Protein-stability in TMAO and Mixed Urea–TMAO Solutions

Pritam Ganguly[1], Jakub Polák[2], Nico F. A. van der Vegt[3], Jan Heyda[2], and Joan-Emma Shea[1,4]

[1]*Department of Chemistry and Biochemistry, University of California at Santa Barbara, Santa Barbara, California 93106, USA.*

[2]*Department of Physical Chemistry, University of Chemistry and Technology, Prague, Technická 5, 166 28 Prague 6, Czech Republic.*

[3]*Eduard-Zintl-Institut für Anorganische und Physikalische Chemie, Technische Universität Darmstadt, Alarich-Weiss-Straße 10, Darmstadt 64287, Germany.*

[4]*Department of Physics, University of California at Santa Barbara, Santa Barbara, California 93106, USA.*

Abstract

Osmolytes are essential for cellular function under ubiquitous osmotic stress. Trimethylamine N-oxide (TMAO) is one such osmolyte that has gained remarkable attention due to its protein-protective ability against urea. This review aims at providing a detailed account of recent theoretical and experimental developments in characterizing structural changes and thermodynamic stability of proteins in the presence of TMAO and urea. New vapor pressure osmometry and molecular dynamics simulation results on urea-TMAO solutions are presented, and a unified molecular mechanism of TMAO counteraction of urea-induced protein denaturation is introduced. In addition, a detailed technical assessment of molecular dynamics force fields for TMAO or for urea-TMAO solutions is presented. The force field analysis highlights how many commonly used force field models are in fact incompatible with solvation thermodynamics and can lead to misleading conclusions. A new optimized force field for TMAO (Shea(m)) is presented and a recently optimized force field for TMAO-urea (Netz(m)) that best reproduces experimental data is highlighted.

1 INTRODUCTION

Organic osmolytes are small molecules which can accumulate in living cells in response to high osmotic stress. An important osmolyte, urea, accumulates in response to hypertonicity in the tissues and extracellular fluid in Elasmobranchii (a subclass of cartilaginous fishes), as well as in mammal renal medullary cells. High concentrations of urea are detrimental to cells as urea unfolds proteins thereby inhibiting critical enzymatic activity. In cartilaginous fishes, such as rays and sharks, the effects of urea is counteracted by the presence of another osmolyte, trimethylamine N-oxide (TMAO).[1,2] Over the last two decades, and in particular in the last decade, the opposing effects of urea and TMAO on protein stability have been heavily explored through experimental and theoretical approaches. The molecular mechanism for the counteraction has been hotly debated in the literature, with often with contradictory mechanisms proposed. In this review, we focus on presenting a unified and consistent picture of the solvation thermodynamics and structural stability of proteins in TMAO and mixed urea-TMAO solutions through an analysis of recent experimental and molecular dynamics (MD) simulations studies. MD simulations provide an important route to understanding molecular mechanisms through which TMAO stabilizes proteins. However, the choice of force field plays a crucial role in dictating the resulting mechanisms. Hence, we include a critical analysis of the strengths and the limitations of existing TMAO and TMAO/urea force fields. We introduce a new force field for TMAO (the Shea(m) force field) and highlight the recently optimized TMAO-urea (Netz(m)). The effect of TMAO and urea on protein structure and stability depends heavily on the concentrations of these osmolytes present, and we address this important consideration in this review through new vapor pressure osmometry and MD simulation studies. The review is structured as follows: First, we discuss TMAO's effects on water and the solvation of hydrophobes and peptides in TMAO-water solutions and present a critical analysis of force fields. Next, we introduce the effect of urea and focus on ternary water-urea-TMAO solutions and the challenges associated with extending TMAO force fields to properly account for TMAO-urea interactions. We present a discussion of the solvation thermodynamics of ternary urea-TMAO-water solutions and address the effects of urea-TMAO solutions on amino acids interactions and on peptide structure.

2 BINARY TMAO-WATER SOLUTIONS

2.1 TMAO's effects on solvation water. TMAO is an amphiphilic molecule which has a strong dipole moment due to the presence of the $N^+ - O^-$ bond. The dipole moment of TMAO has been estimated to be ≈4.55 Debye (DFT calculations)[3] or ≈5.04 Debye in apolar medium (experimental results in dioxane).[4] The strong dipole moment of TMAO makes it an extremely hydrophilic molecule. In infrared spectroscopy experiments, the red shift or the decrease in the frequency of the O-H bond stretching modes of water provides evidence for the formation of strong TMAO-water hydrogen bonds.[5–7] Subsequently, TMAO increases the frequency of the HOH bending mode of water.[7] FTIR spectra also show that TMAO makes the hydrogen-bond network of isotopically labeled water (HDO) more ordered and the TMAO-water interactions are much stronger than the urea-water interactions.[8] Mid-infrared pump-probe spectroscopy shows that TMAO also decreases the O-D stretching frequency of HDO and slows down the dynamics of the O-D groups.[9,10] Raman spectroscopy has shown that TMAO can accept three or more number of hydrogen bonds (up to eight) with water.[11] Earlier MD simulation studies have also predicted strong hydrogen bond formation between TMAO and water.[12,13] It is worth mentioning that few earlier MD simulation studies have reported no significant modification of the water hydrogen-bond network by the addition of TMAO.[14,15] The difference in the observations in these MD simulation studies arises from the choice of the TMAO force-field and this issue will be discussed in detail later in this review.

In contrast to the earlier findings by Stangret and coworkers,[8] using ultrafast optical Kerr effect and THz Raman spectroscopy, Meech and coworkers have shown that TMAO has much smaller effects on the structure and the relaxation dynamics of the solvation water than the other "hydrophilic" solutes such as urea.[16] However, TMAO has small but comparatively greater effects on slowing down the relaxation dynamics of the solvation water than another amphiphilic molecule t-butyl alcohol (TBA).[17] The authors have hypothesized that, at higher concentrations, TMAO molecules self-aggregate and their effects on the structure of the solvation water become rather insignificant.[16] However, earlier infrared spectroscopy experiments have shown no evidence for TMAO-TMAO self-aggregation.[5,6] MD simulations by Laage and coworkers predict a significant slow-down of the dynamics of the solvation water around TMAO.[18] Far-infrared/THz spectroscopy and Raman spectroscopy have also shown evidences for local perturbation of the water structure when TMAO is added to pure water.[19] Laage and coworkers have predicted that the slow-down of the water-dynamics is significantly higher around the hydrophilic part ($\mathrm{N-O}$ group) of the TMAO molecule than around the hydrophobic part (trimethyl group).[18] Recently, similar observations have also been made by Marx and coworkers where the authors have employed ab initio molecular dynamics (AIMD) simulation techniques to theoretically interpret THz spectropscopy results.[20] In another AIMD simulation work, Usui et al. have also found strong directional hydrogen bonds between TMAO and water.[21] From the majority of these aforementioned studies it can be concluded that TMAO forms stable complexes with 2-3 water molecules[10] through strong hydrogen bonds[5,7,10,11] and the water molecules interact with TMAO preferably through the $\mathrm{N-O}$ group of TMAO.[18,20]

2.2 How does TMAO protect proteins? TMAO stabilizes proteins at $\mathrm{pH} > 4.7$, which is the $\mathrm{pK_a}$ of TMAO.[22,23] In this review we will discuss the effects of TMAO at neutral pH. The protein-protective mechanisms of TMAO, as proposed in the literature, can be broadly classified into two categories: a) unfavorable interactions between proteins and TMAO[24] and b) an indirect mechanism where TMAO modifies the structure and the dynamics of the solvation water around protein.[25] The interaction between proteins and TMAO can be manifested by the preferential binding of TMAO with the proteins and the solvation free-energy of the proteins in TMAO solutions (please refer to the Supporting Information for further details). Using dialysis experiments for calculating preferential binding coefficients, Timasheff and coworkers have shown that TMAO is preferentially excluded from peptides.[26] By means of calculating the water-to-solution transfer free energies of amino acids and diketopiperazine (to mimic the peptide backbone), Wang and Bolen have shown that TMAO has unfavorable interactions with the peptide backbone.[27] This work has later been extended to quantitatively demonstrate how the unfavorable interaction between protein backbone and TMAO leads to the folding of proteins in TMAO.[28,29] Alternatively, using 2-D IR spectroscopy with a site-specific IR probe, Gai and coworkers have studied protein folding-unfolding dynamics in TMAO solutions and concluded that TMAO stabilizes proteins by reducing the number of protein-water hydrogen bonds.[25] Along with the enthalpic gain resulting from the disruption of the hydrogen bonded network of the solvation water around protein, TMAO also acts as a molecular crowder which contributes to the protein stability via excluded volume effects.[25] Using scaled particle theory, the excluded volume effects of TMAO are also argued by Graziano.[30]

