Interactions of Trimethylamine *N*−Oxide and Water with *cyclo*-Alanylglycine

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The osmolyte trimethylamine *N*-oxide (TMAO) is one of a family of compounds found in living systems that can stabilize biomolecular tertiary structures. As a step in exploring the interactions between this material and polyamino acids, we have determined intermolecular $^1H\{^1H\}$ nuclear Overhauser effects (NOEs) between the protons of *cyclo*-alanylglycine and protons of solvent components in TMAO−water solutions. Comparison of the results to effects predicted on the basis of the molecular shape of the dipeptide and experimental translational diffusion coefficients suggests that both water and TMAO molecules have properties in the vicinity of the dipeptide that are different from those in the bulk solution. Changes of local concentrations of water and TMAO and changes in the diffusive behavior of these components near the dipeptide are rejected as possible explanations of the discrepancies between observed and calculated Overhauser effects. Rather, it is concluded that TMAO molecules, and the water molecules associated with them, participate to some extent in the formation of long-lived solute−solvent complexes. The aliphatic alcohol *tert*-butyl alcohol is structurally similar to TMAO. Overhauser effect studies of its interaction with *cyclo*-alanylglycine in *tert*-butyl alcohol−water suggest similar kinds of interactions are present in this system but that they are significantly weaker, presumably because of the lower polarity of this alcohol compared to TMAO.

Introduction

Trimethylamine *N*-oxide (TMAO) is one of a collection of organic molecules known as osmolytes that are found in the tissues of organisms subject to environmental stress.[1] Other members of this group include amino acids, amino acid derivatives, polyols, and urea. Often present in high concentrations (up to several molar), these species enable cells to counteract and survive extreme temperatures and loss of water due to hydrostatic pressures or high salinity. Although an effective osmoregulator, urea in appreciable concentrations leads to protein unfolding. Thus, urea in tissues is usually accompanied by other osmolytes that are protein structure-inducing. Such counteracting osmolytes include sugars, other polyols, and TMAO.

The ability of TMAO to enhance the formation of folded peptide and protein structures or encourage protein aggregation has been noted in many in vitro studies. For example, the presence of TMAO has been found to influence favorably the rate and extent of formation of microtubules.[2−4] TMAO significantly accelerates the aggregation of peptides such as Aβ, the principal component of the amyloid plaques found in Alzheimer's disease.[5] Protein crystallization efforts can be aided by the presence of TMAO.[6,7] Studies of the influences of TMAO on the internal dynamics of protein structures have shown that TMAO can stabilize proteins against motions that expose peptide N−H protons to exchange with solvent protons,[8] although studies of the internal flexibilities of several proteins by means of tryptophan phosphorescence experiments indicate that TMAO has no effect on local motion of the tryptophan chromophore in those structures.[9] It has recently been demonstrated that TMAO has a stabilizing influence on RNA tertiary structure.[10] The processes by which TMAO and other osmolytes exert their influences on biological macromolecules are presumably general enough that both proteins and nucleic acids are under their sway.

There have been many attempts to illuminate the reasons for the structure-stabilizing effects of TMAO and other osmolytes. MD simulations and experimental observations show that TMAO probably has an ordering effect on water molecules in aqueous TMAO solutions.[11−14]

There are indications that TMAO is selectively excluded from a protein's surface,[15−19] although studies with other proteins suggest that there can be a slight preference for TMAO interactions at a protein surface.[20]

It seems clear that osmolytes influence macromolecule stability through an excluded volume effect.[18,20,21] Exclusion of TMAO from the volume occupied by a protein increases TMAO−TMAO interactions in the solution, thereby reducing the activity coefficient of the osmolyte. Specific electrostatic, hydrogen-bonding, or van der Waals interactions between the osmolyte and the macromolecule may also play a role.[20] Saunders et al. refer to the first class of interactions as "hard interactions" and to those of the second type as "soft interactions".[21] It is the interplay of hard and soft interactions that determines the overall effect of a given osmolyte on macromolecule structure; excluded volume effects are always stabilizing of folded conformations, while soft interactions can attenuate or enhance these effects.

Thermodynamic considerations alone do not permit disentangling the influence of hard and soft interactions and do not necessarily provide insight at the molecular level regarding the nature of these interactions. Intermolecular nuclear Overhauser effects (NOEs) arise through nuclear spin dipolar interactions and can provide indications of interactions between solvent and solute molecules.[22−27] It is the goal of the present work to use these experiments to examine the interactions between a model peptide, *cyclo*-alanylglycine (I), and both TMAO and water molecules in aqueous solutions of the osmolyte. Our results

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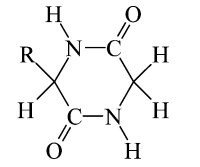
suggest that intermolecular dipole−dipole interactions between this solute and both TMAO and water components of the system are stronger than expected from consideration of bulk solution concentrations and diffusivity behavior.



Cyclo-Alanylglycine (R=CH_3)

I

Solutions of the dipeptide in mixtures of *tert*-butyl alcohol and water at approximately the same molar concentrations as were present in TMAO−water samples were also examined. This alcohol has a molecular shape similar to that of TMAO and presents a similar hydrophobic surface and molecular volume to the remainder of the solution. There are suggestions of enhanced water and alcohol interactions with **I** in *tert*-butyl alcohol−water systems as well, but the effects are considerably smaller than those observed in TMAO−water systems.

Experimental Section

Materials. Trimethylamine *N*-oxide as the dihydrate (>98% purity) and the anhydrous material (98%) were purchased from Aldrich-Sigma and used as received. Proton NMR spectroscopy showed the presence of dimethylformamide (DMF) in the anhydrous TMAO, presumably because the procedure of Soderquist and Anderson had been used for its preparation.[28] The presence or absence of DMF had no significant effect on the measured cross relaxation rates. The dimethylformamide signals generally did not interfere with the Overhauser effect determinations for the dipeptide and provided useful additional reference signals. *tert*-Butyl alcohol (reagent grade) was supplied by Mallinckrodt. 3-(Trimethylsilyl)propionic acid-d_4 (TSP, Aldrich-Sigma) was used as an internal chemical shift reference. *cyclo*-Alanylglycine (alanylglycine diketopiperazine, > 99% purity) was obtained from BACHEM and used as received. Deuterium oxide (99.9% d) was supplied by Cambridge Isotope Laboratories or Aldrich-Sigma. Water used in preparing samples was deionized and then distilled.

Samples for NMR Spectroscopy. Samples were prepared gravimetrically, typically by weighing the desired components into 1- or 2-mL volumetric flasks. Aliquots (700 μL) were transferred to 5-mm-od J. Young tubes (Wilmad Glass) and degassed by several freeze−thaw cycles. All samples contained 10% D_2O to provide a spectrometer lock signal. Unless otherwise stated, all samples were adjusted to pH 6−7 by the addition of hydrochloric acid. pH values were meter readings and were not corrected for the isotopic composition of the solvent.

