Why Do Some Organisms Use a Urea–Methylamine Mixture as Osmolyte? Thermodynamic Compensation of Urea and Trimethylamine *N*-Oxide Interactions with Protein†

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ABSTRACT: Many organisms accumulate low molecular weight substances known as osmolytes when they experience environmental water stress. The main classes of osmolytes are sugars, polyhydric alcohols, amino acids and their derivatives, and methylamines, and all are known to be protein stabilizers. However, marine cartilaginous fishes and the coelacanth use, as osmolytes, a combination of urea and methylamines, i.e., a denaturant and a stabilizer, in a 2:1 molar ratio. Preferential binding and thermal denaturation measurements in the presence of each cosolvent separately and in their mixtures have been carried out using ribonuclease T1 (RNase T1) as the protein. At a 2:1 molar ratio of urea and trimethylamine *N*-oxide (TMAO), the effects of the two cosolvents on the transition temperature (T_m) were found to be essentially the algebraic sum of their effects when used individually. Preferential interaction measurements of urea, TMAO and urea in its 2:1 molar ratio mixture with TMAO, have shown that the presence of TMAO has no effect on the interaction of urea with the protein in either the native or the unfolded (reduced carboxymethylated RNase T1) state. The preferential interaction of TMAO in the presence of urea could not be measured for technical reasons. Calculations of transfer free energy in the two end states of the denaturation reaction have shown that 2 M urea destabilizes RNase T1 by 3.8 ± 0.3 kcal/mol whether 1 M TMAO is present or not. The contribution of 1 M TMAO to stabilization is calculated to be 3.1 kcal/mol in the presence of 2 M urea and is measured to be 2.7 kcal/mol in its absence.

Many prokaryotes, fungi, plants, and animals that live under osmotic stress accumulate small molecular weight compounds for adaptation. These organic substances, principally sugars, polyhydric alcohols, amino acids, and methylamines, are called compatible solutes (Borowitzka & Brown, 1974; Borowitzka, 1985; Yancey et al., 1982; Somero, 1986). Their presence at physiological concentrations does not significantly disturb the structure and function of macromolecules within cells.

Urea, despite its known perturbing effects on the structure and assembly of proteins, is accumulated by some species as the major osmolyte. In fact, it was found in the kidney of a xeric desert rodent, especially in the inner medulla, at 3–4 M concentration during extreme water stress (MacMillen & Lee, 1967). In cartilaginous fish and the coelacanth it reaches a concentration as high as 0.4 M (Lutz & Robertson, 1971). To cope with the high concentration of urea, these fishes simultaneously build up a second set of nitrogenous osmolytes, namely, methylamines such as betaine, sarcosine, and trimethylamine *N*-oxide (TMAO[1]) (Somero, 1986). The physiological ratio of the urea and methylamine concentrations is approximately 2:1. Yancey and Somero (1979, 1980) have showed that the effects of urea on the K_m of enzymes, the exposure of sulfhydryl groups, and the denaturation of proteins are offset by those of TMAO at the 2:1 concentration ratio. Urea is a well-known destabilizer of macromolecules (Gordon & Jencks, 1963; Von Hippel & Schleich, 1969). TMAO increases the midpoint of the thermal unfolding transition (T_m) of ribonuclease A (RNase A) (Yancey & Somero, 1979) and lysozyme (Arakawa & Timasheff, 1985). Bateman et al. (1992) have reported that urea increases the dielectric increment and relaxation time of bovine serum albumin and that betaine reverses its effects. In order to understand the basis of these counteractions of urea and TMAO, we have carried out parallel studies on the effects of urea, TMAO, and their 2:1 mixture on the stability of ribonuclease T1 (RNase T1) and their interactions with the protein individually and in a 2:1 molar mixture.

MATERIALS AND METHODS

Materials. TMAO and methylamine hydrochloride were obtained from Sigma. Urea was purchased from ICN Biomedicals, Inc. RNase T1 with lysine at residue 25 was a generous gift of Dr. C. N. Pace.

Purification of Solvents. Urea was purified essentially by the method of Prakash et al. (1981). Briefly, the urea solution was stirred with activated charcoal, filtered once through Whatman filter paper, and then filtered twice through Millipore filters. Methylamine was added to a concentration of 0.03 M. The solution was passed through Bio-Rad mixed-bed resin AG 501-X8(D). The solution was collected and its concentration was checked densimetrically. It was then diluted to the final working concentration in 0.03 M potassium phosphate buffer (pH 7.0). The solution was stored at 4 °C and used within 1 week.

TMAO was dissolved in water at a concentration slightly higher than the desired one. The solution was stirred with activated charcoal, and the coarse charcoal was removed by filtering through a Whatman paper. The solution was filtered through 3-μm SS and then 0.22-μm GS Millipore filters. It was then passed through Sigma Chelex 100 resin. The eluate was collected and the solution was adjusted to the final

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[1] Abbreviations: TMAO, trimethylamine *N*-oxide; RNase T1, ribonuclease T1; RCM-T1, reduced carboxymethylated RNase T1.

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concentration in 0.03 M potassium phosphate buffer (pH 7.0). The solution was kept at 4 °C and used within 3 days.

The pK_a of TMAO was determined by titration of 0.08 M TMAO in 0.1 M NaCl with 0.41 M HCl. In accordance with the Henderson–Hasselbalch equation, the pK_a of TMAO was found to be 4.56.