An MD simulation study by García and coworkers has pointed at the exclusion of TMAO from proteins.[31,32] However, the authors have also shown that the preferential interaction of TMAO with proteins strongly depends on the choice of TMAO force field.[31] One of the earlier developments of TMAO force fields, the Kast model,[33] significantly over-estimates protein-TMAO preferential interactions.[31,34] Using this force field, Thirumalai and coworkers have demonstrated that the peptide-TMAO preferential interactions depend on the chain-length of the peptides.[35] In that work, it has been shown that TMAO can preferentially bind to shorter peptides, whereas, it is excluded from longer peptides. It has also been argued that TMAO entropically stabilizes proteins via a crowding mechanism,[35] similar to the mechanism proposed by Gai and coworkers.[25] We have earlier shown that the peptide-TMAO preferential interaction can be negative or slightly positive, depending on the peptide composition and the choice of the TMAO and the peptide force fields.[34] Slightly positive preferential interactions between TMAO and peptides are also found in the work of Cremer and coworkers[36] and in the work of Medvedev and coworkers (by comparing the TMAO volume-fraction in the bulk and at the protein surface).[37] These two works use the Kast TMAO model and a variant of the Kast TMAO model which correctly reproduces density of binary TMAO-water solutions (the Shea model,[38] details can be found in the next subsection) respectively. The work by Cremer and coworkers proposes a surfactant-like mechanism for TMAO-induced protein-stabilization, since TMAO reduces the surface tension of the water-air interface.[36] From the above discussion it is clear that the nature of the peptide-TMAO interactions, as predicted by the MD simulation studies, depends heavily on the choice of the force fields. In the next subsection we will discuss various TMAO force fields in detail. After understanding the features and the limitations of these force fields we will again discuss the peptide-TMAO interactions in the light of MD simulations.

2.2.1 TMAO force fields for the MD simulations. In this section, we discuss five all-atom 14-site TMAO force fields developed in the last two decades and compare their reproducibility in terms of six important experimental properties of binary TMAO-water solutions, which are: density, derivative of the TMAO activity coefficient with respect to TMAO molarity (activity derivative), surface tension, number of TMAO-water hydrogen bonds, osmotic coefficient and water self-diffusion coefficient. The five force fields which will be discussed are: the Kast,[33] the García,[31] the Shea,[38] the Netz,[39] and the Hölzl.[40] The last four force fields use the bonded interaction parameters from the Kast model and the nonbonded interaction parameters (Coulomb and van der Waals) are reparameterized to reproduce experimental properties of TMAO-water solutions. Detailed comparisons of these TMAO force fields can be found in the works of Rodríguez-Ropero et al.,[43] Markthaler et al.,[44] and Usui et al.[45] To compare these force fields at once, we have calculated the density and the activity derivatives (in Molar scale) of TMAO-water solutions in this work. The new data are presented in Figure 1 and discussed below. In addition, we introduce a new optimized force field (the Shea(m) force field) that overcomes limitations of existing force fields.

The Kast model: To our knowledge, the Kast model is the first fully-atomistic TMAO force field which uses 12-6 Lennard-Jones function for the van der Waals interactions. The Kast model is developed using quantum chemical ab initio calculations. The model is tested against crystallographic experimental data of TMAO dihydrates. While the Lennard-Jones van der Waals parameters are developed using hydrates of TMAO, the charge distribution on the atoms of TMAO is derived for isolated TMAO molecule in the gas phase. This way, the charge distribution on the TMAO atoms does not guarantee its applicability in the aqueous medium. However, the density of TMAO-water solution has been tested in combination with

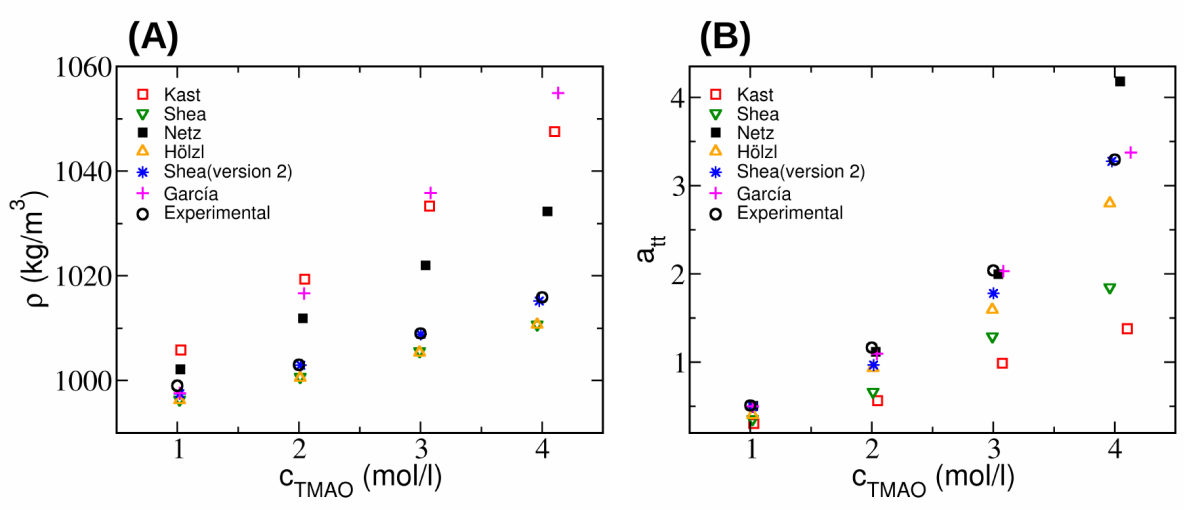

Figure 1: Shown are (A) the density ρ and (B) the activity derivative $a_{tt} = \left(\frac{\partial \ln a_t}{\partial \ln c_{TMAO}}\right)_{T,p}$ of TMAO-water solutions with increasing TMAO concentrations (c_{TMAO}, molar scale) using different TMAO force fields, where a_t is the molar activity coefficient. The Kast,[33] the Shea,[38] the Netz,[39] the Hölzl[40] and the Shea(version 2) models use SPC/E water[41] parameters. The García[31] model uses TIP3P water.[42]

the TIP3P water model.[42] Later, it has been found that the Kast TMAO model has a propensity to self-aggregate and it underestimates TMAO-water interactions resulting in an underestimation of the activity coefficient[39] and the osmotic pressure of TMAO-water solutions.[31] The low activity of this model can also be deduced from the data in Figure 1(B). The Kast model also produces higher density for the TMAO-water solutions at the room temperature[39,45] when the SPC/E water model[41] is used (also see Figure 1A). The density results are closer to the experiments if the TIP3P water model[42] is used, as tested while developing the Kast TMAO force field.[33] It forms the lowest number of TMAO-water hydrogen bonds ($\approx$ 2.7 at 4 M TMAO) among all the five TMAO force fields mentioned earlier.[46] The Kast model qualitatively reproduces the decrease in the liquid-vapor surface tension for the TMAO-water mixtures with increasing TMAO concentrations (however, with the Kast model, the liquid-vapor surface tension slightly increases at very low TMAO concentrations, 0.5 molal).[36,43] Although quantitatively the surface tension is significantly lower than the experimental values when the SPC/E water model[41] is used, the mismatch primarily emerges from the limitation of the water model to reproduce the liquid-vapor surface tension.[47,48]

The García model: The García model for TMAO was developed to overcome the limitation of the Kast force field in reproducing the experimental osmotic coefficients of TMAO-water solutions.[31] In the García model, the charge distribution of the Kast TMAO model is enhanced by 20% and the TMAO-TMAO van der Waals interactions are reduced by 25%. These changes were introduced to increase the TMAO-water interaction and to prevent the TMAO self-aggregation, which cooperatively increase the osmotic pressure of the aqueous TMAO solutions. The García model was developed using the TIP3P water model. TMAO-water solutions modeled with the García model and the TIP3P water model significantly overestimate the density at higher TMAO concentrations ($>$ 2 molal).[44] The García model also predicts a higher self-diffusion coefficient of water.[44] However, the activity derivatives predicted by the García model with TIP3P water show excellent agreement with the experiments (Figure 1B). At 4 M TMAO it forms $\approx$3.2 hydrogen bonds with water.[46] In combination with the TIP3P or the SPC/E water model, the García force field predicts an increase of the surface tension of the liquid-vapor interface with increasing concentration of TMAO, which is qualitatively opposite to the experimental observation.[49] It is worth noting that if the TIP4P/2005 water model[50] is used instead, the liquid-vapor surface tension actually decreases.[36] However, the other properties of the García model have not been tested with the TIP4P/2005 water model.

