Large Hydrogen Bond Mismatch between TMAO and Urea Promotes Their Hydrophobic Association

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SUMMARY

TMAO and urea are both osmolytes found in many marine animals, yet show opposite effects in (de-)stabilizing proteins. Gaining molecular-level insights into the TMAO-urea interaction in aqueous solution is a key step to elucidate their biological roles. Here, combined *ab initio* molecular dynamics simulations, polarization-resolved femtosecond infrared pump-probe spectroscopy, and nuclear magnetic resonance spectroscopy reveal that the interaction between TMAO and urea is governed by their hydrophobic association, rather than through direct hydrogen-bonding. The TMAO-urea hydrophobic association is driven by the large mismatch between the strong TMAO-water H-bonds and the weak urea-water H-bond. Our observations provide a rationale for the counteraction of osmotic pressure and protein denaturation due to urea by TMAO.

INTRODUCTION

Trimethylamine *N*-oxide (TMAO) and urea are both osmolytes, which allow living cells to adjust their osmotic pressure; with high concentrations of both urea and TMAO, marine animals can keep the osmotic pressure comparable to that of seawater.[1] Intriguingly, the effects of these osmolytes on protein structures are opposite; TMAO stabilizes the secondary structure of proteins,[2, 3] and can efficiently counteract protein denaturation due to urea, often at an approximate molecular ratio of 1:2 (TMAO:urea).[4, 5] The molecular mechanism underlying this compensation is however still discussed controversially.[5-10] Thus, detailed molecular-level insight into how TMAO and urea interact in an aqueous environment is a key to understanding their role as chemical chaperones to maintain protein functionality. In the context of synthetic biology, such understanding is a prerequisite for the design of synthetic chaperones.

TMAO-urea interactions in an aqueous environment have been studied by focusing on the TMAO-water interaction,[11-13] urea-water interaction,[13-18] and TMAO-urea interaction, [9, 18-21] using various techniques including pump-probe spectroscopy, neutron scattering, and molecular dynamics (MD) simulations. Also, the effects of TMAO and urea on protein structures in aqueous solution have been examined.[2, 4, 22-28] Regarding TMAO-urea interactions, a previous studies using force field MD (FFMD) simulations[7, 9] and neutron scattering measurements[7] have proposed that TMAO and urea interact through the hydrogen-bond (H-bond) between the N_{UREA}-H_{UREA} groups of urea and the hydrophilic O_{TMAO}-N_{TMAO} group. Here, N_{UREA} (H_{UREA}) denotes the nitrogen (hydrogen) atom of urea and O_{TMAO} (N_{TMAO}) denotes the oxygen (nitrogen) atom of TMAO. Other studies have concluded that TMAO and urea form no direct H-bonds but their effect on water and biomolecules is independent of each other.[6, 20] From a computational

perspective, TMAO and urea force field models substantially influence the simulation results.[29] As such, the precise mode and conformation of TMAO-urea interaction have remained elusive.

Here, by combining free energy calculations using *ab initio* MD (AIMD) simulations together with time-resolved infrared (TR-IR) spectroscopy and nuclear magnetic resonance (NMR) spectroscopy, we elucidate the preferred conformation of the TMAO-urea complex in water and the underlying mechanism for the TMAO-urea interaction. AIMD allows us to sample molecular conformations based on electronic structure theory, providing a more robust description of TMAO solvation dynamics than that obtained with FFMD.[30, 31] Our AIMD simulations indicate that TMAO and urea predominantly interact via hydrophobic interaction, as opposed to recent reports.[6, 7, 9, 10, 21] Experimental TR-IR studies of water dynamics in aqueous TMAO-urea solutions confirm that TMAO preferentially hydrogen-bonds to water, rather than to urea. The NMR experiments provide evidence for the close proximity between urea and the hydrophobic methyl groups of TMAO. Our analysis uncovers that the large discrepancy between the H-bond strength of the strongly accepting O_{TMAO} atom and the weakly donating H_{UREA} atom prohibits the direct H-bonded TMAO-urea interaction.