Instrumentation. All NMR spectra were collected by using a Varian INOVA instrument operating at a proton frequency of 500 MHz. A Nalorac 5-mm IDTX probe equipped with triple-axis gradient coils was used. All data presented in this paper were collected at a sample temperature of 25°. Sample temperatures were determined by using a standard sample of methanol (Wilmad) and are believed to have been constant to better than ±0.1° and accurate to better than ±0.5°. Care was taken to avoid the effects of radiation damping, which were typically present.

Spectral assignments. Signals in the proton NMR spectrum *cyclo*-alanylglycine were assigned on the basis of multiplicity and TOCSY experiments. The approximate chemical shifts were ala-NH, δ 8.41; gly-NH, δ 8.17; ala-CαH, δ 4.14; gly-CαH_2, δ 4.04; ala-CH_3, δ1.45. The shifts for the peptide N−H protons were sensitive to the nature and concentration of the cosolvent present, deviating by up to 0.2 ppm from the values indicated.

Determination of Intermolecular NOES and ROEs. Pulse sequences used for determination of intermolecular NOEs and rotating frame Overhauser effects (ROEs) were local adaptations of published sequences.[29] Double solvent signal suppression during detection was used for samples containing *tert*-butyl alcohol or TMAO in H_2O, following essentially the method of Dalvit.[30] NOEs or ROEs were determined as a function of mixing time; the slope of a plot of the change in signal intensity versus mixing time (t_{mix}) defines the cross relaxation rate σ. Spectrometer macros were used to determine peak intensities. A nonlinear least-squares fitting routine based on the MINPACK package[31] was used to fit the equation $a + bt_{mix} + ct_{mix}^2$ to the data, with the linear coefficient (corresponding to σ) assumed to represent the initial slope of the plot. Replicate experiments were generally reproducible to better than ±15%, which we take to be the approximate experimental uncertainty of the derived cross relaxation parameters. Observed cross relaxation rates were corrected for incomplete inversion of the solvent component signal. The experiments involve difference methods to detect small effects and were extensively signal-averaged to minimize the effects of instrumental instabilities. Intermolecular Overhauser effects on both the DMF and TSP signals were determined in addition to those on the signals of the solute when these materials were present in the samples.

Determination of Translational Diffusion Coefficients. Samples were allowed to equilibrate in the spectrometer probe at the regulated temperature at least 3 h before attempting diffusion measurements. Self-diffusion coefficients were determined by bipolar pulse pair-longitudinal eddy current delay (BPP-LED),[32] bipolar double-stimulated echo (DSTE)[33] and double multiple spin−echo (DMSE)[34] pulsed field gradient methods. The latter two methods suppress the effects of convection within the sample on the measured diffusion coefficient. A standard doped water sample (Wilmad Glass) was used to calibrate field gradient pulses by using the published diffusion coefficient of water.[35] Gradient values and timing parameters, which led to about 2 orders of magnitude change in the intensities of the signals of interest, were used. Diffusion constants derived by the three methods were similar. It has been pointed out that determinations of translational diffusion coefficients in high-resolution NMR probes with pulsed field gradient coils are limited by the linearity of field gradient pulses over the volume of the sample.[36] No attempt was made to correct for these effects in our work. Rather, the experimental conditions for field gradient calibrations and diffusion coefficient determinations were kept closely similar, and it was assumed that any errors present were the same in all experiments. On the basis of these considerations and the reproducibility of diffusion coefficients determined by various methods, the estimated experimental uncertainty for the diffusion coefficients reported is approximately ±6%.

Viscosity Determinations. A falling ball viscometer (Gilmont Instruments) was filled with appropriate solution and equilibrated for 0.5 h in a thermostated water bath (Lauda) operating at 298 K. Falling times for the ball were determined 4−6 times and averaged. Pure water (viscosity 0.897mPa s^{-1} at 298 K) was used as a reference. Solutions were centrifuged and degassed prior to use. Corrections for differences in the density

TABLE 1: Rotational Correlation Times (τ_R)[a]

	90% H_2O, pH 3.3	90% H_2O, pH 7.3	2.53 M TMAO−H_2O, pH 7.0	4.74 M TMAO−H_2O, pH 7.0	4.85 M *tert*-butyl alcohol−H_2O, pH 6.9	2.42 M TMAO−100% D_2O, pH 7.0
peptide	21	18	37 (42[b])	65 (82[b])	60	36
TMAO			49	155		40

[a] In ps, at 25°. [b] The first value given was used in the intermolecular NOE data fitting, while the second value was that estimated from hydrodynamic or relaxation data as indicated in the text.

of the solutions were made by using the data of Auton and Bolen, reported by Zou et al.[11]

Molecular Radii. The apparent radii of the molecules used in this work were estimated from molecular models constructed in SYBYL (Tripos Associates) by using standard bond lengths and angles or by using published crystallographic data (TMAO dihydrate[37]). After minimizing the conformational energy, a van der Waals surface for the model was calculated by using the Connolly method.[38] The radius of the sphere "rolled" over the surface of the model in these calculations was 1.2 Å, corresponding to the van der Waals radii of a covalent hydrogen atom.[39] Distances from the surface defined by the probing sphere to the center of the molecule were calculated and averaged. It was estimated by this approach that the average radii of water, *tert*-butyl alcohol, TMAO, TMAO dihydrate, and AG-DKP are 1.66, 2.73, 2.67, 3.15, and 3.07 Å, respectively. Radii for TMAO used by other workers include 2.7[18] and 2.95 Å,[20] values in reasonable agreement with the radius estimated by the approach described. The radius of the water molecule typically used in calculations of protein surface areas is 1.5 Å,[20,40] somewhat smaller than the estimate produced by our method.

Calculation of Solvent−Solute Cross Relaxation Rates. As in our previous work,[25,26,41] we start with theory for intermolecular relaxation. In the various formulations available in the literature (e.g., Hennel and Klinowski,[42] Ayant et al.,[43] or Halle[44]), the solute proton of interest is considered to be located in a sphere of radius r_H. The solvent spin is similarly situated in a sphere of radius r_X. The intermolecular NOE cross relaxation rate σ_{HX}^{NOE} is given by

$$\sigma_{HX}^{NOE} = \frac{1}{10}\gamma_H^2\,\gamma_X^2 h^2[6J_2(\omega_H + \omega_X) - J_2(\omega_H - \omega_X)] \quad (1)$$

and the intermolecular rotating frame Overhauser effect (ROE) cross relaxation rate σ_{HX}^{ROE} by

$$\sigma_{HX}^{ROE} = \frac{1}{10}\gamma_H^2\,\gamma_X^2 h^2[3J_2(\omega_H) - 2J_2(\omega_H - \omega_X)] \quad (2)$$

where J_2 is a spectral density function defined somewhat differently by various authors but in all cases dependent on the distance of the closest approach, r ($= r_H + r_X$), the concentration of the species containing the solvent spins and the sum of the diffusion coefficients for the molecules D ($= D_H + D_X$) containing the solute proton and the solvent spins.

