The Peptide Backbone Plays a Dominant Role in Protein Stabilization by Naturally Occurring Osmolytes†

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ABSTRACT: Transfer free energy measurements of amino acids from water to the osmolytes, sucrose and sarcosine, were made as a function of osmolyte concentration. From these data, transfer free energies of the amino acid side chains were obtained, and the transfer free energy of the peptide backbone was determined from solubility measurements of diketopiperazine (DKP). Using static accessible surface evaluations of the native and unfolded states of ribonuclease A, solvent exposed side chain and peptide backbone areas were multiplied by their transfer free energies and summed in order to evaluate the transfer free energy of the native and unfolded states of the protein from water to the osmolyte solutions. The results reproduced the main features of the free energy profile determined for denaturation of proteins in the presence of osmolytes. The side chains were found collectively to favor exposure to the osmolyte in comparison to exposure in water, and in this sense the side chains favor protein unfolding. The major factor which opposes and overrides the side chain preference for denaturation and results in the stabilization of proteins observed in osmolytes is the highly unfavorable exposure of polypeptide backbone on unfolding. Except for urea and guanidine hydrochloride solutions, it is shown that all organic solvents (e.g., dioxane, ethanol, ethylene glycol) and solutes (osmolytes) for which transfer free energy measurements have been determined exhibit unfavorable transfer free energy of the peptide backbone. The prevalence of the unfavorable transfer free energy of the peptide backbone, and the fact that the peptide backbone unit is the most numerous group in a protein, makes it an extremely important factor in influencing the character of protein conformations existing in both stabilizing and destabilizing solvents.

Many organisms that have adapted to environmental stresses, such as high temperature, high salt environment, and desiccation, produce small organic molecules which protect their cellular components from the denaturing stresses. These organic molecules, known as osmolytes, are chemically diverse and include such chemical classes as polyols, certain amino acids, and methylamine compounds (Yancey et al., 1982). Osmolyte protection of macromolecular components of the cell against denaturing stresses such as high temperature can be extraordinary, resulting in increases in the thermal denaturation transition temperature of proteins of greater than 20 °C (Santoro et al., 1992). The ability of osmolytes to protect against denaturing stresses is of interest from the viewpoint not only of increasing shelf life of macromolecules, but also in identifying and understanding the principles involved in the protection of macromolecules by such chemically diverse classes of osmolytes.

How are osmolytes able to stabilize proteins against environmental stresses? A major contribution in answering this question comes from the elegant work of Timasheff and colleagues (Arakawa et al., 1990b; Arakawa & Timasheff, 1982a,b, 1983, 1984a−c, 1985; Lee & Timasheff, 1981; Lee & Lee, 1987; Timasheff, 1993, 1994). Over the last 15 years, these authors have shown that naturally occurring osmolytes, and several other compounds and ions which protect proteins against denaturing stresses, are preferentially excluded from the vicinity of the protein. That is, in the zone immediately surrounding the protein, the concentration of osmolyte in this volume element will be less than its concentration in the bulk solvent. By default, the preferential exclusion of the osmolyte means that water concentration in this volume element will be higher than it is in the bulk solvent. Thus, preferential exclusion of osmolyte is equivalent to saying that the protein is preferentially hydrated.

Preferential exclusion of osmolyte (or preferential hydration) is evaluated by thermodynamic means, from which it is possible to show that the presence of osmolyte in the solution increases the chemical potential of the native state of the protein, i.e., osmolyte destabilizes the native state of the protein (Arakawa et al., 1990a). The reason that osmolyte protects proteins against denaturing stresses is that it destabilizes the denatured state much more than it destabilizes the native state, making denaturation more unfavorable in the presence of osmolyte than in water alone (Arakawa et al., 1990a; Lee & Timasheff, 1981). The factor cited to be most important in stabilization of proteins by osmolytes is the increase in surface tension brought about by addition of osmolyte to a solution of protein (Arakawa & Timasheff, 1982b; Lee & Lee, 1981). Solutes which increase the surface tension of water are ones whose concentrations at the air−water interface are depleted relative to their concentration in the bulk solvent (Lewis et al., 1961). Thus, there is a direct parallel between depletion of solute at the air−water interface (surface tension effect) and

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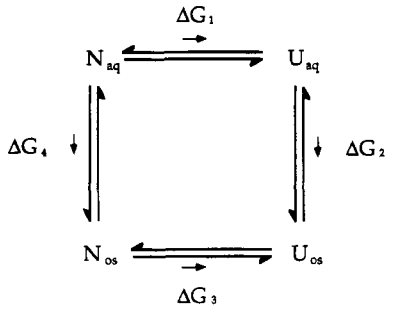
Scheme 1



depletion of solute in the vicinity of the protein (preferential hydration effect). Physically, a solute which increases surface tension will oppose an increase in surface area. This effect is also believed to apply at the molecular level, such that reactions which involve an increase in surface area in aqueous solution will be unfavorable in the presence of osmolyte (Arakawa & Timasheff, 1982b; Lee & Timasheff, 1981; Sinanoglu & Abdulnur, 1964). By definition, the denatured state of a protein exposes a large surface area, so denatured protein would be highly unfavorable (highly destabilized) in the presence of osmolyte.