RESULTS AND DISCUSSION

To explore the free energy landscape of the TMAO-urea interaction and the molecular conformations in aqueous solution, we examine the interaction potential as a function of the separation between TMAO and urea in aqueous solution (each at 0.334 mol/L, consistent with the in *vivo* concentration[32]). Therefore, we calculated the potential of mean force (PMF) by varying the intermolecular distance (r) between the O_{TMAO} atom and the carbon atom of urea (C_{UREA}) (more details are provided in Simulation Procedure and Supplemental Information). First, we compared the calculated free energy landscape of the AIMD and FFMD simulations. Figure 1 shows the simulated PMFs of the TMAO-urea interaction. Both AIMD and FFMD PMFs have a minimum at a distance of 5.3 Å $\leq r \leq$ 5.7 Å (in the green shaded region), while the PMFs differ substantially at shorter TMAO-urea distances (r < 5.3 Å); the AIMD PMF suggests that the TMAO-urea interaction becomes increasingly unfavorable with decreasing r, while the FFMD simulation predicts the most stable TMAO-urea conformation is located at r = 4.1 Å (yellow shaded region). A detailed analysis of the interaction conformation (see Supplemental Information) indicates that the energetic minimum at 5.3 Å $\leq r \leq$ 5.7 Å is governed by the hydrophobic interaction between TMAO and urea; the methyl groups of TMAO are facing urea. Conversely, a direct $O_{TMAO}\cdots H_{UREA}$ H-bond is formed between TMAO and urea for the FFMD energetic minimum at r = 4.1 Å. These conformations are schematically depicted in the lower panels of Figure 1. In the following, we investigate the formation mechanism of the

different structures predicted by the FFMD and AIMD simulations, by varying the charge distributions of TMAO and urea in the FFMD simulations.

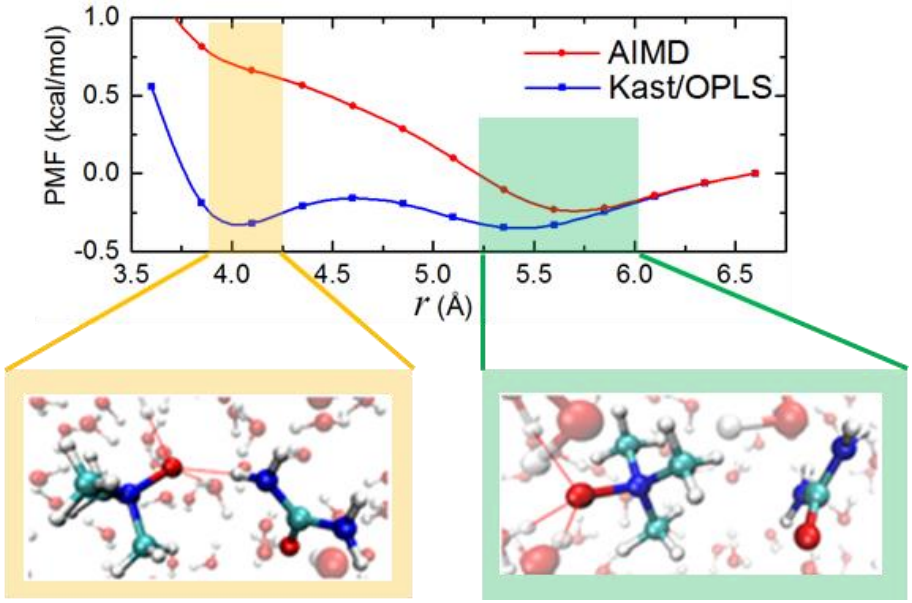

Figure 1. The PMFs obtained from AIMD and Kast/OPLS FFMD simulations and the typical conformations corresponding to the two PMF minima in the FFMD simulation: the direct, H-bonded interaction predicted by FFMD (yellow highlight) is absent for the AIMD results, which predicts hydrophobic interaction (green highlight).

As the partial charge of the O_{TMAO} atom critically affects the H-bond strength of TMAO,[33, 34] we explore how the variation of this charge affects the FFMD PMFs. We calculated the PMFs by systematically varying the charge of O_{TMAO} from -0.65 *e* (as implemented in the Kast model[35]) to -0.91 *e* (used in the Netz model[33]). Here, the charges of the C_{TMAO} and H_{TMAO} atoms were fixed, while the charge assigned to the N_{TMAO} atom was adjusted to ensure charge neutrality of TMAO. The increased partial charge on the O_{TMAO} atom enhances the H-bond accepting strength of TMAO.[31] Nevertheless, counter-intuitively, the enhanced H-bond accepting strength of TMAO destabilizes the TMAO-urea H-bond interaction in water. As can be seen from the simulated PMFs in Figure 2(a), an increase in the partial charge on the O_{TMAO} atom elevates the PMF at r = 4.1 Å, while it does not substantially affect the PMF at r = 5.3 Å. Despite the increase of the H-bonding strength of TMAO, the H-bonded TMAO-urea complex is destabilized, while not affecting the hydrophobic complex.

