different ways: we first notice that all results shown in Figure 1 for water in urea solution are similar to that in the pure water system. The two-body distribution functions of water, including hydrogen-bond angle and length, oxygen−oxygen pair distribution functions, and the three-body O−O−O angle distribution function, are all only slightly perturbed when urea is added, with the most noticeable change being found in the O−O radial distribution function (g_{oo}). These observations are consistent with earlier simulation results and with the notion that urea fits well into the water hydrogen-bond network.[74,89] The analysis of the orientational relaxation of water molecules in a urea solution and in pure water in Figure 2 shows that the two systems are also almost identical, again consistent with the experimental results.[61] However, a noticeable rise of the first valley in the water oxygen−water oxygen radial distribution function can be observed when urea is added as shown in Figure 1a. This change is qualitatively consistent with conclusions drawn from neutron diffraction experiments that urea induces a reduction in the distance of the second shell in the water hydrogen-bonding network, and this change resembles that induced by a high pressure.[90] The slight collapse of the second water shell around a water molecule in urea solution relative to the pure water can also be observed in the spatial density function shown in Figure 3.

TMAO and TMU, in contrast to urea, both have significant effects on the various water distribution functions. We note first that the g_{oo} for water in TMAO and TMU aqueous solutions has a more pronounced first peak at 2.75 Å. As compared to that for pure water, g_{oo} for the TMAO system shows a deeper

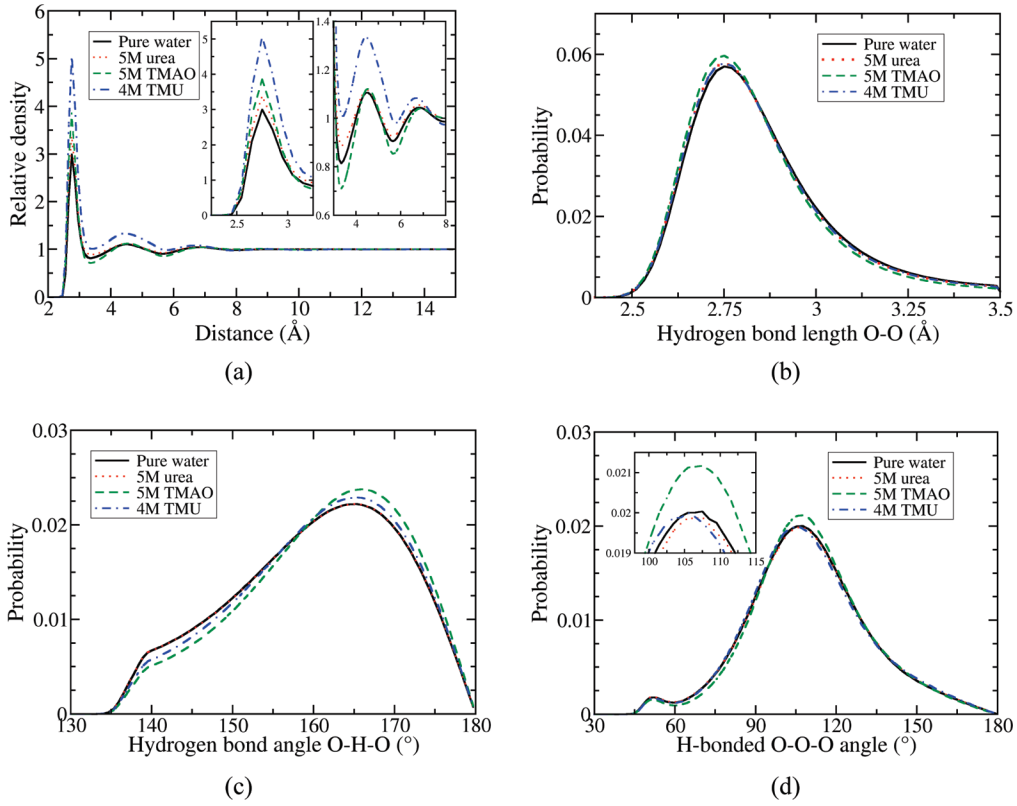

Figure 1. (a) Radial distribution function (RDF) of water oxygen to water oxygen; (b) hydrogen-bond (between water molecules) length distribution; (c) hydrogen-bond (between water molecules) angle distribution; and (d) hydrogen-bonded waters' O−O−O angle distribution.

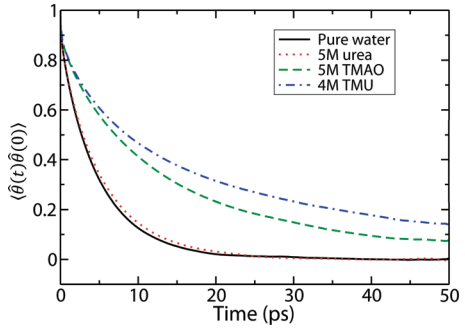

Figure 2. Orientational relaxation of water molecules in pure water, urea, TMAO, and TMU solutions.

valley at the first minimum at 3.4 Å and a more pronounced second peak at 4.5 Å, while TMU makes the first valley shallower. The change of the first two peaks was observed for TMAO in earlier simulations and used as supporting evidence for its structure making property.[48] In addition, the position of the second peak in TMAO solution is ∼0.1 Å further outward than that of the pure water, again suggesting a more ordered water structure, and also a more pronounced and outer-positioned third peak of g_{oo} is also noticeable. On the other hand, although TMU induces a higher first peak for g_{oo}, it leads to a less pronounced second peak and a shallower first minimum. Furthermore, the positions of the second and third peaks are both slightly shifted inward, corresponding a collapse of the water structure (such a collapse of outer shells was also observed in the low to high density water transition induced by increased pressure,[91] as seen in Figure 4).