While the surface tension argument is useful in explaining the overall thermodynamics of stabilization in physical terms, it provides no chemical insight into the causes of the destabilization of both the native and denatured states. To better understand the origin of the thermodynamic effects of osmolyte on stability, we have attempted to evaluate the interaction between osmolytes and exposed chemical groups on native and unfolded proteins. Our approach to this problem is embodied in the thermodynamic cycle described in Scheme 1 and is identical in kind to the approach of Nozaki and Tanford in their effort to understand the protein denaturing effects of urea and guanidine hydrochloride (Nozaki & Tanford, 1963, 1970; Tanford, 1964). Reaction 1 describes the equilibrium unfolding reaction for a protein in aqueous solution, while reaction 3 gives the identical reaction in the presence of osmolyte. The sign and magnitude of the free energy difference between these two reactions ($\Delta G_3 - \Delta G_1$) provide a basis for determining the extent to which protein unfolding is reduced in the presence of osmolyte. The vertical reactions ΔG_2 and ΔG_4 represent the transfer free energy of the unfolded and native state of the protein from water to osmolyte. Since $\Delta G_3 - \Delta G_1 = \Delta G_2 - \Delta G_4$, it is possible to predict the degree of stabilization of protein in osmolyte relative to that in water (i.e., $\Delta G_3 - \Delta G_1$), from evaluation of the transfer free energies of the native and unfolded states ($\Delta G_2 - \Delta G_4$). The object of this report is to use transfer free energy data for the purpose of identifying the roles of the major functional groupings in stabilizing and/or destabilizing the native and unfolded forms of the protein.

We imagine the surface of a protein to be a mosaic of chemical groups with differential affinity for the two solvent components, water and osmolyte. Some protein functional groups will prefer to interact with water, others will tend to interact with groups on the osmolyte, and still others will have no particular preference for water or osmolyte. The net interaction free energy of osmolyte for the protein surface, whether it is the native or unfolded form, may be considered as an algebraic sum of transfer free energy contributions of each of protein component groups with osmolyte (Tanford, 1964). Here, we present basic data on the free energy of transfer of amino acids, amino acid side chains, and the peptide backbone unit from water to various concentrations of the osmolytes, sucrose and sarcosine.

MATERIALS AND METHODS

DKP[1] (diketopiperazine), sucrose, guanidine hydrochloride, and all amino acids and their salts with purity above 99% were purchased from Sigma Chemical Co. and used without further purification. Sarcosine, also from Sigma Chemical Co., with a purity of 98% was crystallized once from water. In order to maintain pH as uniform as practical considering the zwitterionic character of the amino acids, sodium salts of glutamic and aspartic acids and hydrochlorides of lysine and arginine were used in place of the acidic and basic forms of these amino acids.

Solubility measurements were performed in a manner similar to that of Nozaki and Tanford (1963). To eight glass vials, each containing a fixed weight of solvent, was added weighed amounts of an amino acid to provide a series of mixtures with increasing amino acid mass. Each vial was fitted with a Teflon-lined screw cap to produce a watertight seal. The weighed samples were prepared such that approximately four vials would ultimately result in unsaturated solutions and the remaining four in saturated. In the case of solubilities determined in 2, 4, and 6 M sarcosine as solvent, the vials containing the compositions were strapped to a rotating drum in an air bath (LKB Batch Microcalorimeter) maintained at 25.0 ± 0.1 °C. The air bath was housed in a constant temperature room also maintained at 25 °C. The drum was rotated at a rate such that the contents of the vials were inverted about once a minute. After 40 h the rotation was stopped, the supernatant of each vial was removed and microfuged, and the density was measured using an Anton-Parr DMA-602 densimeter, all at 25 °C. For tyrosine and tryptophan, the absorbances of the supernatants were determined in place of density measurements, and solubilities were determined from a plot of absorbance vs vial composition. Solubilities of all other amino acids and amino acid salts were determined from plots of density vs vial composition. The results of amino acid solubilities in aqueous solution were found to agree very well with data reported by Nozaki and Tanford (1963). Seldom did the solubilities disagree by more than 2%.