Preparation of RCM-T1. Reduced carboxymethylated ribonuclease T1 (RCM-T1) was prepared by a modified procedure of Pace and Creighton (1986). RNase T1 (100 mg) was dissolved in a 12 mL solution of 0.2 M Tris-HCl, 2 mM EDTA, 6 M guanidine hydrochloride, and 0.06 M dithiothreitol in the thiol form (pH 8.7), and the reaction was carried out at room temperature for approximately 2 h. Iodoacetic acid (3 mL, 1 M) in 0.2 M Tris-HCl (pH 7.5) was then pipeted into the protein solution, and the reaction was continued for 10 min. RCM-T1 was then isolated by a Sephadex G-25 column in 0.1 M ammonium bicarbonate. The RCM-T1 was shown by electrophoresis to be a single pure protein.

Thermal Denaturation. Thermal unfolding was followed by the change in absorbance with temperature on a Gilford Response II UV/vis spectrophotometer, with a temperature increment of 0.1 or 0.5 °C. The wavelength monitored was 286 nm.

Preferential Interaction Measurements by High-Precision Densimetry. A Precision Density Meter DMA-02 (Anton Paar, Gratz) was used to measure the density of solvents and protein solutions (Lee & Timasheff, 1974; Lee et al., 1979; Gekko & Timasheff, 1981). The apparent partial specific volume, ϕ, was then calculated at each protein concentration from the density of the solution by (Kielley & Harrington, 1960; Cassasa & Eisenberg, 1961)

$$\phi = \frac{1}{\rho_0}[1 - (\rho - \rho_0)/c] \tag{1}$$

where ρ is the density of solution in grams per milliliter, ρ_0 is that of the solvent, and c is the protein concentration in grams per milliliter.

Defining components according to the notation of Scatchard (1946) and Stockmayer (1950) [water, component 1; protein, component 2; the additive, component 3], the densities of protein solutions were determined at conditions at which the solvent composition (isomolal) or the chemical potential (isopotential) was kept identical, in turn, in the protein solution and the reference solvent at 25 °C. The isopotential state was achieved by dialysis equilibrium. The apparent partial specific volumes of the protein, ϕ_2 from the isomolality condition or ϕ_2' from the isopotential condition, were extrapolated to zero protein concentration to obtain ϕ_2^0 and $\phi_2'^0$ respectively. The preferential interaction parameter, ξ_3, is obtained from (Cohen & Eisenberg, 1968)

$$\xi_3 = \left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3} = \rho_0\frac{(\phi_2^0 - \phi'^0_2)}{(1 - \rho_0\bar{v}_3)} \tag{2}$$

where g_i is the concentration of component i in grams per gram of water and $\bar{v}_3$ is the partial specific volume of component 3. The preferential hydration parameter, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$, is related to preferential binding of the additive by (Timasheff & Kronman, 1959; Reisler et al., 1977)

$$\left(\frac{\partial g_1}{\partial g_2}\right)_{T,\mu_1,\mu_3} = -\left(\frac{1}{g_3}\right)\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3} \tag{3}$$

The measured preferential binding is, in fact, a reflection of the mutual perturbations of the chemical potentials of components 2 and 3, since

$$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3} = -\frac{(\partial\mu_2/\partial m_3)_{T,P,m_2}}{(\partial\mu_3/\partial m_3)_{T,P,m_2}} = -\left(\frac{\partial\mu_2}{\partial\mu_3}\right)_{T,P,m_2} \tag{4}$$

where μ_i is the chemical potential of component i, and m_i is the molality of component i. The term $(\partial m_3/\partial m_2)_{T,P,\mu_3}$ is almost equal to the binding measured by the dialysis equilibrium, expressed as moles of ligand per mole of protein, and $(\partial\mu_3/\partial m_3)_{T,P,m_2}$ is the nonideality of the cosolvent, so that

$$(\partial\mu_2/\partial m_3)_{T,P,m_2} = -\left(\frac{\partial g_3}{\partial g_2}\right)_{T,P,\mu_3}\left(\frac{RTM_2}{M_3}\right)\left(\frac{1}{m_3} + \frac{\partial \ln\gamma_3}{\partial m_3}\right) \tag{5}$$

where T is the thermodynamic (Kelvin) temperature, P is pressure, R is the universal gas constant, and M_i and γ_i are the molecular weight and activity coefficient of component i, respectively.

Preferential Interaction Measurements by Differential Refractometry. The refractive index (n) increments of proteins were measured on a Brice photoelectric differential refractometer at 546 nm (Pittz & Bablouzian, 1973; Pittz et al., 1973). The protein solution, either at isomolal or isopotential conditions, was introduced into the back compartment of the differential cell, and the reference solutions into the front compartment. The initial concentrations of the protein were between 5 and 11 mg/mL. The measurements were performed at 25 °C for a series of dilutions down to 0.5 mg/mL. The preferential binding parameter was obtained from (Pittz & Timasheff, 1978):

$$\left(\frac{\partial g_3}{\partial g_2}\right)_{T,P,\mu_3} = \frac{1}{1 - c_3\bar{v}_3}\left[\left(\left(\frac{\partial n}{\partial c_2}\right)^\circ_{T,P,\mu_3} - \left(\frac{\partial n}{\partial c_2}\right)^\circ_{T,P,m_3}\right)\Big/\left(\frac{\partial n}{\partial c_3}\right)_{T,P,m_2}\right] \tag{6}$$

where c_i is the concentration of component i in grams per milliliter of solution. The superscript ° indicates extrapolation to zero protein concentration. The refractive index of the cosolvent solution was measured on a Bausch and Lomb precision refractometer at 589 nm and 25 °C. The refractive index increment of the cosolvent in aqueous solution, $(\partial n/\partial c_3)_{T,P,m_2}$, was obtained from the tangent of the refractive index vs increasing cosolvent concentration plot. For TMAO in aqueous solution, $(\partial n/\partial c_3)_{T,P,m_2}$ is 0.134 mL/gm at 25 °C.

