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Structure and Interaction in Aqueous Urea−Trimethylamine-*N*-oxide Solutions

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Abstract: The structural and energetic properties of solutions containing water, urea, and trimethylamine-*N*-oxide (TMAO) are examined using molecular dynamics simulations. Such systems are of interest mainly because TMAO acts to counter the protein denaturing effect of urea. Even at relatively high concentration, TMAO is found to fit well into the urea−water structure. The underlying solution structure is influenced by TMAO, but these perturbations tend to be modest. The TMAO−water and TMAO−urea interaction energies make an important contribution to the total energy in solutions where counter-denaturing effects are expected. TMAO−water and TMAO−urea hydrogen bonds have the largest hydrogen-bond energies in the system. Additionally, TMAO cannot hydrogen bond with itself, and hence it interacts strongly with water and urea. These observations suggest that the mechanism of TMAO counter denaturation is simply that water and urea prefer to solvate TMAO rather than the protein, hence inhibiting its unfolding.

I. Introduction

Aqueous urea solutions have been of long-standing interest due to their peculiar physical properties. For example, urea increases the solubility of hydrocarbons in water,[1] inhibits micelle formation,[2] and most importantly in high concentration denatures proteins.[3,4] A good deal of effort has been directed toward understanding the mechanism of urea denaturation, but there is still no definitive generally accepted answer to this question, and it remains a subject of active research. Another interesting result concerning chemical denaturation is that the addition of 4 M trimethylamine-*N*-oxide (TMAO) to 8 M urea solution counteracts the denaturing effect of urea, apparently stablizing the folded state.[5] This observation has movitated recent computer simulation studies,[5] including the present work.

Attempts to understand urea denaturation have focused on two concepts that are by no means mutually exclusive. One suggestion is that urea acts indirectly by altering the water structure and consequently the solvation of the denatured protein. A second possibility is that urea stabilizes unfolded states directly by hydrogen bonding with the protein. Recent computer simulations provide at least some evidence for both possibilities.[5−8] Bennion and Daggett[6] carried out molecular dynamics simulations of the protein chymotrypsin inhibitor 2 in 8 M urea solution. They report that the water structure is altered by urea, thereby diminishing the hydrophobic effect and encouraging solvation of hydrophobic groups. Further, because the water structure is weakened by urea, water molecules become free to compete with intraprotein interactions aiding solvation of the unfolded state. Additionally, they note that urea can interact directly with polar groups of the protein, again favoring the denatured state. So, both direct and indirect mechanisms appear to be operative. The existence of both direct and indirect mechanisms of protein denaturation is also supported by the molecular dynamics studies of a ribonuclease A C-peptide analogue reported by Caballero-Herrera et al.[7] We note, however, that both direct and indirect mechanisms are not always equally relevant. For example, Mountain and Thirumalai[8] recently found that direct interaction of urea with site charges was the most important mechanism for the unfolding of hydrocarbon chains in urea−water solution.

There have also been simulation studies of aqueous solutions that include urea, TMAO, and proteins.[5,9] Bennion and Daggett[9] investigated the counteraction of urea-induced protein denaturation by TMAO. They observed that TMAO enhanced water−water hydrogen bonding both in binary TMAO−water mixtures and in ternary urea−TMAO−water solutions. They report that TMAO has a profound effect on the lifetimes of water−water hydrogen bonds, with 1 M TMAO increasing the lifetime by a factor of ∼3.8 as compared to that of pure water. TMAO also strengthened water−urea interactions and led to a decrease in urea−protein hydrogen bonding. They concluded that the influence of TMAO on the water−water and water−urea interactions is the main factor contributing to its ability to counter urea-induced protein denaturation.

In view of their importance, it is of interest to more closely examine the properties of ternary urea−TMAO−water solutions,

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and this is the purpose of the present paper. We note that there have been many simulation studies of urea−water solutions,[10−22] and some of water−TMAO systems.[23−28] However, ternary urea−TMAO−water mixtures have received less attention. Here, we describe a molecular dynamics study of this system. Our main focus is on the structural and energetic (particularly hydrogen bonding) properties, and what these suggest about the origin of the counter-denaturing effect of TMAO. The influence of TMAO on hydrogen-bond dynamics is also discussed and compared to earlier work.

The remainder of this paper is divided into three parts. The model and simulation details are described in section II, results are presented and discussed in section III, and our conclusions are summarized in section IV.