The distributions for water−water hydrogen-bond length and angle in Figure 1b and c show that TMAO and TMU, to a less extent, both increase the ratio of the strong hydrogen bonds indicated by shorter hydrogen-bond lengths and larger hydrogen-bond angles: the probability distribution at the hydrogen-bond length of 2.75 Å and at the hydrogen-bond angle of 165−170° significantly increases in TMAO solution as compared to the water system. These results suggest that on average the individual water−water hydrogen bonds become stronger upon the addition of TMAO or TMU.

However, an analysis of the O−O−O angle distribution function as given in Figure 1d shows that TMAO and TMU have very different effects on the water hydrogen-bond network. TMAO significantly increases the probability of ordered water structures characterized by the tetrahedral angle of 109.47°. It is indicated, from the comparison in Figure 1d, that the addition of TMAO leads to a narrower distribution of the O−O−O angle centered at ∼105°. TMU, in contrast, shifts this distribution to smaller angles and slightly broadens this distribution. (In an earlier study, instead of hydrogen-bonded O−O−O angles but O−O−O angles formed by all four nearest neighbors were used. We note here that due to the hydrogen bonding between water and TMAO, some of the nearest water molecules are inevitably at a rather large distance from the center water molecule and would bias the calculated angle distribution function or the q parameter[11] in direct comparison with the pure water system.) Overall, all of the examined features of the water hydrogen bond in TMAO aqueous solution are consistent with a more ordered water structure. The change of distribution function of the O−O−O angle of water molecules by TMU, however, indicates that it has a "structure breaking" effect on the tetrahedral hydrogen-bonding network, although it appears to strengthen the individual water−water hydrogen bonds.

2. The Step-by-Step Unfolding of GB1 in the Presence of Urea. Two independent 100 ns molecular dynamics simulation trajectories showed that GB1 is easily denatured at room temperature and 1 atm in the 5 or 8 M urea aqueous solutions. The two trajectories show very similar processes for the GB1

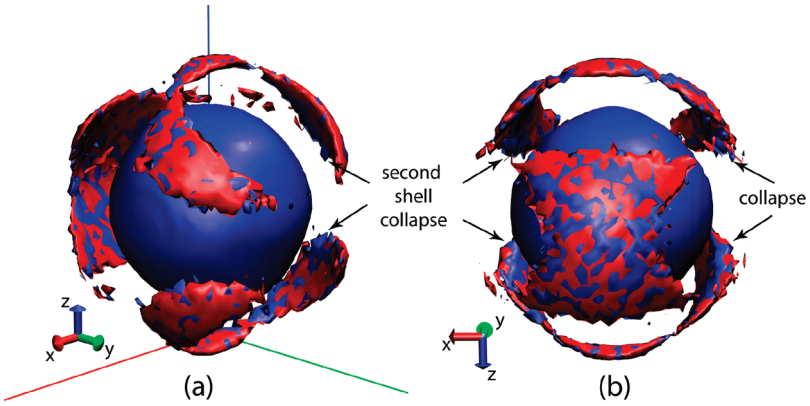

Figure 3. Spatial density function for water in pure water (red) and 5 M urea solution (blue). The spatial density was computed in a box of 30 × 30 × 30 Å^3. The oxygen of a water molecule is positioned at the center of the box, and the water molecule lies in the *yz* plane with the water dipole parallel to axis *y*.

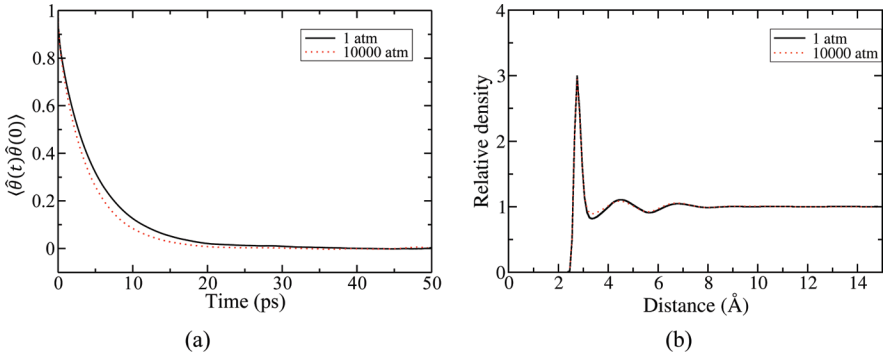

Figure 4. The orientational relaxation (a) and O−O radial distribution function (b) of water at 1 and 10 000 atm, calculated using SPC/E water model at 300 K.

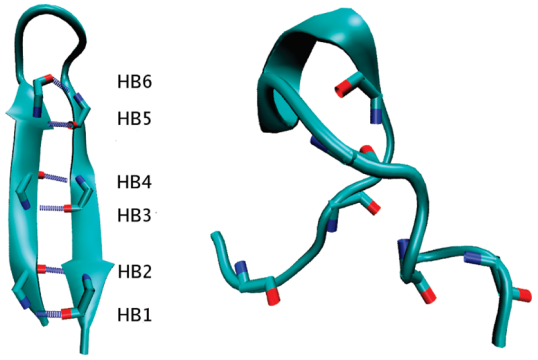

Figure 5. The native (left, with the six native backbone hydrogen bonds labeled on the side) and denaturated (right) structures of GB1.