Equations 1 and 2 are derived by using the assumption that solvent molecules can approach the sphere representing the solute equivalently from all directions. In actual molecules, the solvent will make approaches to the solute from different directions that are nonequivalent due to the shape of the solute molecule. Some solvent approach paths will allow the solute hydrogen and solvent molecule to interact at their van der Waals contact distance, while other approaches will involve interactions at distances longer than this because of steric interference. To take into account the shape of the solute molecule as it interacts with solvent molecules, we used an empirical method that assumes that the contribution to σ_{HX} of a single H−solvent spin interaction over a small element of the solute−solvent contact surface can be computed by using the standard equations. We imagine a large number of equispaced rays extending in all directions from the center of a sphere surrounding the hydrogen atom of interest. Each ray will intersect the surface of the solute molecule at a particular distance from the hydrogen, and at that distance, there will be a characteristic contribution to σ_{HX}. Summing the contributions associated with each ray is assumed to give the aggregate NOE for a solute hydrogen.

The Connolly algorithm was used to obtain a representation of the molecular surface of the solute.[38] This algorithm generates a collection of points that correspond to positions where a spherical solvent molecule is able to make contact with the van der Waals surface of the solute. The number of rays from the solute proton of interest and the molecular surface used in our procedure (~2000) was sufficiently high that there was always a "Connolly surface dot" 0.1 Å or closer to a ray extended from a solute hydrogen atom.

Calculation of Intramolecular Dipolar Interactions. A full relaxation matrix treatment of intramolecular proton−proton interactions was used to calculate the effects of these interactions on the intramolecular cross relaxation rates.[45,46] It was assumed that the cyclic dipeptide could be represented by a sphere that tumbles isotropically with the rotational correlation time τ_R. The rotational correlation times (τ_R) were estimated by using the microviscosity theory of Gierer and Wirtz, the molecular radius developed as described above, and experimental viscosities.[47,48] Additional estimates of τ_R were obtained by using the experimental translational diffusion coefficients and the approximate relation $\tau_R \approx (2\cdot r^2)/(9\cdot D_{tran})$ where D_{tran} is the experimental translational diffusion coefficient,[48] and by considering the T_1 proton relaxation times for the glycine methylene group of **I**. An experimental value for the rotational correlation time of TMAO dihydrate in a 1 M solution (30 ps) obtained in a dielectric relaxation study[49] was used to estimate τ_R for TMAO at higher concentrations of TMAO by assuming that the rotational correlation time is directly proportional to the bulk viscosity. All methods for estimating τ_R agreed within a factor of 2. The values of τ_R used in the calculations done for this paper are given in Table 1.

Conformational flexibility either regarding rotation of the methyl group or interchanges of boat and chair conformations of the six-membered ring was ignored. As was done previously, the neglect of these factors was assumed to lead to differences in calculated relaxation behavior that are minor in comparison to experimental errors.[41] Calculated proton relaxation times for the methyl protons of *cyclo*-alanylglycine were not sensitive to the value of the correlation time for internal rotation of the methyl group (τ_M), and this was set to 10 ps for all calculations.[50]

Calculation of the Cross Relaxation Rate σ_{HX}^{NOE} in the Presence of N−H/Solvent Exchange. Exchange of water protons with the N−H protons of *cyclo*-alanylglycine would lead to detectable Overhauser effects on nearby carbon-bound protons of the cyclic dipeptide.[51] Solvent exchange terms were

TABLE 2: Experimental Translational Diffusion Coefficients ($\times 10^6$ cm^2 s^{-1})[a]

	D_{water}	$D_{peptide}$	$D_{cosolvent}$	D_{TSP}[b]
H_2O, pH 3.5	23.1	8.09		6.34
H_2O, pH 7.3	23.0	8.94		6.94
2.53 M TMAO−H_2O	13.3	5.05	5.22	6.56
4.74 M TMAO−H_2O	5.35	1.69	1.66	1.28
2.42 M TMAO−D_2O		3.67	3.92	3.22
4.85 M *tert*-butyl alcohol−H_2O	7.67	2.64	3.16	2.55

[a] All samples contained 10% D_2O unless otherwise indicated. [b] Diffusion coefficient for the chemical shift reference signal. The diffusion coefficient for DMF when it was present was similar to the value of D_{TSP}.

added to the relaxation matrix treatment to take these effects into account.[52] The terms introduced were dependent on the concentration of peptide, the concentration of water, and the rate of dissociation of a peptide N−H bond. Thus, a full calculation of the apparent cross relaxation term σ_{HX}^{NOE} in the presence of exchange took into account intramolecular effects, intermolecular (solvent) effects, and the rate of solvent exchange. Parameters of the calculation were adjusted manually until good agreement with experiment was obtained, as judged by visual comparison of plots of observed and calculated NOE data. Calculation of the rotating frame cross relaxation rate σ_{HX}^{ROE} in the presence of exchange is feasible[53−55] but is more complex and was not attempted in this work. The contribution of exchange processes to σ_{HX}^{ROE} depends on the rate of exchange, the chemical shifts, and the frequency and magnitude of the spin-locking RF field.[53−55] This contribution will equal or exceed the exchange contribution to an observed σ_{HX}^{NOE}, depending on these conditions.

Results

Translational Diffusion Coefficients. Knowledge of translational diffusion coefficients is necessary for making predictions of intermolecular dipolar interactions that produce NOE and ROE cross relaxation. Translational diffusion coefficients determined for the samples examined in this work are given in Table 2. As a check on these results, the experimental values were compared to the diffusion coefficients calculated by using the microviscosity theory of Gierer and Wirtz,[47] experimental macroscopic viscosities obtained in this work or from the literature, and the molecular radii estimated as described above. The disagreements between observed and calculated values for $D_{peptide}$ and D_{TMAO} averaged ±7%, nearly within the range of experimental uncertainties for these quantities. Diffusion coefficients for water calculated by using microviscosity theory agreed less well with the experimental results, with ±16% deviations. We concluded that that the experimental diffusion coefficients determined by NMR methods are reliable indicators of the bulk translational behavior of the components of the samples examined.

We note that the significant reduction in the translational diffusion of water in the presence of TMAO observed in this work is in line with previous experimental observations[56] and the results of molecular dynamics simulations.[11]

Intermolecular Relaxation of I in Water. Table 3 records observed and calculated NOE and ROE cross relaxation rates between the protons of *cyclo*-alanylglycine and those of water. There were trace amounts of DMF and TSP in the samples examined. Considering the reproducibility of determinations of the cross relaxation rates and the possible errors in the measured translational diffusion coefficients and the estimated molecular radii, it appears reasonable to expect observed and calculated cross relaxation rates to agree within ~20%. The agreement between observed and calculated NOE and ROE cross relaxation terms for the DMF−water and TSP−water interactions in these systems is generally well within this limit and indicate that the approach used to estimate intermolecular solvent−solute dipole−dipole interactions gives reliable predictions of σ_{HX}^{NOE} and σ_{HX}^{ROE}.

