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Methyl Groups of Trimethylamine *N*-Oxide Orient Away from Hydrophobic Interfaces

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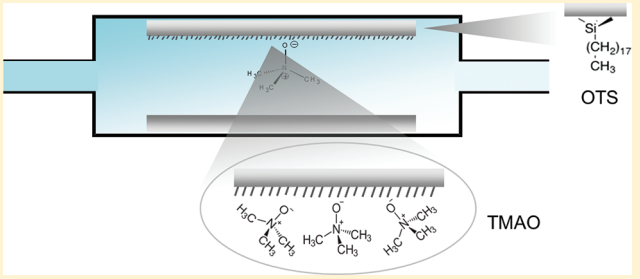
Supporting Information

ABSTRACT: The molecular orientation of trimethylamine *N*-oxide (TMAO), a powerful protein stabilizer, was explored at aqueous/hydrophobic interfaces using vibrational sum frequency spectroscopy (VSFS). The systems studied included the octadecyltrichlorosilane (OTS)/water interface, which represents an aqueous solution in direct contact with a hydrophobic medium. Surprisingly, the measurements revealed that the methyl groups of TMAO pointed into the aqueous phase and away from the OTS. This orientation may arise from the more hydrophilic nature of methyl groups attached to a strongly electron-withdrawing atom such as a quaternary nitrogen. Additional studies were performed at the air/water interface. This interface showed a high degree of TMAO alignment, but the dangling OH from water was present even at 5 M TAMO. Moreover, the addition of this osmolyte modestly increased the surface tension of the interface. This meant that this species was somewhat depleted at the interface compared to the bulk solution. These findings may have implications for the stabilizing effect of TMAO on proteins. Specifically, the strong hydration required for the methyl groups as well as the oxide moiety should be responsible for the osmolyte's depletion from hydrophobic/aqueous interfaces. Such depletion effects should help stabilize proteins in their folded and native conformations on entropic grounds, although orientational effects may play an additional role.



INTRODUCTION

Protecting osmolytes are believed to be vital for stabilizing intracellular proteins against a wide variety of adverse environmental conditions.[1,2] For example, trimethylamine-*N*-oxide (TMAO) has been shown to offset the effects of denaturants such as urea in the kidneys.[3−5] Unlike denaturing osmolytes, protecting osmolytes thermodynamically favor the folded state of proteins. Although the thermodynamic effects of TMAO are as pronounced as those of urea, the molecular level mechanism by which this molecule stabilizes proteins has been much less explored and, hence, continues to remain elusive.[6−9]

Previous studies have explained the stabilizing and denaturing effects of compounds such as urea through depleted volume effects and preferential hydration.[10,11] These models are based on whether a cosolvent favorably partitions to the protein/water interface and often invoke air/water surface tension data. Denaturants typically decrease the surface tension of the air/water interface and accumulate at the protein/water interface, implying their direct interaction with hydrophobic portions of proteins. Protein stabilizers, on the other hand, are generally believed to be depleted at the protein/water interface. Thus, they should increase the surface tension of the air/water interface consistent with more indirect theories of interaction. Although many protein denaturants and stabilizers fit this general model, there are some important exceptions. Urea slightly increases the surface tension of the air/water interface,[12] which would lead to the puzzling conclusion that it does not preferentially accumulate there. Nevertheless, the most widely invoked models assume urea directly interacts with proteins through hydrogen bonding and/or hydrophobic interactions.[13−15]

Unlike denaturing compounds, stabilizing osmolytes are not thought to directly interact with proteins but instead act through more indirect mechanisms. TMAO only modestly influences surface tension, which has made this value somewhat difficult to measure.[12] However, partition coefficients calculated from surface tension data showing a decreasing increment as TMAO is added to solution would require mild accumulation of the osmolyte at the protein/water interface.[16] It has been shown that TMAO uniquely changes water structure at the protein/water interface, which could in turn change the solubility of the protein and stabilize the folded state.[17] However, there is disagreement as to whether TMAO weakens or strengthens water structure at the protein/water interface.[18] In addition, changes in water structure have not been universally observed for other protecting and denaturing osmolytes making it difficult to explain a general mechanism based solely on changes in water structure.