II. Models and Simulation Method

Molecular dynamics simulations of urea−TMAO−water mixtures were carried out at 298 K. For TMAO, we employ a fully rigid version of a model proposed by Kast et al.[26] and recently used by Athawale et al.[28] in simulations of aqueous systems. For urea, we used the all-site model of Duffy et al.,[29,30] often called the DKJ model. Note that recent comparative studies have concluded that the DKJ model for urea−water solutions shows the best overall agreement with experimental results,[18,22] among the rigid models considered. The SPC/E potential[31] was used for water. The interaction between atomic sites of different molecules is expressed as

$$u_{\alpha\beta}(r_{\alpha\beta}) = 4\epsilon_{\alpha\beta}\left[\left(\frac{\sigma_{\alpha\beta}}{r_{\alpha\beta}}\right)^{12} - \left(\frac{\sigma_{\alpha\beta}}{r_{\alpha\beta}}\right)^{6}\right] + \frac{q_\alpha q_\beta}{r_{\alpha\beta}} \qquad (1)$$

where $r_{\alpha\beta}$ is the distance between atomic sites α and β, and q_α is the charge on site α. The Lennard-Jones (LJ) parameters, $\sigma_{\alpha\beta}$ and $\epsilon_{\alpha\beta}$, are obtained using the combining rules $\sigma_{\alpha\beta} = (\sigma_\alpha + \sigma_\beta)/2$ and $\epsilon_{\alpha\beta} = \sqrt{\epsilon_\alpha \epsilon_\beta}$. The values of the potential parameters q_α, σ_α, and ϵ_α for urea, TMAO, and water are summarized in Table 1.

Table 1. Lennard-Jones Parameters and Charges Used in the Models Considered[a]

	atom	σ (Å)	ϵ (kJ/mol)	charge (e)
water (SPC/E)	O	3.166	0.646	−0.8476
	H			+0.4238
urea (DKJ)	C	3.75	0.4365	+0.142
	O	2.96	0.873	−0.390
	N	3.25	0.7067	−0.542
	H	0.0	0.0	+0.333
TMAO	C	3.041	0.2828	−0.26
	N	2.926	0.8368	+0.44
	O	3.266	0.6385	−0.65
	H	1.775	0.0774	+0.11

[a] e is the elementary charge.

Table 2. N_{urea}, N_{TMAO}, and N_{water} Represent the Number of Urea, TMAO, and Water Molecules in the Simulation, and ρ Is the Solution Density in g/cc

system	N_{urea}	N_{TMAO}	N_{water}	ρ
1	100	0	400	1.149
2	100	10	390	1.157
3	100	20	380	1.172
4	100	30	370	1.178
5	100	40	360	1.183
6	100	50	350	1.195

The MD simulations were carried out with 500 molecules (water and solute) in a cubic box of length L. The LJ interactions were spherically truncated at the radius $L/2$. The long-range electrostatic interactions were treated using the Ewald method[32] with the convergence parameter $\alpha = 6.4/L$. The quaternion formulation[32,33] of the equations of rotational motion was employed, and the leapfrog algorithm with a time step of 10^{-15} s was used for the time integration. In the starting configuration, the molecules were located on a face-centered-cubic lattice with random orientations. The box length L was adjusted to give a pressure close to 1 atm, and NVT MD runs of 1 ns were used to equilibrate each system. During the equilibration, the velocities were rescaled to fix the temperature. Finally, each system was run for a further 1.5 ns using the NPT MD method,[32] and these are the results reported. It should be noted that our simulation runs were relatively long. Quantities such as the internal energies equilibrate within a few picoseconds, whereas the stabilization of structural and dynamical properties requires longer simulations. Such peculiarities of aqueous urea solutions have been reported previously.[22,34]

The six systems considered are summarized in Table 2 together with their calculated densities at ∼1 atm pressure. Note that in these systems we have held the number of urea molecules fixed and have simply replaced water molecules with TMAO. The urea concentration ranges from 8.6 M in system 1 to 7.4 M in system 6, and that of TMAO from 0.83 M in system 2 to 3.7 M in system 6.

III. Results and Discussion

A. Structural Properties. Selected site−site radial distribution functions that show the influence of TMAO on the structure of aqueous urea solutions are given in Figures 1−4. Results are shown for a urea−water solution (system 1) and for a ternary solution (system 6). Note that atoms associated with urea, TMAO, and water are denoted by the subscripts u, t, and w, respectively.