The Netz model: The Netz TMAO force field has been developed to reproduce experimental activity coefficient of TMAO-water solutions and the m-value of polyglycine in TMAO-water solutions.[39] The model correctly reproduces the anomaly of polytryptophan (negative m-value) in TMAO-water. In comparison with the Kast model, the Netz model uses a higher dipole moment for the N-O bond of TMAO by assigning more positive charge on the nitrogen atom and more negative charge on the oxygen atom. This model also increases the hydrophobicity of the trimethyl group of TMAO by using a larger atomic radius (in practice it increases the σ-values in the Lennard-Jones interactions) for the carbon and the hydrogen atoms. The model is developed using the SPC/E water model.[41] The Netz model is significantly more hydrophilic than the Kast model and it forms the most number of hydrogen bonds with water ($\approx$ 3.2 at 4 M TMAO),[46] similar to the García model.[44] The self-diffusion coefficient of water in TMAO-water solution is well-reproduced when the Netz model is used.[44] With this TMAO model, the osmotic coefficients of TMAO-water solutions are underestimated at the lower TMAO concentrations, however, it reproduces the experimental osmotic coefficients at relatively higher TMAO concentrations ($\geq$ 1 molal).[43] From Figure 1(B) we can see that the activity coefficients of TMAO-water solutions are well reproduced by the Netz TMAO model with the SPC/E water up to 3 M TMAO concentration; however, the Netz force field predicts a higher activity coefficient than experiment at 4 M TMAO. The original Netz force field paper reports a moderately higher activity derivative at 0.08 mole-fraction (for comparison: 4 M TMAO is equivalent to 0.09 mole-fraction in the current work REWORD).[39] Two other important limitations of the Netz TMAO model are: a) the densities of the TMAO-water solutions are predicted to be significantly higher than the experimental values[44,45] and b) the sur-

face tension of the liquid-vapor interface increases with increasing TMAO concentrations, similar to the results found with the García model.[43,49] As in the García model, the liquid-vapor surface tension decreases upon the addition of the Netz TMAO, if the TIP4P/2005 water model is used.[36]

The Shea model: As mentioned earlier, the Kast TMAO model predicts higher density in conjunction with the SPC/E water model. The Shea model uses similar charge distribution on the TMAO atoms as the Kast model but it assigns larger atomic radius (larger σ) and shallower van der Waals interactions (smaller ϵ in the Lennard-Jones function).[38] In this way it corrects for the higher density predicted by the Kast model and well-reproduces the experimental density.[44,45] A separate version of the Shea force field has been developed for the TIP3P water,[38] however, here we will only discuss the results obtained with the SPC/E version of the Shea model. The Shea TMAO model forms similar number of hydrogen bonds (≈ 2.7 at 4 M TMAO) with water as the Kast model does,[46] which can be attributed to the similar charge distribution on the TMAO atoms for these two force fields. As seen from Figure 1(B), the Shea model predicts lower activity coefficients for the aqueous TMAO solutions. However, the osmotic coefficients are reasonably well-reproduced for the TMAO concentrations < 2.5 molal.[43] The self-diffusion coefficient of water is slightly overestimated by the Shea TMAO model.[44] With this force field, the liquid-vapor surface tension decreases with increase in TMAO concentration.[36,43]

The Hölzl model: The Hölzl TMAO model further modifies the Netz model by reducing the charge separation across the N–O bond and by slightly increasing the van der Waals radius (the σ in the Lennard-Jones function) of the carbon atoms in order to reproduce the experimental activity coefficients and the densities of the TMAO-water solutions.[40] Although the model is primarily developed using the TIP4P/2005 water model, it reproduces correct density of the TMAO-water solutions and the self-diffusion coefficient of water when it is used in conjunction with the SPC/E model as well.[44] With the TIP4P/2005 water, it well-reproduces the experimental activity derivatives. However, with the SPC/E water, it slightly underestimates the activity derivatives (see Figure 1B). With increasing TMAO concentrations, the Hölzl model predicts slight increase in the liquid-vapor surface tension (3% increase at 3 M concentration, results from this work). The Hölzl TMAO model forms ≈ 3 hydrogen bonds (at 4 M TMAO) with water when the SPC/E water model is used.[46] The number of TMAO-water hydrogen bonds is very similar when the Hölzl model is used with the TIP4P/2005 or with the SPC/E water model.[44]

In addition, the dielectric properties of the TMAO-water solutions in terms of the loss peak amplitude and the corresponding loss peak frequency are reasonably well-reproduced by the Netz (with SPC/E water) and the Hölzl model (with SPC/E water); however, the reduced static permittivity is systematically underestimated by the Netz, the Shea and the Hölzl TMAO models where the García model significantly overestimates it.[44] The overall best match with the experimental dielectric spectra[10] is found with the Netz TMAO force field. Apart from the force fields discussed above, another variant of the Kast force field has been developed where three virtual sites with charges are attached to the oxygen atom of TMAO to capture the correct directionality of the TMAO-water hydrogen bonds.[45] This force field, the Usui model, significantly improves the properties of the TMAO solutions when compared with the the Kast model in terms of the experimental viscosity and the TMAO-water hydrogen bond correlation function (with respect to AIMD calculations). However, the Netz model also reproduces these properties of the aqueous solution of TMAO reasonably well.[45]

The Shea(m) model: In order to overcome the limitations of the Netz TMAO force field (higher density with SPC/E water) and the Hölzl model (lower activity derivative and slightly lower density with SPC/E water), here we develop a new TMAO force field to work in conjunction with the SPC/E water model. We start from the Hölzl model, and systematically vary the charges of the N–O dipole. We find that the combination of a 0.85e charge on the oxygen atom and a 0.64e charge on the nitrogen atom, without modifying any other parameter, provides the best match with experiments when the density and the activity derivative of TMAO-water solutions up to 4 M concentrations are compared. The corresponding results, denoted as Shea(version 2) are shown in Figures 1(A) and (B). Similar to the original Hölzl model, with increasing TMAO concentrations, the Shea(version 2) model also predicts slight increase in the liquid-vapor surface tension (2% increase at 3 M concentration, results from this work). With the Shea(version 2) model, each TMAO molecule forms ≈ 3 hydrogen bonds with water at 4 M TMAO concentration.

2.2.2 MD simulation of hydrophobic interactions in TMAO solutions.

Hydrophobic interactions between nonpolar groups of proteins play a crucial role in protein-folding.[51,52] Here we will discuss the effects of TMAO on hydrophobic interactions in the context of small hydrophobic molecules and hydrophobic polymers, starting with the small hydrophobic molecules. Garde and coworkers have shown that TMAO does not have any significant effect on the potential of mean force (PMF) between methane molecules[53] because of entropy-enthalpy compensation.[54] In contrast, Paul and Patey have reported that the PMF between a neopentane pair is significantly destabilized by TMAO.[55,56] However, in the later studies by Sarma and Paul, significantly less pronounced modification to the neopentane-neopentane[57] or methane-methane[58] PMFs has been observed. All the above mentioned studies have been performed using the Kast TMAO model. In a more recent study, we have pointed out the importance of the choice of the TMAO force fields on hydrophobic interactions. By calculating the Kirkwood-Buff integrals (KBIs, details can be found in the Supporting Information) between the neopentane molecules in TMAO-water solution, we have found that the Kast TMAO model slightly disrupts the hydrophobic association between neopentane molecules whereas the Netz model significantly enhances the association.[46] Our inferences have been further supported by the work of Dias and coworkers.[59] The authors of the later paper have shown that the Netz TMAO model stabilizes the PMF between two neopentane molecules whereas the Kast model remains ineffective. We have found that TMAO, modeled with the Netz parameters, is preferentially excluded from neopentane which indicates that the change in the solvation free energy of neopentane upon the addition of TMAO is positive and this makes neopentane "less" soluble in TMAO-water.

