

# Trimethylamine *N*-oxide stabilizes proteins via a distinct mechanism compared with betaine and glycine

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Edited by Peter J. Rossky, Rice University, Houston, TX, and approved January 12, 2017 (received for review August 31, 2016)

**We report experimental and computational studies investigating the effects of three osmolytes, trimethylamine *N*-oxide (TMAO), betaine, and glycine, on the hydrophobic collapse of an elastin-like polypeptide (ELP). All three osmolytes stabilize collapsed conformations of the ELP and reduce the lower critical solution temperature (LCST) linearly with osmolyte concentration. As expected from conventional preferential solvation arguments, betaine and glycine both increase the surface tension at the air–water interface. TMAO, however, reduces the surface tension. Atomically detailed molecular dynamics (MD) simulations suggest that TMAO also slightly accumulates at the polymer–water interface, whereas glycine and betaine are strongly depleted. To investigate alternative mechanisms for osmolyte effects, we performed FTIR experiments that characterized the impact of each cosolvent on the bulk water structure. These experiments showed that TMAO red-shifts the OH stretch of the IR spectrum via a mechanism that was very sensitive to the protonation state of the NO moiety. Glycine also caused a red shift in the OH stretch region, whereas betaine minimally impacted this region. Thus, the effects of osmolytes on the OH spectrum appear uncorrelated with their effects upon hydrophobic collapse. Similarly, MD simulations suggested that TMAO disrupts the water structure to the least extent, whereas glycine exerts the greatest influence on the water structure. These results suggest that TMAO stabilizes collapsed conformations via a mechanism that is distinct from glycine and betaine. In particular, we propose that TMAO stabilizes proteins by acting as a surfactant for the heterogeneous surfaces of folded proteins.**

osmolytes | protein folding | mechanism | spectroscopy | MD simulations

Many organisms use small organic osmolytes to stabilize proteins in harsh environments, such as when the salinity is highly variable (1). In particular, trimethylamine *N*-oxide (TMAO) is known to counteract the denaturing effects of urea as well as salts, and it is present at high concentrations in some aquatic organisms (2). Its effects are often compared with the ions on the left side of the Hofmeister series, which help stabilize the native, folded structures of proteins (Fig. 1).

Because of their fundamental biophysical importance, many studies have investigated the behavior and effects of osmolytes. In particular, Timasheff and coworkers (3, 4) proposed that osmolyte effects result from the relative partitioning of these molecules between the bulk solution and the protein–water interface. Stabilization should occur when osmolytes are depleted from the protein–water interface, but proteins will unfold when osmolytes accumulate at this interface. Accordingly, osmolyte effects are often interpreted in terms of an effective protein–water “surface tension.” In fact, despite the significant differences between protein surfaces and air–water interfaces, osmolyte effects are often, although not always, consistent with their effect on the air–water interfacial tension (5). More recent studies (6, 7) have extended such classical considerations (3, 4, 8) to elucidate the mechanism by which many osmolytes impact protein stability. Nevertheless, the mechanism by which TMAO stabilizes proteins remains controversial.

Solvation studies with model compounds have suggested that TMAO is excluded from unfolded proteins because of its very unfavorable interactions with the peptide backbone (9, 10). Several

molecular dynamics (MD) simulation studies have been consistent with this conclusion (11, 12). However, they have proposed a wide array of explanations for this preferential hydration ranging from unfavorable electrostatic interactions (13, 14) to “nanocrowder” effects (15, 16). By contrast, other simulations have suggested that TMAO stabilizes folded proteins via direct attractive van der Waals interactions (17, 18).

Several studies have investigated the effect of TMAO on the solvent. Garde and coworkers (19) concluded that TMAO has minimal impact on hydrophobic interactions, but Paul and Patey (20) suggested that TMAO actually weakens hydrophobic forces. Other studies suggested that osmolytes stabilize folded states of proteins indirectly by altering the bulk water structure. For instance, MD simulations and IR studies have both suggested that, by strengthening the water hydrogen-bonding network, TMAO impacts protein stability by weakening peptide–water interactions (10, 21–23). In particular, Sharp et al. (24) concluded that TMAO enhances the structure of water, because it induces a red shift in the OH stretch of the IR spectrum. In contrast, Hunger et al. (25) attributed the same red shift to the formation of hydrogen bonds between water and the oxygen of TMAO. Indeed, several simulation studies have observed very strong interactions between TMAO and water (26, 27). Nevertheless, it is not clear that one can draw conclusions about the water structure based solely on shifts in the OH stretch region, because the spectrum represents a convolution of water–water and water–cosolvent interactions. Moreover, Pielak and coworkers (28) have argued that the impact of cosolvents on protein stability and water structure are not correlated. As such, despite many previous studies, the mechanism of TMAO action remains controversial.