Protein Concentration. Protein concentration was measured on a Perkin-Elmer Lambda 3B UV/vis or a Cary 118 spectrophotometer. The extinction coefficients used in these measurements were 1.73 L/g·cm at 277 nm for RNase T1 and 1.39 L/g·cm at 275 nm for RCM-T1.

Activity Coefficients. A Knauer vapor pressure osmometer was used to measure the osmotic coefficients (ϕ_3) of a series of TMAO solutions of increasing molality at 28 °C. The activity coefficients of the TMAO solutions were then calculated by

$$\ln\gamma_3 = (\phi_3 - 1) + \int_0^{m_3}(\phi_3 - 1)\, d\ln m_3 \tag{7}$$

RESULTS

The effects of urea and TMAO on the thermal unfolding of RNase T1, measured by difference UV absorbance

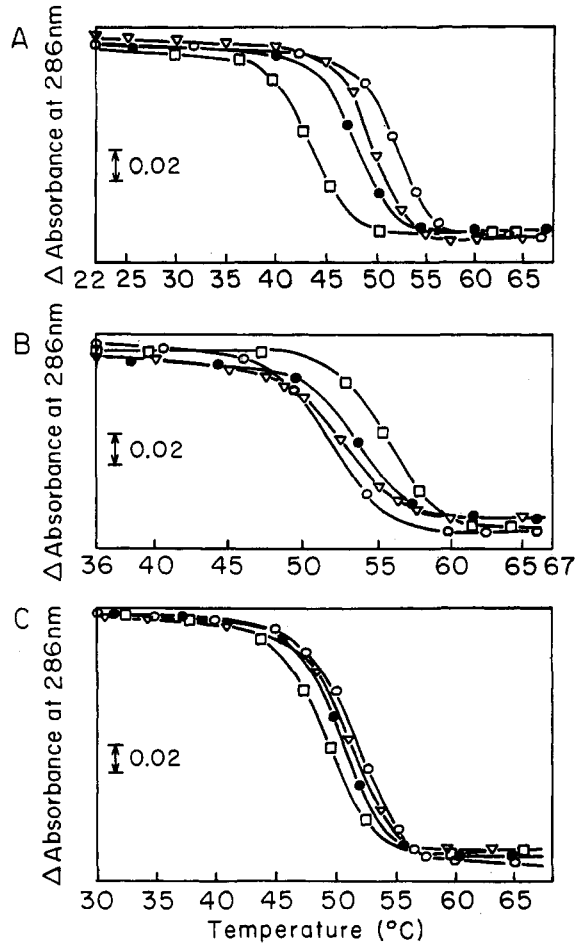

FIGURE 1: Thermal unfolding of RNase T1. (A) Urea: ○, no urea; ▽, 0.5 M urea; ●, 1 M urea; □, 2 M urea. (B) TMAO: ○, no TMAO; ▽, 0.25 M TMAO; ●, 0.5 M TMAO; □, 1 M TMAO. (C) 2:1 molar mixture of urea and TMAO: ○, no urea or TMAO; ▽, 0.5 M urea and 0.25 M TMAO; ●, 1 M urea and 0.5 M TMAO; □, 2 M urea and 1 M TMAO. The buffer for all of these solutions was 0.03 M phosphate (pH 7).

Table 1: Thermodynamic Parameters of Unfolding of RNase T1 in Urea, TMAO, and Their Mixed Solutions

	transition T (°C)	T_m (°C)	$\Delta H°$ at T_m (kcal/mol)	$\Delta G°$ (25 °C) (kcal/mol)
0.03 M phosphate	41.5–58.5	51.9	111 ± 3	9.1 ± 0.3
0.5 M urea	43.2–56.0	49.9	115 ± 2	8.8 ± 0.2
1 M urea	37.4–56.2	48.3	112 ± 2	8.0 ± 0.2
2 M urea	32.7–53.1	44.3	104 ● 1	6.3 ± 0.1
0.25 M TMAO	43.6–60.0	53.0	121 ± 3	10.3 ± 0.3
0.5 M TMAO	47.3–60.0	54.0	136 ± 2	12.0 ± 0.2
1 M TMAO	50.0–62.1	56.0	125 ± 2	11.8 ± 0.2
0.5 M urea + 0.25 M TMAO	43.2–59.1	51.1	115 ± 1	9.3 ± 0.1
1 M urea + 0.5 M TMAO	43.0–57.9	50.4	116 ± 1	9.2 ± 0.1
2 M urea + 1 M TMAO	39.9–57.0	49.3	108 ± 1	8.2 ± 0.1

spectroscopy, are presented in Figure 1. As shown in Table 1, the midpoint of the transition, T_m, is 51.9 °C in dilute buffer at pH 7. Urea lowers T_m, whereas TMAO raises it. In 2:1 urea to TMAO mixtures, the strong destabilizing action of urea is greatly compensated by the stabilizing action of TMAO. In fact, as shown in Table 1, the effect on T_m is essentially equal to the algebraic sum of the effects of urea and TMAO alone (Figure 2). The thermodynamic parameters for the unfolding of RNase T1 at 25 °C in various solvents are listed in Table 1. The values of $\Delta H°$ at T_m, listed in this table, were obtained from van't Hoff plots. The $\Delta G°$ values at 25 °C were calculated by a linear extrapolation of the