Several recent studies have introduced a general mechanism for the protecting or denaturing ability of various osmolytes

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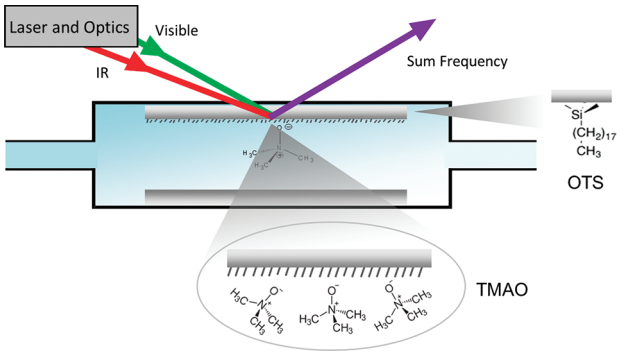

Figure 1. Schematic diagram of the experimental setup for the OTS/water interface. VSFS experiments were run in a Teflon flow cell with quartz windows on the top and bottom. The top quartz window contained an OTS monolayer on its bottom face. The VSFS response was obtained from the OTS/water interface. The spectra revealed that TMAO molecules align with their methyl groups facing away from the interface.

through preferential interactions.[19−21] According to this model, denaturing osmolytes would possess favorable interactions with various polar groups in proteins, while stabilizing osmolytes would have unfavorable interactions with these same groups. However, because of the similar chemical makeup of protecting and denaturing osmolytes, these arguments often invoke changes in the exposed surface area of the protein. In addition, these studies typically focus on polar and charged groups on proteins rather than interactions between osmolytes and hydrophobic groups. Since many studies have shown the importance of hydrophobic groups in protein collapse and folding, we found a strong impetus to carry out a study investigating TMAO interactions at hydrophobic interfaces.[22−25]

Herein, we report molecular-level observations of TMAO at two hydrophobic interfaces, air/water and OTS/water, using vibrational sum frequency spectroscopy (VSFS). VSFS is an interface specific technique, which not only provides a vibrational spectrum of molecules residing at the interface but also affords orientational information.[26,27] In the current study, the molecular orientation of the methyl groups of TMAO was investigated at both the air/water and the OTS/water interfaces. It was found that TMAO is oriented with its methyl groups pointing away from the hydrophobic OTS surface (Figure 1). Moreover, VSFS data from the air/water interface in conjunction with surface tension measurements indicated that TMAO should be depleted from this interface. These observations may provide molecular level clues into the stabilizing nature of TMAO. Namely, methyl groups attached to an electron-withdrawn group, such as a quaternary nitrogen, should make them less hydrophobic than methyl groups at the ends of alkyl chains. The increased need to keep these methyl groups hydrated would cause them to be excluded from protein surfaces and thereby lead to protein stabilization.

MATERIALS AND METHODS

Synthesis of Trimethylamine-d_9 *N*-Oxide Dihydrate-d_2 (TMAO-d_9) and Deuterated Dodecyltrichlorosilane ($CD_3(CD_2)_{11}SiCl_3$ or d-DTS). TMAO-d_9 and d-OTS were synthesized in a fashion similar to their nondeuterated analogues.[28,29] A detailed description of the procedures and NMR spectral features can be found in the Supporting Information.

Preparing TMAO Solutions in D_2O. Trimethylamine *N*-oxide (dihydrate, 98% purity, Fisher Scientific, Waltham, MA) was dissolved in 99.9% D_2O (Cambrige Isotope Laboratories, Inc.). To exchange H_2O bound to the oxygen atom of TMAO for deuterium, TMAO solutions were evaporated using a rotoevaporator, redissolved in D_2O, and evaporated again. This process was repeated 4−5 times until no hydrogenated water was detected in the VSFS spectra between 3000 and 4000 cm^{-1}. Finally, the dried TMAO was redissolved in D_2O, filtered with a 0.45 μm Teflon syringe filter, and used for measurements.