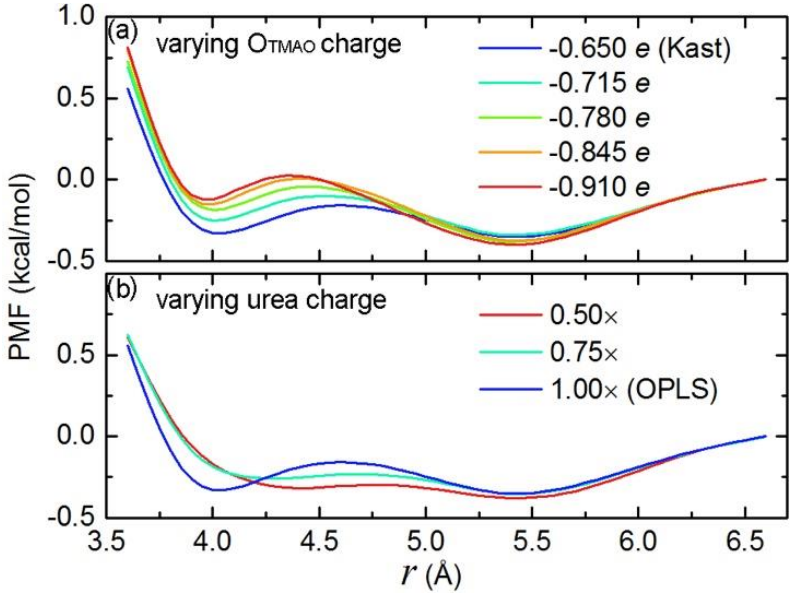

Figure 2. PMFs of the FFMD simulation when (a) varying O_{TMAO} and N_{TMAO} atom charges of the Kast TMAO model, (b) varying atom charges of the OPLS urea model. The Kast TMAO model and the OPLS urea model are labeled in the legends.

Subsequently, we focus on the effects of the charge distribution of urea on the PMFs by scaling the atom charges of the OPLS urea model. We increased the partial charge of H_{UREA}, thereby increasing the H-bond donating strength of urea. The simulated PMFs (Figure 2(b)) show that a higher partial charge on the H_{UREA} atom lowers the PMF at $r = 4.1$ Å; a stronger H-bond donating urea intuitively stabilizes the directly H-bonded TMAO-urea conformation.

Obviously, this opposite effect of increasing the H-bonding strengths of TMAO and urea on the stability of their H-bonded conformation cannot be explained solely from their pair interaction potential; the increase in the absolute value of charges on either O_{TMAO} or H_{UREA} strengthens the $O_{TMAO}\cdots H_{UREA}$ H-bond. Consequently, the data suggest that water plays a key role in the (de-)stabilization of the $O_{TMAO}\cdots H_{UREA}$ H-bond, specifically the $O_{TMAO}\cdots H_W$ and $H_{UREA}\cdots O_W$ H-bond interactions, where H_W and O_W denote the H and O atoms of the water molecule, respectively. To assess the relative strengths of the TMAO-water, water-water, and urea-water H-bonds, we compute the H-bond time correlation function;[36]

$$P_{\mathrm{HB}}(t) = \frac{\langle h(0)h(t)\rangle}{\langle h(0)\rangle}$$

$h(t)$ is unity when 1.59 Å < $r_{O\cdots H}$ < 2.27 Å, 0 otherwise, where $r_{O\cdots H}$ denotes the intermolecular distance between O and H atoms. Slower decay of $P_{\mathrm{HB}}(t)$ indicates a longer-lived, stronger H-bond.

