A Naturally Occurring Protective System in Urea-Rich Cells: Mechanism of Osmolyte Protection of Proteins against Urea Denaturation†

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ABSTRACT: Trimethylamine *N*-oxide (TMAO) is a solute concentrated in the urea-rich cells of elasmobranchs and coelacanth to offset the damaging effects of urea on intracellular protein structure and function. On the basis of transfer free energy measurements, favorable interaction of TMAO with amino acid side chains promote protein denaturation. This effect is more than offset by highly unfavorable TMAO−peptide backbone interactions that not only oppose denaturation but also provide stabilization against denaturation by urea. By combining transfer free energies of side chains and backbone with surface area exposure in the native and unfolded states of ribonuclease T1, the transfer free energies of native and unfolded protein from water to 1 M TMAO are estimated as 1.7 and 5.9 kcal/mol, respectively. These estimates agree favorably with the respective values of 1.2 and 5.4 kcal/mol determined experimentally by Lin and Timasheff [(1994) *Biochemistry 33*, 12695−12701]. The unfavorable transfer free energies of native and unfolded protein from water to TMAO provides a molecular level rationale for preferential hydration of proteins by osmolytes. Promotion of denaturation by urea is found to be offset by TMAO in a manner that is roughly additive of the combined effects of both solutes. The favorable interaction of urea with the backbone provides the dominant driving force for protein unfolding by this denaturant, and the unfavorable interaction of TMAO with backbone is the dominant force opposing urea denaturation. In solutions that contain significant organic solute concentration, the ascendance of the role of the peptide backbone over that of side chains can explain many observed effects in protein denaturation and stability induced by a variety of stabilizing and destabilizing organic solutes.

When isolated proteins are subjected to the severe environmental conditions encountered in the biosphere, many of them are unable to cope with the stress and will denature. Plants, animals, and microorganisms alike have adapted to such environmental conditions by evolving means to protect their proteins and other cell components against denaturing stresses such as extreme temperatures and desiccation (Brown & Simpson, 1972; Yancey et al., 1982). A common mechanism evolved by these organisms for protecting proteins involves synthesis and intracellular accumulation of certain small organic solutes known as "organic osmolytes" (Brown & Simpson, 1972; Hochachka & Somero, 1984; Pollard & Wyn Jones, 1979; Stewart & Lee, 1974; Yancey et al., 1982). These naturally occurring solutes include specific amino acids, certain polyols, and particular methylamine species (Yancey et al., 1982). The ability of organic osmolytes to protect against denaturation is believed to be both generic and independent of the evolutionary history of the proteins (Hochachka & Somero, 1984; Wang & Bolen, 1996; Yancey et al., 1982). That is, an organic osmolyte solution is expected to provide general protection against denaturation to any protein, even if that protein did not evolve in the presence of the organic osmolyte.

Any mechanism offering generalized protection of proteins against denaturation is of fundamental importance to issues of protein folding, stability, and function and of major practical interest in biotechnology, evolutionary biology, and biochemistry. The assumption is that osmolytes were derived through natural selection, and this implies that particular physicochemical properties of stabilizing organic osmolyte solutions were selected for their ability to protect macromolecular and other components of the organism (Hochachka & Somero, 1984; Somero, 1986). Our previous work with the organic osmolytes, sarcosine and sucrose, led us to conclude that the unfavorable interaction between these particular osmolytes and the peptide backbone is responsible for the ability of these solutes to protect proteins against denaturation (Liu & Bolen, 1995). In the work described here, we investigate the properties of a third organic osmolyte, trimethylamine *N*-oxide (TMAO), which has the unusual biological function of protecting proteins against urea-induced inactivation (Yancey et al., 1982).

The coelacanth and marine elasmobranchs (sharks and rays) concentrate both urea and methylamines (including TMAO) in their cells in a ratio from 3:2 to 2:1 (urea:TMAO), with urea reaching concentrations as high as 600 mM in the ray (*Dasyatis americana*) (Forster & Goldstein, 1976; Yancey & Somero, 1980). TMAO is believed to offset the deleterious effects urea has on intracellular protein stability and function (Yancey & Somero, 1979). This osmolyte is fundamentally different from the polyol and amino acid classes of osmolytes in terms of its effect on the activities

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of proteins. Polyol and amino acid osmolytes have little or no effect on biological activity of proteins (e.g. they have little or no effect on K_m or k_{cat} values of enzymes) and are classified functionally as "compatible osmolytes" (Borowitzka, 1985; Borowitzka & Brown, 1974; Brown & Simpson, 1972; Wang & Bolen, 1996; Yancey et al., 1982). But TMAO does alter the biological activity of proteins in a manner that opposes the effect urea has on the protein function. In fact, urea often increases K_m and decreases k_{cat} values of enzymes, and TMAO counterbalances the urea effects by decreasing K_m and increasing k_{cat}. Accordingly, TMAO is classified as a "counteracting osmolyte" in terms of its effects on both function and structure (Yancey et al., 1982; Yancey & Somero, 1979; Yancey & Somero, 1980).