Preparing and Characterizing OTS Monolayers on Quartz. Quartz pieces, (round, 1 in. diameter, $^1/_8$ in. thick) purchased from Quartz Plus, Inc. (Brookline, NH) were soaked in a 50/50 volume mixture of sulfuric and nitric acid for several hours. The clean quartz pieces were then rinsed with deionized H_2O, dried with N_2 gas, and left in a drying oven for ca. 30 min. Next, the quartz pieces were transferred to a 2 M NaOH solution for 15 min at room temperature, washed thoroughly with deionized H_2O, dried under N_2 gas, and left in a drying oven for ca. 30 min. Upon cooling to room temperature, the quartz pieces were transferred to a 1 mM octadecyltrichlorosilane (Sigma-Aldrich, St. Louis, MO) solution in hexanes and left for 2 h. The quartz samples were then cleaned with ethanol, acetone, and deionized H_2O. The samples were stored in deionized H_2O and dried with N_2 gas just prior to use.

OTS monolayers on quartz were characterized by VSFS in air using three different polarization combinations (ssp, ppp, and sps). These designations refer to the polarization of the sum frequency, visible, and infrared beams, respectively. These data are provided in Figure S1 of Supporting Information. The spectra agree well with previous literature reports[30−32] and indicate that the OTS monolayer has nearly an all-trans alkyl chain configuration. This is in agreement with previous studies, which also noted the tilt angle to be close to 10° from the surface normal.[31,33,34]

Vibrational Sum Frequency Spectroscopy. VSFS is a second-order nonlinear spectroscopic technique that involves the spatial and temporal overlap of two incident laser beams. The first beam is of variable infrared frequency and the second is of fixed visible frequency. This produces a third beam whose frequency is at the sum of the two incident beams.[35,36] The process is forbidden in the dipole approximation in bulk centrosymmetric media. Thus, signal only arises when the sample lacks inversion symmetry. The VSFS system, which was employed in these studies, has been described in detail elsewhere.[37,38] Briefly, a mode-locked Nd:YAG laser (PY61C, Continuum, Santa Clara, CA) with a 1064-nm output was used to pump an optical parametric generation/amplification (OPA/OPG) stage (LaserVision, Bellevue, WA) to produce a tunable IR beam between 2700 and 3800 cm^{-1} as well as a fixed frequency beam at 532 nm. The polarization combination used in all the TMAO experiments was ssp unless otherwise noted.

The experimental setup, including a homemade Teflon flow cell, has been described elsewhere.[39,40] A quartz disk coated with OTS was placed onto the flow cell face down so that the OTS was in contact with the solution inside it (Figure 1). Solutions of TMAO were subsequently flowed into the cell using a 60-mL syringe. The input beams were transmitted through the quartz substrate and focused at the OTS/solution interface. The spectra reported herein have been normalized to the nonresonant response from a piece of Z-cut crystalline quartz after background subtraction. The normalized spectra were then fitted to the following equation using Matlab software

$$\chi^{(2)}_{\mathrm{eff}} = \chi^{(2)}_{\mathrm{NR}} + \sum_q \chi^{(2)}_{\mathrm{R}} = \chi^{(2)}_{\mathrm{NR}} + \sum_q \frac{A_q}{\omega_{\mathrm{IR}} - \omega_q + i\Gamma_q} \quad (1)$$

where $\chi^{(2)}_{\mathrm{eff}}$ represents the effective nonlinear susceptibility and $\chi^{(2)}_{\mathrm{R}}$ and $\chi^{(2)}_{\mathrm{NR}}$ are the resonant and nonresonant contributions, respectively. The resonant nonlinear susceptibility is further expressed as a function of the