## Significance

**Although trimethylamine *N*-oxide (TMAO) is perhaps the quintessential protein-stabilizing osmolyte, its mechanism of action has long remained elusive. Our study indicates that, in contrast to betaine and glycine, TMAO forms direct attractive interactions with polypeptides. This work strengthens and extends Berne’s previous conclusions, because we report results for a model polypeptide rather than a hydrophobic polymer. Our results are particularly striking, because we consider a model polypeptide that is enriched in amide groups that are believed responsible for the depletion of TMAO from unfolded proteins. Our study leads to the surprising conclusion that TMAO stabilizes folded conformations, despite interacting with unfolded conformations. We hypothesize that TMAO acts as a unique surfactant for the heterogeneous surface that emerges on protein folding.**

Author contributions: Y.-T.L., A.C.M., M.R.D., W.G.N., and P.S.C. designed research; Y.-T.L., A.C.M., and M.R.D. performed research; Y.-T.L., A.C.M., M.R.D., W.G.N., and P.S.C. analyzed data; and Y.-T.L., W.G.N., and P.S.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1614609114/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1614609114/-DCSupplemental).

## Hofmeister series

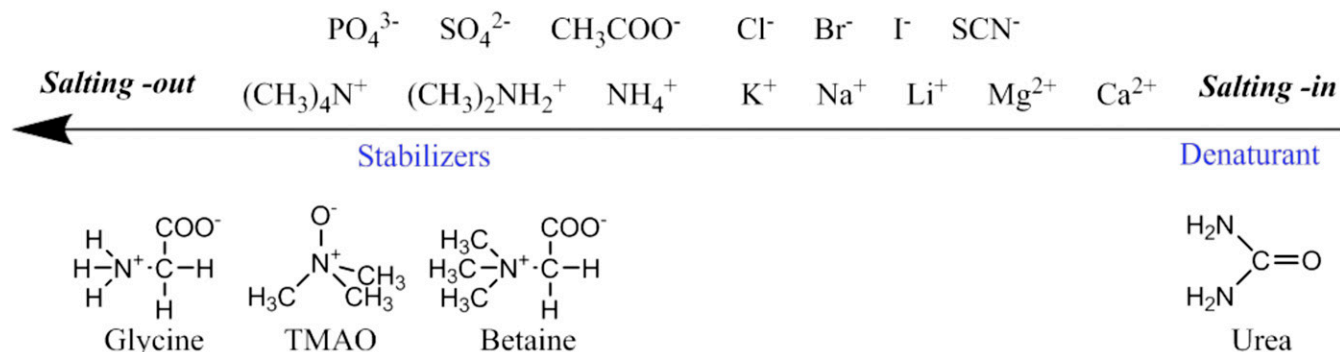


Fig. 1. The rank order of the direct Hofmeister series and common osmolytes typically found for neutral and negatively charged macromolecules.

In this study, we examined contemporary models for the stabilizing influence of TMAO on macromolecular structure in aqueous solutions. Specifically, we looked at the influence of TMAO on the solubility of neutral, hydrophobic elastin-like polypeptides (ELPs), which contain 120 repeat units of the pentapeptide sequence, VPGVG (valine–proline–glycine–valine–glycine), for a total of 600 residues. This biopolymer undergoes hydrophobic collapse above its lower critical solution temperature (LCST) (29). We used a combination of LCST measurements, surface tension measurements, and IR spectroscopy to investigate the nature of TMAO–ELP interactions. To complement these experimental measurements, we also performed atomically detailed MD simulations of a single capped VPGVG repeat in explicit solvent. Fig. 2A presents the chemical formula for this construct, whereas Fig. 2B presents a space-filling model of the ELP fragment, in which water molecules are indicated by a ball and stick representation.