Using the Kast TMAO model, the effect of TMAO on the folding-unfolding transitions of a model hydrophobic chain has been studied by Garde and coworkers.[53] Similar to their findings on the hydrophobic interaction between methane pairs, no significant effects of TMAO on the conformal equilibria of the chain have been found. In contrast, using the same TMAO model, Berne and coworkers have reported TMAO-induced collapse of a model hydrophobic chain,[60] and later of a hydrophobic polymer, polystyrene.[61] The preferential interaction between TMAO and the hydrophobic chain or polystyrene has been found to be positive. However, the TMAO-polymer preferential interaction has been found to be decreasing upon the unfolding of the chain which explains the TMAO-induced collapse of the polymer. Interestingly, in the work by Garde and coworkers, the preferential interaction between the hydrophobic chain and TMAO has also been

reported to be positive, however, increasing upon the unfolding of the chain. To address this issue, a systematic study of the conformational changes of a hydrophobic chain in TMAO at different TMAO concentrations and with various TMAO models has been performed by Rodríguez-Ropero et al.[43] It has been found that the TMAO-induced collapse of the hydrophobic chain is TMAO-concentration-dependent. At low TMAO concentration (1 M), TMAO (modeled with the Kast, Netz, García and the Shea parameters) significantly increases the free-energy difference for the collapsed-to-extended transition of the chain, thus stabilizing the collapsed conformations of the chain. Interestingly, the free-energy difference starts decreasing upon further addition of TMAO (except with the Shea TMAO model) and at higher concentrations of TMAO (3-4 M) it becomes comparable with the unfolding free-energy in pure water. Although these results do not immediately explain why in the work of Garde and coworkers[53] no significant effect of TMAO on the conformational changes of the hydrophobic chain has been observed at an intermediate concentration (2 M) of TMAO, it can be speculated that, at higher concentrations, TMAO may not modulate the conformational equilibria of hydrophobic chains. Also in the work by Rodríguez-Ropero et al., the preferential interaction of TMAO with the hydrophobic chain has been found to be positive. However, it has also been found that the TMAO-hydrophobic chain preferential interaction may decrease or increase upon unfolding of the chain depending on the TMAO concentration and the TMAO force field. Considering all the TMAO force fields, an over-all trend of TMAO-depletion from the surface of the chain at 1 M TMAO has been found, which is consistent with the findings by Berne and coworkers.[60] However, at 4 M TMAO, a general trend of TMAO-accumulation at the hydrophobic surface has been found when the chain extends.

2.2.3 TMAO-induced protein-stabilization through MD simulations. The studies discussed above raise one important question: How do TMAO's effects on hydrophobic polymers translate to the context of protein-stabilization by TMAO? Dias and coworkers have reported positive preferential interaction between hydrophobic amino acid groups and TMAO, which leads to extension of polyleucine in TMAO solutions, and the authors argue that their results are robust across different TMAO force fields.[62] This work shows that the effects of TMAO on pure hydrophobic polymers, as discussed above, do not readily correlate with its effects on hydrophobicity in the context of a peptide. The work by Dias and coworkers also shows that TMAO is excluded from peptide backbone and charged side chains. Unfavorable electrostatic interactions have previously been predicted by Pettitt and coworkers.[15] The exclusion of TMAO from the charged amino acid groups may potentially lead to enhanced stabilization of intrapeptide salt bridges.[34,62] The combined effects of the exclusion of TMAO from peptide backbone and the stabilization of salt bridges protect compact or native conformations of peptides in TMAO solutions.[34,62,63]

3 BINARY UREA-WATER SOLUTIONS

In this section, we briefly discuss urea's effects on protein-solvation. The solution structure and properties of urea-water solutions and the effects of urea on the solvation and aggregation of peptides, and hydrophobic and ionic solutes have been rigorously studied through experiments and computational approaches.[32,64–67] Unlike TMAO, urea has little to no effect on the electrostatic interactions between two oppositely charged ions.[68] However, urea can bind to positively charged ions though urea's carbonyl oxygen[68] and can form strong hydrogen bonds with peptide groups.[69] The effects of urea on hydrophobic interactions are ambiguous.[64,68,70–75] At room temperature, urea slightly decreases the solubility (indicated by positive transfer free-energies) of smaller aliphatic hydrophobic solutes such as methane,[64] but increases the solubility (indicated by negative transfer free-energies) of higher alkanes such as butane and neopentane or nonpolar groups such as toluene.[64,76] In the context of protein-denaturation, urea is known to interact directly with peptide backbone and side chains.[27,77–84]

Several all-atom models for urea have been developed.[85–90] Urea force fields, derived from popular biomolecular force fields, are often inaccurate in capturing the near-ideal behavior of urea-water solutions. For example, the OPLS[85] and the AMBER[86] urea models predict very high urea-aggregations, leading to very low solution activities.[91,92] With the OPLS/AMBER urea force fields, higher urea-aggregations are also observed at the peptide surfaces.[92] This leads to significant overestimation of both the direct and the indirect effects of urea on peptides and predicts an incorrect mechanism for peptide solvation in urea solutions.[92] For these reasons, in our works, we have always used the Kirkwood-Buff-derived urea force field by Weerasinghe and Smith (referred to as the Smith urea force field henceforth), which predicts correct solution KBIs and activity derivatives of binary urea-water solutions.[91]

It is extremely challenging to capture the correct urea-peptide interactions through computer simulations. Peptide and urea force fields are often derived from binary peptide-water and urea-water solutions, respectively. Combining urea and peptide models without any specific modification to peptide-urea interactions often leads to quantitative disagreement with experiments in terms of peptide-urea preferential interactions or transfer free-energies of the peptides to urea solutions.[92,93] Even peptide-water interactions may not be properly captured by the common biomolecular force fields.[94–96] Best and coworkers have taken an important step forward to correctly reproduce experimental peptide transfer free-energies in urea by empirically scaling urea-peptide interaction.[93] However, further tests are required to improve peptide-urea force fields and a similar strategy may be followed to address peptide-TMAO interactions in future.

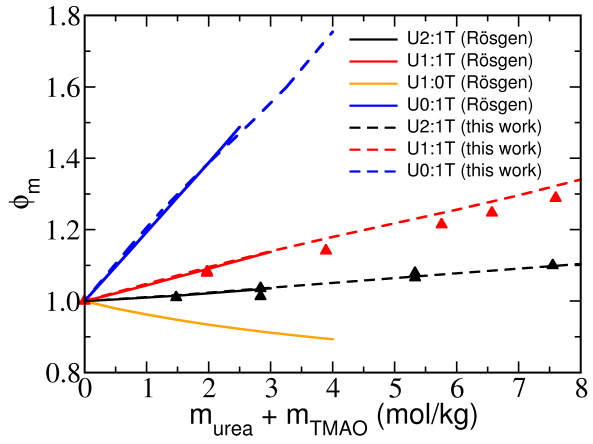

Figure 2: Shown are the molal scale osmotic coefficients (ϕ_m) for binary urea-water, TMAO-water and for ternary urea-TMAO-water solutions. The U:T ratios represent urea:TMAO concentration ratios. The solid lines are fits to the data by Rösgen and Jackson-Atogi.[97] The triangles represent experimental data newly obtained in this work. The dashed lines represent calculated osmotic coefficients using new fitting parameters for activity coefficients (details are in the Supporting Information).

4 TERNARY UREA-TMAO-WATER SOLUTIONS

4.1 Solution properties of urea-TMAO mixtures. TMAO usually counteracts urea-denaturation of proteins at 2:1 to 1:1 urea:TMAO concentrations.[98–101] Thermodynamic properties of these two ternary urea-TMAO mixtures have been thoroughly investigated in Rösgen's group by means of vapor pressure osmometry (VPO) up to total molality of 3 mol/kg.[97,102] Employing a convenient model of the osmotic coefficient, activity coefficients of both urea and TMAO have been determined earlier.[103] The data by Rösgen and Jackson-Atogi[97] shows that the osmotic coefficients (ϕ_m in molality scale) of binary urea-water and TMAO-water are almost exactly linear with concentration. While the ϕ_m of urea-water slightly decreases with concentration (ϕ_m=0.9 at 4 mol/kg), for TMAO-water, it rather steeply rises (ϕ_m=1.5 at 2 mol/kg). Interestingly, their ternary 1:1 and 2:1 mixtures also share this linear character, with a slope that almost exactly matches with the simple addition of the individual effects of urea and TMAO. Consequently, the osmotic coefficient of 2:1 mixture is almost ideal (ϕ_m=1.03 for 2 mol/kg urea with 1 mol/kg TMAO).