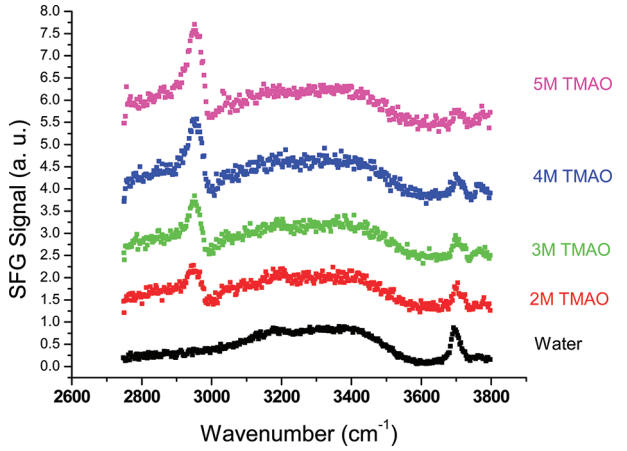

Figure 2. VSFS spectra of the air/water interface using the ssp polarization combination in the presence of varying concentrations of TMAO.

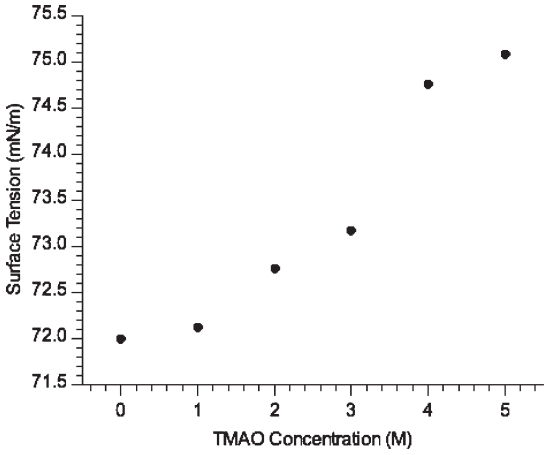

Figure 3. Plot of surface pressure at the air/water interface as a function of TMAO concentration.

oscillator strength, A_q, resonant frequency, ω_q, peak width, A_q, and the frequency of the infrared beam, ω_{IR}.

RESULTS

TMAO Solutions at the Air/Water Interface. Different concentrations of TMAO in aqueous solution were investigated at the air/water interface in a Langmuir trough (Model 601M, NIMA). For all concentrations, prominent peaks in the C−H stretching region near 2950 cm^{-1} were observed, corresponding to the symmetric stretch frequency of the methyl groups (Figure 2). This demonstrated that the osmolyte does in fact reside in a well oriented fashion at the air/water interface. It is normally expected that the methyl groups of organic species will replace the energetically unfavorable dangling O−H bonds from the water molecules at the surface. This is manifested by the disappearance of the stretch at ~3700 cm^{-1} and is readily observed with low concentrations of many surfactants.[41,42] The 3700 cm^{-1} peak also attenuates in the present experiments, albeit much more slowly. Indeed, it is still observable when 5 M TMAO is present in solution.

Fitting the oscillator strength of both the 2950 and 3700 cm^{-1} peaks indicates a roughly linearly increase of the former and a roughly linear decrease of the latter (Figure S2 of Supporting Information). This relatively gradual change in the free OH stretch signal coupled with the relatively high concentrations of TMAO employed is consistent with the idea that there is not substantial accumulation of TMAO at the interface relative to bulk solution. To further test this idea, the surface tension of the air/water interface was monitored as a function of TMAO concentration in a Langmuir trough using a Wilhemly plate. It was found that the tension rose modestly as the osmolyte was added to the solution (Figure 3). This result is consistent with a modest depletion of TMAO at the interface compared to the bulk solution.