The urea−urea functions are plotted in Figure 1. We note that in the absence of TMAO, the urea−urea structure is similar to that observed in earlier studies.[13,14,20] The double peak in the O_u-O_u radial distribution function (rdf) and the three peaks in the O_u-H_u rdf indicate the presence of associated urea species (dimers and trimers) held together through O_u-H_u hydrogen bonds. From Figure 1, we see that even at ∼4 M TMAO has surprisingly little effect on the urea−urea structure. What

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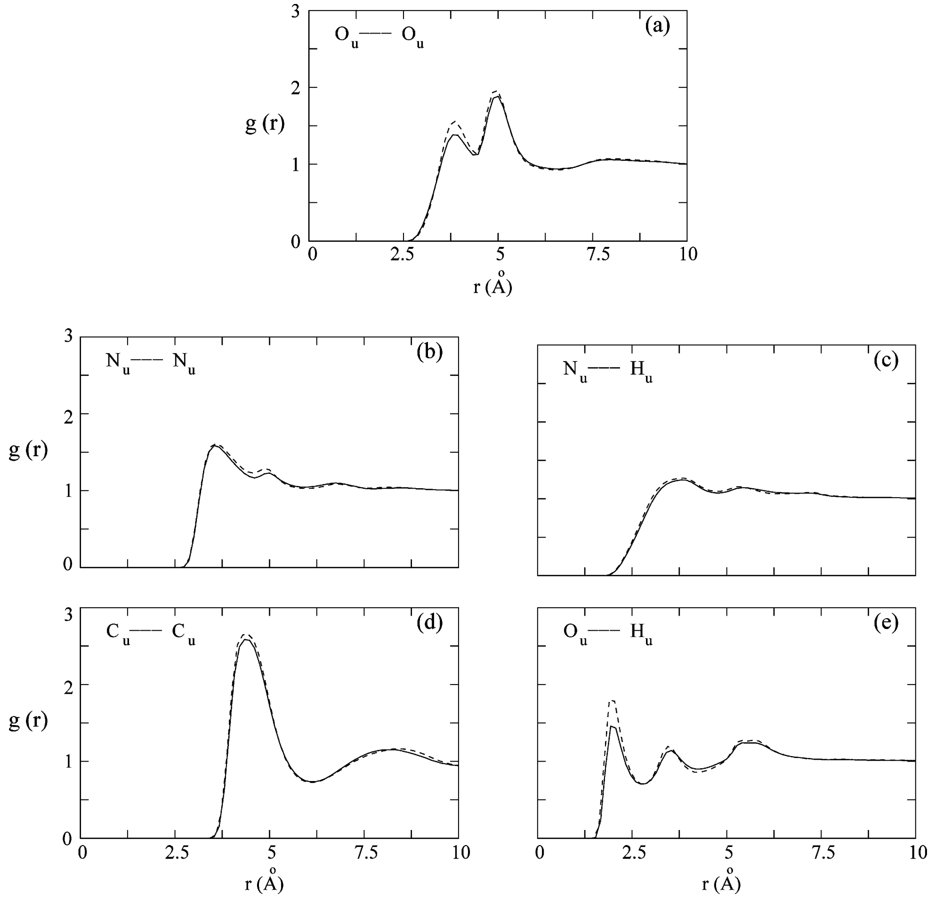

Figure 1. Urea−urea, site−site radial distribution functions. The solid lines represent system 1 (urea−water), and the dashed lines represent system 6 (urea−TMAO−water).

influence there is, is mainly apparent in the O_u−O_u and O_u−H_u rdf's where the peaks are modestly increased in height. Note that the first-shell coordination number obtained by integrating the O_u−H_u rdf increases from 0.34 to 0.38 upon the addition of TMAO. This reflects an increase in the O_u−H_u hydrogen-bond energy (see below). Note that the other three rdf's, N_u−N_u, N_u−H_u, and C_u−C_u, show only slight awareness of the presence of TMAO.

Urea−water, site−site rdf's together with running coordination numbers are shown in Figure 2. Again, the influence of TMAO is not dramatic, and it does not greatly alter the solvation of urea. We do observe that the first peak in the O_u−O_w, H_u−O_w, and O_u−H_w rdf's increases a little in height and that the functions show some water depletion further out, perhaps reflecting the strong affinity for water of TMAO (see ref 25, and the discussion below). We note that the first peak in the O_u−H_w rdf is much stronger than that of H_u−O_w, indicating that water much prefers to be a hydrogen donor in urea−water hydrogen bonds. This is also apparent in the hydrogen-bond energies discussed below.