Our studies indicate that, although TMAO stabilizes the collapsed state of the macromolecule, it is not depleted from either the air–water interface or the polypeptide–water interface. Rather, as Fig. 2C indicates by the silver atoms within the simulated configuration, TMAO molecules appear to form direct, favorable contacts with the ELP fragment. This result stands in striking contrast to the mechanisms observed for other osmolytes, such as glycine and betaine, which seem to stabilize folded conformations via a traditional depletion mechanism as illustrated by the simulated configuration in Fig. 2D. Moreover, we show that the TMAO oxygen significantly impacts the water OH IR spectrum and provide additional evidence that TMAO minimally disrupts the water-bonding network. These results contradict the central tenants of classical theories for protein stabilization by osmolytes. Instead, a new model is required that explains how TMAO stabilizes collapsed conformations, despite forming favorable interactions with extended conformations.

## Results

In a first set of experiments, we wished to directly measure the ability of TMAO, glycine, and betaine to stabilize the collapsed state of a thermoresponsive biopolymer. To this end, we measured the LCST of the elastin  $(\text{VPGVG})_{120}$  as a function of concentration for all three osmolytes. Fig. 3A shows that the LCST decreased linearly with cosolvent concentration in each case. Thus, each of these additives shifts the equilibrium toward the collapsed state with respect to the hydrated state. Glycine was the most effective, whereas betaine was the least effective (Table S1). If we were to assume that the osmolytes have similar (or greater) affinity for the surface of the uncollapsed ELP with respect to the collapsed state, then classical preferential interaction theories suggest that all three cosolvents should be strongly depleted from the ELP–water

interface (3). However, it is curious that, although TMAO has three methyl groups and appears to be similar in structure to betaine, it actually seems to behave more like glycine in terms of its effect on the ELP.

We wished to explore the extent to which each of the osmolytes would partition to the air–water interface. Fig. 3B shows surface tension data as a function of cosolvent concentration for each osmolyte. As expected, glycine increases the surface tension more strongly than betaine (30). In stark contrast to the other two osmolytes, however, TMAO decreases the surface tension at the air–water interface. Such a result suggests that TMAO is actually enriched rather than depleted at hydrophobic interfaces. This finding is consistent with previous measurements (3, 17, 31), although it is perhaps puzzling given the fact that TMAO is

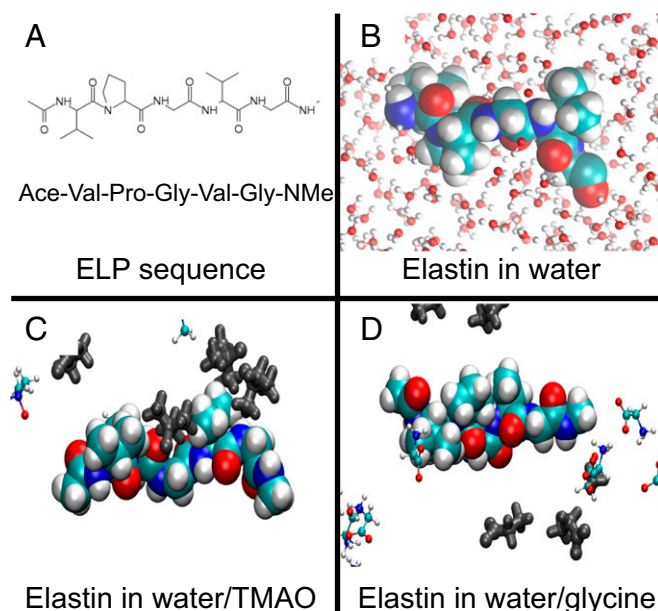
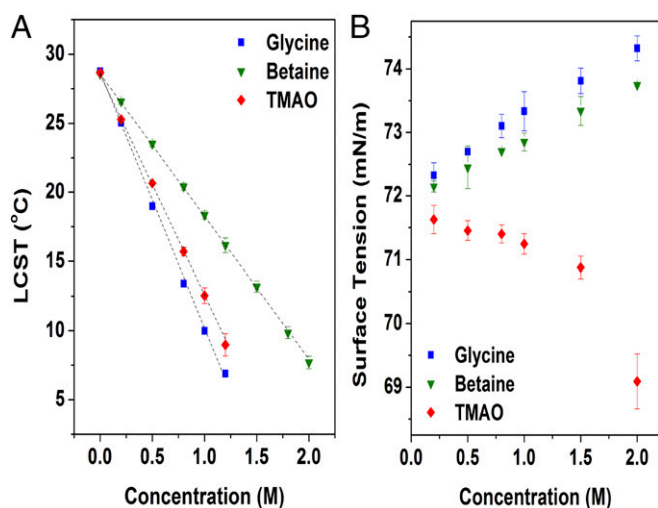