In the aforementioned work, the highest concentration for urea-TMAO mixtures is 3 mol/kg in total. However, to probe denatured/expanded structures of peptides and to demonstrate the effective counteraction by TMAO, a higher concentration of urea (and subsequently of TMAO) is needed. Employing VPO at 37°C, we have measured osmotic coefficients for 1:1 and 2:1 urea:TMAO mixtures at higher concentrations (up to 7.5 mol/kg total osmolyte concentration). The results are presented in Figure 2 and in Table S2 in the Supporting Information. The fit to this extended data series requires small modifications to the parameters by Rösgen (originally valid up to 3 mol/kg in total), and the new parameters are summarized in Table S1 in the Supporting Information. The linearity of the osmotic coefficients for 1:1 and 2:1 mixtures is confirmed up to the highest concentrations examined. Taking into account that at 6 mol/kg about 30% weight consists of the osmolytes for the 2:1 mixture, it is remarkable to find that such a dense complex mixture remains effectively an ideal solution.

Next, we have used the densimetry data by Rösgen and Jackson-Atogi for the densities and the partial molar volumes of urea and TMAO in mixed solutions.[97] A complete Kirkwood-Buff inversion procedure has been performed using a combination of volumetric and activity data (in analytical form) and all six KBIs have been determined.[97,104] Our new data are presented in Figure 3. Among the water-KBIs, the most prominent changes are observed for water-TMAO. This integral starts at ≈ -70 cm^3/mol at infinite dilution and increases rather steeply up to 1 mol/kg (≈ -40 cm^3/mol), and then the slope becomes smaller (≈ -20 cm^3/mol at 4 mol/kg). The slope is steeper for the 2:1 mixture in the whole concentration range. The TMAO-TMAO and the urea-TMAO KBIs change rather linearly with steeper slopes below ≈1.5 mol/kg TMAO concentration, then become flatter and yield a total change of ≈50% for the whole TMAO concentration range studied. The urea-urea KBI starts at 0, becomes weakly negative and values ≈ -50 cm^3/mol at the highest urea/TMAO concentration.

4.2 Effects of TMAO on protein-urea interactions. Having discussed urea-TMAO solutions without proteins/solutes, we now focus on their interactions with proteins. Preferential binding and thermal denaturation measurements of ribonuclease T1 in urea-TMAO solutions (up to 2 M urea and 1 M TMAO concentrations) have shown that TMAO does not modify protein-urea preferential interactions.[26] CD experiments with notch ankyrin domain and barnase proteins show that TMAO (0 to 1 M) does not have any effect on the m-values of the urea-denaturations of these proteins.[105] Using MD simulations of staphylococcal nuclease at 2 M urea with 1 M TMAO solutions, Medvedev and coworkers have found that TMAO does not significantly affect urea-structure around the protein; the addition of urea to TMAO solutions depletes TMAO from the protein surface.[37] Urea-induced depletion of TMAO from protein surface has been predicted by later nanoelectrospray ionization mass spectrometry (nESI-MS) experiments as well.[106] Through MD simulation studies of a polyalanine chain at fixed conformations, Pettitt and coworkers have computed the peptide-urea preferential interactions for different polyalanine conformers.[15] Their results show that depending on the polyalanine conformation, the addition of 4 M TMAO to 8 M urea solutions may decrease or may not alter peptide-urea preferential interactions.

In the work by Wang and Bolen,[27] it has been argued that the transfer free energies of the amino acids in mixed urea-TMAO solutions can be closely estimated by the arithmetic summation of the transfer energies obtained in pure urea or TMAO solutions. In other words, these inferences indicate additivity of the individual effects of urea and TMAO on amino acids and may potentially discard the idea that urea and TMAO modify each other's solvation effects on amino acids. However, a closer inspection to their data reveals that, for a number of amino acids, this aforementioned additivity may only be inferred if a significant margin of error is accepted. In Figure 4, using the data by Wang and Bolen, we replot (Figure 3 in the original paper) the apparent transfer free energies (details are in the Supporting Information) of the amino acid side chains in mixed 2 M urea and 1 TMAO solutions (Δg_{tr}^{ut}) along with the arithmetic sums of that in 2 M urea and 1 M TMAO ($\Delta g_{tr}^{u} + \Delta g_{tr}^{t}$). 20% to 70% deviation from additivity (indicated by the dashed line) has been found for methionine, leucine, asparagine, serine, sodium aspartate, sodium glutamate, histidine, and phenylalanine (data not plotted). Interestingly, for the majority of the amino acids, Δg_{tr}^{ut} has systematically been overes-

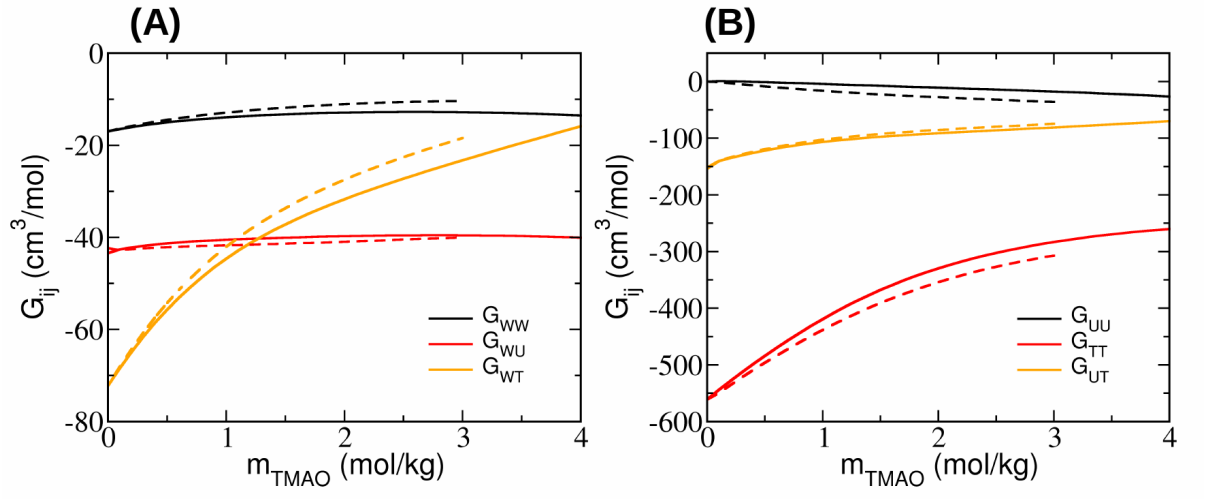

Figure 3: KBIs in 1:1 (m_{TMAO}=0-4 mol/kg, solid lines) and in 2:1 (m_{TMAO}=0-3 mol/kg, dashed lines) urea/TMAO mixtures. (A) water KBIs and (B) osmolyte-osmolyte KBIs.

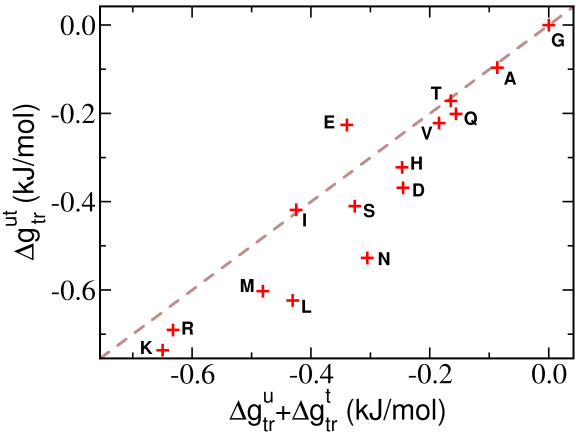

Figure 4: Plotted are the apparent transfer free energies of the amino acid side chains in mixed 2 M urea and 1 M TMAO solutions (Δg^{ut}_{tr}) along with the arithmetic sums of that in 2 M urea and 1 M TMAO ($\Delta g^{u}_{tr} + \Delta g^{t}_{tr}$). The data obtained from the work by Wang and Bolen.[27]

timated by the additivity approximation, while for tryptophan (not shown in Figure 4) and sodium glutamate, it underestimates Δg^{ut}_{tr}. The deviation of Δg^{ut}_{tr} from $\Delta g^{u}_{tr} + \Delta g^{t}_{tr}$ sets the scope for exploring the non-additive effects of urea and TMAO on amino acids and peptides in mixed osmolyte solutions.