TMAO−urea and TMAO−water rdf's are given in Figure 3 for system 2 (0.83 M in TMAO) and system 6 (3.7 M in TMAO). The O_t−O_u and O_t−H_u functions are of particular significance. It is evident that their general features are strikingly similar to the corresponding urea−urea rdf's, O_u−O_u [Figure 1a] and O_u−H_u [Figure 1e]. The qualitative similarity of these rdf pairs indicates that TMAO acts as a hydrogen-bond acceptor for urea and appears to simply "fit" into the solution structure much as would additional urea molecules. The very weak concentration dependence exhibited by these functions further supports this interpretation. This is likely why TMAO is accommodated by the urea−water solution with so little effect on the urea−urea structure.

The O_t−O_w and O_t−H_w rdf's [Figure 3] demonstrate the strong affinity of TMAO for water coming through the strong TMAO−water hydrogen bond (see below). Note that the O_t−H_w rdf is similar in shape to the corresponding urea function, O_u−H_w [Figure 2c], but that the first peak is much stronger in the TMAO case reflecting the stronger hydrogen bond. Again, these rdf's show only very weak dependence on TMAO concentration.

The influence of the solutes on the water−water rdf's is shown in Figure 4. Consistent with earlier work,[13] we note that the presence of urea alone tends to increase the height of the first peak in the O_w−O_w rdf and of the first and second peaks in the O_w−H_w rdf, signaling some perturbation of the water structure. We see that the addition of TMAO does nothing to decrease the solvent structural perturbation induced by urea, rather it appears to be enhanced by the TMAO, with the rdf peaks further increasing in height.

B. Energy Considerations. In our discussion of the solution structure given above, we noted that TMAO molecules seem to fit well into the water−urea structure. The average interaction energies for various species are given in Table 3. These results lead to several interesting observations. We see that the total interaction energy per mole of solution becomes more negative as TMAO is added. The urea−urea, urea−water, and water−water contributions become smaller, but this is more than

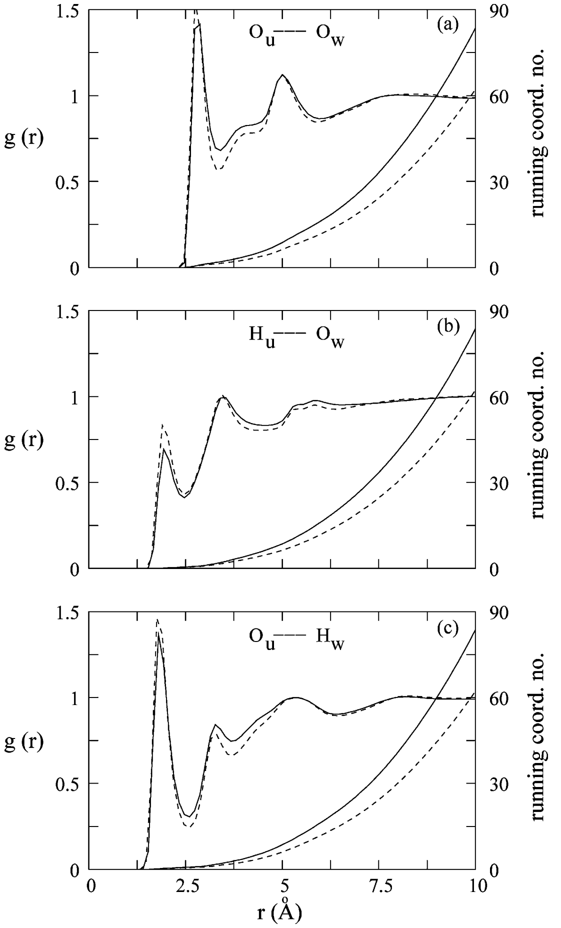

Figure 2. Urea−water, site−site radial distribution functions and running coordination numbers. The line types are as in Figure 1.

compensated by the TMAO−urea and TMAO−water energies. Indeed, the most striking and likely the most significant observation is the strength of the TMAO−urea and TMAO−water interactions. Note that for system 6, the TMAO−urea interaction is −57.0 kJ per mol of TMAO, as compared to a urea−urea interaction of −34.3 kJ per mol of urea. Similarly, for TMAO−water, one has −89.7 kJ per mol of TMAO, as compared to −66.7 kJ per mol of urea for urea−water, and −30.6 kJ per mol of water for the water−water case. Unlike urea, TMAO cannot hydrogen bond with itself, and hence the TMAO−TMAO interaction is relatively weak, −8.1 kJ per mol of TMAO in system 6. This means that TMAO is "free" to interact strongly with both urea and water, and this may be the reason it acts to counter the protein denaturing effect of urea. If denaturation depends upon water−protein and/or urea−protein interactions as has been suggested,[5,8] the TMAO obviously provides strong competition for these interactions. It is possible that in ternary water−urea−TMAO solutions, the water and urea components simply "prefer" to solvate TMAO rather than the unfolded protein.