Interpretation of observed cross relaxation rates for the carbon-bound protons of cyclic dipeptide **I** is complicated by the ability of the N−H protons of the molecule to undergo exchange with the protons of the solvent. Calculations of cross relaxation rates for these protons depend on intermolecular interactions between water protons and the protons of the cyclic dipeptide, intramolecular dipolar interactions, and the rate of solvent exchange of the peptide N−H protons. At pH 3.5, exchange is slow, and separate signals are observed for the amide protons. Analysis of the NOE data indicated that the rate constants for dissociation of the alanyl and glycyl N−H protons were 0.096 and 0.14 s^{-1}, respectively, values that are of the expected order of magnitude.[57] As shown in Table 3, exchange of N−H protons has only minor effects on the observed cross relaxation rates of **I** at low pH, with the observed values of σ_{HH}^{NOE} and σ_{HH}^{ROE} being essentially those predicted to result from intermolecular dipolar interactions with the bulk solvent molecules.

At pH 7.3, exchange of the N−H protons of *cyclo*-alanylglycine with solvent water protons is rapid enough ($k_{ex} \sim 1000$ s^{-1}) that there is no observable signal for these protons in the PMR spectrum. The effects of N−H/water exchanges on the observed solvent proton−solute proton cross relaxation rates are more pronounced than was the case at the lower pH. Calculations of σ_{HX}^{NOE} show that inclusion of exchange between N−H and water protons in the calculation of intermolecular dipolar interactions with solvent molecules produces apparent cross relaxation terms that are in reasonably good agreement with experiment (Table 3). Values observed for σ_{HH}^{ROE} at pH 7.3 are greater than those predicted from consideration of intermolecular effects, as would be expected if there is an exchange contribution to the experimental σ_{HH}^{ROE} parameters.

Calculations show that solvent cross relaxation terms for the backbone C−H (α) protons of **I** are influenced by solvent exchange but that exchange effects are absent from solvent cross relaxation terms for the methyl protons of **I**. That is, the larger than expected values for σ_{HH}^{NOE} and σ_{HH}^{ROE} at pH 7.3 for methyl proton−water interactions are not the result of solvent exchange with the dipeptide N−H protons.

Intermolecular Relaxation of I in Water−TMAO Mixtures. Cross relaxation parameters for interactions of the protons of I with both water and TMAO in solutions of these components were determined (Table 4). A comparison of predicted and experimental cross relaxation terms is given in the table as well. As before, agreement of observed and calculated cross relaxation terms for interactions of the trace amounts of DMF or TSP present in the samples with water in the mixtures suggest that the approach and assumptions used to compute the predicted values σ_{HH}^{NOE} and σ_{HH}^{ROE} produces reasonably reliable results (Table 4).

Although separate signals for the glycyl and alanyl amide protons of **I** were observed in the spectra, it became clear that exchange of these protons with the water component of the solvent was sufficiently rapid that the observed cross relaxation rates between carbon-bound protons and water were significantly influenced by exchange processes. As before, the experimental NOE data was fit to a model that included relaxation due to intermolecular dipolar effects of the water, intramolecular

TABLE 3: Solvent−Dipeptide Intermolecular Cross Relaxation Rates in Water (25°)[a]

	at pH 3.5		at pH 7.3	
	$\sigma_{HH}^{NOE} \times 10^3\ s^{-1}$	$\sigma_{HH}^{ROE} \times 10^3\ s^{-1}$	$\sigma_{HH}^{NOE} \times 10^3\ s^{-1}$	$\sigma_{HH}^{ROE} \times 10^3\ s^{-1}$
ala-NH	−73 (−67, 20)	nd	*b*	*b*
ala-CαH[c]	29 (19, 19)	22 (25)	48 (45, 19)	36 (25)
ala-CH_3	24 (20, 20)	27 (27)	35 (20, 19)	38 (25)
gly-NH	−100 (−110, 21)	nd	*b*	*b*
gly-CH_2[c]	29 (20, 20)	26 (26)	43 (39, 20)	32 (25)
DMF methyl	18 (20)	24 (25)	nd	nd
TSP	19 (20)	23 (26)	28 (20)	28 (26)

[a] The first number given in parentheses is the calculated initial cross relaxation rate; its value depends on intermolecular dipolar interactions with water as well as exchange with protons of water. The second number is the calculated contribution of intermolecular dipolar interactions only. When only a single number is present in parentheses, it is the calculated contribution of the intermolecular dipolar interactions. The calculated rotating frame cross relaxation rates includes only the effects of intermolecular dipolar interactions with water. [b] Signal not observed due to rapid exchange with solvent. [c] Average value for the resolved lines of the multiplet.

TABLE 4: Intermolecular Cross Relaxation Rates in TMAO−H_2O Solutions (pH 7.0, 25°)[a]

	invert H_2O		invert TMAO	
	$\sigma_{HH}^{NOE} \times 10^3\ s^{-1}$	$\sigma_{HH}^{ROE} \times 10^3\ s^{-1}$	$\sigma_{HH}^{NOE} \times 10^3\ s^{-1}$	$\sigma_{HH}^{ROE} \times 10^3\ s^{-1}$
		2.54 M TMAO		
ala-NH	−1.5 (−1.6, 25)	−2.0 (36)	12 (5.7)	nd (12)
ala-CαH	55 (55, 24)	76 (35)	10 (5.4)	16 (11)
ala-CH_3	36 (24, 25)	49 (35)	6.9 (5.5)	13 (12)
gly-NH	−1.6 (−1.6, 26)	−1.9 (36)	7 (5.6)	nd (10)
gly-CH_2[c]	48 (48, 25)	72 (35)	9.8 (5.2)	14 (12)
water			10 (4.6)	nd
TMAO	20 (19)	nd		
DMF formyl	20 (25)	45 (34)	5 (5.6)	17 (11)
DMF methyl	31 (25)	40 (34)	8 (5.5)	11 (11)
		4.74 M TMAO		
ala-NH	−1.5 (−1.6, 41)	nd (77)	37 (7.8)	44 (56)
ala-CαH	91 (96, 38)	146 (75)	17 (6.7)	55 (55)
ala-CH_3	65 (38, 39)	87 (76)	16 (7.2)	50 (55)
gly-NH	−1.6 (−1.6, 42)	nd (79)	40 (8.4)	53 (57)
gly-CH_2[b]	90 (87, 40)	137 (76)	11 (7.4)	46 (56)
water			34 (15)	36 (35)
TMAO	52 (40)	56 (77)		
DMF methyl	46 (40)	54 (73)	14 (10)	35 (50)
TSP	27 (38)	77 (77)	6.3 (4.3)	42 (60)

[a] The first number given in parentheses is the apparent initial cross relaxation rate; its value depends on intermolecular dipolar interactions with water as well as exchange with protons of water. The second number is the calculated contribution of intermolecular dipolar interactions only. When only a single number is present in parentheses it is the calculated contribution of the intermolecular dipolar interactions. [b] Average value for the four lines of the glycine CH_2 multiplet.

dipolar interactions between the protons of **I**, as well as exchange. Figure 1 illustrates that it was possible to achieve agreement between observed and calculated signal intensities in a NOE experiment by holding intermolecular relaxation contributions at the value predicted from eq 1, reckoning intramolecular relaxation by using the rotational correlation times in Table 1 and adjusting only the rate of solvent exchange. The analysis of the data indicated that the rate constants for dissociation of the alanyl and glycyl N−H protons were 21 and 23 s^{-1}, respectively, at pH 7.0 in 4.74 M TMAO.