Solubility data in 0.5 and 1.0 M sucrose at 25.0 °C were determined in the same manner as with sarcosine except that a Tronac water bath equipped with a submerged reciprocating (30 cycles/min) shaker platform was used in place of the air bath. The water bath maintained temperature control at 25 °C to better than ±0.001 °C, and the vials, each between half and two-thirds filled, were constantly mixed by placing each vial horizontally in the direction of the back and forth reciprocating motion of the platform. After 40−48 h, the vials were wiped dry and placed in a vertical position, and solution from each vial was withdrawn through a separate syringe each equipped with a glass fiber filter. The extracted solutions were kept at 25.1 °C, and the solution density was determined for each amino acid and DKP at 25.0 °C. The solubilities of tyrosine and tryptophan in sucrose solutions were determined spectrophotometrically as described above.

[1] Abbreviations: ribonuclease A, RNase A; guanidine hydrochloride, GdnHCl; diketopiperazine, DKP.

From the solubility limits, expressed in the molar concentration scale, the transfer free energy data were calculated as described in the Results section.

RESULTS

The free energy of transfer of an amino acid from one solvent system to another can be based on the solubility of that amino acid in the two solvent systems (Edsall & Wyman, 1958; Nozaki & Tanford, 1963). At the solution concentration representing the solubility limit of the amino acid in the solvent of interest, two phases are in equilibrium, the crystalline amino acid and the solution phase. At equilibrium, the chemical potential of the amino acid in the crystalline phase must be equal to the chemical potential of the amino acid in solution. Since the chemical potential of an amino acid in its crystal is invariant, regardless of the solvent with which it is in contact, and since this chemical potential is the same in both solvent systems, then the chemical potential of the amino acid at the saturation point in one solvent must equal its chemical potential at the saturation point in the second solvent (Nozaki & Tanford, 1963). Equating the two chemical potentials and expanding the relationship in terms of standard chemical potentials and activities of the amino acids in the two solvents (water and osmolyte solution) gives:

$$\Delta\mu^\circ = \Delta G^\circ_{tr} = RT \ln(a_w/a_{os}) \quad (1)$$

Here, ΔG°_{tr} represents the difference in unit molar standard state chemical potentials of the amino acid transferred from water to osmolyte solution, and a_w and a_{os} are the chemical activities of amino acid at the point of saturation of the amino acid in water and osmolyte, respectively. Equation 1 can be expressed in terms of molar concentrations (C_w and C_{os}) and activity coefficients (γ_w and γ_{os}), as given in eq 2. It

$$\Delta G_{tr} = RT \ln (C_w/C_{os}) + RT \ln (\gamma_w/\gamma_{os}) \quad (2)$$

should be noted that the transfer free energies reported by Tanford and Nozaki are based upon the mole fraction scale rather than the molar scale that we are using (Nozaki & Tanford, 1963, 1965, 1971).

Obtaining activity coefficients for amino acids in aqueous osmolyte mixtures (three components) is extremely difficult and has rarely been reported (Schönert & Stroth, 1981; Uedaira, 1972, 1977). Two approaches have been taken in dealing with the activity coefficient problem in studies involving transfer free energy measurements, but in the final analysis both of the approaches end up evaluating the transfer free energy change by use of only the first term on the right of eq 2 (Nozaki & Tanford, 1963, 1970; Robinson & Jencks, 1965a). There have been some efforts to estimate the contribution due to activity coefficients in different solvent systems (Nozaki & Tanford, 1963, 1965, 1971). However, this practice was not continued in later work (Nozaki & Tanford, 1970, 1971). Taking the lead of workers who have evaluated transfer free energies in a variety of aqueous organic solvents, we are forced to ignore the activity coefficient term on the right side of eq 2 and declare the quantities reported here as apparent transfer free energies (Lapanje et al., 1978; Nozaki & Tanford, 1963, 1965, 1970, 1971; Pittz & Bello, 1971; Tanford, 1970).