It is challenging to determine the absolute orientation of the methyl groups at the TMAO/water interface because one would have to also know the relative phase of the water near the 2950 cm^{-1} TMAO resonance. Since the free OH stretch is still present even at higher TMAO concentrations, one might assume that the water resonances and phases are similar to those at the neat air/water interface. If this assumption is made, then the water should have positive phase below 3200 cm^{-1}. If this is the case, the methyl groups on TMAO would have an opposite phase relative to the dangling OH and point down into the bulk solution (Figure S3 of Supporting Information). A further check employing the maximum entropy (MEM)[43] to calculate the sign of the imaginary portion of $\chi^{(2)}$ also indicated that the methyl peaks from TMAO possessed the opposite sign from the dangling OH (Figure S4 of Supporting Information). This again would be consistent with the methyl groups on TMAO pointing into the aqueous solution and away from the air.

OTS Monolayers in 6 M TMAO Solution. Because of the difficulty in determining the orientation of TMAO at the air/water interface, we wished to find a hydrophobic interface where the phase of the peak from the TMAO methyl groups could be more easily referenced. For this purpose, the orientation of the osmolyte was tested at the OTS/water interface, which is a model oil/water interface. The OTS monolayer contains terminal methyl groups with a known orientation facing away from the substrate and into the water phase (Figure 1). The interference between these methyl groups and those on TMAO can be used to determine the orientation of the osmolyte with respect to the interface. To perform these experiments, OTS monolayers on quartz were transferred to a flow cell containing a D_2O solution. The data revealed the presence of two main peaks at 2876 cm^{-1} and 2933 cm^{-1}, corresponding to the CH_3 symmetric stretch of the terminal methyl group as well as a Fermi resonance (Figure 4a, black spectrum). Additional features are sometimes also observed for this system near 2840 cm^{-1} and 2960 cm^{-1} due to the CH_2 asymmetric stretch and the CH_3 asymmetric stretch.[30,31] Such resonances represent disorder in the OTS monolayer and were quite small in the present case.

When 6 M TMAO in D_2O was flowed into the cell, the higher frequency peak increased in intensity by more than 10% and became somewhat blue-shifted (Figure 4a, red spectrum). On the other hand, the 2876 cm^{-1} peak remained unchanged. There were three possibilities to explain the differences in the spectra in Figure 4a. First, the increased intensity of the higher frequency peak could be due to a reordering of the OTS monolayer. Second, the changes could reflect the presence of the osmolyte. Third, a combination of intensity from the osmolyte and a reordering of the OTS monolayer could be responsible for the observed changes. To investigate this, the OTS/D_2O spectra were repeated in a 6 M deuterated TMAO solution and compared to the identical monolayer taken in pure D_2O (Figure 4b). As can be seen, the spectra in this case are essentially identical. This is in good