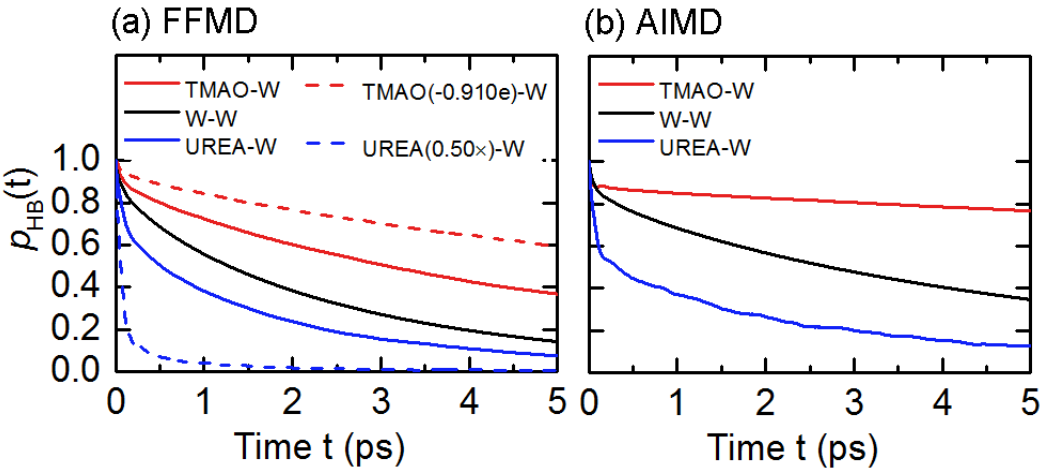

Figure 3. H-bond time correlation functions for different hydrogen-bonded pairs, in (a) FFMD and (b) AIMD of aqueous TMAO solutions (TMAO-W), aqueous urea solutions (UREA-W), and pure water (W-W), where W stands for water.

Figure 3(a) shows the FFMD data of the $O_{TMAO}\cdots H_W$, $O_W\cdots H_W$, and $H_{UREA}\cdots O_W$ H-bond time correlation functions. The H-bond lifetime increases in the order of $\tau_{UREA\text{-}W} < \tau_{W\text{-}W} < \tau_{TMAO\text{-}W}$, indicating that the H-bond strength increases in the order of $H_{UREA}\cdots O_W$, $O_W\cdots H_W$, and $O_{TMAO}\cdots H_W$. When the absolute value of the O_{TMAO} partial charge is increased, the $O_{TMAO}\cdots H_W$ H-bond dynamics slows down even more. Similarly, the decrease in H_{UREA} partial charge accelerates the $H_{UREA}\cdots O_W$ H-bond dynamics. As such, an increase in the absolute value of the O_{TMAO} charge (decrease in the H_{UREA} charge) enhances the difference between $O_{TMAO}\cdots H_W$ ($H_{UREA}\cdots O_W$) and $O_W\cdots H_W$ H-bond strength and lifetimes. Apparently, as the PMFs in Figure 2 reveal, the enhanced differences in the H-bond strengths of $O_{TMAO}\cdots H_W$ and $O_W\cdots H_W$ and those of $H_{UREA}\cdots O_W$ and $O_W\cdots H_W$ both destabilize the directly H-bonded TMAO-urea conformation.

Why does such enhanced difference of TMAO-water or urea-water H-bonds relative to water-water H-bonds destabilize the TMAO-urea conformation? This can be understood as follows: an increase in the absolute value of the O_{TMAO} charge makes the $O_{TMAO}\cdots H_W$ interaction more favorable than the $O_{TMAO}\cdots H_{UREA}$ interaction, as the difference in the interaction energy increases due to the enhanced electrostatic contributions. The decrease in the H_{UREA} charge also makes the $O_{TMAO}\cdots H_W$ interaction more favorable than the $O_{TMAO}\cdots H_{UREA}$ interaction, since the $O_{TMAO}\cdots H_{UREA}$ interaction is destabilized.

Based on this understanding, we turn our focus to the AIMD H-bond dynamics in Figure 3(b). The differences between the $O_{TMAO}\cdots H_W$, $O_W\cdots H_W$, and $H_{UREA}\cdots O_W$ H-bond dynamics our more pronounced in the AIMD simulation than in the FFMD simulation. Based on the above notion, the enhanced difference

in the H-bond strengths of TMAO and urea in the AIMD simulation leads to an even more unfavorable H-bonded interaction of TMAO-urea, consistent with the PMF (Figure 1). As such, our AIMD results show that the O_{TMAO} atom is nearly exclusively H-bonded to water molecules in aqueous solutions of TMAO and urea, in contrast to previous reports based on FFMD results.[7, 9, 29] Because of the unstable H-bonded TMAO-urea conformation, the hydrophobic association of TMAO and urea becomes the only favorable TMAO-urea conformation.