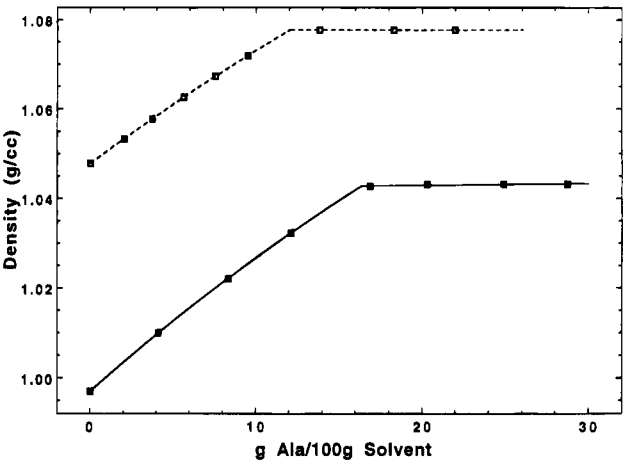


FIGURE 1: Densities of supernatants of alanine in 2 M sarcosine (□) and water (■). The supernatants were extracted from vials containing the compositions given. The contents of the vials had been continuously mixed for 40−48 h at 25 °C.

Amino acid solubility limits in water and in aqueous osmolyte solutions were determined by evaluation of the solution phases of several mixtures, each containing increasing amounts of crystalline amino acid added to a fixed amount of solvent, as described in the Materials and Methods. To ensure equilibrium solubilization, the sealed vials containing the mixtures were placed in a constant temperature bath for 24−48 h and gently agitated as described. The density of solution obtained from each vial was plotted as a function of the composition of the vial and the solubility limit of the amino acid determined at the point of intersection in the solubility plot as illustrated in the example in Figure 1. The solubilities of amino acids reported in Table 1 are given in grams of amino acid soluble in 100 g of each solvent, water, and 0.5 and 1.0 M sucrose. Table 2 provides amino acid solubility data in the solvents 2, 4, and 6 M sarcosine. The apparent transfer free energy changes for amino acid from water to the particular osmolyte solutions of interest are calculated from eq 2, taking the activity coefficient ratio as 1. Also listed in Tables 1 and 2 are the transfer free energy changes for amino acid side chains (Δg_{tr}). These quantities are obtained in the manner of Nozaki and Tanford, viz., by subtracting ΔG_{tr} of glycine from ΔG_{tr} of the amino acid (Nozaki & Tanford, 1963, 1965, 1971). We have used diketopiperazine (DKP), also known as cyclic glycylglycine, as a model for the peptide backbone in a protein. Transfer free energy changes for DKP in sucrose and sarcosine were evaluated, and these quantities, which are presented at the bottoms of Tables 1 and 2, need to be divided by 2 to get the transfer free energy on a per mole peptide backbone unit basis.

Graphical representations of the side chain and backbone free energy changes in the two osmolytes are given in Figures 2 and 3. The hydrophobic amino acid side chains are displayed in order of decreasing hydrophobicity to illustrate the magnitude and dependence of side chain transfer free energy on the hydrophobicity scale; polar and charged amino acid side chains are also provided in the figures.

According to Scheme 1, the transfer free energy change for the native and unfolded states of a protein can be estimated from the sum of free energy contributions of the component parts of the protein (Tanford, 1964). By way of

Table 1: Amino Acid Solubilities and Apparent Transfer Free Energies from Water to Sucrose Solutions

	solubility (g of AA/100 g solvent)			density at solubility limit			ΔG_{tr} (cal/mol) water to sucrose		Δg_{tr} (side chain) water to sucrose	
		sucrose			sucrose					
AA	water	0.5 M	1.0 M	water	0.5 M	1.0 M	0.5 M	1.0 M	0.5 M	1.0 M
Gly	25.1	20.7	16.9	1.08302	1.12996	1.17770	67.8	144.5		
Ala	16.6	13.4	10.8	1.04295	1.09623	1.14994	80.9	166.6	13.1	22.1
Phe	2.82	2.42	2.30	1.00528	1.06772	1.13057	52.6	48.2	−15.2	−96.3
Trp	1.33	1.27	1.34	1.01	1.06591	1.12974	−4.90	−70.8	−72.7	−215.3
His	4.30	3.94	3.64	1.01206	1.07546	1.13727	13.8	25.9	−54.0	−118.6
Tyr	0.0469	0.0373	0.0199	0.99705	1.06250	1.12710	98.0	435.1	30.2	290.6
Met	5.75	4.80	4.00	1.01340	1.07429	1.13522	67.1	137.9	−0.70	−6.60
Val	5.73	4.65	3.71	1.00951	1.07057	1.13162	82.9	178.5	15.1	34.0
Ile	3.35	2.75	2.20	1.00345	1.06646	1.12900	77.4	173.6	9.60	28.1
Gln	4.08	3.51	3.02	1.01133	1.07342	1.13531	50.6	102.6	−17.2	−40.8
Thr	9.73	7.95	6.42	1.02896	1.08653	1.14411	77.8	165.3	10.0	20.8
Leu	2.16	1.74	1.40	1.00090	1.06473	1.12804	89.0	181.6	21.2	37.1
Asn	2.76	2.25	2.00	1.00746	1.07077	1.13414	82.0	116.2	14.2	−28.3
Ser	42.9	35.2	28.3	1.12805	1.16468	1.20862	65.2	141.5	−2.7	−3.5
Pro	181.5	149.6	123.5	1.19459	1.20951	1.23541	35.9	71.5	−32.0	−73.5
NaAsp	77.9	66.0	54.9	1.28523	1.30346	1.32472	48.8	107.6	−19.0	−37.3
NaGlu	62.4	53.4	44.7	1.21785	1.24463	1.27334	45.3	102.2	−22.5	−42.8
LysHCl	71.3	59.4	54.1	1.12780	1.15612	1.12010	50.8	104.9	−17.0	−39.6
ArgHCl	85.3	70.3	64.4	1.15905	1.18469	1.22009	51.6	65.3	−16.2	−79.7
DKP	1.68	1.48	1.36	1.00252	1.06688	1.13037	37.1	52.2		