The analyses showed that the intensity of an N−H proton signal as a function of mixing time was primarily dependent on the rate of exchange of these protons with water. Half or more of a predicted σ_{HH}^{NOE} cross relaxation parameter for the C_α-H protons could be assigned to the effects of exchange, suggesting that it would be difficult to estimate accurately the portion of an experimental cross relaxation parameter that was due to intermolecular (nonexchange) interactions with solvent. However, calculations suggested that these nonexchange contributions probably were not more than ∼50% different from those predicted through eq 1 by consideration of solute shape and the mutual diffusion of the solute and water molecules. Thus, our calculations do not lead to an accurate determination of σ_{HH}^{NOE} for intermolecular interactions of the dipeptide C_α-H protons with water in TMAO−water solutions, although they indicate that the values of σ_{HH}^{NOE} for these interactions cannot be extremely different from predictions based on diffusion coefficients and the shape of the solute.

The rotating frame cross relaxation parameters for interactions of **I** with the water component of the TMAO−water mixture are larger than those predicted, presumably because of the solvent exchange process.

The protons of the methyl group of **I** are distant from the exchangeable N−H protons, and simulations showed that their experimental cross relaxation parameters are not influenced markedly by exchange. Thus, as was the case in pure water, even considering experimental uncertainties, the observed σ_{HH}^{NOE} and σ_{HH}^{ROE} for interactions of the methyl protons of **I** with water in water−TMAO lead to cross relaxation effects that are somewhat larger than expected.

Cross relaxation rates for interaction of the protons of **I** with the protons of dissolved TMAO were also determined. Backbone N−H and C_α-H protons show values for σ_{HH}^{NOE} that are appreciably larger than expected in 2.5 M TMAO, even more so in 5 M TMAO. Cross relaxation between methyl protons of

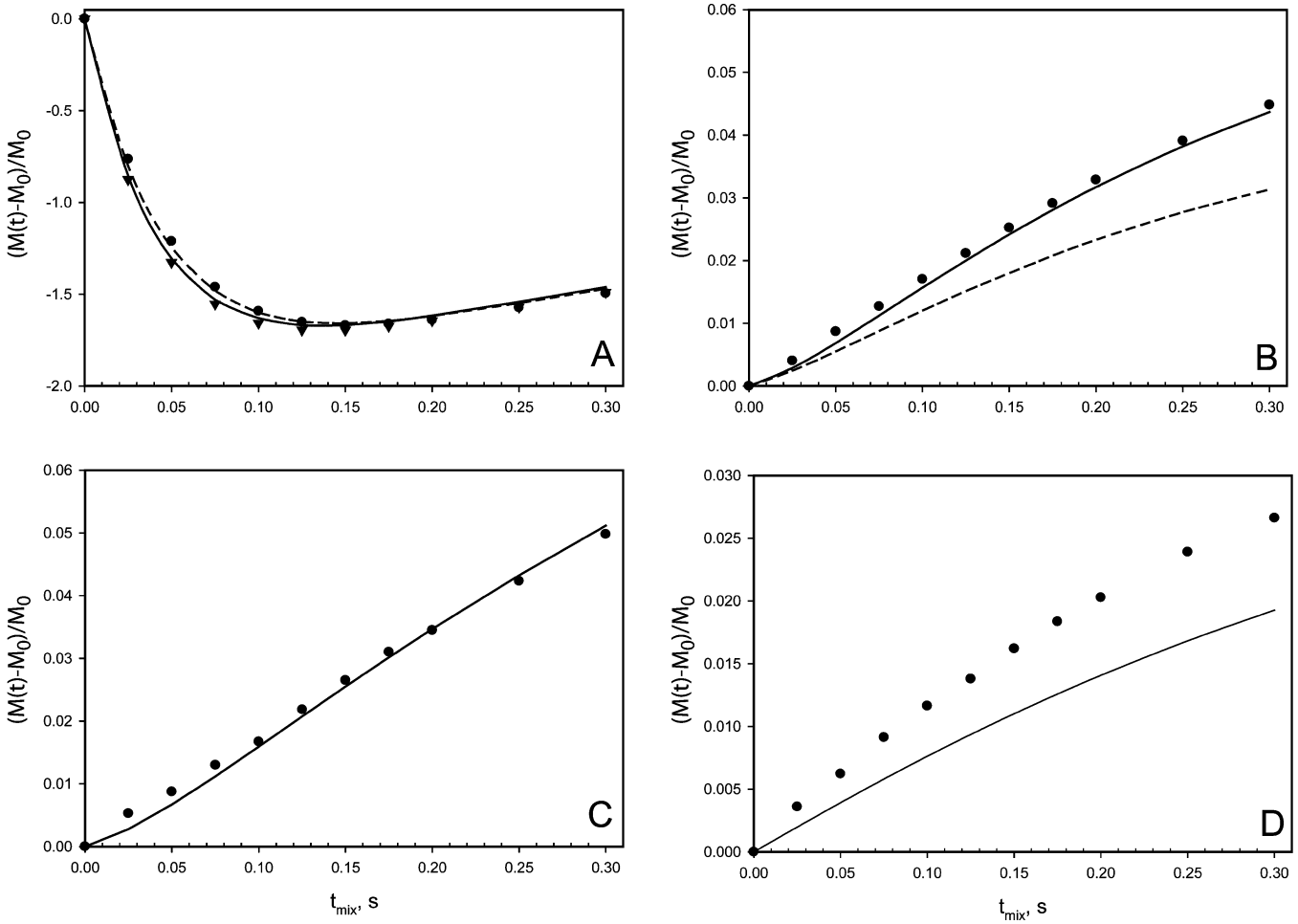


Figure 1. Comparison of observed and calculated NOE cross relaxation effects for *cyclo*-alanylglycine (I) dissolved in 4.74 M TMAO, pH 7, 25° when the water signal of the sample is inverted. Solid points correspond the experimental values of the NOE, determined as a function of mixing time (t_{mix}). The solid or dashed continuous curved are calculated by using a model that includes intermolecular solute proton−solvent proton interactions, proton−proton interactions within the dipeptide, and exchange of the N−H protons with the solvent, as described in the text. (A) Alanine (circles, dashed line) and glycine (triangle, solid line) N−H protons; (B) Glycine C_α-H, solid and dashed lines corresponds to the two protons of the CH_2 group; (C) Alanine C_α-H; (D) Alanine methyl group.