Experiments support the conclusion that TMAO and urea interact hydrophobically, rather than through H-bonding interaction in the aqueous mixture. As discussed below, polarization-resolved femto-second infrared pump-probe experiments[37, 38] reveal the TMAO hydrogen bonds preferentially with water, rather than with urea. The result of NMR experiments are consistent with the hydrophobic interactions between TMAO and urea.

In the femtosecond infrared experiments, the effect of TMAO and urea on the dynamics of water is investigated. In these experiments, an intense infrared pulse excites the O-D stretch vibration for HOD molecules diluted in H_2O. Since O-D oscillators parallel to the laser pulse polarization are preferentially excited, the excitation is anisotropic. Due to the random orientational motion of water, the anisotropy decays (with a decay time of ~2 ps for pure water).

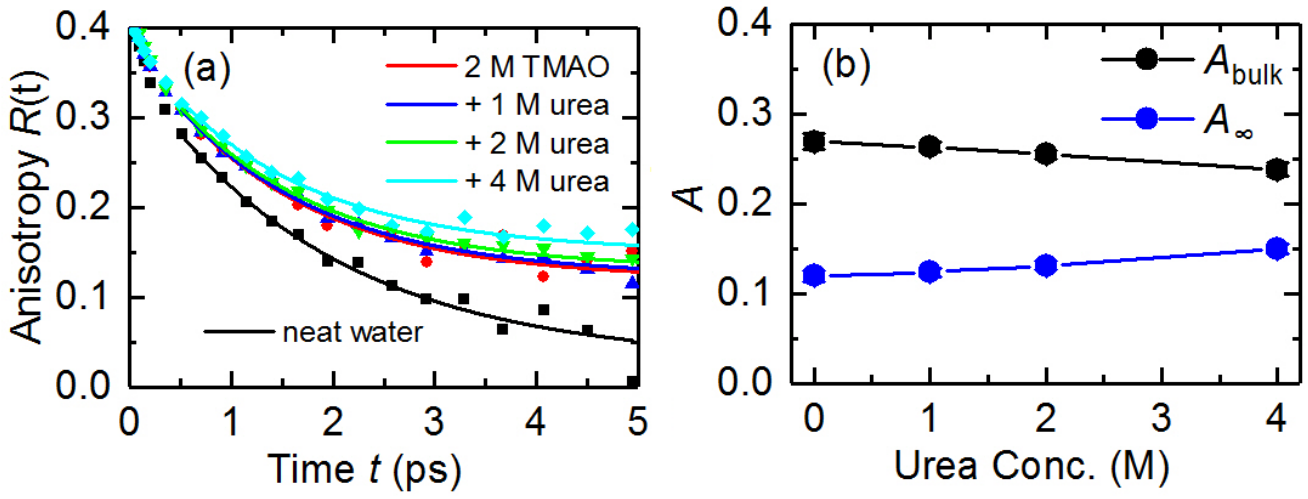

Figure 4. (a) Decay of the excitation anisotropy $R(t)$ of the OD stretching vibration (2470 – 2530 cm^{-1}) for neat water (black), 2 M TMAO (red), 2 M TMAO/1 M urea (blue), 2 M TMAO/2 M urea (green) and 2 M TMAO/4 M urea (cyan). The solid lines show fits of a mono-exponential decays with an offset ($R(t) = A_{bulk} \exp(-t/\tau_{rot}) + A_\infty$) to the data.[37] (b) Values of A_∞, and A_{bulk} for aqueous TMAO solutions (2 M) with different concentrations of urea.

The anisotropy decay data are displayed in Figure 4(a). The comparison of neat water (black) and aqueous TMAO solution (red) indicates that the presence of TMAO causes the orientational motion of a significant fraction of the O-D groups to be almost immobilized, with no reorientation discernible on the experimentally accessible time window of ~5 ps.[37] This "immobilization" is caused by the formation of strong, long-lived $O_{TMAO}\cdots H_W$ H-bonds.[11, 38] To quantify the fraction of these immobilized water, we fit a mono-exponential decay with an offset (A_∞) to the experimental data, where A_∞ is proportional to the number of immobilized water molecules.[16] These fits confirm the qualitative observations: 2M TMAO immobilizes 30% of the water molecules (A_∞ = 0.12 out of 0.4). As the observed immobilization of water dynamics due to TMAO originates from the strong H-bonds that are formed between O_{TMAO} and water, the insensitivity of water rotational motion towards urea indicates that the long-lived $O_{TMAO}\ldots H_W$ H-bonds stay intact for all studied solutions. Thus, in line with the AIMD results, the rotational dynamics of water as measured with infrared pump-probe spectroscopy provides experimental evidence for the absence of direct H-bonds formed between TMAO and urea, which would release HOD molecules bonded to TMAO and thus speed up water dynamics. Contrarily, we find a similar effect of urea on water dynamics in solutions of TMAO as found for aqueous solutions of only urea.[19] Thus, the effect of TMAO and urea on water dynamics is found to be simply additive.