Lin and Timasheff determined the individual and combined effects of urea and TMAO on the thermodynamics of denaturation of ribonuclease T1 (RNase T1)[1] (Lin & Timasheff, 1994). They found that the thermodynamic effects of TMAO and urea on protein stability are roughly additive, namely, the free energy change brought about by a mixture of TMAO and urea is approximately the algebraic sum of the free energy changes contributed by TMAO and urea individually. Compatible osmolytes protect against extremes of temperature, dehydration, and high salt environment while counteracting osmolytes like TMAO protect cellular proteins against urea inactivation (Hochachka & Somero, 1984; Yancey et al., 1982). Given that these denaturing stresses are very different from one another, there is no reason *a priori* to expect compatible and counteracting osmolytes to have the same mechanisms for protecting proteins.

Our purpose here is to develop an understanding of the stabilization of protein structure by TMAO, and to investigate the phenomenon of the thermodynamic additivity effects by urea and TMAO at the molecular level. In order to evaluate the physicochemical properties leading to protein stabilization, we need to determine the propensities of TMAO and urea to interact with various parts of the protein. The propensities are evaluated from measurements of the free energies of transfer of amino acid side chains and backbone: from water to TMAO, from water to urea, and from water to a 2:1 ratio of urea:TMAO.

MATERIALS AND METHODS

DKP (diketopiperazine) and all L-amino acids were purchased from Sigma Chemical Company, but L-histidine was from Bachem and L-proline was from CalBiochem. Ultrapure Urea was purchased from ICN, and TMAO-dihydrate was synthesized by oxidation of trimethylamine (from Fluka) with hydrogen peroxide (from Fisher Scientific Co.) as described by Hickinbottom (1936). TMAO-dihydrate was recrystallized from aqueous solution and the crystals were dried and kept in a desiccator in the dark at room temperature. The compound was found to be prue by NMR, melting point, and elemental chemical analysis. Because TMAO-dihydrate is deliquescent (Hickinbottom, 1936), a standard curve was prepared and used to evaluate TMAO concentrations conveniently. To construct the standard curve, TMAO-dihydrate samples were weighed and solutions were prepared in volumetric flasks under nitrogen gas protection. The refractive index (n) was measured for each of the TMAO aqueous solutions, and a standard curve was obtained and mathematically described by polynomial fitting of TMAO (molar) concentration as a function of Δn, the refractive index difference between buffered TMAO solution and the buffer itself. The resulting relationship is given in eq 1.

$$[\text{TMAO}] = -0.0038 + 103.3151\Delta n - 259.43\Delta n^2 \quad (1)$$

The solubilities of L-amino acids and DKP were measured at 25.10 °C in 1 M TMAO, in 2 M urea, and in the mixture of 1 M TMAO plus 2 M urea, following the procedure of Liu and Bolen (1995). Briefly, eight to nine sample vials were prepared, each with an increasing weight of a given amino acid and weighed amounts of either 2 M urea, 1 M TMAO, or 2 M urea plus 1 M TMAO was added to the vials. Of the nine vials, four to six contained low enough weights of amino acid such that unsaturated solutions resulted. The remaining vials contained sufficient amino acid weights to produce saturated solutions. The sealed samples were shaken gently in a water bath at 25.10 °C for at least 24 h, and the solution from each sample was extracted through a glass-fiber filter into a syringe and the filtered solution was then injected into a DMA-602 densimeter (Anton Paar USA) for evaluation of density.

For analysis, the density of each sample was plotted as a function of its composition (g of the amino acid per 100 g of solvent), and the density versus composition data of the unsaturated solutions were fitted to a polynomial (from 2 to 4 degrees), while the data of the saturated solutions were fitted to a straight line. The solubility limit was evaluated at the intersection of the two fitted lines, and the amino acid concentration and density of solution at the solubility limit were recorded.

Solubility limits for 10 amino acids in 2 M urea were reported by Nozaki and Tanford (1963). For these amino acids, the density at the solubility limit was obtained by measuring the density of a slightly saturated amino acid solution (in 2 M urea) at 25.10 °C. Solubility limits for an additional eight amino acids in 2 M urea were determined using the method described above.

RESULTS

Solubility Limits of Amino Acids and DKP in 1 M TMAO, in 2 M Urea, and in a Mixture of 1 M TMAO and 2 M Urea at 25.10 °C. The solubility limits of each amino acid in 2 M urea, 1 M TMAO, and 2 M urea plus 1 M TMAO were obtained from density measurements at 25.10 °C. As an example, Figure 1 shows the density profiles of valine in the three different solute-containing solutions. The solubility limit (expressed as g of valine per 100 g of solvent) and the density (g/mL) at the solubility limit were obtained at the intersection of the two fitted lines in each experiment. These results are representative of the quality of the data obtained by using this method.

Table 1 summarizes the solubility limits and corresponding densities for DKP (diketopiperazine) and all the amino acids except tyrosine, cysteine, and cystine. The solubility of tyrosine is too small to be measured accurately using densimetry, while cysteine was not used because it readily