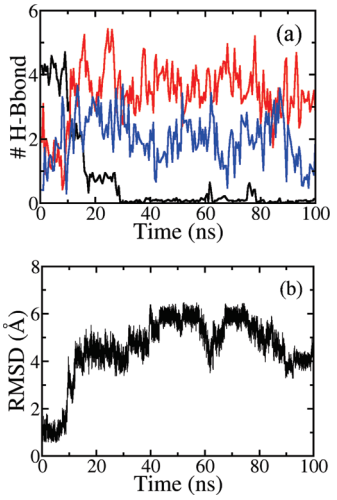

Figure 6. (a) The average number of native backbone hydrogen bonds (black), the numbers of hydrogen bonds formed with water (red), and with urea (blue), as a function of time. (b) The backbone root-mean-square displacement (rmsd) from the native structure as a function of time.

denaturation, and only results from the trajectory of the 5 M urea system are presented in detail. The denaturation of the β-hairpin structure of GB1 in urea aqueous solution is clearly seen from the breaking of the native hydrogen bonds (Figure 5). The same native structure remains stable in a control simulation in pure water at 330 K for more than 50 ns. The evolution of the number of native backbone hydrogen bonds and the root square displacement (rmsd) as a function of time are shown in Figure 6 to illustrate the denaturing process, and it indicates that the denaturing of GB1 in 5 M urea solution is a quick and spontaneous process. All native backbone hydrogen bonds break in less than 30 ns and remain broken during the rest of the 100 ns simulation. Moreover, the breaking of backbone hydrogen bonds is coupled to the hydrogen-bond formation between the polypeptide backbone and urea/water. In the denatured structure, both urea and water form a significant number of hydrogen bonds with the backbone, with hydrogen bonds to water being preferred, presumably due to the higher density of water (although the ratio between the number of the two types of hydrogen bonds remains almost the same for 8 M urea solution). Figure 6 also shows that the breaking of the native backbone hydrogen bonds is more strongly coupled to the formation of hydrogen bonds between water and the backbone, although the initial 10 ns trajectory is characterized by an increase of hydrogen bonding with urea and a slight decrease of hydrogen bonding with water; these trends are the same for both trajectories obtained in this study and similar to

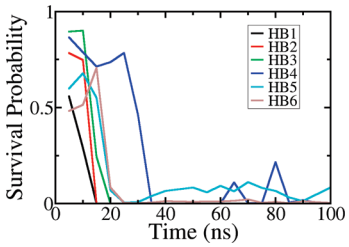

Figure 7. The survival probability of individual native hydrogen bonds during GB1 denaturation in 5 M urea solution.

that for the denaturation of lysozyme by 8 M urea solution[64] studied by Hua et al.

Furthermore, the small size and the relatively simple structure of GB1 allow us to analyze in detail its unfolding process, in particular, how each individual backbone hydrogen bond is broken. Both denaturing trajectories in 5 and 8 M urea solutions are consistent with a roughly step-by-step mechanism for the breaking of the native backbone hydrogen bonds. The order of hydrogen bond breaking, as indicated from Figure 7, which shows the time evolution of the survival probability of native hydrogen bonds 1−6, is roughly from the tail to the turn (1→2→3→5,4). Hydrogen bond (H-bond) 6, due to its configuration constrained by the nearby β-turn, is the least stable and is out of this order. The order of hydrogen bond breaking is consistent with their order of stability as revealed by calculations of the folding free energy landscape, H-bond 1 being the least stable and H-bond 5 being the most stable, although that calculation was performed using an implicit solvent model.[92]

To obtain more details of the denaturing mechanism of GB1, we analyzed the hydrogen-bond formation between solvent/cosolvent and the carbonyl and amino groups of the residues that form native hydrogen bonds 1−6, shown in Figure 8. The result shows that the breaking of the native hydrogen bonds is typically associated with the formation of the hydrogen bond between the protein C═O group with water, and to a less extent with urea. Although hydrogen bonds with both urea and water can form before the breaking of backbone hydrogen bonds, the formation of hydrogen bonds between water and the protein backbone N−H group likely occurs after the formation of backbone C═O/water (or urea) hydrogen bond. The formation of the hydrogen bond involving NH is more likely a consequence of the breaking of the backbone hydrogen bonds and does not appear to be required for the breaking of the amide hydrogen bond as indicated by the sequence of events shown in Figure 8. These results suggest that the breaking of the native hydrogen bonds is most likely initiated by the attack of the amide C═O group by water and/or urea. Overall, although both urea and water can serve as the hydrogen donor, water is more likely to initiate the process of breaking the backbone hydrogen bonds. The penetration of water into the interior of a protein, which initiates denaturation, was also observed in a molecular dynamics simulation for urea denaturation of chymotrypsin inhibitor at an elevated temperature.[42] All of these results suggest that the change of water−water interaction by urea could play an important role in the denaturing power of the urea. In the following, we further explore the physical origin of this indirect effect of urea at the atomic level.

3. Interactions of Water, Urea, TMAO, and TMU with the Amides. ***3.1. The Effects of Cosolvents on the Hydration of a Small Model Compound.*** The observation that urea promotes the capability of water in breaking amide hydrogen bonds intrigues us to investigate the effect of cosolvents, being a protein structure denaturant or protectant, on the interactions