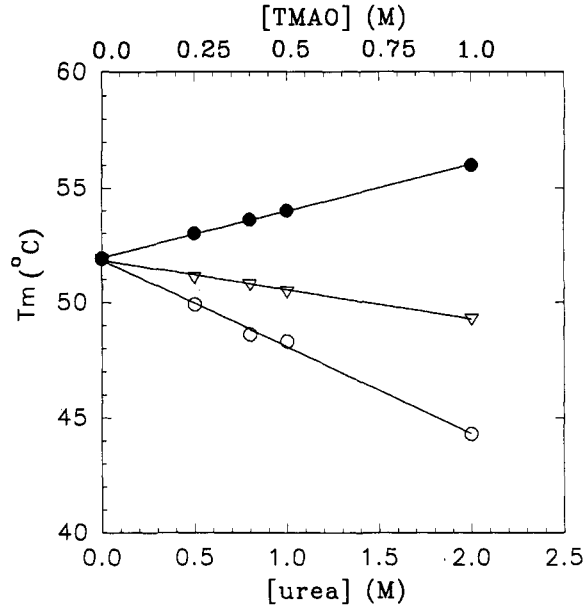

FIGURE 2: Midpoint of the thermal unfolding transition of RNase T1 in urea (○), TMAO (●), urea and TMAO at a molar ratio of 2:1 (▽).

van't Hoff plots, since these showed no apparent deviation from linearity within the limits of the experimental data.

What determines the effect of a cosolvent on the stability of a protein relative to water? It is the difference between its interactions with the protein in the denatured and native states. The strength of the protein–solvent interaction is expressed by the transfer free energy of the protein from water to the given system:

$$\Delta\mu_2 = \mu_2(\text{cosolvent}) - \mu_2(\text{water}) \quad (8)$$

For denaturation, the effect of an additive on the standard free energy of denaturation, $\Delta G°$, is then equal to:

$$\delta\Delta G° = \delta\Delta\mu_2 = \Delta G°_{m_3} - \Delta G°_w = \Delta\mu_2{}^D - \Delta\mu_2{}^N \quad (9)$$

where the subscripts w and m_3 refer to protein in water and in a cosolvent solution of molal concentration m_3, and the superscripts D and N refer to the transfer free energies of the protein in the denatured and native states, respectively. The determination of a transfer free energy requires measurements of the preferential interactions between the protein and the cosolvent system over a cosolvent concentration range from zero to the working solvent composition, since

$$\Delta\mu_2 = \int_0^{m_3} (\partial\mu_2/\partial m_3)_{T,P,m_2}\, dm_3 \quad (10)$$

Therefore, the preferential interactions of urea and TMAO with native and unfolded RNase T1 were determined. For the unfolded state of the protein, we used RNase T1 with the disulfides linking residues 2–10 and 6–103 (Heinemann & Saenger, 1982) reduced and carboxymethylated (RCM-T1) since, when these are broken, the protein unfolds (Oobatake et al., 1979; Pace et al., 1988). Electrophoresis in polyacrylamide gel of our RCM-T1 clearly demonstrated a single band that ran much more slowly than native RNase T1 at 12 °C, consistent with the observation of Pace and Creighton (1986). At pH 7, the thermal unfolding of RCM-T1 displayed poor reversibility. Its unfolding between 8.5 and 20.6 °C, however, permitted its use at 25 °C as the unfolded form of the protein.

The preferential interactions of the proteins with the cosolvents were measured by dialysis equilibrium, with the cosolvent concentrations inside and outside the bag determined

Table 2: Thermodynamic Parameters of Urea and TMAO in Aqueous Solutions at 25 °C

solvent	m_3 (mol/kg)	g_3 (g/g)	ρ_0 (g/mL)	$\bar{v}_3$[a] (mL/g)	γ_3	$(\partial \ln \gamma_3/\partial m_3)$ (1/mol)	$(\partial\mu_3/\partial m_3)_{T,P,m_2}$ [cal/(mol·mol)]
0.5 M urea	0.51	0.031	1.0084	0.733	0.960[b]	−0.076	1095
1.0 M urea	1.05	0.063	1.0160	0.735	0.924[b]	−0.068	517
2.0 M urea	2.20	0.132	1.0298	0.740	0.859[b]	−0.059	235
0.25 M TMAO	0.25	0.019	1.0029	0.970	0.992[c]	−0.051	2295
0.5 M TMAO	0.52	0.039	1.0034	0.968	0.965[c]	−0.147	1054
1.0 M TMAO	1.08	0.081	1.0058	0.960	0.883[c]	−0.167	449

[a] Measured at 20 °C. [b] Taken from Scatchard et al. (1938). [c] Measured at 28 °C.