I and TMAO is also enhanced over the expected values but not as dramatically. Although experimental σ_{HH}^{ROE} terms in 2.5 M TMAO appear to be larger than those predicted, the differences are barely outside the experimental errors. However, the diffusion constants of TMAO and the peptide are such that spectral density function σ_{HH}^{ROE} is highly sensitive to the value of the mutual diffusion coefficient for these samples. For example, in 4.7 M TMAO, a 20% increase in D leads to a ∼20% reduction in predicted σ_{HH}^{ROE}, but a ∼40% increase in σ_{HH}^{NOE}. Thus, the computed cross relaxation terms for the 4.7 M TMAO system may be underestimated in the case of the NOE results, but overestimated in the case of the ROE parameters. There was no reasonable adjustment of the diffusion coefficients that produced agreement of observed and calculated NOE and ROE data for either TMAO−water system, and the qualitative conclusion remains that the water and TMAO cross relaxation terms for interaction with the peptide are larger than expected only on the basis of diffusive motions of these solution components.

Trimethylamine *N*-oxide interacts strongly with water molecules, to the extent that a dihydrate of the compound can be isolated and crystallized.[12,37] Thus, there is reasonable concern that solute NOEs or ROEs produced by inversion of the TMAO magnetization could arise because polarization of TMAO spins leads to polarization of water spins, which would then produce the observed Overhauser effects. This possibility was addressed in two ways. The experimental cross relaxation rates between water and TMAO were used in calculations of solute cross relaxation rates in which both solvent components were present. These calculations showed that there was virtually no effect of water−TMAO cross relaxation interactions on the predicted values of σ_{HH}^{NOE}. Additionally, a series of experiments was run for samples of **I** dissolved in deuterium oxide−TMAO. Replacement of protium by deuterium would be expected to reduce any water−TMAO cross relaxation effects by a factor of ∼49. Typical results for TMAO−D_2O experiments are shown in Table 5. (The data in Tables 4 and 5 are not directly comparable due to the differences in the diffusion coefficients of the systems.) A small enhancement of σ_{HH}^{NOE} over the value predicted for an intermolecular effect is probably present, such as was the case with the TMAO−H_2O system at this concentration of TMAO (Table 4). Regardless, it is clear that replacement of the H_2O component of the solvent mixture by D_2O has at best a minor effect on TMAO spin−solute spin Overhauser effects. We conclude that cross relaxation between the water and TMAO components of these systems has essentially no influence on the observed Overhauser effects arising between either solvent component and the dipeptide.

Intermolecular Relaxation in *tert*-Butyl Alcohol−Water. The molecular shape and composition of *tert*-butyl alcohol is

TABLE 5: Intermolecular Cross Relaxation Rates in 2.42 M TMAO−D_2O Solution (pH 7, 25°)[a]

	invert TMAO	
	$\sigma_{HH}^{NOE} \times 10^3\ s^{-1}$	$\sigma_{HH}^{ROE} \times 10^3\ s^{-1}$
ala-CαH	8.9 (5.5)	15 (14)
ala-CH_3	6.0 (5.7)	15 (15)
gly-CH_2[b]	7.8 (5.8)	15 (15)
DMF formyl	7.7 (5.8)	

[a] Cross relaxation rates calculated by the method described in the text are give within parentheses. The NH protons were not detected because of the rapid exchange with the solvent spins. The TSP reference signal in this sample was too weak to give reliable cross relaxation data. [b] Average value for the lines of the glycine CH_2 multiplet.

similar to that of TMAO. It presents a "hydrophobic face" to water molecules and solutes that should be similar to that of the amine oxide. Intermolecular NOE measurements were used to examine the interactions between the protons of *cyclo*-alanylglycine and those of water and *tert*-butyl alcohol in a mixture containing 4.85 M of the alcohol at pH 6.9. The results are presented in Table 6. Again, the exchange of amide N−H protons of the solute with water dominates the observed cross relaxation between water and the solute backbone protons. Analysis of the data indicated that the rate of solvent exchange with the alanine and glycine N−H were 6.2 and 7.0 s^{-1}, respectively. The water-induced cross relaxation rates are generally smaller than those observed in corresponding TMAO system. It is clear that σ_{HH}^{NOE} and σ_{HH}^{ROE} for the methyl protons are greater than the predicted values as was the case in TMAO−water and pure water cases. While σ_{HH}^{NOE} values for the interaction of *tert*-butyl protons with the solute spins are somewhat larger than expected, the enhancements are not nearly as large as are seen with TMAO present at the same concentration.

A variety of experimental techniques confirm that *tert*-butyl alcohol is extensively aggregated over the mole fraction (X) composition range 0.1−0.3.[14,58] The sample examined in the present work had $X = 0.149$; on the basis of the work of Nishikawa et al.[58] and Shulgin and Ruckenstein,[59] the clusters would be expected to contain roughly 20−30 molecules of alcohol. Our observations suggest that it is unlikely that the dipeptide is selectively solvated by the alcohol aggregates, as is the case in mixtures of trifluoroethanol and water,[60,61] because NOE cross relaxation rates for both water and *tert*-butyl alcohol exceed those expected on the basis of the bulk composition of the solvent. If the solute were localized in an aggregate of alcohol molecules, it would be expected that the concentration of water within the aggregate would be less than that of the bulk, leading to a reduction in σ_{HH}^{NOE} compared to its value calculated on the basis of the bulk concentration.

Discussion

The experimental cross relaxation rates reported here are derived from inherently small effects on signal intensities. While values for σ_{HH}^{NOE} were typically reproducible to ±15% in replicate experiments, the accuracy and reliability of these parameters is a limitation on any interpretations that can be made of our results.

Calculated cross relaxation rates are subject to any uncertainties in measured diffusion coefficients, experimental errors that would affect the determined concentrations of the solvent components and the approximations inherent in the theory and computational method used. The general agreement between observed and calculated cross relaxation data in many instances suggests that the computational methods used to predict the cross relaxation give reasonably reliable results and indicates that the models and methods used for the calculations at least approximately correspond to the nature of the sample.

Our data show that interactions between TMAO and water are probably more complex than those envisioned by the interacting spheres model used to predict cross relaxation rates. If that model were correct, then the ratio of σ_{HH}^{NOE} values obtained in an experiment in which the water magnetization is inverted and the TMAO spins observed and the opposite experiment (TMAO spins inverted and water protons observed) would be in the ratio of the molar concentrations of the TMAO and water protons in the sample. The results presented in Table 4 show that this is not the case. To explain this discrepancy, we suggest that all of the TMAO molecules present in water−TMAO solutions are in the form of the dihydrate and that the observed σ_{HH}^{NOE} when TMAO protons are inverted include *intramolecular* dipole−dipole interactions within the dihydrate plus *intermolecular* interactions between the TMAO protons in the dihydrate and the remaining (uncomplexed) bulk water. Calculations using the crystal structure of the dihydrate[37] as a model for the structure of TMAO·$2H_2O$ in solution and the rotational correlation times for TMAO given earlier produce intramolecular cross relaxation rates consistent with this notion and with the observed values of σ_{HH}^{NOE} for both the water inverted−TMAO observed and TMAO inverted−water observed NOE experiments.