Fig. 2. Simulated ELP systems. A depicts the chemical sequence of the simulated ELP construct. B presents a space-filling model of the ELP construct while representing water molecules with a ball and stick representation. C and D present configurations that have been sampled from atomically detailed, explicit solvent simulations of the ELP fragment in water–TMAO and water–glycine solutions, respectively. In C and D, osmolyte atoms within 10 Å of the ELP solute have been highlighted in silver, whereas more distant osmolyte atoms are indicated with ball and stick representations. The simulated water molecules in C and D have been omitted for clarity.



**Fig. 3.** The effect of osmolytes on (A) the ELP LCST and (B) the surface tension of water.

nearly as effective as glycine at depressing the LCST of the ELP (Fig. 3A). However, as illustrated in Fig. 2, the elastin surface presents both polar amide moieties and hydrophobic surfaces. As such, although the air–water surface tension results suggest that TMAO may accumulate at apolar surfaces, the LCST data could still be consistent with a classical depletion effect if this accumulation at nonpolar surfaces is offset and overcome by an even stronger repulsion from the amide groups (3). Consequently, we performed MD simulations to explore this possibility.

In the following calculations, we described water with the extended simple-point charge model (SPC/E) (32) and TMAO with the popular model by Kast and coworkers (33). We modeled both the ELP fragment and glycine with the CHARMM27 force field (34) while using the correction map (CMAP) (35). We followed the procedure of Ma et al. (36) to parameterize a betaine model that reasonably reproduces experimental measurements of the betaine preferential interaction coefficient with a model peptide. *SI Materials and Methods* describes our models and simulations in detail. Moreover, *SI Materials and Methods* indicates that the conclusions of these simulations seem quite robust (Figs. S1–S7).

In a first set of computational experiments, we assessed whether these simulation models were consistent with the experimentally observed trends for air–water interface surface tension measurements. Our simulations with the SPC/E water yielded an air–water interfacial tension value of  $58.8 \pm 0.15$  mN/m, which is consistent with previous results for this model (37, 38), although  $\sim 17\%$  lower than the experimentally measured value. Importantly, the addition of 1 M betaine and 1 M glycine both significantly increased the simulated surface tension to  $61.0 \pm 0.15$  and  $60.1 \pm 0.20$  mN/m, respectively. The addition of 1 M TMAO, in contrast, reduced the simulated air–water surface tension to  $58.2 \pm 0.20$  mN/m (39). Indeed, although the simulated models do not quantitatively reproduce the surface tension measurements, they seem capable of qualitatively reproducing the trend that betaine and glycine both increase the air–water surface tension, whereas TMAO reduces it.

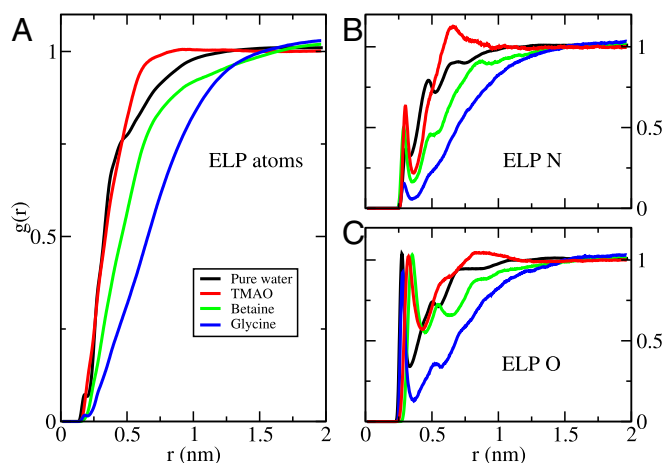
Next, we used MD simulations to investigate the partitioning of the three osmolytes to the ELP–water interface. Accordingly, we simulated a single capped ELP repeat (Fig. 2A) in a 0.55 M solution of each osmolyte. To explore the competition between water and the various cosolvents for the surface of the polymer, we introduced a weak potential that biased the ELP to sample relatively extended conformations.