Table 3: Preferential Interaction Parameters of RNase T1 and RCM-T1 in Urea and Urea–TMAO Solutions at pH 7, 25 °C

solvent	ϕ_2^0[a] (mL/g)	$\phi_2'^0$[a] (mL/g)	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial\mu_2/\partial m_3)_{T,P,m_2}$ [cal/(mol of protein·mol of cosolvent)]
			RNase T1			
0.03 M phosphate	0.7149[b]	0.7068[b]				
0.5 M urea	0.7154	0.7010	0.0244	4.49	−0.793	−4915
1.0 M urea	0.7190	0.7023	0.0345	6.36	−0.549	−3289
2.0 M urea	0.7207	0.6977	0.0647	11.93	−0.490	−2801
0.5 M urea + 0.25 M TMAO[d]	0.7154[c]	0.7018	0.0213	3.92	−0.693	−4293
1.0 M urea + 0.5 M TMAO[d]	0.7190[c]	0.7038	0.0286	5.27	−0.455	−2724
2.0 M urea + 1.0 M TMAO[d]	0.7207[c]	0.6987	0.0610	11.24	−0.462	−2639
			RCM-T1			
0.03 M phosphate	0.7346[b]	0.7281[b]				
0.5 M urea	0.7410	0.7281	0.0247	4.66	−0.806	−5096
1.0 M urea	0.7412	0.7211	0.0542	10.19	−0.862	−5268
2.0 M urea	0.7405	0.7005	0.1445	27.18	−1.094	−6382
0.5 M urea + 0.25 M TMAO[d]	0.7410[c]	0.7281	0.0248	4.67	−0.808	−5108
1.0 M urea + 0.5 M TMAO[d]	0.7412[c]	0.7231	0.0465	8.76	−0.741	−4526
2.0 M urea + 1.0 M TMAO[d]	0.7405[c]	0.7009	0.1442	27.14	−1.093	−6372

[a] The standard deviations in the ϕ_2^0 and $\phi_2'^0$ measurements were ±0.001. [b] The difference between ϕ_2^0 and $\phi_2'^0$ in buffer alone was used as an additive correction in calculations of preferential interaction parameters. [c] The values were taken to be the same as ϕ_2^0 in the urea solutions. [d] The densities of the mixed solutions in the absence of the protein (ρ_0) are as follows: 1.009 g/mL for 0.5 M urea + 0.25 M TMAO; 1.016 g/mL for 1 M urea + 0.5 M TMAO; 1.032 g/mL for 2 M urea + 1 M TMAO.

Table 4: Preferential Interaction of RNase T1 and RCM-T1 with TMAO at pH 7, 25 °C.

[TMAO] (M)	$(\partial n/\partial c_2)_{T,P,m_3}$[a] (mL/g)	$(\partial n/\partial c_2)_{T,\mu_1,\mu_3}$[a] (mL/g)	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial\mu_2/\partial m_3)_{T,P,m_2}$ [cal/(mol·mol)]
			RNase T1			
0	0.1787	0.1798				
0.25	0.1760	0.1761	0.0008	0.12	−0.044	−283
0.5	0.1793	0.1780	−0.0096	−1.42	0.247	1495
1.0	0.1803	0.1748	−0.0450	−6.63	0.553	2973
			RCM-T1			
0	0.1634	0.1626				
0.25	0.1635	0.1604	−0.0238	−3.59	1.247	8232
0.5	0.1630	0.1597	−0.0258	−3.89	0.663	4099
1.0	0.1484	0.1475	−0.0069	−1.03	0.084	462

[a] The standard deviations in the measurements of the refractive index gradient were ±0.001. The reproducibility in the difference between the refractive index gradients measured at isomolal and isopotential conditions varied between 0.0005 and 0.001.

densimetrically for the urea and mixed urea–TMAO systems or refractometrically for TMAO alone. The thermodynamic parameters of the cosolvents are shown in Table 2. The preferential interaction parameters for RNase T1 and RCM-T1 with urea are listed in Table 3, and those with TMAO are listed in Table 4. Urea is found to preferentially bind to both states of the protein. As shown in Figure 3, the extent of the preferential binding is low for the native form and much larger for the denatured protein, i.e., protein unfolding is accompanied by an increase in the interaction with the denaturant and a concomitant increase in the exclusion of water. Closer examination of Table 3 reveals that the preferential binding of urea to both states of the protein increases with urea concentration, and this increase is much greater with the unfolded protein. In 0.5 M urea the values are almost identical at ~4.5 mol of urea bound per mole of protein, while in 2 M urea the binding to the denatured protein is essentially twice that to the native molecule. The values of the parameter $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ for RNase T1 and RCM-T1 in urea are listed in column 7 of Table 3. The negative values indicate favorable interactions of urea at all concentrations with both states of the protein. The near constancy of the parameter in the case of the unfolded protein points to an invariant affinity of the ligand for the macromolecule, while the decrease in the absolute values for interactions with the native protein shows gradual saturation of the interaction and an increasing contribution from nonexchangeable water molecules on the protein surface (Timasheff, 1992, 1993). The transfer free energies were calculated with eq 10 from a linear fit of the $(\partial\mu_2/\partial m_3)$ values. They are listed in Table 5. The values of $\Delta\mu_2 = -8.4$ and −12.0 kcal/mol for RNase T1 and RCM-T1 in 2 M urea indicate that the binding of urea (at 2 M) to RNase T1 destabilizes the protein by 3.6 kcal/mol relative to water, as $\delta\Delta\mu_2 = 3.6$ kcal/mol.