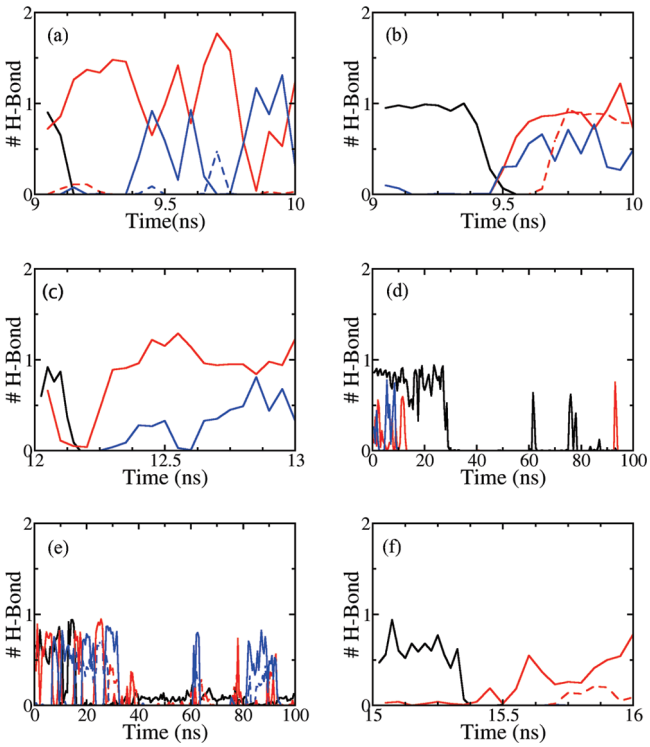

Figure 8. The breaking and formation of hydrogen bonds for residues involvied in each of the six native backbone hydrogen bonds (a−f: HB1−6): black (number of backbone hydrogen bond); red (number of hydrogen bonds formed with water); blue (number of hydrogen bonds formed with urea). The solid lines represent hydrogen bonds in which water or urea served as the hydrogen donors, and the dashed ones are for hydrogen bonds in which water or urea are proton acceptors.

between water molecules and the amide group, in particular, the carbonyl group. Simulations were first performed for a simple molecule, CH_3−NH−CO−CH_3 (NMA), solvated in water, 5 M urea, 4 M TMU, and 5 M TMAO aqueous solutions. As shown earlier, the addition of urea, TMAO, or TMU results in a modification of the water−water interactions, in particular, its hydrogen-bonding interactions. From these studies, it is expected that urea and TMU, in particular the latter, weaken the interaction between water molecules and thus render the water molecules stronger interactions with the solute, whereas TMAO has the opposite effect. Among the water−protein interactions, the hydrogen-bonding interactions are strong and more specific. The hydrogen bonds between water molecules and the carbonyl were characterized, and the radial distribution of water with respect to the oxygen atom in NMA for the different systems mentioned above is shown in Figure 9a. It is clearly seen from the significantly reduced and shifted first peak in Figure 9a that TMAO weakens the interaction between water and the carbonyl group, while TMU increases this interaction as shown by the significantly elevated first peak. Judging by this radial distribution function, urea also appears to increase the interaction of water with carbonyl, although its effect is much weaker. The effects on water−carbonyl interactions of all three molecules are in perfect accordance with their effects on water−water interactions.

To further test whether TMU strengthens the water−carbonyl interaction, the hydrogen-bond angle distribution for C═O···HOH was also calculated, shown in Figure 10, from which one sees that the angle distribution for C═O···HOH is shifted to larger angles, which is consistent with stronger hydrogen bonding between the two species. Next, the radial distribution of water around the NH group of the model compound NMA was also

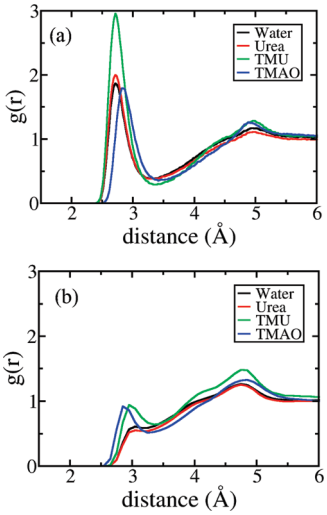

Figure 9. (a) The radial distribution function (RDF) of water O (a) around O of the amide CO group and (b) around the amide N atom in NMA in water, urea, TMU, and TMAO solutions.

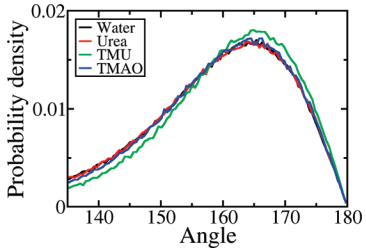

Figure 10. The calculated hydrogen-bond angle distribution function for the hydrogen bond formed between water and the amide CO group for NMA in water, urea, TMU, and TMAO solutions.

calculated and is shown in Figure 9b. As seen from the low first peaks for all four solutions, the hydration of NH is much weaker as compared to the CO group. The other difference of the water−NH interaction from the water−CO interaction is that both TMAO and TMU increase the water−NH interaction. We believe that this phenomenon is a result of the break of the balance of the numbers of proton acceptors and donors. Through the addition of TMAO and TMU, one introduces to the system extra good proton acceptors (the CO group of TMU and the NO group of the TMAO) without changing the proton donors. As a result, the NH group of the model compound NMA is more likely to be recruited as the proton donors. Nevertheless, the overall hydration of the amino groups remains weaker as compared to the carbonyl even in the presence of these extra proton acceptors. On the other hand, the stronger interaction between the carbonyl group and water after the introduction of extra proton acceptors through the addition of TMU makes the effect of TMU on water−carbonyl interaction even more impressive.