In an earlier work from our labs, we have probed deeper and addressed the question on how urea and TMAO affect the solvation structure of amino acid residues in mixed urea-TMAO solutions.[107] Using the Kirkwood-Buff theory of solution[108,109] and MD simulations, we have calculated the KBIs and the preferential interaction of the amino acids with urea and TMAO. When the results for the binary urea-water or TMAO-water solutions have been compared with the results for the ternary urea-TMAO-water solutions, we have found that urea and TMAO both exclude each other from the amino acids in mixed osmolyte solutions. Qualitatively similar results, obtained with amino acids of different types of side chains (uncharged/charged, polar/hydrophobic, and aliphatic/aromatic), have strongly indicated at the TMAO-induced modification of peptide-urea solvation properties. Our results correlate well with a later MD simulation study on villin headpiece protein where it has been shown that the addition of 2.4 M TMAO to 4.8 M urea solution significantly decreases the number of protein-urea hydrogen bonds.[110] A reduction in the protein-urea hydrogen bonds in presence of TMAO has also been observed by earlier MD studies.[111]

4.3 Force fields for ternary urea-TMAO-water solutions: the optimized Netz(m) model

All the MD studies reviewed in Section 4.2 use urea and TMAO models which are developed using binary urea-water or TMAO-water solutions. In our earlier study,[46] we have shown that different TMAO models have markedly different affinities for water and urea (modeled with the Smith urea force field[91]). In urea solutions, the solvent-separated interactions (urea-mediated) between neopentane molecules stabilize their hydrophobic association.[74] We find that depending on their affinities for urea and water, TMAO models can enhance or decrease hydrophobic associations between neopentane molecules in urea-TMAO solutions.[46] Motivated by this, we have revisited the solvation thermodynamics of the urea-TMAO mixtures in terms of the solution KBIs.[34] We have found that the urea-urea, urea-TMAO and the TMAO-TMAO KBIs are poorly represented by the existing TMAO/urea models.

The Netz TMAO model and the Smith urea model[91] reproduce solution KBIs in binary TMAO-water and urea-water solutions. However, when mixed, these models produce a significantly lower urea-TMAO KBI and a significantly higher TMAO-TMAO KBI than experiments.[97] Based on these models, we have developed a force field for urea-TMAO mixtures, by correcting the van der Waals interactions between urea and TMAO and between TMAO and TMAO, with simple scaling factors of 1.1 and 0.9, respectively. The new force field,[34] termed as the Netz(m) force field for urea-TMAO solutions, reproduces solution KBIs (urea-urea, urea-TMAO, TMAO-TMAO, urea-water, TMAO-water and water-water) when compared with the experimental values. Our force field, originally developed at the mixture of 2 M urea and 1 M TMAO (Figure 5A), is transferable at a

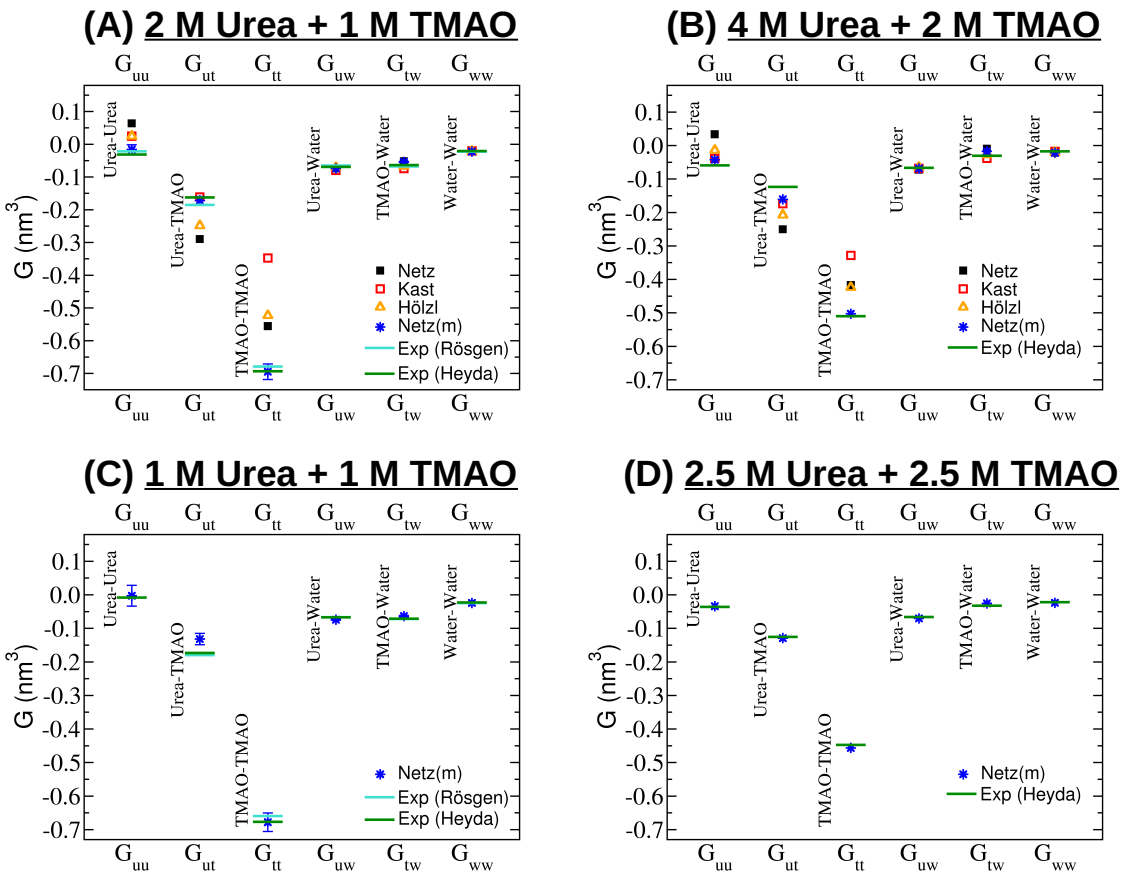

Figure 5: Shown are the urea-urea (G_{uu}), urea-TMAO (G_{ut}), TMAO-TMAO (G_{tt}), urea-water (G_{uw}), TMAO-water (G_{tw}), and water-water (G_{ww}) KBIs for ternary urea-TMAO-water solutions at different urea-TMAO compositions. The results are compared for different urea/TMAO force fields with the experimental results, denoted by Exp (Rösgen) and Exp (Heyda). The Exp (Rösgen) data are obtained from the work by Rösgen and Jackson-Atogi.[97] The panels (A) and (C) are reprinted (adapted) with permission from J. Am. Chem. Soc. 2018, 140, 483. Copyright (2018) American Chemical Society. Exp (Heyda) data are newly obtained in this work. Experimental data are converted from $\mathrm{cm}^3/\mathrm{mol}$ or l/mol to nm^3.

higher urea-TMAO concentration (4 M urea with 2 M TMAO, Figure 5B). The Netz(m) model reproduces solution KBIs for 1:1 urea-TMAO solutions at low (1 M-1 M, Figure 5C) and high (2.5 M-2.5 M, Figure 5D) concentrations.

The correct representation of the urea-TMAO and the TMAO-TMAO interactions in the urea-TMAO force fields has immense effect on the conformational characteristics of peptides. We have found that the uncorrected combination of the Netz TMAO model and the Smith urea model enhances urea-induced extension of a model peptide, polyalanine, by increasing urea-aggregation at the peptide-surface.[34] In contrast, the Netz(m) force field works towards preventing the action of urea, representing a more realistic scenario.

4.4 Counteraction mechanism for TMAO against urea.

After developing a urea-TMAO model which properly captures urea/TMAO/water interactions, we study the impacts of urea and TMAO on solvation thermodynamics and conformations of peptides.[34] As a model intrinsically disordered peptide, we choose the second repeat (R2 fragment, ^{273}GKVQIINKKLDL^{284}) of the Alzheimer's disease-related tau protein. Using enhanced sampling replica-exchange MD simulations, we find that the peptide chain assumes compact configurations in water and in TMAO-water solutions. In presence of urea, the peptide explores extended configurations. In urea-TMAO solutions at 2:1 concentration ratio, modeled with the Netz(m) force field, we find that the peptide predominantly explores the compact conformations. After successfully demonstrating counteraction to urea-induced configurational changes in the peptide by TMAO, we examine the molecular mechanism behind this counteraction.