Since solutions of TMAO have densities near 1, ($\bar{v}_3 \simeq 1$, see Table 2), the preferential binding of this cosolvent could

Table 5: Analysis of Transfer Free Energies and Unfolding Free Energies of RNase T1 at 25 °C

	$\Delta\mu_2$, RNase T1 (cal/mol)	$\Delta\mu_2$, RCM-T1 (cal/mol)	$\delta\Delta\mu_2$ (cal/mol)	$\delta\Delta G°$ (cal/mol)
2 M urea	−8439 ± 500	−12000 ● 500	−3561 ± 1000	−2820 ± 400
1 M TMAO	1197 ± 500	5364 ± 1500	4167 ± 2000	2650 ± 500
2 M urea in (2 M urea + 1 M TMAO)	−7355 ± 500	−11427 ± 500	−4072 ± 1000	
2 M urea + 1 M TMAO				−960 ± 400

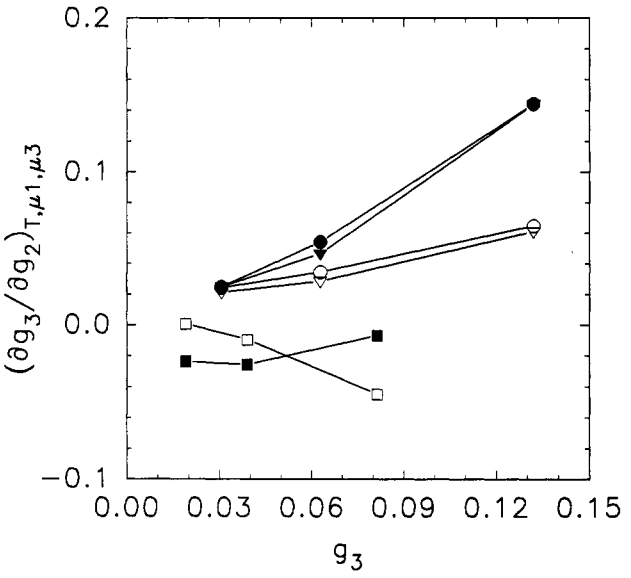

FIGURE 3: Cosolvent concentration dependence of the preferential binding of cosolvent to protein: RNase T1 in urea (○); RCM-T1 in urea (●); RNase T1 in TMAO (□); RCM-T1 in TMAO (■); RNase T1 in solutions of urea and TMAO with a molar ratio of 2:1 (▽); RCM-T1 in solutions of urea and TMAO with a molar ratio of 2:1 (▼). The g_3 values for the ternary solvents indicate the concentration of urea.

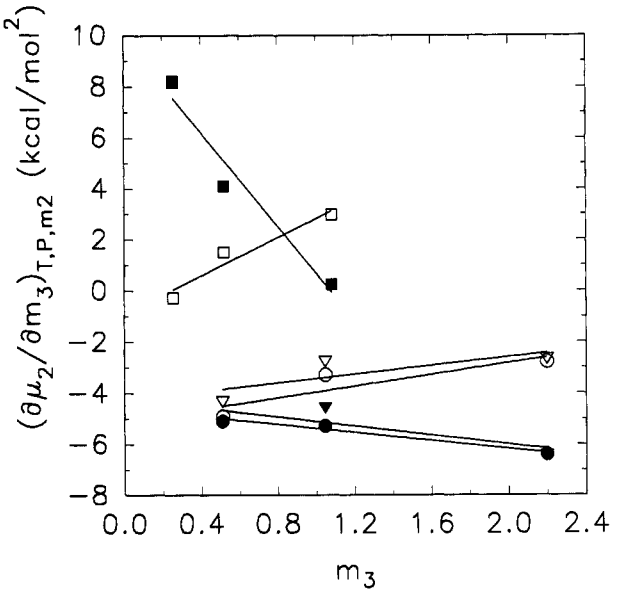

FIGURE 4: Dependence of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ on cosolvent concentration. The symbols are the same as those described in the legend for Figure 3. The m_3 values for the ternary solvents indicate the concentration of urea (filled triangles are not drawn at 0.5 and 2 M urea, since these values coincide exactly with the filled circles).

not be measured densimetrically. It was determined, therefore, by differential refractometry on a photoelectric differential refractometer at 546 nm and 25 °C (Pittz & Timasheff, 1978). As shown in Table 4, at all TMAO concentrations, the refractive index increment measured at isomolal conditions was greater than the isopotential value, which means that the preferential binding of TMAO to both RNase T1 and RCM-T1 is negative. This indicates preferential exclusion of TMAO from the protein (Figure 3), i.e., preferential hydration. The variations with solvent composition, however, follow opposite trends. The values of $(\partial g_3/\partial g_2)$ listed in column 4 stem from the small differences between the two refractive index gradient measurements in each case. Since we have found these differences to be reproducible to within 0.0005 dn/dc_2 units, we regard the observed trends as real, the deviations not being greater than 25%. The small number of points available due to the extreme difficulty of the measurements precludes a complete statistical analysis. The finding that the preferential exclusion increases for RNase T1 as the cosolvent concentration becomes higher, while for RCM-T1 it decreases, suggests that TMAO can bind to the denatured protein, possibly due to its partially hydrophobic nature (Arakawa & Timasheff, 1985). This is brought out clearly by the variation of the preferential interaction parameter, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ with TMAO concentration (Figure 4). The positive values testify to unfavorable interactions between TMAO and both states of the protein. Their trends with cosolvent concentration, however, point to an increase in this effect for the native protein, while for the unfolded one this is obviously compensated by an increasing favorable interaction, translated by the increasing occupancy of loci on the protein surface by TMAO and a gradual overcoming of the nonexchangeable water. The transfer free energies were calculated with eq 10 from the data of Table 4 using the best straight line through each set of points. As shown in Table 5, in 1.0 M TMAO, $\Delta\mu_2$ shifts from 1.2 to 5.4 kcal/mol when the protein becomes unfolded, i.e., the protein is stabilized by $\delta\Delta\mu_2 = 4.2$ kcal/mol relative to water.

