Effects of Trimethylamine-*N*-oxide on the Conformation of Peptides and its Implications for Proteins

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To provide insights into the stabilizing mechanisms of trimethylamine-*N*-oxide (TMAO) on protein structures, we perform all-atom molecular dynamics simulations of peptides and the Trp-cage miniprotein. The effects of TMAO on the backbone and charged residues of peptides are found to stabilize compact conformations, whereas effects of TMAO on nonpolar residues lead to peptide swelling. This suggests competing mechanisms of TMAO on proteins, which accounts for hydrophobic swelling, backbone collapse, and stabilization of charge-charge interactions. These mechanisms are observed in Trp cage.

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Biochemical reactions in living systems take place in aqueous environments containing small organic molecules [1,2]. These molecules affect osmosis in cells and they are known as protecting or denaturing osmolytes depending on how they modulate the stability of proteins [2–4]. Denaturing osmolytes, e.g., urea and guanidine, are widely used in protein folding studies to destabilize the native state [5]. The effects of these molecules emerge from favorable protein-osmolyte interactions, which enhance the preference for protein conformations with greater solvent exposed surface area, i.e., the unfolded state [6–11]. In contrast, protecting osmolytes, e.g., trimethylamine-*N*-oxide (TMAO) and proline, favor the native state of proteins [12]. These osmolytes counteract effects of water stresses enabling organisms to cope with extreme conditions [1]. For example, deep-sea animals counteract effects of hydrostatic pressure by increasing the concentration of TMAO in their muscles [13,14]. Despite intensive studies on the effects of TMAO in proteins, its molecular mechanisms remain a question of debate [15].

The stabilizing effect of TMAO on native structures is often explained by its exclusion from the vicinity of the protein surface [16–18]. Exclusion is possible because TMAO is more strongly attracted to water [19–25] than to the protein surface. Moreover, exclusion may be more pronounced close to the main chain of the protein, i.e., the backbone [25–29], which explains the adoption of more compact conformations by polyglycine (which is commonly used as a model of the protein backbone) in aqueous TMAO solution compared to pure water [30]. Despite these results, TMAO exclusion from the protein surface as a mechanism to protect the native state has been challenged by recent studies. In particular, computer simulations and experiments have shown that TMAO stabilizes compact conformations of some nonpolar polymers through direct interactions [31,32]. Recently, TMAO's effect on proteins was reported to emerge from favorable interactions of this osmolyte with the heterogeneous protein surface that emerges upon folding [33]. Other studies are suggesting that protecting effects of TMAO emerge because this molecule acts as a crowding agent reducing the conformational entropy of the unfolded state [34,35] and/or by weakening the strength of hydrogen bonds between the protein and water molecules [35].

In this Letter, we highlight the effects of TMAO on the molecular forces stabilizing native protein structures by studying small peptides and the Trp-cage miniprotein. Consistent with other studies [30,34], we find that TMAO favors compact conformations of a peptide model of the protein backbone, i.e., polyglycine. However, the addition of even the smallest nonpolar side chain (i.e., the $-CH_3$ group of alanine) to the backbone counteracts this effect while larger nonpolar side chains account for peptide swelling. This suggests that TMAO can destabilize the hydrophobic core of proteins. We also study conformations of nonpolar peptides with charged terminal residues. We find that these peptides become more compact when TMAO is added to water due to stronger interactions between charged residues. In light of these results, we hypothesize that competition of TMAO's effect on hydrophobic and charged interactions accounts for its net stabilizing role in proteins. Extensive replica exchange molecular dynamics (REMD) simulations of the small Trp-cage protein in pure water and TMAO solution are performed to test this hypothesis. Accordingly, we find that residues that form the hydrophobic core of Trp cage sample more extended conformations while distances between its charged residues decrease when TMAO is added to water.