[a] The densities of 0.5 and 1.0 M sucrose are 1.062532 and 1.127100 g/cm^3, cc, respectively. ΔG_{tr} for DKP is reported for the entire molecule. To obtain ΔG_{tr} per mole of peptide bond, divide the number listed by 2.

Table 2: Amino Acid Solubilities and Apparent Transfer Free Energies from Water to Sarcosine Solutions

	solubility (g/100 g of solvent)				density at solubility limit				ΔG_{tr} (cal/mol) water to sarcosine			Δg_{tr} (side chain) water to sarcosine		
		sarcosine				sarcosine								
AA	water	2 M	4 M	6 M	water	2 M	4 M	6 M	2 M	4 M	6 M	2 M	4 M	6 M
Gly	25.1	18.8	13.2	8.98	1.08302	1.10787	1.13402	1.16227	127.2	443.3	485.4			
Ala	16.6	12.0	8.19	4.96	1.04295	1.07768	1.11256	1.14844	149.0	462.3	596.3	21.8	19.0	110.9
Phe[b]	2.82	2.25	1.6		1.00528	1.055	1.09851		101.9	387.5		−25.3	−55.2	
Trp	1.33	1.50	1.59	1.64	1.01	1.06	1.0992	1.14144	−98.9	−42.3	−194.8	−226.1	−485.6	−680.2
His	4.3	3.53	2.54	1.71	1.01206	1.05915	1.10248	1.14382	85.6	366.7	458.9	−41.6	−76.6	−26.5
Tyr	0.0469	0.0393	0.0386	0.033	0.99705	1.04930	1.09570	1.13890	74.4	167.7	129.4	−52.8	−275.6	−356
Met	5.75	4.26	2.9	1.73	1.0134	1.05843	1.09713	1.14243	143.5	452.1	617.6	16.3	8.8	132.2
Val	5.73	3.94	2.36	1.39	1.00951	1.05472	1.09865	1.14069	185.8	567.7	742	58.6	124.4	256.6
Ile	3.35	2.23	1.15	0.77	1.00345	1.05115	1.09684	1.13883	207.1	677.5	781.1	79.9	234.2	295.7
Gln	4.08	3.23	2.28	1.45	1.01133	1.05805	1.10184	1.14299	106.8	398.5	525.2	−20.4	−44.8	39.8
Thr	9.73	7.59	5.39	3.60	1.02896	1.07041	1.10978	1.14784	112.1	404.7	490.2	−15.1	−48.6	4.8
Leu[c]	2.16	1.45	0.872		1.00090	1.04962	1.09630		203.8	584.9		76.6	141.6	
Asn	2.76	2.43	2.01	1.69	1.00746	1.05664	1.10205	1.14366	45.3	245.5	209.3	−81.9	−197.8	−276.1
Ser	42.9	35.3	26.9	19.6	1.12805	1.15102	1.17069	1.19036	70.8	328.0	325.8	−57.4	−71.4	−160.3
Pro	181.5	139	115.1	76.6	1.19459	1.19942	1.20849	1.21182	58.7	328.0	226.4	−69.5	−114.5	−259.8
NaAsp	77.9	59.9	44.0	31.0	1.28523	1.27165	1.25883	1.25335	98.6	498.3	380.1	−29.6	55.9	−106.1
NaGlu	62.4	48.0	34.6	24.1	1.21785	1.21473	1.21464	1.21945	101.5	470.0	403.2	−26.7	27.5	−82.9
LysHCl	71.3	57.3	46.1	39.2	1.12780	1.14058	1.16332	1.18548	72.3	325.0	201.9	−54.9	−118.3	−283.5
ArgHCl	85.3	69.6	48.9	20.8	1.15905	1.16960	1.18036	1.17565	62.4	385.8	380.1	−65.7	−56.6	87.1
DKP	1.68	1.18	0.797	0.503	1.00252	1.05273	1.09773	1.14007	177.4	493.3	631.4			