To probe the urea–TMAO compensation seen in the unfolding experiments, the preferential interaction of urea with the protein was measured in the presence of TMAO. For a four-component system, when measured densimetrically, the preferential bindings of cosolvents to the protein are related to the experimentally measured parameters by (Cassassa & Eisenberg, 1964)

$$\rho_0(\phi_2^0 - \phi_2'^0) = (1 - \bar{v}_3\rho_0)\left(\frac{\partial g_3}{\partial g_2}\right)_{T,P,\mu_3,\mu_4} + (1 - \bar{v}_4\rho_0)\left(\frac{\partial g_4}{\partial g_2}\right)_{T,P,\mu_3,\mu_4} \quad (11)$$

where $(\delta g_3/\delta g_2)$ and $(\delta g_4/\delta g_2)$ are the preferential bindings of the two cosolvents (urea and TMAO in the present case) to the protein, expressed as grams of ligand per gram of protein. The partial specific volumes of components 3 and 4, $\bar{v}_3$ and $\bar{v}_4$, are listed in Table 2. Since the partial specific volume of TMAO, $\bar{v}_4$ in eq 11, is close to 1, the product $\bar{v}_4\rho_0$ assumes values between 0.98 and 0.99 and the last term of eq 11 vanishes. The quantity on the left-hand side of the equation reflects then, within experimental error, the preferential interaction of the protein only with urea in the four-component system. The results presented in Table 3 show that the preferential binding of urea to both RNase T1 and RCM-T1 in the 2:1 molar ratio mixture of urea and TMAO is essentially

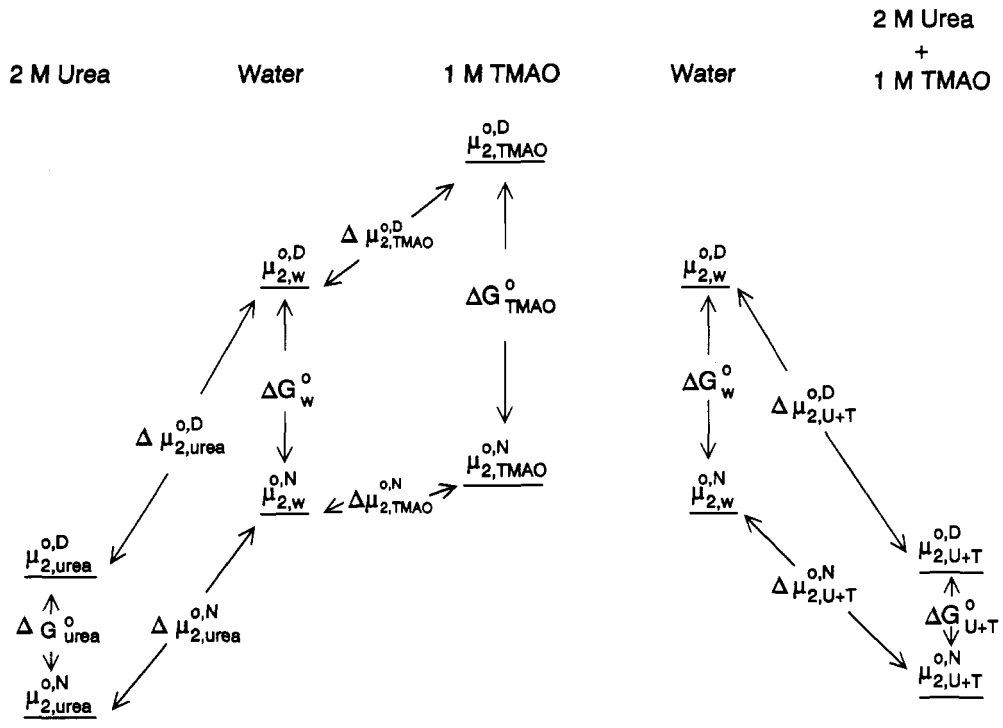

FIGURE 5: Free energy diagram of the effect of cosolvents on protein stability. The standard chemical potentials of native (N) and denatured (D) proteins in water (w) and cosolvents are indicated by the horizontal lines. In the mixed solutions, urea and TMAO are denoted by U and T, respectively. $\Delta G°$ is the unfolding free energy of RNase T1. In the diagram on the right, the transfer free energies refer to the contribution of urea in the mixed solvent.

identical to that found for urea solutions alone. This is clearly seen in Figures 3 and 4. The contribution of urea at 2 M to the transfer free energy in the four-component system is −7.4 kcal/mol for the native protein and −11.4 kcal/mol for the unfolded molecule, i.e., the same as for the pure urea system within experimental error. Their difference, −4.1 kcal/mol, indicates (see eq 9) that urea destabilizes the protein to essentially the same extent whether TMAO is present or not.

DISCUSSION

The unfolding results reported in this paper are essentially identical with those previously reported in the literature. Kiefhaber et al. (1990) reported T_m = 51.9 °C and ΔH = 480 kJ/mol = 114 kcal/mol in the absence of cosolvents, identical to our values listed in Table 1. Shirley et al. (1989), using a ΔC_p of 1650 cal/mol·deg, obtained a $\Delta G°$ value of 6.9 kcal/mol at 25 °C, in the absence of urea from their thermal unfolding study of RNase T1. Using the same value of ΔC_p, we obtain a $\Delta G°$ of 7.3 kcal/mol at zero cosolvent concentration. We chose to use a linear extrapolation in the calculation of $\Delta G°$'s at 25 °C because of the uncertainty in the effects of urea and TMAO on ΔC_p. Nevertheless, the $\delta\Delta G°$, which we obtained between 2 and 0 M urea (−2820 cal/mol), is the same within experimental error as that reported by Shirley et al. (1989): −2420 cal/mol.