Equilibrium conformations of peptides in this work are determined using *NPT* molecular dynamics simulations (1 atm and 298 K) of at least two independent simulations [36]. Peptides are terminated with COOH and NH_2 groups, and they are initially in an extended conformation. To study equilibrium configurations of the Trp-cage miniprotein we use REMD simulations. Trp cage has one positive net charge that is neutralized by adding one chloride ion to the solvent. Simulations are performed in pure water and 5*M*

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TMAO solution at a constant pressure of 1 atm. We employ 32 replicas with temperatures between 298.00 and 402.26 K that provide a replica-exchange rate of 0.2 [36,39]. Exchanges between neighboring replicas are attempted at every 750 steps. Simulations are extended over 300 ns per replica and the last 200 ns are used for analysis. All the simulations are performed using GROMACS 4.5 [40] with the AMBER99SB-ildn force field to describe peptides [41], TIP3P water, and the Kast [42] model to mimic the behavior of TMAO molecules [36]. To show that results from simulations are force-field independent, we also use the Osmotic [43] and Netz [44] models for TMAO as well as SPCE water [36]. We use high concentrations of TMAO molecules to allow for significant changes in protein conformations within the time scale of simulations. At these high concentrations, Kast model tends to underestimate the effective repulsion between TMAO molecules [43].

Figure 1(a) shows extended conformations of deca-homopeptides made of glycine (G_{10}), alanine (A_{10}), valine (V_{10}), and leucine (L_{10}). The side chain of glycine is made of one hydrogen atom and, therefore, polyglycine is commonly used as a model of the protein backbone. The side chain of alanine is made of a small nonpolar group ($-CH_3$) whereas valine and leucine are decorated with large nonpolar groups ($-C_3H_7$ and $-C_4H_9$). Thus, L_{10} and V_{10} are more hydrophobic in nature than A_{10}. Distributions of the radius of gyration R_g of backbone atoms of these peptides are studied in Figs. 1(b)–1(e) in pure water (black) as well as $3M$ (red) and $7M$ (blue) TMAO solutions.

Figure 1(b) shows that compact and extended conformations of G_{10} become more and less populated, respectively, when TMAO is added to water. Previous studies have suggested that this effect of TMAO is the dominant stabilizing effect of this osmolyte on native protein structures [26]. In Figs. 1(c)–1(e) we probe effects of TMAO on nonpolar peptides. Distributions of R_g for the A_{10} peptide are not strongly affected by TMAO; see Fig. 1(c). This is consistent with computational studies showing that the interaction between nonpolar compounds that are comparable in size to alanine's side chain ($-CH_3$), i.e., methane molecules (CH_4), is only weakly affected by TMAO [45]. In contrast, V_{10} and L_{10} become more extended as the concentration of TMAO increases; see Figs. 1(d)–1(e). In Fig. 1(e), we also show distributions of R_g for L_{10} in aqueous solutions containing Osmotic and Netz TMAO molecules (in green). Independent of the TMAO force field, Fig. 1(e) shows that the population of extended conformations of L_{10} increases when TMAO is added to water. Swelling of L_{10} is also observed with a different water model, i.e., SPCE water, using Netz TMAO molecules; see Fig. S7b [36]. These results show that TMAO's collapsing effect on the backbone can be overcompensated by its effect on hydrophobic residues which causes peptides to swell. This is consistent with the hydrophobic effect becoming weaker in aqueous TMAO solutions as reported for nonpolar compounds that