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

Table 1: Amino Acid Solubility Limits (g/100 g of solvent) and Densities (ρ) (g/mL) at the Solubility Limits (25.10 °C)

	water		1 M TMAO		2 M urea		1 M TMAO + 2 M urea	
amino acid	concn	ρ	concn	ρ	concn	ρ	concn	ρ
Trp	1.36[c]	1.01[c]	1.273	1.003 51	1.98[d]	1.033 38	1.697	1.033 74
Phe	2.805[a]	1.005 28[c]	2.091	1.005 25	3.42[d]	1.036 24	2.649	1.037 06
Leu	2.15[b]	1.000 9[c]	1.545	1.002 58	2.37[d]	1.031 72	1.793	1.033 46
Ile	3.35[c]	1.003 45[c]	2.548	1.004 50	3.479	1.034 04	2.568	1.035 09
Val	5.73[c]	1.009 51[c]	4.159	1.008 94	5.617	1.039	4.023	1.038 75
Met	5.69[b]	1.013 4[c]	4.226	1.011 97	6.19[d]	1.044 73	4.693	1.043 14
Ala	16.65[a]	1.042 95[c]	12.13	1.034 22	15.3[d]	1.067 24	10.90	1.059 85
His	4.3[a]	1.012 06[c]	2.911	1.009 98	4.66[d]	1.043 31	3.164	1.041 27
Thr	9.74[b]	1.028 96[c]	6.972	1.022 88	9.56[d]	1.057 63	6.680	1.052 04
Ser	42.9	1.130 28	31.73	1.103 92	41.94	1.153 31	31.31	1.128 05
Gly	25.09[b]	1.083 02[c]	17.69	1.062 74	22.7[d]	1.102 66	15.59	1.084 07
LysHCl	71.3[c]	1.127 8[c]	59.45	1.114 79	70.97	1.146 42	59.78	1.135 16
ArgHCl	85.3[c]	1.159 05[c]	70.56	1.140 11	84.08	1.177 86	69.08	1.160 83
NaGlu	62.4[c]	1.217 85[c]	50.30	1.182 94	55.96	1.221 22	40.98	1.185 87
NaAsp	77.9	1.285 39	59.81	1.237 07	68.62	1.282 84	55.24	1.234 24
Gln	4.19[a]	1.011 33[c]	2.767	1.009 29	4.49[d]	1.042 19	2.932	1.041 03
Asn	2.64[a]	1.007 46[c]	1.831[e]	1.006 98	2.89[d]	1.030 92	2.119[e]	1.038 72
Pro	181.5	1.194 18	152.14	1.183 94	176.1	1.203 28	148.42	1.194 59
DKP	1.68[c]	1.002 52[c]	1.240	1.003 75	2.063	1.034 98	1.500	1.035 49

[a] Average value from Nozaki and Tanford (1963) and Liu and Bolen (1995). [b] Average value from Nozaki and Tanford (1963), Liu and Bolen (1995), and Sarker and Bolen (unpublished). [c] Liu and Bolen (1995). [d] Nozaki and Tanford (1963). [e] Asn solutions in TMAO and the mixture of TMAO and urea became yellow-colored during the 24-h incubation at 25.10 °C.

oxidizes. The data in Table 1 are used to calculate the molar transfer free energy, as described below.

Transfer Free Energies of Amino Acids, Amino Acid Side Chains, and DKP from Water to 1 M TMAO, to 2 M Urea, and to a Mixture of 1 M TMAO and 2 M Urea. The standard transfer free energy ($\Delta G°_{tr}$) of an amino acid from water (w) to a solution containing a substantial amount of solute (s) may be calculated from the activities of the amino acid at the solubility limits in the two solvents (a_w and a_s) as given in eq 2 (Liu & Bolen, 1995; Tanford, 1964). The

$$\Delta G_{tr} = RT \ln \frac{a_w}{a_s} \quad (2)$$

$$\Delta G_{tr} = RT \ln \frac{C_w}{C_s} + RT \ln \frac{\gamma_w}{\gamma_s} \quad (3)$$

activity of an amino acid can also be represented as the product of the amino acid concentration (C) and activity coefficient (γ), as in eq 3. Because activity coefficients of amino acids in three- or four-component system are unavailable and estimates show $RT \ln(\gamma_w/\gamma_s)$ to be small (Nozaki & Tanford, 1963), we used only the first term on the right side of eq 3 for evaluation of the *apparent* transfer free energies of amino acids (ΔG_{tr}) from water to the solute-containing solutions listed in Table 2. From these data, the apparent transfer free energy (Δg_{tr}) for an amino acid side chain is obtained by subtracting ΔG_{tr} of glycine from ΔG_{tr} of the amino acid of interest (Nozaki & Tanford, 1963, 1970). The Δg_{tr} for the peptide backbone unit is obtained by determining the transfer free energy of the cyclic glycylglycine dipeptide, diketopiperazine (DKP), and dividing the result by 2. We've used DKP as a model for the peptide backbone in other studies and find that the peptide backbone transfer free energy falls well within the range of values obtained with other peptide backbone models (Liu & Bolen, 1995). The ΔG_{tr} and Δg_{tr} values are listed in Table 2.

Predicted Free Energy Profiles of Proteins. Apparent transfer free energies of amino acid side chains and the