3.2. Interaction of Water with the GB1 Backbone Amide Groups. Next, the interactions between water/cosolvents and the protein GB1 were analyzed. In this study, to minimize the effects of intramolecular interactions of protein, we focus on the cosolvent effects on the denatured GB1 structure. Simulations were performed on denatured GB1 solvated in pure water, 5 M urea, 5 M TMAO, or 4 M TMU solutions. During the simulation time of 20 ns, the protein structure remains largely stable and unchanged in all cases. Through these analyses, we are again most interested in the change of water−protein interaction by the addition of cosolvents. For this purpose, the radial distribution functions for water around the backbone C═O

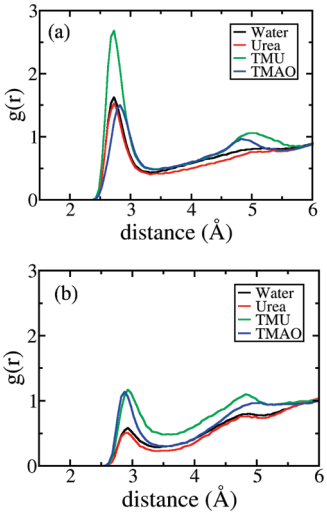

Figure 11. (a) The radial distribution function of water O around O of the amide CO group averaged for GB1 residues; (b) the radial distribution function of water O around the amide N atom averaged for GB1 residues in water, urea, TMU, and TMAO solutions.

TABLE 1: Average Number of Hydrogen Bonds[a] Formed by Residue 2 of GB1 with Various Molecules in Water, or 5 M Urea, 4 M TMU, or 5 M TMAO Aqueous Solutions

		solution		
H-bond type (protein/solvent)	water	urea	TMU	TMAO
C═O/water−OH	1.38	1.00	1.42	1.14
C═O/cosolvent−NH		0.46		
sum	1.38	1.46	1.42	1.14
NH/water−O	0.70	0.46	0.68	0.57
NH/cosolvent		0.27	0.07	0.11
sum	0.70	0.73	0.75	0.68
total	2.08	2.19	2.17	1.82

[a] The hydrogen bonds are defined with the following criteria: The results shown in this table depend on the definition of the hydrogen bonds. The purpose of this table is to show that hydrogen bonds with the amide CO group are more populated than those with the amide NH group. The results shown here are for residue 2, which is most exposed to the solution. The results for different residues are different because of their different local environment including the side chains and solvent and cosolvent distributions near their amide groups.

oxygen were calculated and shown in Figure 11 for the four different solution systems and averaged for all C═O groups of the protein backbone. It is clearly seen from Figure 11a that different cosolvents have significantly different effects on this radial distribution function: as compared to the case of pure water, when TMU or urea are present in the solution, water approaches to a shorter distance to the C═O oxygen, and the first peak of the radial distribution function increases with more so for TMU than for urea. These changes again indicate that the hydrogen-bonding interactions between water and the protein C═O group are noticeably strengthened by the indirect effects of TMU, as in the model compound. The presence of TMAO, on the other hand, quite dramatically weakens the hydrogen-bonding interaction between water and the carbonyl group, as indicated from the shift of the O−O radial distribution function to longer distances. The effects of the cosolvents can also be seen from the total number of hydrogen bonds formed between water molecules and the backbone amide groups, as listed in Table 1 for one of the well-hydrated residues (residue 2). In TMU solution, the average number of hydrogen bonds is 1.42 for the carbonyl group, which is higher than the value of 1.38

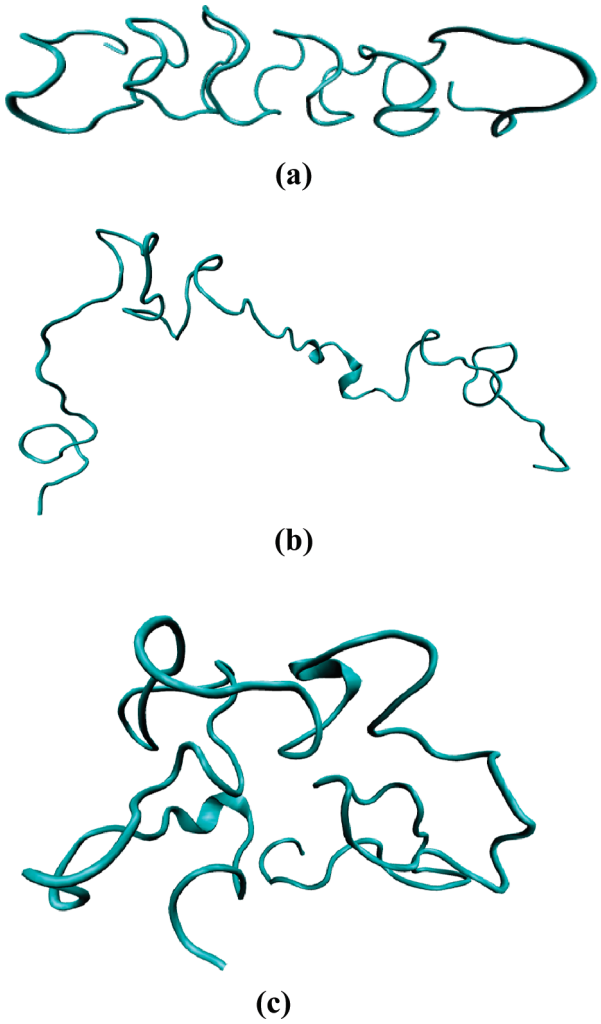

Figure 12. The structure of ELP in TMU solution (b) and TMAO (c) solutions after 50 ns simulation. The β-spiral structure is shown in (a).

found in pure water or the value of 1.14, in the TMAO solution. Taking into account the hydrogen bonds formed by the NH group, the averaged total number of hydrogen bonds for the amide C═O and N−H in TMU solution (2.17) is again higher than that in pure water (2.08), and both are higher than that calculated for the TMAO solution (1.82).