[a] The densities of 2, 4, and 6 M sarcosine solutions are 1.0492165, 1.0956046, and 1.1388736 g/cm^3, respectively. ΔG_{tr} for DKP is reported for the entire molecule. To obtain ΔG_{tr} per mole of peptide bond, divide the number listed by 2. [b] Phenylalanine in 6 M sarcosine forms a gel-like material under constant agitation at 25 °C; no solubility data were obtained under these conditions. [c] The solubility of leucine in 6 M sarcosine was too low to be measured accurately, so its Δg_{tr} is taken to be the same as the Δg_{tr} for isoleucine.

illustration, we have calculated the transfer free energy change for the native (ΔG_4) and unfolded states (ΔG_2) of ribonuclease A from water to osmolyte solution using eq 3.

$$\Delta G_{2 \text{ or } 4} = \sum n_i \alpha_i \Delta g_i \quad (3)$$

Here, Δg_i represents the transfer free energy of the peptide backbone unit or the amino acid side chain of amino acid i, n_i is the number of amino acids i in the protein, and α_i is the fractional exposure of the n_i amino acid side chains or peptide backbone units in the (native or unfolded) protein species of interest. The α_i values for the native state are obtained using the static accessible surface algorithm of Lee and Richards as modified by Lesser and Rose, with a probe size of 1.4 Å (Lee & Richards, 1971; Lesser & Rose, 1990). The unfolded state was assumed to be the fully exposed polypeptide chain, resulting in α_i values all equaling unity. The results of these calculations are presented in Table 3, in which the contributions to the transfer free energies of the native and unfolded states of RNase A are dissected into the side chain contributions which collectively favor transfer, the contributions from side chains which collectively are unfavorable to transfer, and the contributions due to transfer of the polypeptide backbone. Since $\Delta G_2 - \Delta G_4 = \Delta G_3 -$

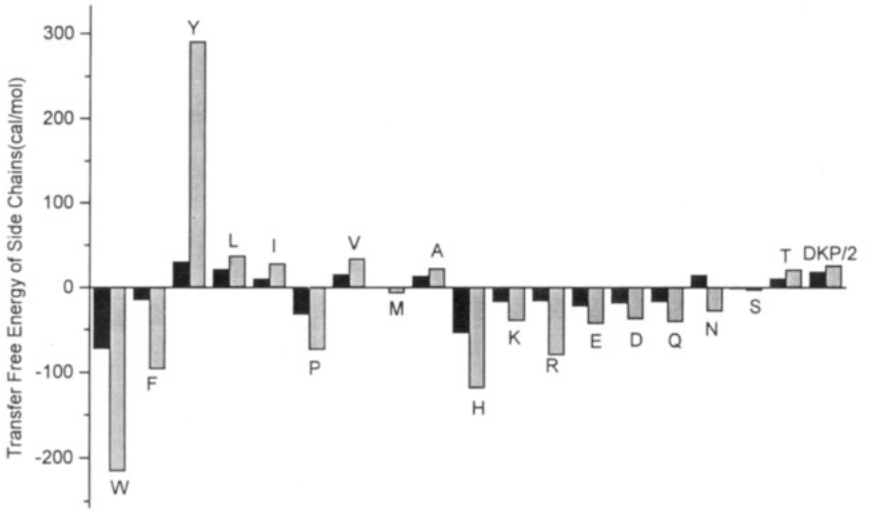


FIGURE 2: Display of the side chain and peptide backbone unit transfer free energies (Δg_{tr}). Solid columns and stippled columns represent Δg_{tr} of the side chains and peptide backbone from water to 0.5 M sucrose and water to 1 M sucrose, respectively. Data taken from Table 1.