Further insight into the nature of the interactions in urea−TMAO−water solutions can be obtained by considering the strength and number of the hydrogen bonds between various species. Following earlier workers,[35−39] we adopt a set of geometric criteria to define hydrogen bonds. Two water molecules are taken to be hydrogen bonded if their inter-oxygen distance is less than 3.41 Å and, simultaneously, the hydrogen−oxygen distance is less than 2.38 Å, and the oxygen−oxygen−hydrogen angle is less than 45°. These oxygen−oxygen and oxygen−hydrogen distances were determined from the positions of the first minimum in the corresponding radial distribution functions. For water−urea (or TMAO) hydrogen bonds, we again selected cutoff distances according to the location of the first minimum of the appropriate radial distribution function. This gives: 3.41 Å for O_u-O_w, 2.64 Å for O_u-H_w, 2.51 Å for O_w-H_u, 3.45 Å for O_t-O_w, and 2.64 Å for O_t-H_w. For urea−urea and urea−TMAO hydrogen-bond calculations, the same cutoff distance rule was employed. An angle of 45° was used to define all hydrogen bonds.

Hydrogen-bond energies together with average hydrogen-bond numbers (given in brackets) are summarized in Table 4. The hydrogen-bond numbers are expressed per the second species mentioned in the column labels; that is, for W−T it is per TMAO molecule, and for W−U it is per urea molecule, etc. From Table 4, we see that the W−T and T−U hydrogen bonds are the strongest in the system by significant amounts. For system 6, the W−T hydrogen-bond energy is 9.1 kJ/mol more attractive than the W−W bond and 7.8 kJ/mol more attractive than the W−U (water donor) case. Similarly, the T−U hydrogen bond is 4.6 kJ/mol more attractive than the U−U bond. This explains the relative magnitudes of the average interaction energies discussed above. We note that the hydrogen-bond energies are not strong functions of TMAO concentration. The W−W hydrogen-bond energy of system 6 is just 0.7 kJ/mol more negative than that of system 1. The number of W−W hydrogen bonds decreases as TMAO is added to the solution, and we note that the decrease is nearly proportional to the number of water molecules replaced by TMAO. These observations suggest that TMAO is having only a modest effect on the water−water interactions. The W−T hydrogen-bond energy becomes a little more attractive as the TMAO concentration is increased, but this is likely not of much significance. The remaining hydrogen bonds show only small nonsystematic variations with TMAO concentration. We note that the number of U−U hydrogen bonds decreases by nearly the number of T−U bonds formed as TMAO is added to the solution. This suggests that U−U hydrogen bonds are simply being exchanged for T−U bonds, with the total number of U−U and T−U hydrogen bonds remaining essentially constant.

C. Dynamical Aspects. It has been reported[9] that TMAO has a profound effect on the lifetime of water−water hydrogen bonds. This appears to have been taken as partial evidence that TMAO strongly enhances water−water hydrogen bonding, rendering water less able to solvate unfolded protein states, hence leading indirectly to counter denaturation in urea−TMAO−water solutions. On the basis of a particular model simulation, Bennion and Daggett[9] have reported that the water−water hydrogen-bond "lifetime" in bulk water increases from 0.8 ps in pure water to 3.0 ps in 1 M TMAO to 3.8 ps in 4 M TMAO. Given the rather weak TMAO dependence of the water−water hydrogen-bond energies described above, the