Our results show that, in all solvent systems studied, intermolecular dipole−dipole interactions between protons of the solute **I** and water lead to cross relaxation effects that are

TABLE 6: Solvent−AGDKP Intermolecular Cross Relaxation Rates in 4.85 M *t*-Butyl Alcohol−H_2O (pH 6.9, 25°)[a]

	invert H_2O		invert *tert*-butyl alcohol	
	$\sigma_{HH}^{NOE} \times 10^3\ s^{-1}$	$\sigma_{HH}^{ROE} \times 10^3\ s^{-1}$	$\sigma_{HH}^{NOE} \times 10^3\ s^{-1}$	$\sigma_{HH}^{ROE} \times 10^3\ s^{-1}$
ala-NH	−0.88 (−0.87, 24)	nd (40)	nd[b] (11)	nd[b] (36)
ala-CαH[c]	63 (64, 23)	109 (39)	21 (10)	44 (35)
ala-CH_3	34 (21, 24)	45 (39)	14 (11)	38 (36)
gly-NH	−0.95 (−0.95, 25)	nd (41)	24 (12)	26 (37)
gly-CH_2[c]	63 (50, 24)	87 (40)	18 (11)	45 (36)
water			18 (13)	32 (24)
tert-butyl alcohol	27 (25)	31 (38)		
TSP	∼10[d] (22)	∼21[d] (38)	∼16[d] (10)	∼49[d] (35)

[a] Apparent cross relaxation parameters computed from slopes at $t_{mix} \sim 100$ ms appear first in parentheses. Cross relaxation rates in the absence of exchange calculated by the method described in the text are given second. [b] An artifact in the spectrum probably related to the *tert*-butyl alcohol signal overlapped this part of the spectrum and made reliable determination of σ impossible. [c] Average value for the lines of the multiplet. [d] A weak signal for TSP precluded accurate evaluation of this cross relaxation term.

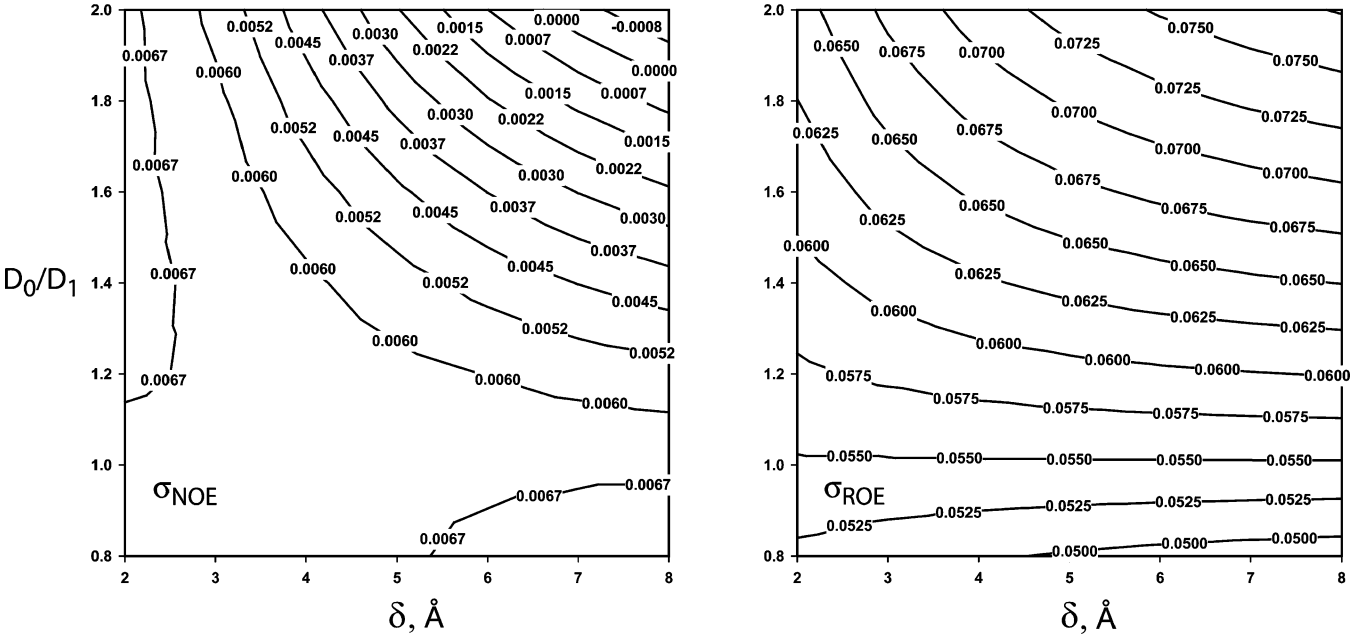


Figure 2. Calculated dependence of σ_{HH}^{NOE} and σ_{HH}^{ROE} on the thickness δ of a special layer of TMAO molecules adjacent to the solute. The TMAO molecules in this layer have a translational diffusion coefficient D_1 when the bulk diffusion coefficient is D_0. The value of D_0 used in the calculation corresponds to the diffusion coefficient of TMAO in 5 M TMAO.

larger than expected for the methyl protons of the solute. This could also be the case for water interactions with the other protons of the dipeptide, but because of the competing effects of exchange, definite conclusions in this regard cannot be made. The data indicate that interactions between **I** and TMAO are appreciably stronger than predicted, with the effects appearing to be dependent on the concentration of TMAO. When TMAO is replaced with a similarly structured aliphatic alcohol, the cross relaxation terms between the alcohol protons and the solute are still larger than expected, but the effects are smaller than observed in the case of TMAO interactions.

Intermolecular cross relaxation between solute spins and solvent spins depends on the number density (concentration) of the solvent and the mutual diffusion of the solute and solvent. An increase in a cross relaxation parameter could arise because the local concentration of solvent species near a proton of interest is increased over the bulk concentration of that species. "Solvent sorting" in mixed solvents is well-known and has been confirmed by means of intermolecular NOE experiments.[23] An excess of either TMAO or *tert*-butyl alcohol in the vicinity of the solute would be consistent with our observations of enhanced cross relaxation rates for these components. However, a high local concentration of TMAO or *tert*-butyl alcohol would imply that the local concentration of water is reduced and a concomitant reduction in water−solute cross relaxation would be expected. This is contrary to observations, and we thus conclude that selective solvation or solvent sorting is not the primary reason for the enhanced cross relaxation effects observed in our systems. However, if TMAO is present as the dihydrated molecule, any factor that alters the local concentration of one of these components would necessarily simultaneously influence the concentration of the other.