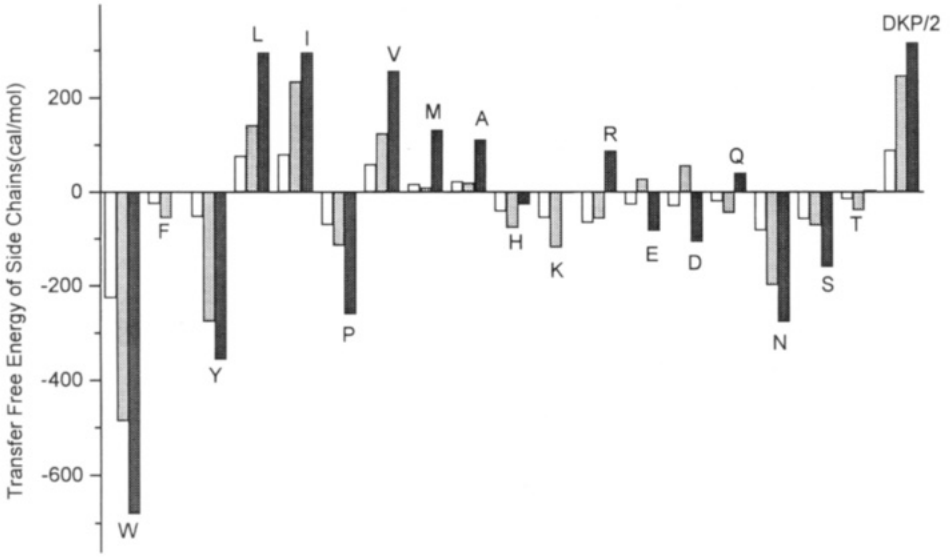


FIGURE 3: Display of the side chain and peptide backbone unit transfer free energies (Δg_{tr}). White, stippled, and solid filled columns represent Δg_{tr} of the side chains and peptide backbone from water to 2, 4, and 6 M sarcosine, respectively. Data taken from Table 2. Side chain transfer free energy data for tryptophan in 6 M sarcosine were not measured. Side chain transfer free energy data for leucine are approximated as being equal to those of isoleucine.

Table 3: Transfer Free Energies of Native and Denatured States of RNase A[a]

osmolyte solution	osmolyte (M)	RNase A state	side chains favoring transfer: $\sum_j n_j \alpha_j \Delta g_j$	side chains opposing transfer: $\sum_k n_k \alpha_k \Delta g_k$	peptide backbone: $\sum_i n_i \alpha_i \Delta g_i$	ΔG_4	ΔG_2	stabilization: $\Delta G_2 - \Delta G_4$
sucrose	0.5	N	−396	255	542	401		
	0.5	U	−996	759	2245		2008	1607
sucrose	1.0	N	−1098	730	763	395		
	1.0	U	−2820	2597	3158		2935	2540
sarcosine	2.0	N	−1713	74	2593	954		
	2.0	U	−3626	729	10733		7836	6882
sarcosine	4.0	N	−3286	213	7212	4139		
	4.0	U	−7283	1625	29851		24193	20054
sarcosine	6.0	N	−5520	648	9229	4357		
	6.0	U	−11187	3805	38200		31495	27138

[a] N and U represent the native and fully unfolded states of RNase A, respectively. Other symbols have the meaning ascribed in the text.

ΔG_1 and ΔG_1 and ΔG_3 represent the unfolding reactions in water and osmolyte, respectively, the calculations show it is more unfavorable to unfold RNase A in the presence of osmolyte than it is in water, a result well established by experimental means (Arakawa & Timasheff, 1982b; Lee & Timasheff, 1981; Santoro et al., 1992).

DISCUSSION

Amino acid transfer free energy measurements have had a long and important history, and they have played a key role in understanding the effect of denaturants and organic solvents on protein stability (Edsall & Wyman, 1958; Nozaki & Tanford, 1963; Tanford, 1964; Wetlaufer et al., 1964). The basic idea for use of these quantities lies in the reductionist view that the whole is equal to the sum of its parts (Tanford, 1964). That is, the sum of the free energy of transfer of the solvent exposed component parts of a protein from one solvent to another equals the free energy of transfer of the whole protein. This view is based on the assumption that the contributions of component parts of the protein are additive, and this assumption is one of the weaknesses of the model. Several investigators have struggled with the question of additivity with much, but not all, of the data supporting the concept of additivity of side chain contributions (Edsall & Wyman, 1958; Lapanje et al., 1978; Nandi & Robinson, 1984; Nozaki & Tanford, 1963, 1965, 1971; Schrier & Schrier, 1976; Tanford, 1964; Wetlaufer et al., 1964). Similarly, additivity of the peptide backbone unit appears to depend on chain length, and while some have presented data indicating the transfer free energy of peptide backbone unit is additive, others suggest nonadditivity (Lapanje et al., 1978; Nandi & Robinson, 1984; Nozaki & Tanford, 1963, 1965, 1971). Due to the long history of the issue of additivity, it is doubtful that a convincing case, one way or the other, could settle the issue without extensive studies using a wide variety of compounds and solvent systems. Our position on this question is that additivity for side chain and backbone units must hold to a significant extent; otherwise, the ability to obtain reasonable unfolding free energy changes based upon side chain and backbone transfer free energy data would not be as successful as it continues to be (Pace, 1975; Staniforth et al., 1993). It should also be noted that additivity of side chain transfer free energy data of Nozaki and Tanford is incorporated as an essential part of theories which have been successful in modeling the thermodynamics of protein unfolding (Alonso & Dill, 1991).