4. The Interaction of Water, TMAO, and TMU with Elastin-like Protein (ELP). ***4.1. The Structure of ELP in Pure Water, TMAO Solution, and TMU Solutions.*** To further test the effects of cosolvent on molecular interactions in protein solutions, simulations were performed on a segment of ELP containing 90 residues. The simulations showed that TMU and TMAO have dramatically different effects on the ELP structure. The final structures of ELP in 4 M TMU and 4 M TMAO aqueous solutions after ~50 ns simulations are shown in Figure 12. It is clearly seen that the ELP structure in the TMU solution is much more extended than that in the TMAO solution, in which the ELP structure takes a collapsed form. The time evolution of the solvent approachable surface area (SASA) in different solutions given in Figure 13 also indicates that TMU favors while TMAO disfavors the exposure of the protein surface. The decrease of SASA and radius of gyration in the presence of TMAO is also consistent with simulations on a prion protein by Daggett and co-workers.[93] It is interesting to note that the extended structure of ELP obtained from the simulations in TMU solution at 300 K is very similar to that obtained in

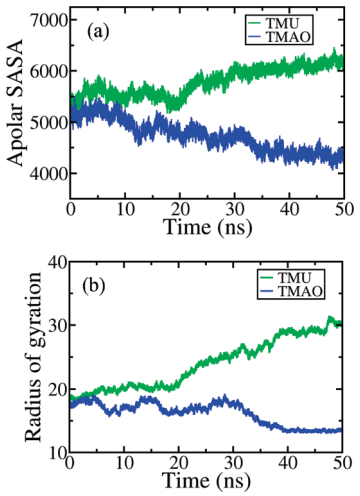

Figure 13. Time evolution of the nonpolar side-chain solvent-accessible surface areas (SASA) (a) and the radius of gyration (b) in 4 M TMU (green) and 5 M TMAO (blue) solutions.

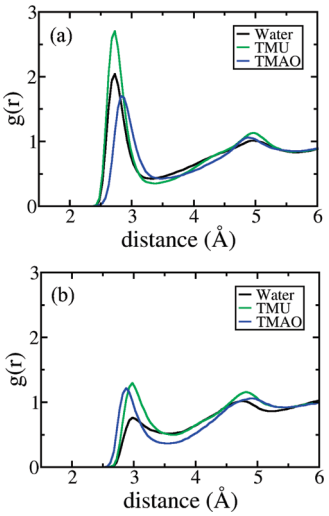

Figure 14. (a) The radial distribution function of water O around O of the amide CO group averaged for ELP residues; (b) the radial distribution function of water O around the amide N atom averaged for ELP residues in water, urea, TMU, and TMAO solutions.

pure water at 283 K, whereas the ELP structure in TMAO closely resembles that obtained from simulations at 328 K in pure water, which takes a compact structure.[86] These temperature and cosolvent dependences of the ELP structure are consistent with the existence of a low critical solution temperature (LCST) for its solvation in water as well as the TMU and TMAO dependence of the LCST (the former increases and the latter decreases the LCST).[94]

4.2. The Solvation of ELP Backbone. To understand the molecular details of the effects of TMU and TMAO on the ELP solution structure, we again analyzed the hydration of the amide groups by water. Because of the lack of hydrogen donors of TMU and TMAO, the exposed C═O groups should be primarily stabilized by hydrogen bonding to the water molecules. Figure 14 shows the radial distribution functions for the distance between the ELP backbone C═O and water oxygen atoms calculated for the three different systems (pure water, the TMU and the TMAO solutions). It is clearly seen from the short-distance radial distribution function that TMU enhances while TMAO depletes the water molecules closely surrounding the C═O group. The results thus show that TMU favors while

TMAO disfavors the hydration of the carbonyl group of ELP, consistent with that obtained for GB1 and the model compound NMA.

Discussion

In this study, molecular dynamics were used to investigate the effects of three common cosolvents, urea, TMU, and TMAO, on water structure and dynamics. The simulations showed that urea, TMU, and TMAO molecules affect the water orientation dynamics and water−water interactions in significantly different ways. Urea has little effects on any of the water structural properties studied, and TMAO and TMU both strengthen individual hydrogen bonds but show opposite effects on the tetrahedral hydrogen bonding of water. Urea was also shown to have very little effects on the orientational relaxation of water molecules, while both TMAO and TMU significantly slow the reorientation of water. These results are consistent with earlier simulations and experiments, and suggest that the denaturants, urea and TMU, might function through different mechanisms: direct mechanism plays a more important role in urea denaturation, while TMU has a stronger indirect effect, by which the water−water interaction is changed so that water−amide interaction becomes more favorable, and thus TMU helps the hydration of amide groups in the bulk aqueous solution. On the other hand, although both TMAO and TMU strengthen the water hydrogen bonds, TMAO also strengthens the water hydrogen-bond network and makes the water more "ice-like". The different effects of TMAO and TMU on the longer-range structure of water are consistent with them being protein renaturant and denaturant, respectively.

The similarities and differences between TMAO and TMU also showed that the dynamics properties of the system, such as the orientational relaxation time, and some of the structural elements, such as the stability of individual hydrogen bonds, might be inadequate for the evaluation of the effects of cosolvents on water−water interactions. For example, the orientational relaxation was shown to be dominated by exchange of hydrogen bonds between different waters[95,96] and thus reflects mainly the availability of neighboring hydrogen-bonding agents but is less indicative of the hydrogen-bonding quality.