Fig. 4 presents the radial distribution functions (RDFs) quantifying the simulated density of TMAO, betaine, and glycine about

the ELP repeat. Although it can form favorable interactions with the ELP backbone carbonyl groups, glycine (blue curves in Fig. 4) is strongly depleted from the ELP surface. Compared with glycine, betaine (green curves in Fig. 4) interacts slightly more favorably with the NH groups of the ELP backbone but is also significantly depleted from the ELP surface. However, Fig. 4 presents strikingly different results for TMAO (red curves in Fig. 4). Although TMAO and betaine show similar affinity for the amide groups along the peptide backbone, TMAO is not depleted from the ELP surface. Instead, as suggested by the surface tension measurements, TMAO actually accumulated slightly at the simulated ELP interface and preferentially solvated the polymer relative to water. In remarkable contrast to the expectations of simple depletion arguments, TMAO seems to stabilize collapsed conformations, despite binding to the polymer.

Next, we examined whether the influence of osmolytes on the bulk water structure correlated with changes in the relative stability of the collapsed vs. uncollapsed conformation of the ELP. Changes in the OH stretch spectra are often used to probe water structure and in particular, the water hydrogen-bonding environment (40–45). Unfortunately, interpreting the water vibrational spectrum is not straightforward because of strong inter-/intramolecular vibrational coupling. Consequently, a single uncoupled OH stretch vibration for HOD in  $D_2O$  solution is often used as a hydrogen-bonding network reporter. A red shift in the OH stretch to lower frequency suggests stronger intermolecular hydrogen bonding and weaker intramolecular OH bonds. In particular, OH stretch vibrations centered at 3,200 and 3,400  $cm^{-1}$  are often empirically associated with more and less structured water, respectively, by comparison with vibrational spectra of ice and liquid water (46–48).

To explore the influence of TMAO on water structure, we performed FTIR measurements of samples containing 0–4 m (molarity) TMAO in a 3 mol%  $H_2O$  in  $D_2O$  solution (Fig. 5A). As can be seen from the data, the contribution to the intensity at lower frequencies increased with increasing TMAO concentration, whereas the contribution at higher frequencies decreased. We note that, although the solution became increasingly basic as TMAO was added, control experiments with Raman spectroscopy (Fig. S8 and



**Fig. 4.** RDFs characterizing the solution around the ELP in pure water (black) and 0.55 M solutions of TMAO (red), betaine (green), and glycine (blue). The RDFs in A were calculated using all atoms from the ELP construct, the cosolvents, and water. B and C characterize the density of hydrogen-bonding groups around the ELP backbone NH and CO sites, respectively. B considered distances from the nitrogen atoms of four ELP valine and glycine residues to the oxygen atoms of the cosolvents and water. C considered distances from the carbonyl oxygen atoms of five ELP residues to the nitrogen atom of glycine, the methyl carbon atoms of betaine and TMAO, or the oxygen atom of water.

Table S2) indicate that this red shift is not caused by the increasing solution pH. Consequently, the red shift observed in Fig. 5A primarily reflects interactions of water with TMAO itself rather than indirect pH-induced changes that resulted from addition of the osmolyte to solution.

Next, we wished to determine if the changes in the OH stretch region of the IR spectra in Fig. 5A were more sensitive to the methyl groups of TMAO or the NO dipole. Accordingly, the sample at 4 M TMAO was studied as a function of pD (the scale for the negative log of the deuterium ion concentration in solution) from 8.8 down to 0.8 (Fig. 5B). The pD value was adjusted by adding DCl to acidify the solution. It should be noted that the pD values were determined with a standard pH meter and recorded as  $pD = pH + 0.4$  (49). Significantly, as the pD value was decreased, the spectrum continuously blue-shifted toward the spectrum for pure water (black curve in Fig. 5B). Because the  $pK_a$  of TMAO is 4.65 (50), the osmolyte became increasingly deuterated as the solution was acidified. Thus, Fig. 5B indicates that deuteration of TMAO negated the red shift because of the addition of TMAO. Consequently, we conclude that the TMAO-induced red shift in the OH spectra is very sensitive to hydrogen bonding of water to the lone pairs on TMAO's oxygen and affected only to a minor extent by the methyl groups. Additional control experiments show that acidification caused the OH spectrum to red shift (Fig. S10). Consequently, the pronounced blue shift observed in Fig. 5B was being partially masked by a competing red shift that results from an increasingly stronger water H-bonding network as the solution was acidified. Therefore, we conclude that direct hydrogen bonds between water and the TMAO oxygen atom make the predominant contribution to the observed spectral shifts.