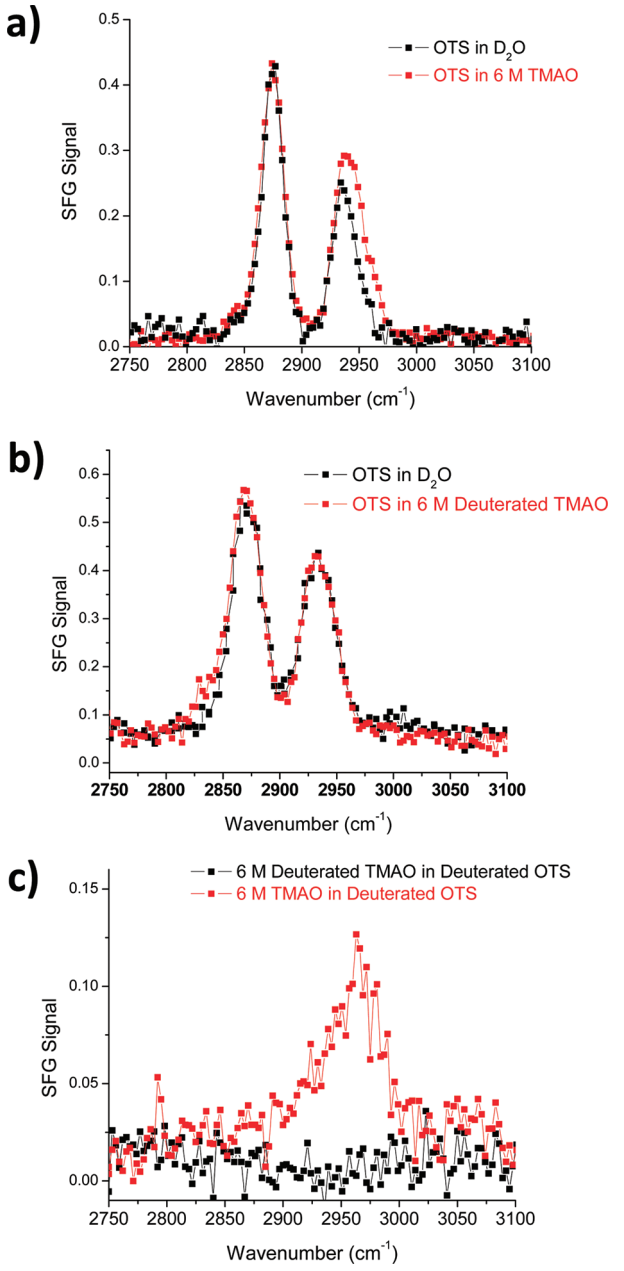

Figure 4. VSFS spectra of the OTS/D_2O interface, taken in a quartz flow cell. (a) Spectra in the presence and absence of 6 M TMAO. (b) The same system as in (a), but with 6 M perdeuterated TMAO. (c) The same system as in parts a and b but with hydrogenated TMAO and perdeuterated OTS.

agreement with the notion that the presence of the osmolyte did not disrupt the tightly order, well-packed OTS monolayer.

As an additional control, an experiment was performed with deuterated OTS and hydrogenated TMAO. No signal was obtained in the CH stretch region when just the deuterated OTS monolayer was present at the quartz/water interface (Figure 4c, black spectrum); however, a weak and broad peak somewhat above 2950 cm^{-1} was observed when 6 M TMAO was added to the solution (Figure 4c, red spectrum). This is consistent with the small rise and blue-shift observed in Figure 4a if the two resonances constructively interfere (e.g., have the same phase). This can only be the case if the methyl groups on TMAO have the same orientation as the terminal methyl groups in the OTS monolayer. Since the methyl groups on the OTS monolayer point toward the water, these data indicate that the methyl groups from TMAO also point toward the water. This is depicted schematically in Figure 1. As depicted in the diagram, the constructive interference between the methyl group resonances from TMAO and the OTS layer only requires that the methyl groups have the same net orientation with respect to the surface normal. The TMAO may, however, be tilted as long as its net orientation is away from the surface.

Finally, the orientation of the methyl groups from TMAO at the OTS/D_2O interface was checked by fitting the red spectrum in Figure 4a. Both the 2876 and 2933 cm^{-1} resonances from the OTS monolayer can be arbitrarily assigned to a negative sign. Figure 5a shows a fit to the data in which the additional resonance from TMAO near 2950 cm^{-1} has the same sign (negative), while Figure 5b shows the fit if this resonance is assumed to have the opposite sign (positive) of the OTS peaks. The difference is small because the TMAO oscillator strength was weak. Nevertheless, the fit with the same sign was superior in agreement with the idea that the methyl groups from TMAO faced toward the aqueous solution rather than toward the OTS layer. MEM calculations were also performed which confirmed that the imaginary part of $\chi^{(2)}$ becomes more negative near 2950 cm^{-1} after 6 M TMAO was introduced to the D_2O/OTS interface (Figure S5 of Supporting Information). Fittings are also provided in the Supporting Information for the other TMAO/OTS spectra in Figure 4 (Figure S6 of Supporting Information).