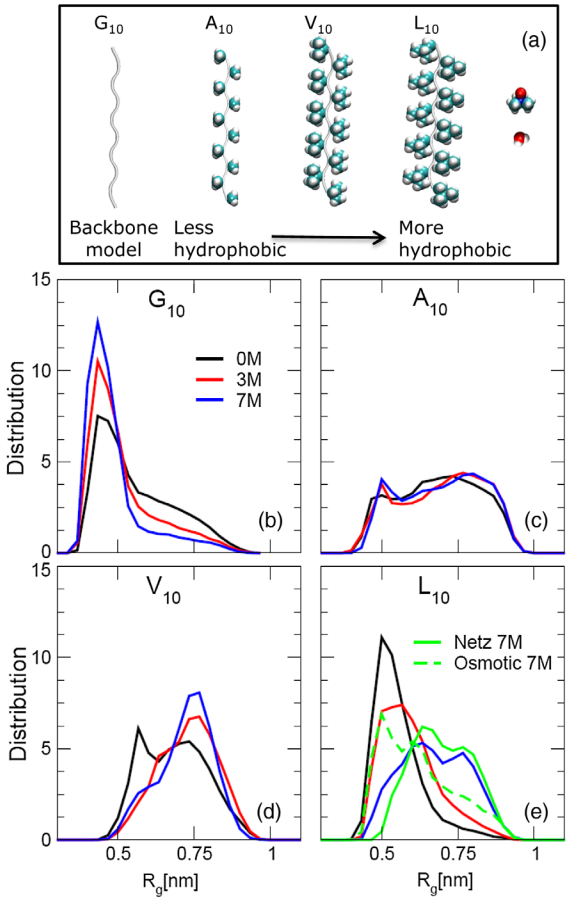

FIG. 1. Conformations of homopeptides in different aqueous solutions. (a) Schematics of homopeptides using a cartoonlike representation for the backbone and a van der Waals representation for side chains highlighting the weaker hydrophobic nature of polyalanine compared to polyvaline and polyleucine. TMAO (C_3H_9NO) and water (H_2O) are also shown. Distributions of the radius of gyration R_g of backbone atoms for (b) polyglycine, (c) polyalanine, (d) polyvaline, and (e) polyleucine in pure water as well as $3M$ and $7M$ TMAO solutions at 298 K and 1 atm.

are comparable in size to leucine's side chain ($-C_4H_9$), e.g., neopentane (C_5H_{10}) [31,46–49]. Moreover, since the hydrophobic core is key to protein folding this result suggests that, in addition to the backbone, other elements of the protein may also play an important role in TMAO's stabilizing effect.

Insights into another stabilizing effect of TMAO can be obtained by studying the $A\beta_{16-22}$ peptide (**KLVFFAE**) which is made of five nonpolar residues (bold letters) flanked by opposite charged residues (underline letters). Based on results from Fig. 1, the addition of TMAO to water is expected to cause the $A\beta_{16-22}$ peptide to swell due to its highly nonpolar nature. However, all-atom molecular dynamics simulations using the CHARMM22 force field with the CMAP modification have reported that TMAO favors compact conformations of the $A\beta_{16-22}$ peptide possible due to a coil-helix transition [34]. In our simulations

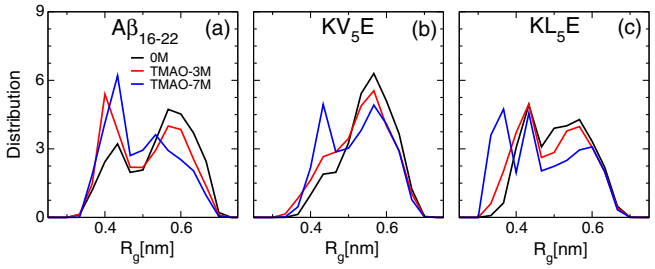

FIG. 2. Effects of TMAO on the conformation of nonpolar peptides flanked by opposite charged residues. Distribution of the radius of gyration R_g of backbone atoms for (a) $A\beta_{16-22}$, (b) KV_5E, and (c) KL_5E in different aqueous solutions at 298 K and 1 atm.

using the AMBER99SB-ildn force field, the peptide also becomes more compact when TMAO is added to water [see Fig. 2(a)] but without favoring any type of secondary structures; see Table S3 and Fig. S2 [36]. An analysis of the structures of $A\beta_{16-22}$ shows that TMAO favors conformations where charged residues are close to each other; see Fig. S3 [36]. Therefore, we speculate that charged residues at the end of $A\beta_{16-22}$ are responsible for the collapsing effect of this peptide. To verify this idea, we flank short polyvaline and polyleucine peptides [which adopt more extended conformation in the presence of TMAO; see Figs. 1(d)–1(e)] with opposite charged residues: $\underline{K}\mathbf{L}_5\underline{E}$ and $\underline{K}\mathbf{V}_5\underline{E}$. Results from simulations of these peptides are shown in Figs. 2(b)–2(c). These simulations show that the presence of charged residues makes TMAO to favor compact peptide conformations. A similar result is also observed using SPCE water and the Netz model for $A\beta_{16-22}$; see Fig. S7a [36]. It suggests that TMAO enhances the magnitude of charge-charge interactions. Accordingly, in Fig. S1 [36] we compute the potential of mean force (PMF) for the interaction between Na^+ and Cl^- in pure water and 7M TMAO solution [36]. The PMF to form a contact between these ions increases significantly (~2 kJ/mol) when TMAO is added to water. Thus, effects of TMAO on charge-charge interactions contribute to overcome TMAO's swelling effect on nonpolar peptide segments and they may play an important role in stabilizing compact protein structures.