We also obtained FTIR spectra in the presence of betaine (Fig. 6A) and glycine (Fig. 6B). It should be noted that betaine causes the pH of water to rise but to a lesser extent than TMAO (Table S2). Also, because glycine is somewhat less soluble than betaine, we obtained data only up to 3.0 M glycine. Interestingly, the OH peak hardly shifted as betaine was added to solution, but glycine caused much more pronounced changes as observed for TMAO. These data provide additional evidence that the osmolyte methyl groups are not the primary origin for the observed red shift in the OH stretch spectra. Indeed, like TMAO, betaine has three methyl groups, whereas glycine has none. Nevertheless, changes in the OH stretch spectra almost certainly reflect a combination of hydrogen bonding to the oxygen lone pairs (carboxylate moieties on betaine and glycine), hydrophobic hydration of the methyl groups, and potentially, any additional very small bulk water contributions that

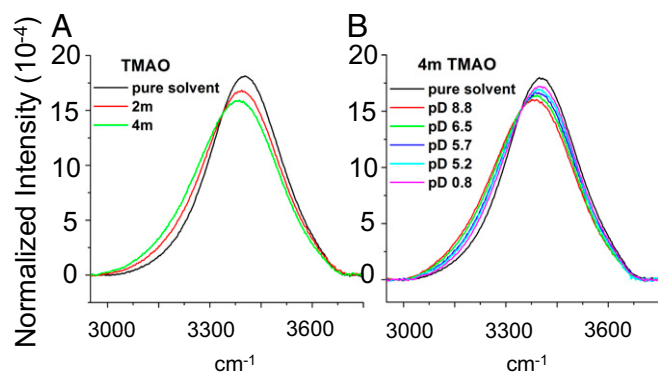


Fig. 5. IR spectra of 3 mol%  $H_2O$  in  $D_2O$  with TMAO as (A) a function of osmolyte concentration and (B) a function of pD at 4 M TMAO. In all cases, the spectra have been normalized to have the same area under each curve. The spectrum for pure water is shown in each case as a reference. Spectra in the OH stretch range over a wider range of TMAO concentrations are provided in *SI Materials and Methods* (Fig. S9).

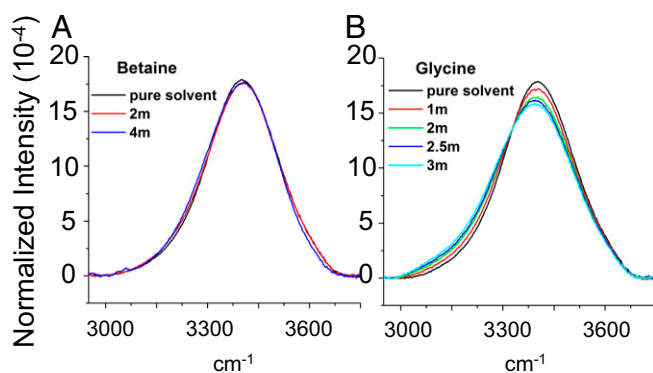


Fig. 6. OH stretch peak from the FTIR spectra for (A) betaine and (B) glycine as a function of osmolyte concentration. The data represent 3 mol%  $H_2O$  and 97%  $D_2O$ .