DISCUSSION

TMAO is often considered to be the quintessential example of a stabilizing osmolyte. Yet, it is only mildly depleted from the air/water interface. Therefore, the molecular level details of its interactions at hydrophobic interfaces may shed light on the mechanism of protein stabilization. Vanderkooi and co-workers have classified TMAO as a hydrophobic solute.[44,45] They note that the addition of this osmolyte to aqueous solutions increased the infrared absorption of the OH stretch band of water on the red side of the peak. Moreover, the population of water with less distorted hydrogen bond angles in its first hydration shell was increased, while the population with more distorted hydrogen bonds was decreased. Adding TMAO to water also led to a positive change in the hydration heat capacity. These properties are classically associated with increasing the "icelike" properties of bulk water and may play a role in TMAO's propensity to partition to the air/water interface despite its relatively high solubility in aqueous solution.

The current studies show that when TMAO resides at a hydrophobic/aqueous interface, it orients to have its methyl groups facing away from the hydrophobic phase and toward water. This is perhaps somewhat surprising as one might have expected these methyl groups to orient toward the hydrophobic interface and away from the surrounding aqueous environment. However, TMAO is zwitterionic, and the positively charged trimethylammonium moiety partially resembles the tetramethylammonium cation, which like most cations is excluded from the air/water surface.[46,47] Moreover, the methyl groups on TMAO should not necessarily be considered hydrophobic.[48] This is because these groups are directly attached to an electron-withdrawing substituent. As such, the CH_3 moieties may be better able to interact with surrounding water molecules than methyl groups

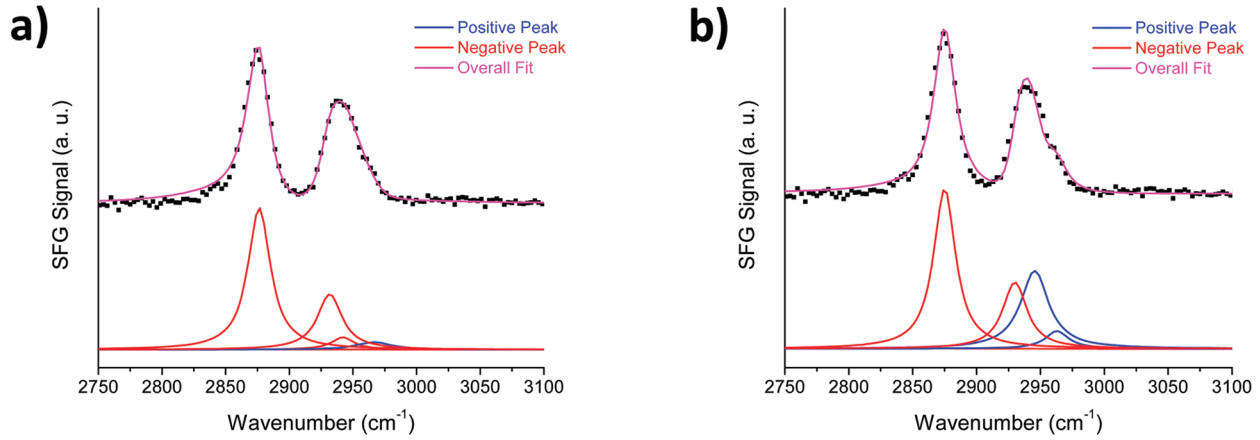

Figure 5. Fits to the VSFS data in Figure 4a (red spectrum). Both the 2876 and 2933 cm^{-1} features were assigned a negative sign, while the 2960 cm^{-1} resonance was assigned a positive sign. No resonance at 2840 cm^{-1} was used as its inclusion did not sufficiently improve the spectral fits. (a) The fit where the peak near 2950 cm^{-1} from TMAO is assigned a negative sign and (b) the same fit but with the 2950 cm^{-1} resonance assigned to a positive sign.

attached, for example, to a methylene unit.[49] In fact, recent thermodynamic studies of TMAO have determined that TMAO's methyl groups have hydrophilic rather than hydrophobic properties.[50] Therefore, it is energetically costly to dissociate water from them in order for direct interactions with hydrocarbon surfaces like OTS to take place.