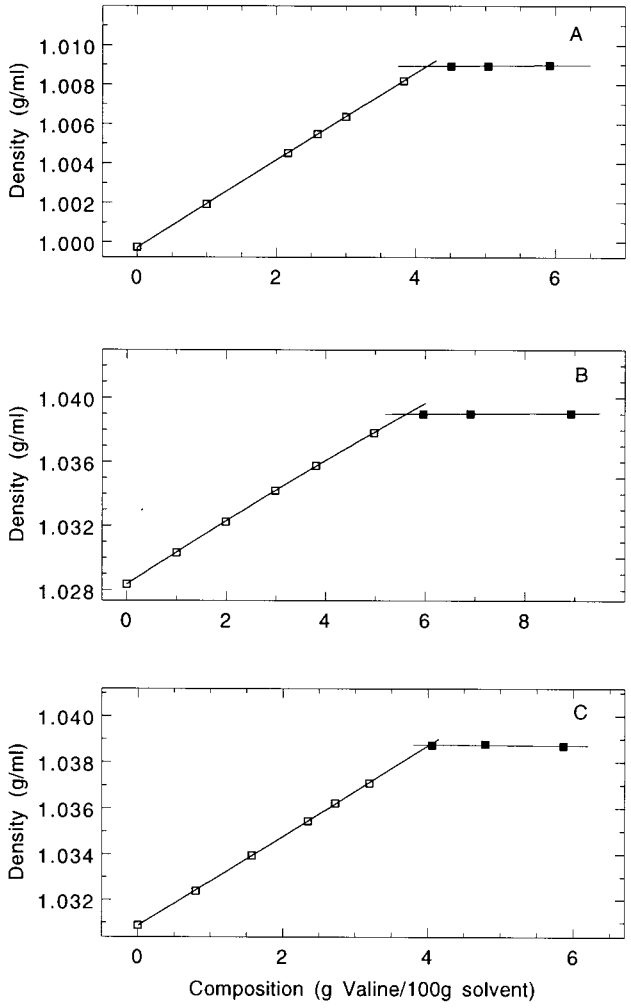


FIGURE 1: Representative solubility profiles of valine in 1 M TMAO (panel A), in 2 M urea (panel B), and in a mixture of 1 M TMAO and 2 M urea (panel C) at 25.10 °C. The solid lines are polynomial best fits of the unsaturated (□) and saturated (■) data. The solubility limits and the densities at the solubility limit are obtained at the intersection of the fitted lines, and are reported in Table 1.

Table 2: Apparent Transfer Free Energies from Water to Various Solute-Containing Solutions (kcal/mol)

	ΔG_{tr}			Δg_{tr}		
amino acid	to 1 M TMAO	to 2 M urea	to 1 M TMAO + 2 M urea	to 1 M TMAO	to 2 M urea	to 1 M TMAO + 2 M urea
Trp	42.5	−232	−143	−140	−269	−378
Phe	170	−132	14.6	−12.0	−169	−220
Leu	191	−74.4	86.5	9.0	−112	−149
Ile	157	−39.4	135	−25.0	−76.6	−100
Val	181	−5.89	182	−1.0	−43.1	−53.0
Met	169	−65.1	91.4	−13.0	−102	−144
Ala	169	29.6	212	−13.0	−7.67	−23.0
His	224	−63.6	158	42.0	−101	−77.0
Thr	186	−6.20	194	4.0	−43.4	−41.0
Ser	144	−2.83	137	−38.	−40.0	−98.0
LysHCl	72.1	−8.09	59.3	−110	−45.3	−176
ArgHCl	73.0	−4.92	70.0	−109	−42.1	−165
NaGlu	99.1	38.9	181	−82.9	1.70	−54.0
NaAsp	116	44.6	147	−66.0	7.40	−88.0
Gln	239	−57.1	187	57.0	−94.3	−48.0
Asn	212	−65.8	109	30.0	−103	−126
Pro	44.4	1.89	44.9	−138	−35.3	−190
Gly	182	37.2	235	0.00	0.00	0.00
DKP	177	−138	46.9			
DKP/2				88.5	−69.0	23.5

[a] ΔG_{tr} = amino acid transfer free energy. [b] Δg_{tr} = side chain transfer free energy.

backbone from water to a solvent can be used to predict the transfer free energy of the native (ΔG_4) or unfolded (ΔG_2) states of a protein ($\Delta G_{2\text{ or }4}$, eq 4) under the assumption that

$$\Delta G_{2\text{ or }4} = \sum n_i f_i \Delta g_i \qquad (4)$$

the transfer free energy of the whole protein molecule can be estimated from the sum of transfer free energy contributions of its component parts (Liu & Bolen, 1995; Tanford, 1964). To evaluate the transfer free energy of the native state of a protein such as RNase T1, the static accessible surface area of the native protein is determined using an algorithm originating from Lee and Richards (1971) and adapted by Lesser and Rose (1990), and the fractional exposure of each amino acid side chain and backbone is evaluated. The area of a partially exposed amino acid side chain *i* is averaged over the total area of all such side chains in the protein to give the fractional area f_i. This is multiplied by the side chain transfer free energy and the number of residues *i* in the protein as described in eq 4. These quantities for native RNase T1 are shown in Figure 2A. The same procedure is carried out for peptide backbone exposure (also shown in Figure 2A) and the sum of backbone and side chain contributions is taken to represent the transfer free energy of native RNase T1 from water to the solute-containing solution of interest as illustrated in Table 3. The same process is repeated with the fully extended protein sequence to represent the unfolded state of RNase T1 (Figure 2B), permitting estimation of the free energy of transfer of the unfolded state also given in Table 3. For comparison, the experimentally determined values for such transfers are given in parentheses (Lin & Timasheff, 1994).