Because TMAO and TMU share quite similar molecular structures, both containing multiple methyl groups and a large dipole, it is likely that their common methyl groups are responsible for the strengthening of the individual hydrogen bonds, as more so for the slowing of the orientational relaxation of individual water molecules. To further illustrate the limitation of such dynamics information on revealing structural information, the orientational relaxation of water at 300 K and 10 000 atm was also calculated. As shown in Figure 4, although water at such a pressure has a much higher density ($\sim$1.3 g/cm^3) and a dramatically different pair distribution function (Figure 4b) that is consistent with the experimental results of high density water,[91] its orientational relaxation behavior is remarkably close to that of water at 300 K and 1 atm. To fully understand the effects of cosolvents on water hydrogen-bonding properties, more cooperative features, such as the tetrahedral hydrogen-bonding network of the liquid structure, should be also examined.

The molecular dynamics simulations performed in the present study showed that GB1 is quickly and spontaneously denatured in the 5 M (or 8 M) urea solution at the room temperature and 1 atm. Because of the relatively simple structure of the protein GB1 (in particular, from the tail to the turn of the β-hairpin structure, the backbone hydrogen bonds, named 1−6, become less exposed to the aqueous solution), we were able to follow the order in the breaking of protein native backbone hydrogen bonds. The analyses of the breaking of the backbone hydrogen bonds and the making of those between the protein backbone amide and water as well as urea molecules are consistent with both direct and indirect effects of urea. Specifically, through an indirect mechanism urea allows water to interact more favorably with the carbonyl group. At the same time, urea also has a high probability of forming hydrogen bonds with the C═O group of protein, although the attacking of water molecules appears to be more strongly coupled to the breaking of the protein hydrogen bonds. The breaking of the protein backbone hydrogen bonds and the opening of space by water molecule makes the C═O group form a hydrogen bond with urea more easily and also allows the corresponding N−H group to be hydrogen-bonded with water, or to a less extent, with urea; for example, see Table 1. Without urea, it is however more difficult, both thermodynamically and kinetically, for water molecules to escape the hydrogen-bonding network and penetrate into the proximity of the amide groups, and the folded GB1 remains stable in water.

The effects of urea on the water structure, on the other hand, appear to be short-ranged, and only two (or three) water layers are influenced, as shown from the water radial distribution function for the urea aqueous bulk solution. This lack of long-range effect is consistent with the observation that high concentrations of urea are needed for it to have a significant effect on protein structure or solubility. Therefore, only when urea molecules are close to amide groups is water affected significantly enough to facilitate the breaking of the protein hydrogen bond. This short-range effect of the cosolvent on water structure could also be partially responsible for the relative stability of the GB1 structure in the presence of 4 M TMU in a 100 ns simulation, during which only one hydrogen bond is broken: although TMU has a stronger effect on the surrounding water structure, its large size prevents its easy approach to the protein, in particular the more buried domains including the backbone hydrogen bonds, and thus the denaturation of protein by TMU could be thermodynamically more favorable (as seen from Figure 10, the presence of TMU substantially increases the interaction/hydration of both CO and NH groups of the protein backbone), but kinetically slow. The strong effect of TMU on the carbonyl−water interaction can also be seen from the distribution function of the hydrogen bond formed between them for the simple amide compound (Figure 11). The current analysis focuses on the analysis of hydration of the non-hydrogen-bonded amide groups and showed that TMU leads to a better hydration of such species. Studies are also needed to characterize the effects of TMU (as well as the other cosolvents) on the strength of amide hydrogen bonds (as well as hydrophobic interactions). Nevertheless, based on these analyses, the mechanisms of urea and TMU denaturations and their effects are considerably different, although they both stabilize the open and better hydrated structures of proteins. Their effects on the local protein structures, in particular, on the competition between hydrogen-bonding interactions and hydrophobic contacts, are expected to be different, and the denatured proteins may be expected to take different conformations.

TMAO, in contrast to both urea and TMU, induces ordering of its closely neighboring water molecules, consistent with a water structure making effect. The analyses of the distribution of water molecules around the carbonyl groups showed that the TMAO in the aqueous solution substantially "dehydrates" the carbonyl. Because of the lack of the hydrogen donor of TMAO,

the depletion of water molecules from the close proximity of the C═O group thus results in a significant loss of its hydrogen bonds. On the other hand, due to the excessive strong proton acceptors brought in by TMAO, the NH of the amide group, as a proton donor, becomes better hydrated. Yet the change of hydration is more severe for the CO (becomes more poorly hydrated, and has a potential of forming two hydrogen bonds) than for the NH group (becomes better hydrated, but has the potential of forming only one hydrogen bond) group. The overall dehydration of the entire amide group makes it more likely to form an intramolecular protein hydrogen bond. This observed desolvation of the amide group is also consistent with the transfer free energy measurement, which shows that the transfer of the amide group from water to TAMO solution is associated with a rather large free energy penalty.

Simulations and analyses of the ELP in TMAO and TMU solutions again revealed the effects of the two cosolvent molecules on the interaction between water molecules and the protein backbone, which are in perfect agreement with those seen on GB1 and inferred from their effects on aqueous bulk solutions. In particular, TMU induces the hydration of the protein, and TMAO has an opposite effect on the protein structure. These observations are consistent with the effects of TMU and TMAO on ELP solubility: TMU increases and TMAO decreases the solubility of this protein in the experiments. Finally, the effects of urea, TMAO, and TMU on the hydration of protein amide groups are also substantiated by an analysis of hydration of a simpler and cleaner system, NMA, in the presence of pure water and aqueous solutions of the various cosolvents.