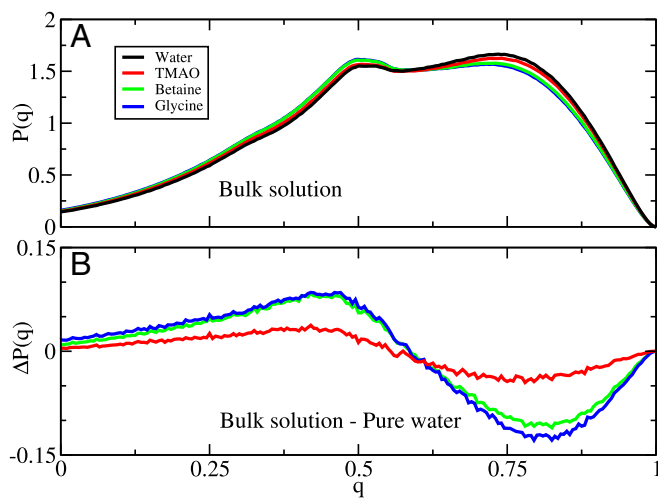
might arise beyond the inner hydration shells. However, because glycine and TMAO cause a substantial shift in the water peak, whereas betaine does not, it would seem that changes to the OH spectrum are not well-correlated with the stabilization or destabilization of the collapsed vs. extended conformations of ELPs.

We also used MD simulations to directly examine the effect of each osmolyte on the water structure. In particular, Fig. 7 quantifies the impact of each osmolyte on the tetrahedrality of the water hydrogen-bonding network (51). Fig. 7A presents the probability distribution of tetrahedral order ( $q$ ) sampled by water molecules. In each osmolyte solution, there exists a population of water molecules with a relatively ordered H-bonding solvation shell ( $q \sim 0.75$ ) and a second population of water molecules with a relatively disordered H-bonding solvation shell ( $q \sim 0.5$ ). Fig. 7B quantifies the change in  $P(q)$  caused by each osmolyte. All of the osmolytes disrupt the intermolecular hydrogen-bonding network, although they exert differing effects. TMAO seems to have relatively little impact on the H-bonding network. Betaine has a considerably larger effect, whereas glycine is most disruptive to the hydrogen-bonding network. Consequently, our MD simulations provide additional evidence that the red shifts observed in the IR spectra primarily reflect direct osmolyte-water interactions rather than indirect effects on the hydrogen-bonding network, which should, instead, blue-shift the spectra.

## Discussion

TMAO, betaine, and glycine act as protecting osmolytes that stabilize the collapsed state of ELPs. As would be expected from prevailing preferential solvation theories for cosolvent effects, glycine and betaine are significantly depleted from the surface of the well-hydrated, uncollapsed state of the ELP. Indeed, both MD simulations and air-water surface tension measurements are consistent with this conclusion. By sharp contrast, however, these same measurements suggest that TMAO actually slightly accumulates at the surface of the uncollapsed state. Such a result is still consistent with basic statistical thermodynamics for preferential solvation as long as TMAO is even more accumulated at the surface of collapsed ELPs.

Interestingly, Berne and coworkers (17, 18) have recently observed similar results for hydrophobic polymers, such as polystyrene and simulated polymers of uncharged Lennard-Jones beads. In particular, they suggested that TMAO accumulates at the surface of extended hydrophobic polymers but still stabilizes collapsed conformations, because TMAO accumulates to an even greater extent at the surface of the collapsed polymer (17, 18). However, in contrast to the purely hydrophobic polymers studied by Berne and coworkers (17, 18), we have studied ELPs, which are polypeptides with backbone amide groups. In fact, glycine residues, which lack a hydrophobic side chain, account for 40% of the amino acids in the studied ELP systems. Thus, if TMAO is repelled from polypeptide



**Fig. 7.** Characterization of the tetrahedral-order parameter,  $q$ , quantifying the hydrogen-bonding network around water molecules in pure water (black) and 0.55 M solutions of TMAO (red), betaine (green), and glycine (blue). **A** presents the distribution  $P(q)$  sampled by bulk water molecules in each solution. **B** presents the difference between the distributions sampled by the bulk solution in the absence and presence of each cosolvent. Fig. S11 presents similar results for other TMAO models.

backbones and if we assume that collapsed ELP conformations completely bury their hydrophobic groups while exposing their polar backbone, then it is rather difficult to envision why TMAO would accumulate to an even greater degree about collapsed ELP conformations. It is certainly possible, however, that ELPs adopt collapsed conformations that bury some of their polar backbone by forming beta hairpins and other hydrogen-bonding secondary structures (52). Consequently, the collapsed conformations may actually expose a relatively larger fraction of their hydrophobic surface compared with the extended conformation, which may then trigger the formation of large, insoluble ELP aggregates.