To show that the results obtained for peptides in Figs. 1 and 2 also apply to proteins that fold into a native state; we show in Fig. 3 results from REMD simulations of the Trp-cage miniprotein. The key charge-charge interaction of Trp cage flanks the loop region of this protein while the hydrophobic core holds one side of the α helix bonded to the straight segment in the native state [50]; see Fig. 3(a). Experimental and computational studies have highlighted the importance of these residues in accounting for the stability and folding of Trp cage [51,52]. Results from our simulations of peptides (see Figs. 1 and 2) predict that adding TMAO to water destabilizes the hydrophobic core of Trp cage while it increases the stability of the charged residues. To verify this prediction, distributions of the radius of gyrations of hydrophobic R_g^{hydro} and charged R_g^{charge} residues are shown in Figs. 3(b)–3(c) for simulations performed in pure water and 5M TMAO solution. These

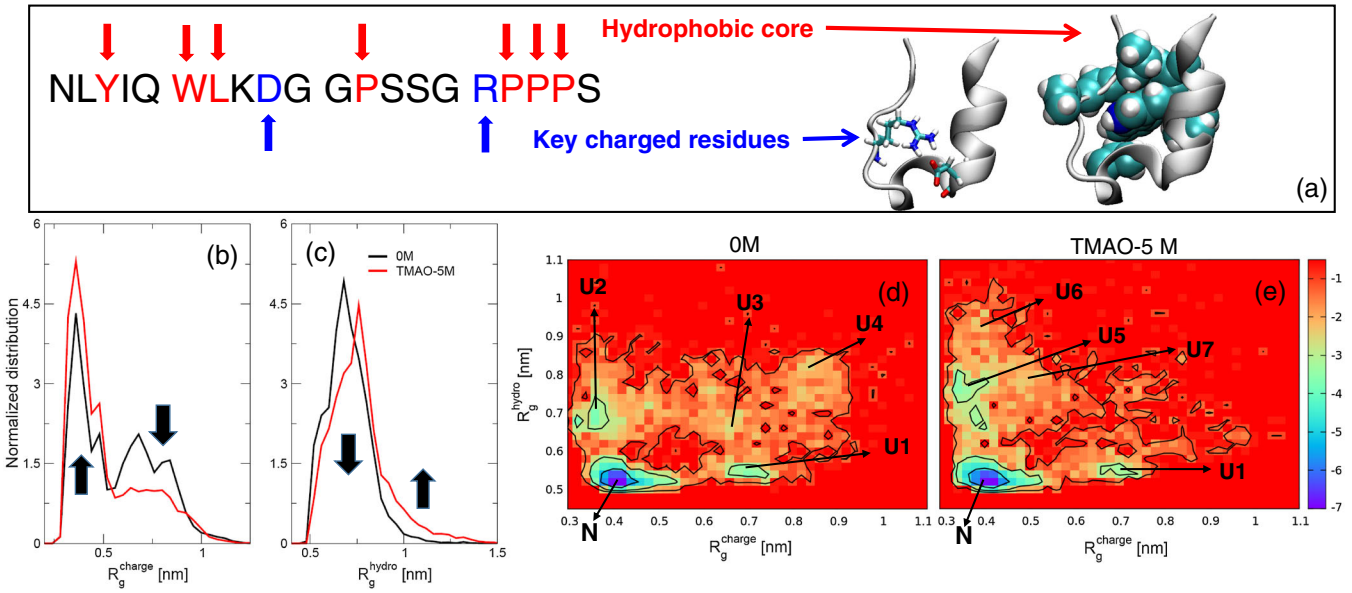

FIG. 3. Effects of TMAO on the Trp-cage miniprotein. (a) Amino acid sequence of Trp-cage highlighting residues that form the hydrophobic core and a key charge-charge interaction of this miniprotein. The native structure of Trp cage with side chains of charged and the hydrophobic residues are depicted on the right-hand side. (b)–(c) R_g distributions of charged and hydrophobic residues are depicted for simulations performed in pure water and 5M TMAO solution. (d)–(e) Free-energy landscape of Trp cage divided by the thermal energy as a function of hydrophobic and charge R_g in pure water and 5M TMAO solution.

distributions are shown for the unfolded state at the coexistent temperature $T_e = 350$ K, where native and unfolded states are equally populated [53]. Figures 3(b)–3(c) show that when TMAO is added to water charged and hydrophobic residues sample more compact and extended conformations, respectively. This is consistent with results from Figs. 1 and 2. To provide further detail, we show the free-energy of Trp cage divided by the thermal energy as function of R_g^{hydro} and R_g^{charge} in pure water [Fig. 3(d)] and TMAO solution [Fig. 3(e)] at T_e. They show that in pure water, the unfolded state of Trp cage samples mostly conformations in which charged residues are far apart, i.e., states U3, and U4 in Fig. 3(d). In contrast, the