Considering the protein backbone or the amide group, the present study shows that the cosolvents significantly affect the hydration of the carbonyl group; given that TMU and TMAO cannot serve as proton donors, the quite large changes of the hydrogen bonding to the carbonyl group due to the addition of TMAO or TMU must be a result of an indirect effect through which water activity is changed. In addition, although both TMU and TMAO can serve as proton acceptors for the NH group, the current simulations showed that NH groups are dominantly hydrogen-bonded with water. The effects on the NH hydrogen bonding with the solvent are again indirect. Therefore, it is likely that TMU and TMAO affect the hydration of the protein backbone dominantly through indirect effects. Urea, on the other hand, represents a much more complicated scenario, in which both direct and indirect effects appear to play important roles, although the detailed analysis of the sequence of events in the breaking of backbone hydrogen bonds showed its noticeably stronger coordination with the formation of hydrogen bonds between water molecules and the carbonyl group than that with the hydrogen-bond formation between urea and the amide. From this latter point of view, in which urea activates water molecules for the attack of the protein hydrogen bonds, the indirect effects of urea also play important roles in the breaking of the protein amide hydrogen bonds.

We have to point out, however, that although the present study focuses on the effects of cosolvent on water structure and water interaction with other molecules and the analyses on the effect of cosolvent on both water−water hydrogen bonding and water−amide hydrogen bonding show significant indirect effects, this study does not rule out the direct effects of the cosolvent in affecting protein structures, nor does it provide a quantitative comparison between the importance of direct and indirect effects and between the effects on hydrophilic and hydrophobic groups. The analysis of the interaction of side chains with the cosolvent molecules showed preferred binding between most of them, and an earlier careful analysis by Berne and co-workers showed that after unfolding, the interaction between urea and side chains increases significantly.[64] We also performed analyses for the denatured GB1 for urea, TMAO, and TMU solution systems. Although the radial distribution functions of the cosolvent molecules around the side chains are quite complicated and heavily dependent on the nature of the side chain and the conformation of the polypeptide, it appears that all three molecules tend to accumulate to some extent near the side chains. The overall conclusion we obtain for urea solution is similar to earlier studies, which shows that urea tends to interact strongly with side chains of a strong van der Waals interacting strength, such as the aromatic ones. In particular, also consistent with a detailed analysis of Grubmuller and co-workers,[45,67] urea tends to more accumulate near a glycine than near a valine or an alanine. Urea but not TMU or TMAO tends to accumulate near the charged residues (both positively and negatively charged, shown in Figure 15 in the Supporting Information). The strong interactions between urea and the charged groups are again consistent with earlier simulations. All of these observations point to the possible importance of urea−protein direct interactions in protein denaturation, which unfortunately complicates the analysis of the denaturing mechanism but warrants further and more detailed studies, in particular a direct comparison of the molecular dynamics simulations with thermodynamics including transfer free energy and preferred binding measurements.

Summary

In summary, using molecular dynamics, we thoroughly examined the effects of three different cosolvents, two denaturants, urea and TMU, and one renaturant, TMAO, on the water and protein structure, as well as on their interactions. The simulations revealed important indirect cosolvent effects on protein backbones that, to the best of our knowledge, have not been studied carefully by earlier studies. This study led to a number of new discoveries that are summarized as follows.

First, the present study presents the first thorough analysis of the indirect effects of urea on the hydration of protein backbone. Indirect effects have been discussed in earlier studies mainly on hydrophobic solvation. Here, we observed and discussed the origin of the indirect effect on protein/water hydrogen bonding. Because backbone is likely to play a much more important role than side chains, as concluded from the transfer free energy measurement, this indirect effect of urea is of great interest in the understanding of urea denaturation, as well as for the renaturing effect of TMAO. The understanding of the hydration of protein backbone is also very important in the understanding of the roles of backbone hydrogen bonds in protein structure formation. Therefore, the present study adds an essential element in the mechanism of protein structure formation and the effects of cosolvents.

Second, to the best of our knowledge, this is the first report containing a detailed analysis of the molecular mechanism of protein hydrogen bond breaking in urea denaturation. In particular, this study reveals the step-by-step breaking of the native hydrogen bonds in GB1 when it is denatured in urea aqueous solution. These analyses also allowed an atomic-detailed understanding of kinetic process of protein denaturation, including the sequential events in native hydrogen bond breaking, the attacking of carbonyl group by water, the subsequent hydrogen-bond formation between the carbonyl group and urea, and the final hydrogen-bond formation between

the solvent/cosolvent and the NH group. This analysis not only lays the ground for the further understanding of the physical interactions that determine protein structures but also leads to explicit molecular mechanisms of urea denaturation that can be directly tested by experiments.

Third, a thorough analysis of the effects of cosolvents (both denaturants and renaturants) on water structure and dynamics was performed to further understand their effects on the hydration of protein backbone, in particular the carbonyl group. The molecular-detailed connection between their effects on water and those on model amide compounds and proteins yields a clear physical picture on the solvation of proteins. These integrated analyses allowed a self-consistent understanding of the "indirect" effects of each individual cosolvent studied.

Finally, the present study provided a detailed and unified understanding of the effects of both denaturants (both urea and tetramethyl urea) and renaturant (trimethylamine *N*-oxide) on protein and water structures. The direct comparison of the different effects of these cosolvents provides further evidence for the indirect effects on protein hydration. In particular, it provides a possible explanation of the effects of TMAO on the transfer free energy of protein backbone.

In particular, it is known that protein structure renaturants, such as TMAO, are preferentially expelled from the protein surfaces,[55] which makes a direct interacting mechanism less favored. The present study provides a reasonable explanation for the strong effect of TMAO on protein backbones,[55] which involves the hydration weakening of the backbone of unfolded proteins.

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Supporting Information Available: Distribution function of urea, TMAO, and TMU around charged residues during GB1 denaturation in 5 M urea are shown in Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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