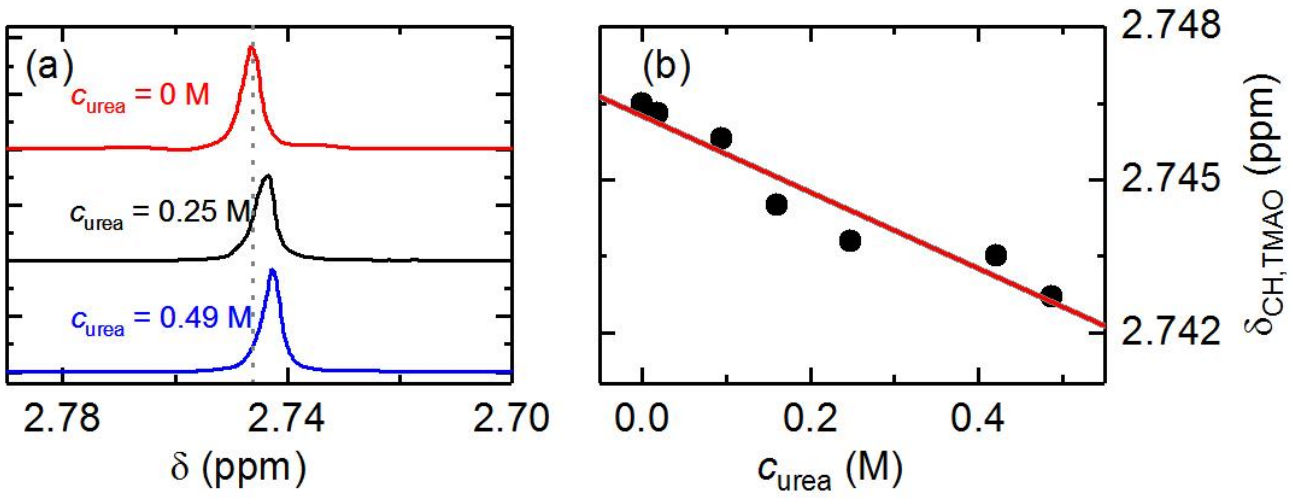

Figure 5. (a) ^{1}H-NMR spectra for solutions of TMAO (c_{TMAO} = 0.35 M) with increasing concentration of urea. The peaks correspond to the CH_3 protons of TMAO, which shift up-field upon addition of urea. (b) Extracted chemical shift of the CH_3 protons as a function of c_{urea} at a constant concentration of TMAO (c_{TMAO} = 0.35 M). The red line is a guide to the eye.

Experimental and computational NMR data further support the conclusion that TMAO and urea interact via the hydrophobic CH_3 groups of TMAO. ^{1}H NMR spectra of solutions with 0.35 mol/L TMAO indicate that the chemical shift of the CH_3 group of TMAO shifts up-field with increasing concentration of urea (from 0 to 0.49 mol/L urea, see Figure 5, for experimental details see Supplemental Information). *Ab initio* calculation for the chemical shift of TMAO ^{1}H chemical shift (see Table S4 of the Supplemental Information) indicates that this up-field arises from the proximity of urea to TMAO's CH_3 groups, while for substitution of a water-TMAO ($H_W \ldots O_{TMAO}$) hydrogen-bond by a urea-TMAO ($H_{UREA} \ldots O_{TMAO}$) a down-field shift of the TMAO's CH_3 groups would be expected. Although it is generally extremely challenging to directly prove intermolecular interactions using NMR for such weak association,[39] the NMR spectra together with the chemical shift calculations provide strong evidence for the proximity of urea to TMAO's CH_3 groups. Thus, the NMR chemical shifts are consistent with the hydrophobic interaction between TMAO and urea.