By calculating the preferential interactions between urea and the peptide, we find that the peptide adopts extended conformations by preferentially binding to urea over water. The addition of TMAO decreases the peptide-urea preferential interactions. We also find that in water and in TMAO solutions, the peptide retains its compact forms through intrapeptide hydrogen bonds and a stable salt bridge between the Lys274 and the Asp283 residues. The addition of urea significantly suppresses the formation of the hydrogen bonds and the salt bridge. Our simulations further suggest that the addition of TMAO to urea solutions does not perturb the intrapeptide hydrogen bonding network, but it markedly enhances the probability of the formation of the Lys274-Asp283 salt bridge.[34] To check whether the enhanced stability of the salt bridge is a cause or an indirect effect of the compaction of the peptide by TMAO, we have reanalyzed the data for only the compact structures. Even for the compact structures we find a TMAO-induced stabilization of the salt bridge (Figure S2 in the Supporting Information). Thus, with a combined mechanisms of the

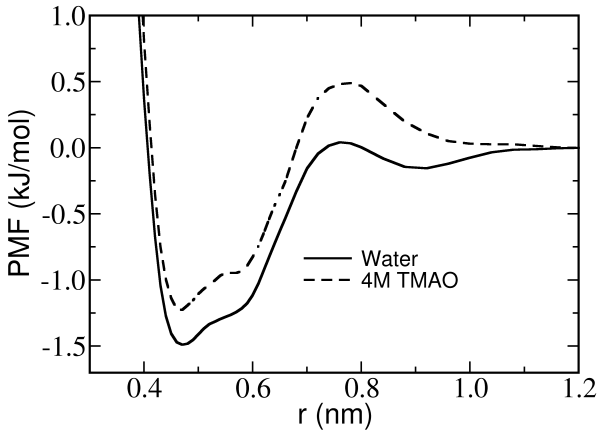

Figure 6: Shown are the potentials of mean force (PMFs) between the center of masses of a urea molecule and an alanine molecule in water and in 4 M TMAO. The Netz(m) urea-TMAO force field has been used for the 4 M TMAO solution.

reduction in peptide-urea preferential interactions and the stabilization of the intrapeptide salt bridge, TMAO counteracts the effects of urea on the conformations of the peptide.[34,63]

To further understand the effects of TMAO on the amino acid-urea interactions, in this work, we have calculated the PMF between the center of masses of an alanine monomer and a urea molecule with and without TMAO. In pure water (with one molecule of alanine and one molecule of urea), the alanine-urea interactions are favorable, as seen from the first minima of the PMF (results are shown in Figure 6). The addition of 4 M TMAO reduces alanine-urea interactions, which agrees well with our hypotheses of TMAO-induced urea-removal from amino acid surfaces.

4.5 Interactions between urea and TMAO. Since our MD simulation results strongly indicate that the TMAO-driven displacement of urea from the surfaces of amino acids and peptides plays a major role in the stability of proteins in urea-TMAO solutions, it is important to understand the interactions between a urea and a TMAO molecule in aqueous medium. Neutron-scattering experiments by Koch and coworkers have indicated possible hydrogen bond formation between urea and the oxygen atom of TMAO in 1:1 urea-TMAO solutions at 2.5 M concentration.[113] In that work, the radial distribution function between the oxygen atom of TMAO and the nitrogen atom of urea shows a direct solvation peak at 0.28 nm. However, the height of the peak has been found to be slightly larger than unity, which is justified by the steric hindrance for the formation of urea-TMAO conjugates.[97] A later combined X-ray scattering and neutron-scattering study by Koch and coworkers has demonstrated weak-association between urea and TMAO at low concentrations (2.5 M 1:1 urea-TMAO).[114] In the same paper, it has been shown that for a fixed concentration of TMAO (2.5 M), urea gradually replaces water from the hydration shell of TMAO with increasing urea concentration (2.5 M to 6.7 M). By reanalyzing these data with a theoretical exchange-model, Rösgen and Jackson-Atogi have predicted stable binding between urea and TMAO at high urea concentrations (2.5 M TMAO to 7.5 M urea). Our MD simulations of 8 M urea with 4 M TMAO solutions (with neopentane) have also indicated the formation of nearly one hydrogen bond between urea and TMAO on average.[46] A more recent Raman spectroscopic study shows blue shift in the H–N–H symmetric bending mode of urea in presence of TMAO (at high urea-TMAO concentrations), which suggests direct urea-TMAO interactions.[115]

In contrast, using MHz to THz dielectric spectroscopy, Hunger and coworkers have shown no hydrogen bond formation between urea and TMAO at 1:1 3.5 M urea-TMAO solutions.[116] Their experiments with gradually increasing urea concentration at a fixed TMAO concentration indicates that the strong TMAO-water hydrogen bonds are not replaced by the formation of TMAO-urea hydrogen bonds. The authors propose a possible water-mediated interaction mode between urea and TMAO. We note that the maximum urea concentration in this study has been kept lower than that in the studies by Koch et al.[114] or by Rösgen et al.[97] An earlier MD simulation study of urea-TMAO solution by Paul and Patey shows ambiguous results regarding urea-TMAO hydrogen bond formation.[13] Interestingly, the authors find that the strength of a TMAO-urea hydrogen bond is comparable with that of a TMAO-water hydrogen bond. However, the average number of urea-TMAO hydrogen bonds (0.21) has been found to be significantly lower than the average number of TMAO-water hydrogen bonds (2.0) in a mixed 7.4 M urea with 3.7 M TMAO solution. We note that this work uses the Kast TMAO model and the OPLS urea model,[85,117] as opposed to the Smith urea model[91] used in our simulations.[46] As discussed earlier, the OPLS urea model shows unphysical urea-urea self-aggregation, diverging from the near-ideal behavior[118] of binary urea-water systems.[91] Higher self-aggregation of urea may potentially lead to a lower number of urea-TMAO hydrogen bonds.

In a recent study, the interaction between urea and TMAO has been addressed through AIMD and MD simulations, time-resolved infrared pump-probe spectroscopy and NMR spectroscopy.[112] The potential of mean force (PMF) between the carbon atom of urea and the oxygen atom of TMAO in water (1 molecule of urea and TMAO each in the solution), calculated by AIMD simulations, shows a shallow minimum between 0.53 nm and 0.57 nm. The PMFs and the corresponding molecular snapshots, obtained with MD simulations (1 molecule of urea and TMAO each in the solution, 0.3 M concentration) where the Coulombic and the van der Waals interactions between urea and TMAO are varied systematically, indicate that the aforementioned minimum in the urea-TMAO interaction corresponds to the van der Waals interactions between urea and TMAO through the methyl groups of TMAO. In addition, the position in the AIMD PMF, which would correspond to urea-TMAO hydrogen bond formation, is found to be energetically unstable. The hydrophobic association between TMAO and urea arises from the mismatch between high TMAO-water hydrogen bonding propensities and comparatively low urea-water hydrogen bonding propensities, it has been hypothesized.

In the aforementioned work, two TMAO force fields (the Kast[33] and the Netz[39]) and two urea force fields (the OPLS[85] and the Smith[91]) are used for the MD simulation studies. When the urea-TMAO PMFs are compared, we can see that the Netz TMAO model, with both the Smith and the OPLS urea models, provides the closest match with the results obtained with the AIMD simulations. The Kast TMAO model, when combined with either of the OPLS and the Smith urea models, indicates possible stability for the urea-TMAO hydrogen bond formation, which does not harmonize with the findings from the AIMD simulations. We have recomputed the PMF between the carbon atom of urea and the oxygen atom of TMAO using the combination of the Netz TMAO model and the Smith urea model and using the Netz(m) urea-TMAO model as well. The corresponding results, along with the AIMD results and the results obtained by using the Netz TMAO and the Smith Urea model in the work by Na-