unfolded state in TMAO solution is mostly characterized by hydrophobic residues that are far apart from each other whereas charged residues remain as compact as the native state; see states U5, U6, and U7 in Fig. 3(e). A detailed analysis of the different structures adopted by Trp cage in pure water and TMAO solution is provided in the Supplemental Material [36]; see Fig. S6.

To provide insight into how TMAO destabilizes hydrophobic interactions while enhancing the magnitude of charge-charge interactions, we compute the preferential interaction defined as [43,54]

$$\Gamma(r) = \left\langle N_{\mathrm{TMAO}}(r) - \left(\frac{N_{\mathrm{TMAO}}^{\mathrm{bulk}}}{N_{\mathrm{water}}^{\mathrm{bulk}}}\right) N_{\mathrm{water}}(r) \right\rangle, \quad (1)$$

where $N_{\mathrm{TMAO}}(r)$ and $N_{\mathrm{water}}(r)$ are the number of TMAO and water molecules with minimal distance to peptide atoms between 0 and r. $N_{\mathrm{TMAO}}^{\mathrm{bulk}}$ and $N_{\mathrm{water}}^{\mathrm{bulk}}$ are numbers of TMAO and water molecules in the bulk [55]. $\Gamma(r)$ compares the number of TMAO molecules within a distances r from the protein with the expected number of TMAO in a similar water region in the bulk [33,56,57]. It has been proposed that osmolytes which accumulate in the vicinity of proteins and, therefore, are characterized by positive $\Gamma(r)$ values, interact favorably with the protein surface favoring the unfolded state. In contrast, osmolytes that are repelled from the protein surface [i.e., $\Gamma(r) < 0$] favor the folded state of proteins [56,57]. Insights into how osmolytes are partitioned close to the air-water interface can be obtained from measurements of surface tension which are often, but not always, consistent with their effects on proteins [58]. Upon addition of TMAO to water, the air-water surface tension decreases suggesting that this osmolyte accumulates at this interface [33,57,59]. In contrast, positive transfer free-energies of proteins from pure water to TMAO solutions imply that this osmolyte is repelled from the vicinity of the protein [60,61].

In Fig. 4(a), we show that for polyglycine $\Gamma(r)$ is negative implying that TMAO is excluded from the surface of this peptide. For the other peptides, we provide insights into how backbone and side chain atoms contribute to $\Gamma(r)$ by assigning solvent molecules to a particular group