The observation of the hydrophobic interaction between TMAO and urea has two major implications. (1) The (ensemble) averaged strength of all H-bonds that water forms is correlated to the solution osmotic coefficient. In urea solution, the H-bond strength of water is reduced, as urea-water H-bonds are weaker than water-water H-bonds and thus increase the osmotic pressure. TMAO can counter this increase in the osmotic stress due to urea, by forming stronger H-bonds to water than water-water H-bonds. Our findings thus provide a rationale for the counteracting effects of TMAO and urea on the osmotic stress. This counteraction occurs via independent interaction of urea and TMAO with water, which is in line with the conclusions from experimental measurements of the osmotic pressure for TMAO-urea solutions.[10] (2) While the stabilizing effect of TMAO on proteins may partially originate from the strong binding of water to TMAO, our results indicate that it cannot fully account for TMAO's counteraction for protein destabilization due to urea. We observed that the direct TMAO-urea interactions are exclusively hydrophobic. When TMAO is added to the protein-urea solution, it can be expected that the hydrophobic urea-protein interaction is weakened due to TMAO, since TMAO offers additional sites for hydrophobic interaction with urea. This is consistent with a previous report suggesting that urea denatures proteins via hydrophobic interaction with the protein.[40] Furthermore, Rösgen and Jackson-Atogi have shown that TMAO behaves like hard-sphere with two hydration sites which can be replaced by urea.[10] Here, we show that the hydration sites where urea replaces water are located at the hydrophobic methyl groups of TMAO instead of the previously supposed hydrophilic part. Note, that the hydrophobic interaction between TMAO and urea can also explain the non-ideal behavior of the viscosity and the molecular rotation times

of TMAO and urea in aqueous solution, which some of us have ascribed previously to water mediated interactions between TMAO and urea.[21]

In summary, our combined AIMD, ultrafast polarization-resolved infrared measurement, and NMR measurement show that TMAO does not directly H-bond with urea in aqueous solution, in contrast to conclusions from previous FFMD studies. The absence of direct TMAO-urea interactions can be traced to the large difference in their H-bonding abilities; TMAO-water H-bonds are much stronger than urea-water H-bonds. Water molecules H-bonded to TMAO are therefore *not* replaced by urea, and the TMAO-urea interaction is dominated by hydrophobic interaction between the methyl groups of TMAO and urea. Our results show that the different, compensatory osmotic effect of TMAO and urea stems from their individual and independent interaction with water. Additionally, the direct, hydrophobic interaction of TMAO and urea can prevent urea from hydrophobic binding to proteins – and thereby possibly prevent protein denaturation.

SIMULATION PROCEDURE

To explore the energy landscape of the TMAO-urea interaction and the molecular conformations in aqueous solution, we used AIMD and FFMD simulations. AIMD simulation employed the BLYP/TZV2P level of theory together with the Grimme's D3 van der Waals correction.[41] For FFMD simulation, we adopted the Kast model[35] for TMAO, the OPLS model[42] for urea, and the SPC/E model[43] for water. To obtain the distance-dependent interaction energy, we calculated the potential of mean force (PMF) using thermodynamic integration.[44] We took the intermolecular distance (r) between the O_{TMAO} atom and the carbon atom of urea (C_{UREA}) as an order parameter. The concentration of each osmolyte in the simulation is 0.334 mol/L which is consistent with the in *vivo* concentration.[32] Furthermore, to characterize the interaction between the solutes and water, we computed the H-bond dynamics with AIMD and FFMD simulations. Details of the simulation protocols are given in Supplemental Information.

EXPERIMENTAL PROCEDURE

Details of the polarization-dependent time-resolved infrared measurements, description of the analysis of the transient spectra, and the procedure how the thermal contribution to the spectra has been accounted for are given in Supplemental Information. Furthermore, NMR measurement protocols are also given in Supplemental Information.

SUPPLEMENTAL INFORMATION

The Supplemental Information includes computational and experimental details, supporting data of simulations, and NMR data can be found online with this article at http://dx.doi.org/

AUTHOR CONTRIBUTIONS

WJX, JH, YN, and MB conceived the research idea, WJX, TO, WM, and YN performed the simulation, SC and MW performed the experiment, WJX, SC, JH, YN analyzed the data, and all the authors wrote the manuscript.

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