The results of Figure 2 and Table 3 show that TMAO stabilizes proteins and helps to identify the relative roles played by side chains and backbone in protein stabilization by TMAO alone. It has long been known that urea promotes denaturation, so the question arises as to how a mixed solution of urea and TMAO affects protein stability. There are numerous reports that the effects of TMAO and urea in

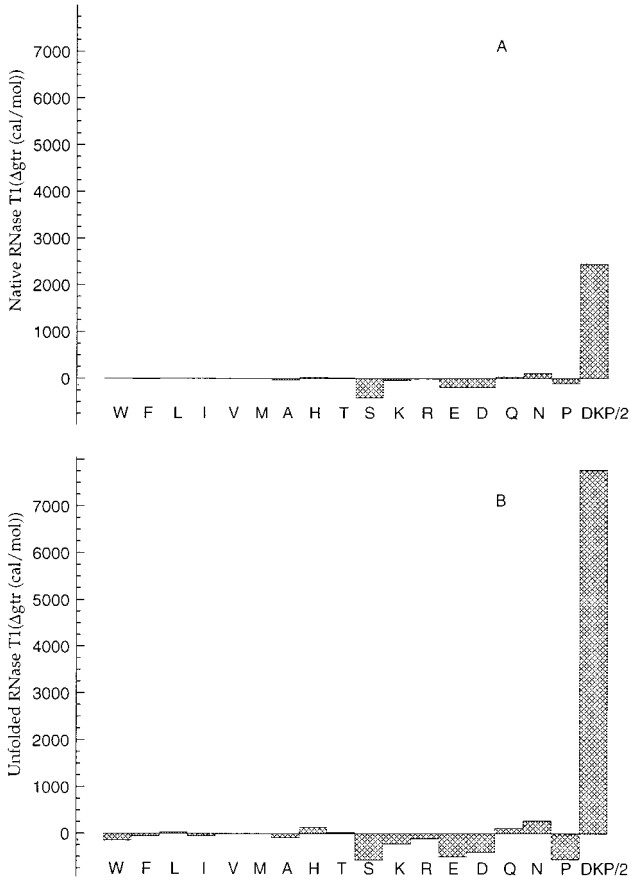


FIGURE 2: Side chain and backbone contribution to the transfer free energy of RNase T1 from water to 1 M TMAO. Panel A shows free energy contributions of the various amino acid side chains and backbone (DKP/2) exposed in native RNase T1 using eq 4. Panel B provides data on contributions to unfolded RNase T1 using eq 4.

combination appear to be the algebraic sum of their effects individually on both the structure and function of the protein (Gopal & Ahluwalia, 1993; Lin & Timasheff, 1994; Yancey et al., 1982; Yancey & Somero, 1979, 1980). Figure 3

Table 3: Transfer Free Energies of Native and Unfolded RNase T1 from Water to Various Solute-Containing Solutions (kcal/mol)[a]

	to 1 M TMAO		to 2 M urea		to 1 M TMAO in 2 M urea	
free energy contributions	native	unfolded	native	unfolded	native	unfolded
$\Delta G_2 - \Delta G_4$	1.7 (1.2)	5.9 (5.4)	−3.2 (−8.4)	−10.6 (−12.0)	−1.9	−5.6
from backbone	2.5	8.0	−1.9	−6.3	0.7	2.1
from side chains	−0.8	−2.1	−1.3	−4.3	−2.5	−7.7
$\Delta G_3 - \Delta G_1$	4.2 (4.2)		−7.4 (−3.6)		−3.7	
from backbone	5.5		−4.4		1.5	
from side chains	−1.3		−3.1		−5.2	

[a] Values in parentheses are quantities experimentally determined by Lin and Timasheff (1994). $\Delta G_2 - \Delta G_4 = \Delta G_3 - \Delta G_1$.

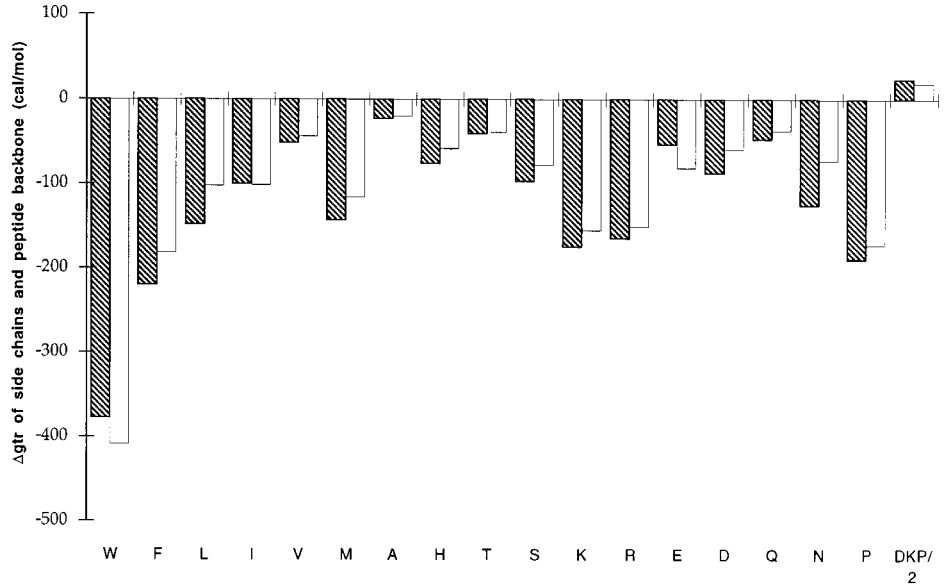


FIGURE 3: Apparent transfer free energies (cal/mol) of amino acid side chains and the peptide backbone (modeled by DKP/2) at 25.10 °C. Cross-hatched bars represent the transfer free energy of side chain or backbone transferred from water to a mixture of 1 M TMAO and 2 M urea. Open bars represent the result of adding the free energy contribution of backbone or a particular side chain on transfer from water to 1 M TMAO with that for transfer from water to 2 M urea. That is, adding the free energy entry in column 5 of Table 2 with the entry in column 6 for that particular side chain.

provides a comparison of the transfer free energies of side chains and backbone from water to a combined solution of 1 M TMAO plus 2 M urea, with that contributed from the sum of the transfers to 1 M TMAO and to 2 M urea. The results appear to show rough additivity for the individual parts of a protein.