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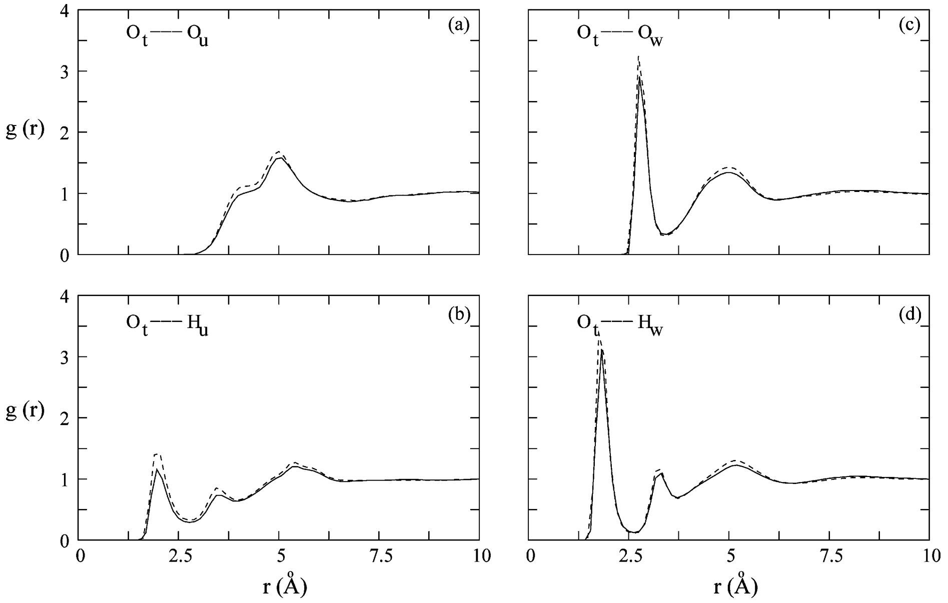

Figure 3. TMAO−water and TMAO−urea, site−site radial distribution functions for the urea−TMAO−water solutions labeled system 2 (−) and system 6 (− − −).

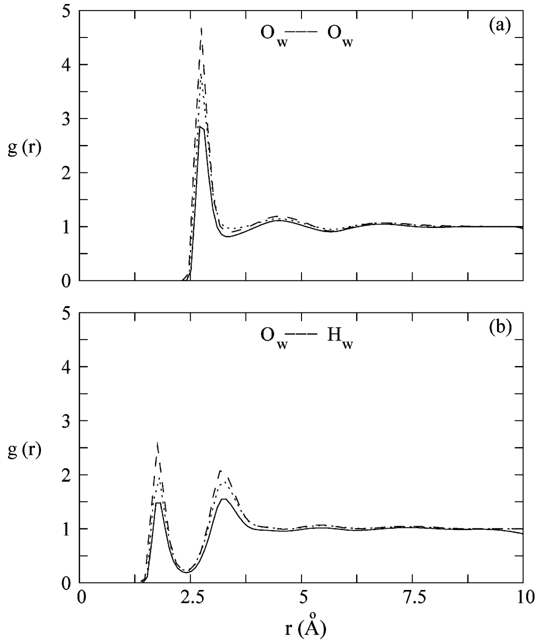

Figure 4. Water−water, site−site radial distribution functions for pure water (−), system 1 (• • •), and system 6 (− − −).

reported changes in lifetime are difficult to rationalize. Note that in 1 M TMAO, the water−water hydrogen-bond energy is only ∼0.15 kJ/mol (0.06RT at 298 K) more attractive than that of pure water. In view of this, we have also considered the dynamics of hydrogen bonds in water−TMAO and water−urea−TMAO solutions.

Hydrogen-bond dynamics was investigated using an approach employed by earlier workers.[36−40] We define two hydrogen-bond population variables $h(t)$ and $H(t)$. $h(t)$ is unity if a particular tagged pair of particles is hydrogen bonded (according to the definition adopted above) at time t and is zero otherwise. $H(t)$ is unity if the tagged pair of particles remains continuously hydrogen bonded from time 0 to time t, and is zero otherwise. We then define the continuous hydrogen-bond time correlation function $S_{HB}(t)$ as[35−40]

$$S_{HB}(t) = \frac{\langle h(0)H(t)\rangle}{\langle h\rangle} \qquad (2)$$

where ⟨⟩ denotes an average over all hydrogen bonds that are present at $t = 0$. Clearly, $S_{HB}(t)$ describes the probability that a pair of particles, which was hydrogen bonded at $t = 0$, remains continuously bonded up to time t. For all systems considered, once past the inertial regime ($t \gtrsim 0.2$ ps), $S_{HB}(t)$ exhibited single-exponential decay. The time integral of $S_{HB}(t)$ denoted τ_{HB} describes the average time that a hydrogen bond survives after it is chosen at $t = 0$. Because the hydrogen bonds are chosen randomly without imposing any condition on when they were created, τ_{HB} is the average persistence time (life expectancy) of a randomly chosen hydrogen bond.[36] We note that other times characterizing different aspects of hydrogen-bond dynamics can be defined.[36] Here, we are mainly interested in changes introduced by TMAO, and τ_{HB} as defined above is sufficient for that purpose.