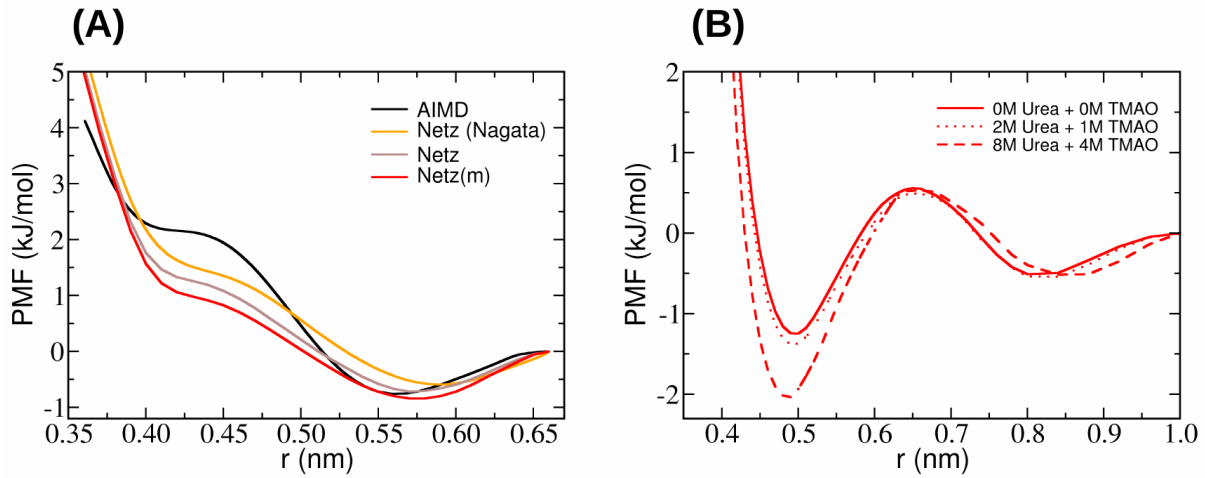

Figure 7: (A): Shown are the potentials of mean force (PMFs) between the carbon atom of urea and the oxygen atom of TMAO for a single molecule of urea and TMAO in water at 380 K temperature. "AIMD" denotes the results obtained from the ab initio MD studies by Nagata and coworkers.[112] Results obtained with the MD simulation using the combination of the Netz TMAO model[39] and the Smith urea model[91] are also shown from the work by Nagata and coworkers[112] (denoted by Netz (Nagata)). "Netz" denotes our simulation results as obtained from the MD simulation using the combination of the Netz TMAO model and the Smith urea model. The results using the Netz(m) urea-TMAO model[34] are shown as well. (B): Shown are the PMFs between the center of masses of a urea molecule and a TMAO molecule in 2:1 urea-TMAO solutions at 2 M and 8 M urea concentrations at 300 K temperature, along with the results obtained with only one urea and one TMAO molecule present in the system (indicated by 0 M urea and 0 M TMAO). The Netz(m) urea-TMAO force field is used.

gata and coworkers,[112] are shown in Figure 7(A). We find that the depth of the PMF obtained with the AIMD simulation is closely reproduced both by the uncorrected Netz TMAO force field and the Netz(m) urea-TMAO force field. We note that there is no TMAO-TMAO interaction present in the system for this set of PMFs. The small difference in the data for the Netz force field, when compared between the work by Nagata et al.[112] and our simulations, can be attributed to the difference in system size. The linear dimension of our system is $\approx$ 3 nm, as opposed to 1.7 nm in the work by Nagata et al.

In order to understand the overall interaction between urea and TMAO at finite concentrations of urea and TMAO, in this work, we calculate the PMFs between the center of masses of a urea molecule and a TMAO molecule in 2:1 urea-TMAO solutions at 2 M and 8 M urea concentrations. Figure 7(B) shows the corresponding results, along with the results obtained with only one urea and one TMAO molecule present in the system (indicated by 0 M urea and 0 M TMAO). We find that the interaction between urea and TMAO significantly increases at higher urea-TMAO concentrations, when compared with that in the solutions with lower urea-TMAO concentrations.

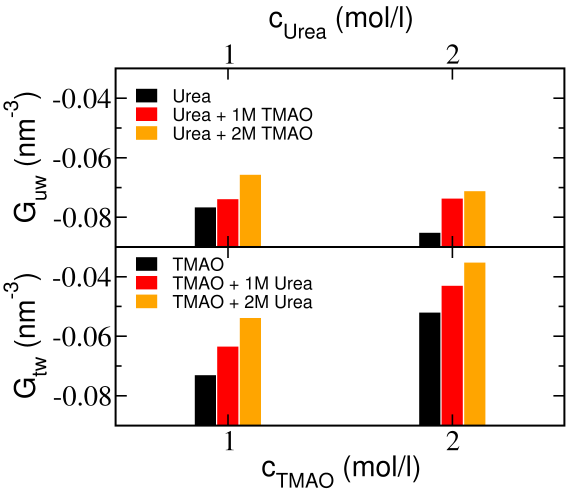

Figure 8: Shown are the urea-water (upper panel, G_{uw}) and TMAO-water (lower panel, G_{tw}) KBIs for binary urea-water/TMAO-water and ternary urea-TMAO-water solutions. The Netz(m) urea-TMAO force field has been used for the mixtures of urea and TMAO.

4.6 Osmolyte-hydration in mixed solutions. An indirect effect, related to the hydration of the osmolytes, may contribute to the TMAO-induced removal of urea from protein surface. Earlier dielectric spectroscopic measurements have also indicated increase in the number of "bound water" in mixed urea-TMAO solutions.[116] Our MD simulations of binary urea-water/TMAO-water and ternary urea-TMAO-water systems show that in the mixed-osmolyte solutions, both urea and TMAO increase their water-affinity. Comparing the urea-water (and TMAO-water) KBIs in Figure 8 for binary and ternary osmolyte mixtures, we find an increase in the osmolyte-water KBIs, when the other osmolyte is added to the solution. Our earlier simulations of polyalanine in urea-TMAO solutions have also predicted enhanced urea-water KBIs when TMAO is added.[34] The increase in TMAO-water KBI upon the addition of urea is also evident from our new experimental KBI results presented in Figure S1 in the Supporting Information. However, the increase in urea-hydration by TMAO is much smaller than the increase in TMAO-hydration by urea and at dilute urea concentration, this increase in urea-water KBI is almost indistinguishable.[97] However, by comparing the experimental results of binary urea-water solution by Chitra and Smith[119] and our KBI results of urea-TMAO-water solutions, we find an increase in the urea-water KBI from $\approx$-45 cm^3/mol to $\approx$-40 cm^3/mol when 2 M

TMAO is added to 4 M urea. This extra hydration of the osmolytes in mixed solutions potentially may lead to inhibited protein-osmolyte interactions at finite urea-TMAO concentrations.

5 CONCLUSIONS

In this review we have addressed the counteraction mechanism by TMAO to urea-denaturation of proteins. The fact that TMAO and urea both decelerate water dynamics indicates that the stability of proteins in urea/TMAO solutions should be discussed beyond simple ideas of kosmotropes and chaotropes.[120–122] The consensus view that arises from this review supports the idea that TMAO protects functional structures of proteins by stabilizing intrapeptide ionic interactions (salt bridges) and by being excluded from the protein backbone (or by being effectively more excluded from the non-native/extended conformations than from the native/compact conformations). Urea, which denatures proteins by its favorable interactions with protein backbone and side chains, interacts with TMAO at high urea-TMAO concentrations and the nature of this interaction is predominantly hydrophobic. While the solution activity remains additive when TMAO and urea are mixed, their individual effects on proteins do not. TMAO and urea molecules are mutually excluded from amino acids in mixed solutions which inhibits the denaturing effects of urea. The hydration of the osmolytes increases in mixed solutions and at finite osmolyte concentrations, the hydration of the osmolytes and the direct urea-TMAO interactions may work in synergy to remove the osmolytes from the amino acid residues. Stabilization of intrapeptide ionic interactions by TMAO is evident even in the presence of urea and this contributes positively to the counteraction mechanism.

6 OUTLOOK

The thermodynamic aspect of protein stability in urea-TMAO solutions at ambient conditions is highlighted in this review. Two other topics, which have not been covered in this review, are important for broader understanding of the role of osmolytes in extremophiles and higher order organisms. These are: A) Osmolyte effects on proteins under high hydrostatic pressure and in high or low temperature[123] and B) Protein-Protein interactions in osmolytes.[124] A small number of experimental and theoretical works have addressed the role of TMAO (or osmolytes in general) on protein stability under high pressure.[125–130] Computer simulation of osmolytes at high pressure/temperature is hindered mostly by the scarcity of available force fields suitable for extreme conditions.[40] There have been few promising simulation studies for TMAO/urea at high pressure,[40,131–133] but further inspection is required. In addition, the applicability of the TMAO models at higher temperatures needs to be tested to shed lights on how TMAO stabilizes proteins against temperature-denaturation. TMAO is known to modulate aggregation and liquid-liquid phase separation in proteins.[124,134,135] A deeper understanding of these processes is extremely important for identifying the pathogenesis of various proteopathies driven by protein-aggregation. MD simulations have indicated how water reorganization around amino acid residues can lead to higher aggregation of Alzheimer's disease related tau peptides in TMAO.[134] Recent studies[136,137] indicating association of TMAO with various cardiovascular diseases and chronic kidney diseases in humans bring further attention to this molecule and its interplay with other cellular entities.

ACKNOWLEDGMENTS

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SUPPORTING INFORMATION AVAILABLE

The theory of solvation, experimental and simulation details, experimental KBIs and osmotic coefficients, salt bridges of the R2 fragment of tau in water and osmolytes.

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