Finally, Figure 4 shows the contributions of side chains and backbone to the difference in the free energy for transfer of unfolded and native RNase T1, ($\Delta G_2 - \Delta G_4$) from water to the solute solutions of interest. The figure also compares RNase T1 protein stability estimated for unfolding the protein in 1 M TMAO plus 2 M urea with that estimated from additivity of the unfolding in 1 M TMAO and in 2 M urea.

DISCUSSION

The transfer model has been a fixture in biophysical chemistry since at least the 1930s (McMeekin et al., 1935, 1936), and it has contributed prominently to the concept of hydrophobic interactions as well as to understanding protein denaturation and protein folding (Cohn & Edsall, 1943; Kauzmann, 1959; Tanford, 1964). For purposes of our discussion, it is useful to consider a thermodynamic cycle that provides an experimental means of using transfer free energies to understand solute-induced protein stabilization and destabilization. The horizontal reactions given in Scheme 1 represent native to unfolded equilibria in the presence of water (w) and solute (sol) with respective free energy changes ΔG_1 and ΔG_3. The vertical reactions represent the transfer of native and unfolded protein from water to the solute-containing solution, characterized respectively by ΔG_4 and ΔG_2. Because $\Delta G_3 - \Delta G_1 = \Delta G_2 - \Delta G_4$, the difference in protein stability in water versus stability in solute ($\Delta G_3 - \Delta G_1$) can be evaluated from knowledge of the transfer free energies of unfolded and native states from water to solute-containing solution ($\Delta G_2 - \Delta G_4$).

The results of evaluation of the transfer free energy of native and unfolded RNase T1 from water to 1 M TMAO (Figure 2) suggest which molecular interactions are important in the stabilization of proteins by TMAO. It is clear with both native and unfolded protein that the unfavorable interaction of the peptide backbone with TMAO dominates the free energy contributions of the side chains. It is also clear that the hydrophobic amino acid side chains do not contribute energetically either to the native or the unfolded state of RNase T1, rather, it is the interactions of TMAO with the polar and charged groups that make up most of the side chain contributions.

Table 3 suggests how the unfavorable interaction of TMAO with peptide backbone contributes to protein stability. The data show that it is unfavorable to transfer the native state from water to 1 M TMAO by 1.7 kcal/mol, but it is much more unfavorable (5.9 kcal/mol) to transfer the unfolded state. Thus, in TMAO the native to unfolded

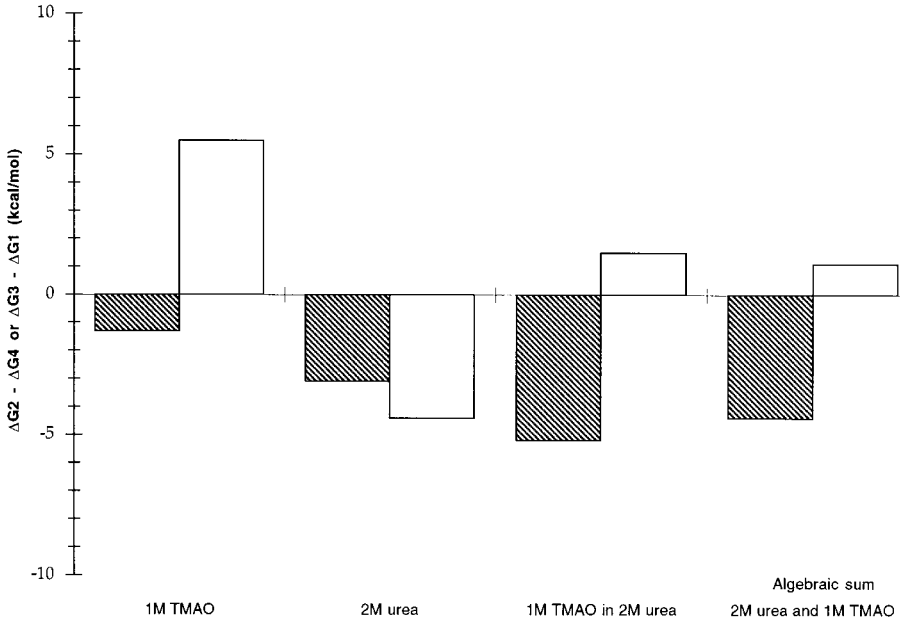
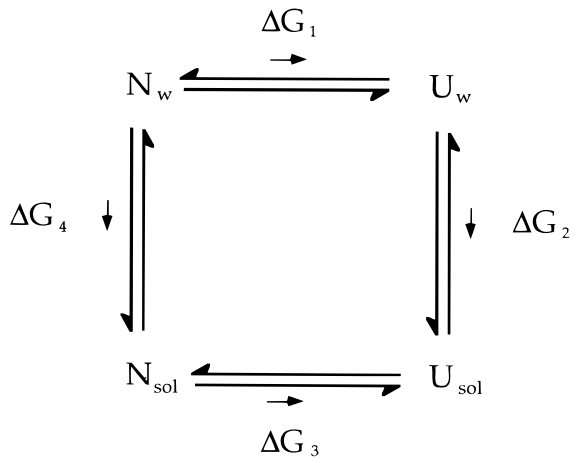