The effects of urea, TMAO, and their mixture on the stability of RNase T1 are summarized in Table 5. The relations between the transfer free energies and the unfolding free energies are depicted in the diagram of Figure 5. The transfer free energy of RNase T1 decreases by 3.6 kcal/mol upon unfolding in 2 M urea solution. This agrees with the thermal unfolding results of $\delta\Delta G°$ = −2.8 kcal/mol. In 1 M TMAO solution, the transfer free energy of RNase T1 increases by 4.2 kcal/mol, which again is consistent, within experimental error, with the thermal unfolding results of $\delta\Delta G°$ = 2.7 kcal/mol. Therefore, urea destabilizes the protein, while TMAO increases its stability. Mixing of urea and TMAO in a 2:1 ratio does not significantly change the effects of the individual osmolytes. This is illustrated by the observation that the T_m's of RNase T1 in the mixed solutions are essentially the algebraic sums of the effects of the individual solvents (Figure 2). Furthermore, the preferential binding of urea and the chemical potential changes of the protein induced by urea in urea–TMAO solutions are also close to those of pure urea solutions (Table 3, Figures 3 and 4). Therefore, TMAO does not affect the interaction of urea with the protein, and the two cosolvents interact independently of each other with the protein.

The observation that the preferential hydration of both RNase T1 and RCM-T1 in the presence of TMAO increases with cosolvent concentration for the native protein and decreases for the denatured protein has led to the conclusion that TMAO must bind to the denatured protein. In the native state, the interaction of polar regions of the protein with water should render the exchange of water with TMAO unfavorable (Timasheff & Kronman, 1959; Schellman, 1987, 1990; Timasheff, 1992, 1993). In denatured proteins, the exposure of new nonpolar regions to solvent should make possible their interactions with the methyl groups of TMAO. This would decrease the preferential hydration. Such complexities of cosolvent binding and exclusion are also found in dimethyl sulfoxide (DMSO) solutions. At low concentrations of DMSO, proteins are preferentially hydrated (T. Arakawa and S. N. Timasheff, unpublished) as a consequence of the high affinity of the sulfoxide group of DMSO for water (Jacob et al., 1971). At high cosolvent concentrations, DMSO preferentially binds to proteins, probably due to the interaction between the methyl groups and the nonpolar regions of the proteins (Arakawa et al., 1990), as well as to the depression of the surface tension of water by DMSO (Kita et al., 1994).

What is the thermodynamic effect of the mixed cosolvents on protein stability? As shown in Table 5, the interaction of

2 M urea with RNase T1 in the presence of 1 M TMAO is characterized by a transfer free energy of −7.4 kcal/mol, a value similar to that of 2 M urea alone. For RCM-T1 the transfer free energy of −11.4 kcal/mol in 2 M urea in the presence of 1 M TMAO again is close to the $\Delta\mu_2$ = −12.0 kcal/mol value in 2 M urea alone. Therefore, urea contributes a $\delta\Delta\mu_2$ = −4.1 kcal/mol in the mixed solvent, essentially the same as in the absence of TMAO (Table 5).

The contribution of TMAO to the transfer free energies in the urea–TMAO mixture cannot be measured due to the technical reason that TMAO and urea both perturb the refractive index of water. Any measurement, therefore, would contain contributions from the interactions of both cosolvents with the protein. For the four-component system, eq 9 becomes

$$\delta\Delta G^\circ = \delta\Delta\mu_2(\text{urea}) + \delta\Delta\mu_2(\text{TMAO}) \qquad (12)$$

where the $\delta\Delta\mu_2$ terms are the contributions of urea and TMAO in the presence of each other to the change in the transfer free energy, respectively. Therefore, $\delta\Delta\mu_2$ for TMAO in the presence of urea can be inferred from the thermal transition of RNase T1 in the mixed solvent. For the mixture of 2 M urea and 1 M TMAO, the ΔG° values of Table 1 give $\delta\Delta G^\circ$ = −1.0 kcal/mol. When combined with the change in transfer free energy on denaturation measured for urea in the 2:1 mixture, $\delta\Delta\mu_2$ = −4.1 kcal/mol (Table 5), this leads to a contribution from TMAO of 3.1 kcal/mol to the stabilization of the protein in the mixed solvent. This value is strikingly similar to that of 1 M TMAO alone, for which $\delta\Delta G^\circ$ = 2.7 kcal/mol.

The results of both the preferential interaction and thermal transition studies demonstrate that the actions of urea and TMAO are thermodynamically additive. The free energy diagram of Figure 5 clearly shows that the contributions of urea and TMAO are essentially equal in magnitudes but opposite in sign, while the urea diagram remains essentially unaffected by the addition of TMAO. The mutual independence of the transfer free energies and their additivity in a mixture fully account for their effect on protein stability and for the observed compensation of the urea destabilization of proteins by TMAO stabilization. This additivity of the thermodynamic interactions of the two cosolvents with the protein can explain, therefore, why nature selected their mixture as an effective osmolyte system. Urea is a naturally available metabolite. Its use alone, however, could destabilize the biological system. The use of a methylamine alone might have the unfavorable consequence of imparting too much stability, i.e., too much rigidity, to the system. Their combined use, however, gives the desired osmotic effect, while mutually neutralizing their perturbations of biological function.

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