Results obtained for water−water hydrogen bonds in water−TMAO solutions are given in Table 5. Note that the TMAO concentration ranges from 0 to ∼4.5 M. Values of τ_{HB} (given in brackets) for water−urea−TMAO solutions, where 100 water molecules have been replaced by a corresponding number of urea molecules (systems 2−6), are also included. We see that τ_{HB} increases from 1.16 ps in pure water to 1.24 ps in ∼1 M TMAO, to 1.77 ps in ∼4.5 M TMAO, and that similar increases occur in the water−urea−TMAO system. The TMAO-induced increases in τ_{HB} that we observe are consistent with the increases in the hydrogen-bond energies also given in Table 5, but appear to be at odds with the results reported by Bennion and Daggett.[9] They report that the water−water hydrogen-bond lifetime in 1 M TMAO solution is 3.8 times larger than that of pure water, whereas we find only a factor of 1.1. It is difficult to compare directly with the results of Bennion and Daggett because neither hydrogen bonds nor hydrogen-bond "lifetimes" are precisely defined in ref 9, and furthermore the models employed are

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Table 3. Average Interaction Energies (in kJ/mol of Solution) for the Systems Considered[a]

system	E_{UU}	E_{UT}	E_{UW}	E_{TT}	E_{TW}	E_{WW}	E
1	−7.62		−17.47			−28.44	−53.53
2	−7.52	−1.20	−16.25	−0.03	−2.05	−27.15	−54.20
3	−7.32	−2.45	−15.60	−0.13	−3.88	−25.63	−55.01
4	−7.08	−3.48	−15.44	−0.28	−5.83	−23.78	−55.89
5	−7.05	−4.64	−14.00	−0.52	−7.40	−22.80	−56.41
6	−6.85	−5.70	−13.34	−0.81	−8.97	−21.45	−57.12

[a] E is the total configurational energy, and E_{UU}, E_{UT}, E_{UW}, E_{TT}, E_{TW}, and E_{WW} are the average urea−urea, urea−TMAO, urea−water, TMAO−TMAO, TMAO−water, and water−water contributions.

Table 4. Hydrogen-Bond Energies (kJ/mol of Solution) and Average Hydrogen-Bond Numbers (Given in Brackets) for the Systems Considered[a]

system	W–W	W–T	W–U (water donor)	W–U (water acceptor)	T–U	U–U
1	−19.05 (2.90)		−20.61 (1.36)	−14.93 (0.99)		−24.42 (0.86)
2	−19.18 (2.80)	−27.75 (2.0)	−20.74 (1.27)	−15.04 (1.16)	−29.74 (0.03)	−23.97 (0.81)
3	−19.40 (2.77)	−28.29 (2.05)	−20.85 (1.25)	−15.12 (1.34)	−29.54 (0.10)	−23.71 (0.67)
4	−19.52 (2.72)	−28.62 (2.03)	−20.91 (1.21)	−15.15 (1.28)	−29.85 (0.13)	−24.70 (0.69)
5	−19.67 (2.62)	−28.70 (1.98)	−21.00 (1.13)	−15.25 (1.23)	−31.21 (0.19)	−25.27 (0.64)
6	−19.78 (2.53)	−28.93 (2.0)	−21.11 (1.09)	−15.29 (1.29)	−29.45 (0.21)	−24.87 (0.69)

[a] In the column headings, W, T, and U refer to water, TMAO, and urea. The hydrogen-bond numbers are defined with respect to the second species mentioned in the column headings (i.e., per water molecule in column 2, per TMAO molecule in column 3, etc.).

Table 5. Values of τ_{HB} for Water−Water Hydrogen Bonds in TMAO−Water Solutions Together with the Corresponding Hydrogen-Bond Energies E_{HB} (kJ/mol of Solution)[a]

N_{TMAO}	N_{water}	τ_{HB} (ps)	E_{HB}
0	500	1.16	−18.96
10	490	1.24 (1.29)	−19.10
20	480	1.35 (1.42)	−19.22
30	470	1.49 (1.55)	−19.38
40	460	1.60 (1.71)	−19.52
50	450	1.77 (1.87)	−19.69

[a] τ_{HB} results for urea−TMAO−water solutions with 100 water molecules replaced by 100 ureas (systems 2−6) are given in brackets.