Of course, it is also energetically costly for the negatively charged oxide moiety of TMAO to face toward an apolar surface. The question is which orientation is less energetically costly. The current VSFS experiments clearly indicate that an orientation in which the methyl groups face into the aqueous solution is more favorable. As such, dehydration of the methyl groups may cost the molecule more potential favorable water interactions than the loss of water at the oxygen. Moreover, the VSFS data merely show that the absolute orientation of these methyl groups is away from the surface. As noted above, their angle with respect to the surface normal should vary somewhere between 0 and 90°. A tilted orientation of the TMAO may accommodate at least some hydrogen bonding between water and the oxide moiety. In fact, recent MD simulations seem to suggest something closer to a side-on orientation.[50]

Implications for the Stabilization of Protein Structure. Although proteins contain charged and polar groups, their hydrophobic content is sufficiently large to yield a dielectric constant that is generally considerably lower than the surrounding water. Most estimates of protein dielectric constants at 25 °C are between 2 and 20,[51] which is much less than the value of 78 for water. The dielectric constant of an OTS monolayer is approximately 2.[52] Thus, in terms of hydrophobicity and bulk dielectric, an OTS monolayer resembles proteins to at least some extent. It is therefore conceivable that TMAO would orient in a similar manner at the more hydrophobic portions of the protein/water interface. This would in turn require the oxide moiety to point toward the hydrophobic portions of a protein, which would be energetically unfavorable. The fact that TMAO is depleted from the hydrophobic/aqueous interface probably provides the main driving force for protein stabilization on entropic grounds.[53,54] However, its orientation when present at the protein/water interface may help favor native folded protein structure.

Further, evidence that osmolyte orientation at hydrophobic interfaces may be a factor in protein stabilization/denaturation behavior comes from the fact that denaturants show markedly different interfacial behavior compared with TMAO. VSFS measurements of methylated urea compounds, such as tetramethylurea (TMU), at the TMU/OTS interface demonstrate that TMU is aligned with its methyl groups pointing toward the hydrophobic surface, not away from it (data not shown). Moreover, the denaturing efficacy of urea-like compounds scales directly with the hydrophobic content of these molecules.[55] Thus, the ability of urea-like compounds to denature protein structure directly corresponds to both their hydrophobic content and orientational properties. Of course, TMU should be accumulated rather than depleted at hydrophobic interfaces, which certainly contributes to its denaturing properties.[12]

TMU's orientation is in agreement with recent studies suggesting that its methyl groups are relatively hydrophobic.[56,57] This is expected, as they are attached to nitrogen atoms that are not quaternary and apparently do not provide a sufficient electron-withdrawing propensity to render the methyl groups hydrophilic. Moreover, molecules similar to TMAO but possessing more hydrophobic cationic groups, such as triethylamine *N*-oxide, have less ability to stabilize protein structure.[55] In fact, these types of osmolytes can simply behave like surfactants, which typically denature proteins.[58,59] Thus, the data presented herein indicate that the efficacy of the powerful protein stabilizer, TMAO, may partly lie in its specific unfavorable interactions with hydrophobic groups on proteins. Recent studies have implicated hydrophobicity as being a crucial factor in many biological and chemical processes ranging from micelle formation[60−62] and enzyme catalysis[63−65] to protein folding.[66−68]

■ ASSOCIATED CONTENT

Supporting Information. Details on the synthesis of deuterated TMAO and OTS analogs, OTS characterization on quartz, MEM calculations, and spectral fitting parameters. This material is available free of charge via the Internet at http://pubs.acs.org.

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