FIGURE 4: Difference in free energy of transfer of unfolded (ΔG_2) and native (ΔG_4) RNase T1 from water to various solutions. Results are given in terms of side chain (cross-hatched bars) and backbone (open bars) contributions, to the differences in free energy of transfer ($\Delta G_2 - \Delta G_4$) from water to 1 M TMAO (first pair of bars), water to 2 M urea (second pair of bars), water to 1 M TMAO in 2 M urea (third pair of bars), and from water to the algebraic sum of the first two pairs of bars. From the thermodynamic cycle, $\Delta G_3 - \Delta G_1 = \Delta G_2 - \Delta G_4$.

Scheme 1



conversion is 4.2 kcal/mol less favorable than it is in water, and it is the unfavorable interaction of TMAO with the backbone that is responsible for the stabilization. The side chains play no role in stabilization, in fact, the small contribution from the side chains actually promotes unfolding. Considering the errors involved, the unfavorable transfer of the native state as estimated by the transfer model (1.7 kcal/mol) compares well with the 1.2 kcal/mol transfer free energy measured by Lin and Timasheff (1994) using preferential interaction data. And the 5.4 kcal/mol transfer free energy of unfolded RNase T1 from water to 1 M TMAO determined by Lin and Timasheff compares favorably with the value (5.9 kcal/mol) determined using the transfer model. From their transfer free energy values for native and unfolded protein, Lin and Timasheff show RNase T1 is 4.2 kcal/mol more stabilized in 1 M TMAO than it is in water. This is identical to the stability ($\Delta G_3 - \Delta G_1$) obtained using the transfer model.

An unfavorable transfer of native or denatured state from water to TMAO means that, on the whole, the native or denatured state of the protein prefers to interact more with water than with TMAO, that is, the transfer model predicts preferential hydration of both the native and the denatured states of RNase A in the presence of TMAO and provides a molecular rationale for the origin of preferential hydration while identifying the major chemical moiety (backbone) responsible for the ability of TMAO to stabilize proteins.

When 2 M urea is used as a solute, the transfer free energies from the model and experiment do not agree well for native state transfer but agreement is acceptable for transfer of the unfolded state (see Table 3). Perhaps part of the reason the values for native state transfer don't agree more closely has to do with the constraints imposed on the transfer model. In the transfer model, we treat the native state as an impenetrable object in evaluating the surface area exposed, but experimentally the native state can be penetrated by denaturant. Hydrogen exchange experiments in denaturants indicate that the native state becomes penetrable by solvent at concentrations of denaturant in the predenaturational range (Bai et al., 1995; Chamberlain et al., 1996; Mayo & Baldwin, 1993; Qian et al., 1994). Experimentally determined preferential interaction measurements in 2 M urea then, should reflect additional interaction free energy as a result of solvent penetration that is not accounted for in the transfer model. An additional point to be made from the 2 M urea data in Table 3 is that the backbone interaction with urea again dominates the side chains, but unlike TMAO the interaction of backbone with urea is thermodynamically favorable.

The midpoint of urea denaturation of RNase T1 with both of its disulfide bonds intact is 4.3 M urea (Pace et al., 1988). This means that 2 M urea should not unfold this protein. The reason that the transfer model and the measurements of Lin and Timasheff show RNase T1 to be unfolded in 2 M urea is that in both these analyses, the model of the denatured state has no disulfide bonds. The transfer model deals only with interactions and makes no allowances for entropic

effects that disulfide bonds would have on the energetics of protein folding. Consequently, application of the transfer model will always treat the N ⇄ U equilibrium as involving no cross-links in the denatured state. In the case of Lin and Timasheff's preferential interaction measurements involving the unfolded state of RNase T1, the measurements were performed on reduced and carboxymethylated RNase T1, a species at 25 °C that exists as an unfolded state even in the absence of urea (Lin & Timasheff, 1994). Pace et al. provide urea-induced unfolding data on RNase T1 with zero, one, and two disulfide bonds from which one can estimate an unfolding free energy change of −4.5 to −6.6 kcal/mol for native to unfolded conversion in 2 M urea, with the unfolded state having no intact disulfide bonds (Pace et al., 1988). The estimates of Pace et al. fall between our evaluation of −7.4 and Lin and Timasheff's value of −3.6 kcal/mol for the free energy change for unfolding in 2 M urea.

Table 3 and Figure 4 show that the major difference between the free energy of RNase T1 unfolding in 2 M urea and in a mixture of 2 M urea and 1 M TMAO is in the contributions from the backbone. The presence of 1 M TMAO in 2 M urea causes a net unfavorable interaction between the combined solutes and the peptide backbone. Because the side chains contribute even more favorably to unfolding in the TMAO−urea mixture than in urea alone, any stabilization of the protein in the mixture is solely due to the contributions from the backbone. Thus, the origin of the stabilization arises from a source (backbone) almost never considered of importance for protein folding in water, and the side chains (which are of paramount importance to protein folding in water) are a minor factor that contribute unfavorably to net protein stabilization.