Table 6. Translational Diffusion Coefficients in Urea−TMAO−Water Solutions[a]

system	$D_{water} \times 10^5$	$D_{urea} \times 10^5$	$D_{TMAO} \times 10^5$
1	2.02	0.78	
2	1.78	0.59	0.57
3	1.50	0.47	0.42
4	1.43	0.43	0.40
5	1.29	0.36	0.36
6	1.17	0.28	0.30

[a] The values are given in cm^2 s^{-1}.

different. However, we note that in pure water our result (1.16 ps) does not differ greatly from theirs (0.8 ps). This agreement for pure water would appear to rule out the different water models and/or differences in hydrogen-bond related definitions as possible sources of the large discrepancy. Given this, and the fact that so few TMAO molecules are present in a ∼1 M solution ($N_{TMAO} = 10$ and $N_{water} = 490$ in our simulations), the large increase in the water−water hydrogen-bond lifetime reported by Bennion and Daggett remains difficult to rationalize physically.

We have also estimated the translational diffusion constants of the various solution components by integrating the velocity−velocity autocorrelation functions in the usual manner.[32] The results obtained for water, urea, and TMAO are given in Table 6. It is clear that TMAO reduces the diffusion constants of both water and urea. Note that $D_{water} \times 10^5$ is reduced from 2.02 to 1.17 cm^2 s^{-1} when 50 water molecules are replaced by TMAO. However, this observation is not very dependent on the nature of the solute. If instead of TMAO, 50 additional urea molecules replace 50 waters, $D_{water} \times 10^5$ is reduced to 1.25, very close to the TMAO value. The influence of TMAO on $D_{urea} \times 10^5$ is more specific, reducing it from 0.78 to 0.28 cm^2 sec^{-1}, whereas 50 additional ureas only reduce it to 0.52. This likely reflects the very strong urea−TMAO interaction discussed above. It is also interesting to observe that the urea and TMAO diffusion constants are very similar in all solutions.

IV. Summary and Conclusions

In this paper, we discuss MD simulation results for model urea−TMAO−water solutions. The systems considered were near 8 M in urea, and the TMAO concentration was varied up to ∼3.8 M. We found that even at the highest TMAO concentration considered, the TMAO molecules “fit” well into the urea−water structure. The urea−urea, site−site rdf’s showed little impact of the TMAO, and the “corresponding” urea−TMAO and urea−urea rdf’s were rather similar. The urea−water functions also were not greatly perturbed by TMAO; moreover, the urea−urea and urea−water hydrogen-bond energies showed little sensitivity to its presence. The water−water rdf’s showed increased structure in the more concentrated TMAO solutions, and the water−water hydrogen-bond energies were ∼4% stronger in 3.8 M TMAO than in the binary urea−water system. This increase in the water−water structure and interaction is qualitatively similar to earlier observations, but we do not find large increases in the water−water hydrogen-bond lifetimes as previously reported.[9] We observe only modest TMAO-induced lifetime increases consistent with the modest increases in the hydrogen-bond energies. Therefore, it seems unlikely that the main counter-denaturation effect of TMAO comes through its effect on water−water structure and interaction.[5,9]

The most striking result of our calculations is the strength of the TMAO−water and TMAO−urea interactions. The TMAO−urea hydrogen bond is the strongest in the system, followed closely by the TMAO−water bond. Further, unlike urea and water, TMAO cannot hydrogen bond with itself, and this leaves it “free” to interact strongly with both water and urea, leading to relatively large average interaction energies. This suggests that TMAO might counter denaturation simply through a preferential solvation mechanism. Because of their very strong hydrogen bonding to TMAO, we suggest that water and urea simply “prefer” to solvate TMAO molecules rather than the protein, hence inhibiting its unfolding in aqueous urea solutions. It is also worth remarking that we have investigated the influence of TMAO on the hydrophobic interaction of a pair of neopentane molecules in urea−TMAO−water solutions. TMAO is found to have little effect on the neopentane−neopentane potential of mean force, and the hydrophobic interaction remains reduced as compared to pure water, much as it is in urea−water systems.[41] Thus, it is unlikely that TMAO counters the denaturing effect of urea by altering interactions between hydrophobic parts of the protein.

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