It may be that the diffusion behavior of water and TMAO (or TMAO dihydrate) in the solutions is not the same throughout the sample. Attractive or repulsive interactions of these components with the solute could lead to a change in the mutual diffusion behavior in the vicinity of the solute. Halle has provided a basis for exploring this possibility.[44] His theory of nonuniform diffusion posits a solvent layer of thickness δ within which the mutual diffusion coefficient D_1 is different from that in the bulk solvent (D_0). Calculations were carried out by using his approach for $2 < \delta < 8$ Å (corresponding to one or two layers of TMAO·$2H_2O$ molecules on the surface of the solute) and $0.8 < D_0/D_1 < 1.6$. Values of D_0/D_1 greater than one are anticipated for interactions of the solute with water.[44] Calculations showed that σ_{HH}^{NOE} and σ_{HH}^{ROE} cross relaxation rates for interactions of **I** with TMAO in 2.5 M TMAO are relatively insensitive to changes in δ or D_0/D_1, with any combination of these parameters predicting cross relaxation rates that are smaller than those observed experimentally. As shown in Figure 2, the situation is more dire for solute−TMAO interactions in 5 M TMAO, with predicted cross relaxation terms more dependent on the values of δ and D_0/D_1. However, reduced or even negative values for σ_{HH}^{NOE} are predicted, in sharp contrast to experimental observations. We conclude that microscopic variations in diffusion behavior cannot account for the augmented solute−TMAO cross relaxation rates reported in Table 4.

There are many indications of strong amide group−water interactions.[62−66] If such interactions were long-lived enough, interacting solvent molecules would take on the dynamics of dissolved *cyclo*-alanylglycine to some extent and this would enhance solvent−solute cross relaxation rates beyond those predicted on the basis of diffusive encounters.[44] The cyclic peptide presents a number of opportunities for hydrogen-bonding interactions or other dipolar interactions with water or TMAO molecules.

It is possible that water, TMAO, or *tert*-butyl alcohol molecules interact strongly enough with *cyclo*-alanylglycine that dipolar interactions between the solvent spins and spins of the solute are modulated to some extent by the rotational motion of the solute−solvent molecule complex rather than by diffusive encounters. That is, the cross relaxation arising from solvent spin−solute spin interaction becomes, in essence, an intramolecular process. For such an interaction to have an appreciable effect on cross relaxation, the complex would have to persist for times that are long compared to the rotational correlation time (τ_R) of the complex. Halle has indicated that the contribu-

tion of such a long-lived interaction to an observed dipolar cross relaxation rate is given by [44]

$$(\gamma^2 h)^2 \sum_{k=1}^{N} \frac{1}{r_k^6}\left(\frac{\tau_{C,k}}{1+\omega^2 {\tau_{C,k}}^2}\right) \quad (3)$$

where N is the number of solvent molecules interacting with the solute in this way, r_k is the solvent spin−solute spin distance for the kth interaction, $\tau_{M,k}$ is the mean residence time for that interaction, and a correlation time $\tau_{C,k}$ given by

$$\frac{1}{\tau_{C,k}} = \frac{1}{\tau_R} + \frac{1}{\tau_{M,k}} \quad (4)$$

A variety of models for complexes between the peptide and TMAO were considered. Generally, these led to calculated contributions to σ_{HH}^{NOE} and σ_{HH}^{ROE} that were too large if values of $\tau_{M,k}$ longer than τ_R are assumed. For example, one can propose that a TMAO molecule becomes hydrogen-bonded to a peptide N−H. Given a hydrogen bond length of 2.8 Å, the averaged distance between TMAO methyl protons and the N−H proton in this model is about 4 Å. However, for this model, the calculated contributions to the cross relaxation parameters are about an order of magnitude too large for both the 2.5 and 5 M TMAO systems when $\tau_{M,k} \sim \tau_R$. A complex in which the distances between the TMAO molecule and the peptide are increased by 50% would be consistent with the results in 5 M TMAO; such an increase in distance would be consistent with a water molecule intervening between the TMAO and the peptide N−H.

We could find no single model for a TMAO−dipeptide complex that was consistent with both observed σ_{HH}^{NOE} and σ_{HH}^{ROE} for all solute protons. However, not all solute molecules need be complexed to water, TMAO, or TMAO·$2H_2O$ in the same way. Indeed, the apparent dependence of cross relaxation effects on the concentration of TMAO present suggests that an equilibrium between complexes and uncomplexed solute molecules is present. It is possible that a variety of structurally and dynamically different solute−TMAO complexes are present and interconverting rapidly. Thus, we conclude that the formation of solute−TMAO complexes that have a lifetime longer than that characteristic of a collisional (diffusive) encounter of these partners seems to be the most likely explanation of the solvent−solute cross relaxation effects that we have observed.

This conclusion is consonant with the chemical nature of TMAO. The molecule has a large dipole moment ($\mu = 5$ D[12]) and is strongly interactive with other polar molecules such as water and, presumably, **I**. These interactions are strong enough to have an appreciable effect on the viscosity of solutions of TMAO and the diffusion coefficients.[12,56,67]

Aliphatic alcohols can also stabilize peptide and protein conformations, although *tert*-butyl alcohol is less potent in this regard that smaller alcohols.[68] At a concentration of 5 M, the presence of *tert*-butyl alcohol has a considerably smaller effect on solution viscosity or molecular diffusion than TMAO (Table 2). MD simulations of TMAO−water and *tert*-butyl alcohol−water show that water is more tightly coordinated to the amine *N*-oxide than to *tert*-butyl alcohol.[12] The cross relaxation rates of the protons of either water or the alcohol with **I** are smaller than the corresponding effects produced by an equivalent concentration of TMAO, consistent with the smaller dipole moment of *tert*-butyl alcohol ($\mu = 1.7$ D[39]) and the greater number of opportunities for hydrogen bonding presented by the dihydrate of TMAO. Solute−solvent complexes involving the alcohol would be expected to have a more fleeting existence and probably a less-well-defined structure than complexes formed with TMAO.

Conclusions

Intermolecular Overhauser effects have been used to explore the interactions between protons of *cyclo*-alanylglycine and protons of the components of the solvent. The cross relaxation rates for the dipeptide are typically larger than those predicted by a computational approach based on the shape of the dipeptide and the mutual diffusion of this material and the solvent components. The differences between observed and calculated cross relaxation rates for the dipeptide are particularly striking in the case of TMAO−water mixtures. Possible reasons for the enhanced dipole−dipole interactions between solute protons and those of TMAO are considered. It is concluded that the formation of solute-hydrated TMAO complexes with lifetimes longer that the rotational correlation time of the complex is the most likely reason for the observed effects. It may be that formation of a single, structurally well-defined complex of dipeptide and TMAO may not be present, but rather, a collection of complexes. Because TMAO interacts strongly with water molecules, water molecules would be expected to be part of the composition of any such complexes, accounting for the larger than expected cross relaxation rates between solute protons and water. Our results provide no support for the notion that TMAO is excluded from the surface of the dipeptide, but rather, indicate that an aquated form of this molecule interacts rather strongly. These interactions involved appear to produce a complex or complexes that contain the dipeptide, water molecules, and TMAO.

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