Due to technical reasons, Lin and Timasheff were unable to evaluate the free energy contribution of TMAO to RNase T1 unfolding in the presence of a 2 M urea:1 M TMAO mixture (Lin & Timasheff, 1994). But they estimate that the presence of 1 M TMAO in 2 M urea will stabilize RNase T1 by 3.1 kcal/mol. The transfer model shows that in the mixture of 2 M urea and 1 M TMAO, RNase T1 is stabilized by 3.7 kcal/mol relative to RNase T1 stability in 2 M urea alone. Consequently, in all cases except for transfer of native RNase T1 from water to 2 M urea, there is good agreement between the values obtained using the transfer model and those provided by Lin and Timasheff through preferential interaction measurements.

The problem with the transfer model is that there are several points at which the model could break down. Some of these include the validity of subtracting the transfer free energy of glycine from other amino acids to obtain side chain transfer free energies, the validity of assuming group additivity, the validity of assuming full exposure of all groups in the unfolded state, the approximation of ignoring electrostatic effects and of ignoring activity coefficients in (eq 3) in evaluating amino acid transfer free energies. But despite these possible problems, the agreement between the free energy results from the transfer model and experimentally determined values is quite reasonable, at least within a range which gives confidence that the major factors responsible for stabilization by osmolytes can be identified from transfer data.

It has been observed that stabilizing effects of TMAO and destabilizing effects of urea are approximately additive (Gopal & Ahluwalia, 1993; Lin & Timasheff, 1994; Yancey & Somero, 1979). The results in Figure 4 show the side chain and backbone contributions to RNase T1 stability in 1 M TMAO, 2 M urea, a mixture of 1 M TMAO + urea, and the stability calculated by adding the contributions from 1 M TMAO and 2 M urea individually. It is clear from the last four columns in Figure 4 that rough additivity holds. The additivity is reflected at the level of individual side chain transfer free energies as seen in Figure 3. This latter figure shows that in most cases the side chain transfer free energy determined for the combined TMAO−urea mixture is greater than the sum of transfer free energies derived from 2 M urea and 1 M TMAO solutions individually. Figures 3 and 4 and Table 3 show that, to a large extent, TMAO and urea act rather independently in terms of their effects on side chains and backbone. These results are consistent with experimental observations on additive effects of urea and TMAO on the Tms of proteins (Gopal & Ahluwalia, 1993; Lin & Timasheff, 1994; Yancey & Somero, 1979).

TMAO has recently been shown to offset the denaturing effects of urea, and on the basis of the presence of methyl groups in TMAO, it has been proposed that this osmolyte interacts favorably with the hydrophobic side chains in unfolded RNase T1 and unfavorably with polar regions of the protein (Gopal & Ahluwalia, 1993; Kita et al., 1994; Lin & Timasheff, 1994). The transfer free energy data of Table 2 show, however, that TMAO has no particular propensity to interact with the hydrophobic residues, and in fact, interacts most favorably with charged and polar side chains to give an overall favorable interaction between side chains and TMAO. Though the histidine side chain and (amide-containing) side chains of asparagine and glutamine interact unfavorably with TMAO, it is the (amide-containing) backbone, by its unfavorable interaction with TMAO, that is responsible for the ability of this osmolyte to protect proteins. The indifference of hydrophobic residues in contributing to stabilization or unfolding in two other osmolytes, sucrose and sarcosine, has been noted previously, so the phenomenon is not peculiar to TMAO (Liu & Bolen, 1995). Thus, attempts to explain the effects of TMAO by invoking concepts of hydrophobicity are not supported by the free energy data involving transfer of amino acid side chains to this osmolyte. With respect to protein folding in water, hydrophobicity is the force of dominant importance, with hydrophobic side chains being responsible for this force. What is suggested by the transfer free energy measurements is that hydrophobicity and side chains are not the focal points in the stabilization of proteins by the naturally occurring osmolytes; rather, the backbone becomes the structural component of importance and its unfavorable interaction with osmolyte becomes the basis for the protein stabilization.

Intracellular proteins in urea-rich cells are said to have the same susceptibility to the deleterious effects of urea as the proteins in cells without urea (Yancey et al., 1982). To be evolutionarily advantageous, a solute such as TMAO that will offset the effects of urea on all proteins must oppose the structural changes induced by urea. So ideally, TMAO should affect one or more structural features of the protein that are characteristically altered on urea denaturation of any protein and the degree of exposure of this feature (or features) is changed upon urea-induced denaturation. Potentially, both the peptide backbone and side chains qualify as structural features fitting these requirements. But the advantage of using backbone interactions with TMAO to achieve protein

stabilization in urea rather than using side chains, is that stabilization would be uniformly applied to all proteins, regardless of how hydrophobic or hydrophilic the protein might be. In addition, because protein folding in water is driven by hydrophobic collapse with side chain interactions playing the dominant role in the process, by not involving the side chains in the mechanism of stabilization the rules for proper folding of a protein in the cellular environment are less likely to be compromised. The natural selection of a counteracting osmolyte in urea-rich cells appears to be a remarkably simple means of evolutionary adaptation, with stabilization deriving from a structural entity (peptide backbone) seldom considered to be of importance in the conventional paradigm of protein folding.

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