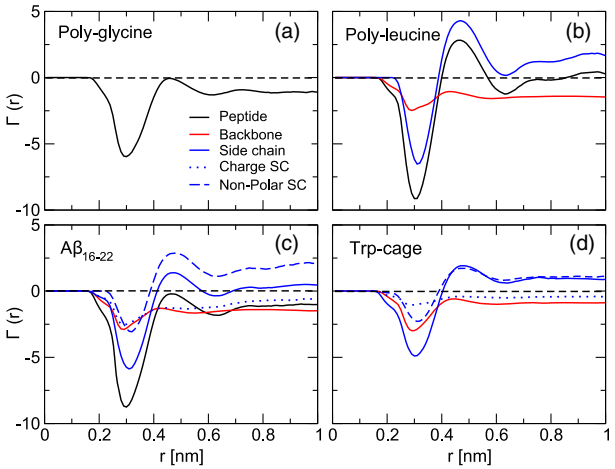

FIG. 4. Preferential interaction $\Gamma(r)$ of polyglycine, polyleucine, $A\beta_{16-22}$, and Trp cage. (b)–(d) $\Gamma(r)$ is decomposed into contributions from backbone (red) and side chain (blue) atoms. (c)–(d) $\Gamma(r)$ of side chains is further analyzed in terms of contributions from polar (doted blue), and nonpolar (dashed blue) side chains. Only key residues comprising charge-charge interactions and the hydrophobic core [see Fig. 3(a)] are used to compute contributions of polar and nonpolar side chains of Trp cage.

(backbone or side chain) if it is closest to that group. This assignment is independent of r. Solvent molecules assigned to a group are used to compute $\Gamma(r)$ of that particular group. Consistent with our results for polyglycine, $\Gamma(r)$ computed for solvent molecules associated to backbone atoms of polyleucine, $A\beta_{16-22}$, and Trp-cage [red lines in Figs. 4(b)–4(d)] are negative confirming that TMAO is excluded from the backbone of proteins. Similarly, TMAO is excluded from the proximity of charged residues of $A\beta_{16-22}$ and the Trp-cage protein as $\Gamma(r)$ for these groups [dotted blue lines in Figs. 4(c)–4(d)] are negative. In contrast, $\Gamma(r)$ computed for solvent molecules associated with nonpolar side chain atoms of polyleucine [blue line in panel (b)] as well as of $A\beta_{16-22}$ and Trp cage [dashed blue lines in panels (c), (d)] are positive for distances greater than 0.4 nm implying that TMAO is attracted to nonpolar groups of proteins. Notice that the exclusion of TMAO from backbone and charged amino acids is consistent with peptides adopting more compact structures in Figs. 1(b) and 2. Also, attraction of TMAO to nonpolar residues is consistent with nonpolar peptides adopting more extended conformation in Figs. 1(d)–1(e).

In summary, we find that TMAO accounts for swelling of nonpolar peptides suggesting that it can destabilize the hydrophobic core of proteins. Accordingly, our simulations of the Trp-cage protein reveal that its nonpolar residues adopt more extended conformations in TMAO solutions. This result may provide rationalization for the R_g of the Snase protein which was found to be larger in TMAO solutions (17.3 ± 1.5 Å) than in water (15.6 ± 0.2 Å) measured using SAXS [62]. Moreover, we find that effects

of TMAO on backbone and charged residues are found to stabilize compact peptide structures. Traditionally, the former has been related to TMAO's main stabilizing mechanisms [26,30]. However, the observed swelling of nonpolar peptides in our simulations provide evidence that at least for these amino acid sequences, TMAO's effects on the backbone are not enough to counteract its effects on nonpolar residues. For nonpolar peptides flanked by charged residues as well as the Trp-cage miniprotein, we find that charged residues contribute significantly to counteract effects of TMAO on nonpolar residues. Evidence that charged residues contribute to the stability of globular proteins is provided by thermophilic proteins [63,64]. While hydrophobic interactions are the main interaction accounting for the increased stability of thermophilic proteins, 68% of these proteins showed an increased number of salt bridges when compared to their mesophilic homologs [65]. These additional salt bridges contribute to enable thermophilic proteins to function at higher temperatures [66]. Despite the novel insights brought up by our simulations, this work does not exclude the existence of other stabilization mechanisms of TMAO, e.g., the recently proposed surfactant mechanism which may explains TMAO's effect on elastin that is made of alternating glycine and nonpolar residues with no charged amino acids [33].

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