These considerations also suggest a possible mechanism for the stabilizing effect of TMAO on globular proteins by interacting more favorably with folded than unfolded conformations. First of all, protein folding generally sequesters the peptide backbone from the solvent. Because TMAO interacts unfavorably with the peptide backbone (9, 10), it will provide a stabilizing effect for any protein (19). Second, although it is generally accepted that hydrophobic forces provide the dominant driving force for protein folding (53), folded proteins do not completely sequester all hydrophobic groups from the solvent. Instead, the resulting surface of folded proteins is a heterogeneous patchwork that exposes both polar and nonpolar moieties (54). Water molecules likely outcompete TMAO for the polar features when these regions are isolated. However, because TMAO has a significant dipole and also forms favorable interactions with hydrophobic surfaces (2, 3, 9, 10, 14), it may be that amphiphilic TMAO molecules more effectively interact with the protein when polar and nonpolar regions are brought into close proximity on the surface of a folded protein. Because folded protein surfaces emerge as the coalescence of nonpolar and polar groups from many different amino acids, one can imagine that this stabilizing mechanism is both quite general for globular proteins and

also, quite difficult to quantify or predict from consideration of individual amino acids, assumptions of additive behavior, short peptide constructs, or even, studies of backbone mimics.

In addition, our studies also provide insight into the effect of osmolytes on water structure. Our FTIR experiments indicate that TMAO generates a particularly large red shift in the OH spectrum, which is consistent with the strong water–TMAO interactions observed in experiments (25) and MD simulations (26). Our FTIR experiments show that glycine produces a similar red shift, whereas betaine minimally impacts the OH stretch spectra. Thus, these red shifts seem essentially uncorrelated with the stabilizing effects of osmolytes on the hydrophobic collapse of ELPs. Additionally, by varying the pH, we have shown that the TMAO-induced red shift is very sensitive to direct H-bonding interactions between water and the lone pairs of the TMAO oxygen. In particular, this red shift is not caused by the effect of the cosolvents on the solution pH. Moreover, as previously observed by Gai and coworkers (22, 23), our MD simulations indicate that the osmolytes all disrupt rather than strengthen the water tetrahedral H-bonding network, although TMAO seems the least disruptive to the water network (19, 26). Consequently, we conclude that the observed red shifts primarily reflect direct interactions between water and the osmolytes. Indeed, if the effects of the osmolytes on the water–water hydrogen-bonding network were to predominate the IR spectrum, then we would expect a blue shift in the OH stretch spectra. These results refute the notion that osmolytes indirectly stabilize proteins by altering water structure [i.e., through kosmotropic (structure-making) or chaotropic (structure-breaking) effects (21)].

## Conclusions

Herein, we have shown that TMAO, like glycine and betaine, stabilizes the collapsed and aggregated structure of ELPs. Both glycine and betaine stabilize collapsed conformations via a classical preferential depletion mechanism. Namely, the osmolytes are strongly partitioned away from the polymer–water interface and thus, cause collapse and aggregation via a depletion effect. TMAO, however, drives collapse via a different mechanism. Indeed, TMAO seems to accumulate at both the hydrophobic air–water interface and also, the polymer–water interface of the extended macromolecule. Our results indicate that TMAO has less influence on water structure than the other osmolytes. As such, its mechanism of action seems to involve neither its partitioning away from the peptide–water interface nor its impact on the hydrogen-bonding network of bulk water. Instead, we suggest a nonclassical mechanism, whereby TMAO interacts with the extended state of the macromolecule but interacts even more strongly with the collapsed state. In particular, we speculate that TMAO may act as a surfactant at the interface of polar and nonpolar regions of folded protein surfaces.

## Materials and Methods

**SI Materials and Methods** describes the IR spectrum of water in the presence of TMAO at basic pH, the effects on the water spectra at higher osmolyte concentrations, and the effects of acidification on the IR spectrum of bulk water along with additional details and analysis of the MD simulations.

**ACKNOWLEDGMENTS.** Fig. 1 was made with VMD (Visual Molecular Dynamics). VMD is developed with NIH support by the Theoretical and Computational Biophysics Group at the Beckman Institute, University of Illinois at Urbana–Champaign. This work was funded by National Science Foundation Grants MCB-1053970 (to W.G.N.), CHE-1565631 (to W.G.N.), and CHE-1413307 (to P.S.C.).

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