In evaluating transfer free energies, Nozaki and Tanford determined the concentration of amino acid at the solubility limit by potentiometric titration methods (Nozaki & Tanford, 1963). Since one of our osmolytes (sarcosine) is a titratable amino acid itself, we had to develop another means of detection. We found density measurements to be a useful and convenient method, as illustrated by the solubility profiles in Figure 1. The density method was used to determine solubility limits of numerous amino acids in water and urea solutions, and excellent agreement with literature values was found (Nozaki & Tanford, 1963).

Tables 1 and 2 show that, except for tryptophan, it is unfavorable to transfer amino acids from water to sucrose or sarcosine solutions. The contribution of the side chain of an amino acid to its transfer free energy is defined as the effect on the amino acid transfer free energy of substituting a side chain for a hydrogen atom (Nozaki & Tanford, 1963). Operationally, a particular side chain transfer free energy is obtained by subtraction of the transfer free energy of glycine from the transfer free energy of the amino acid of interest.

Listings of these quantities are provided in Tables 1 and 2, and their graphical representations, given in Figures 2 and 3, illustrate several aspects of transferring component parts of a protein molecule from water to osmolyte. First, all of the side chain free energy contributions are quite small in comparison with side chain transfer from water to urea or GdnHCl (Nozaki & Tanford, 1963, 1970). This shows that the interactions of osmolyte with the protein fabric are quite modest and that water, in its abundance, will compete very strongly with osmolyte for interactions with the protein, even with side chains that favor interaction with osmolyte. Second, the amino acid side chains from tryptophan to alanine are given in rank order of decreasing hydrophobicity, and the patterns exhibited for both osmolytes show the hydrophobicity scale to be indifferent to transfer, with the most hydrophobic side chains and proline preferring to interact with osmolyte and the less hydrophobic side chains favoring interaction with water. In general, the figures show that the charged and polar uncharged side chains prefer to interact with osmolyte, though it should again be emphasized that these preferences are very modest. It is observed that sarcosine and sucrose do differ in sign of the transfer free energy of some side chains, but these differences are small in number. What is striking is that the overall transfer free energy patterns for these two chemically different osmolytes are rather similar. Finally, the most significant finding is that it is unfavorable to transfer a peptide backbone unit from water to osmolyte solution. This result is illustrated by the positive transfer free energy of diketopiperazine, also known as cyclic glycylglycine. We have used this compound as a model of an internal peptide backbone, because it has no charged ends as do the peptide backbone models used by Nozaki and Tanford (1963, 1970, 1971). Since DKP has two peptide units, the transfer free energy for the peptide backbone unit is obtained by dividing the transfer free energy of DKP by 2. A rationale for use of DKP as a model is provided below.

Table 3 gives the results of evaluating the transfer free energy of the native (ΔG_2) and unfolded (ΔG_4) states, using Scheme 1, eq 3, and the solvent accessible surface areas, and provides a breakdown of the various contributions to the change in free energy of these states upon transfer from water to osmolyte. First, ΔG_2 and ΔG_4 are both positive, showing that it is unfavorable to transfer the native and unfolded states of RNase A from water to osmolyte. Columns 4 and 5 of Table 3 give, respectively, the sum of the contributions of those amino acids contributing favorably to transfer and the sum of those contributing unfavorably. Taking any horizontal line in Table 3 shows that the sum of the entries from columns 4 and 5 is always negative in sign. Thus, the collective contribution from the side chains for transfer of either the native or unfolded state from water to osmolyte is always favorable. That is, as far as the side chains are concerned, they would (collectively) rather be exposed to osmolyte than to water. Opposing this favorable transfer of the side chains is the unfavorable transfer of the peptide backbone (column 6). This quantity, in all cases, dominates the collective side chain contribution, leaving the native or unfolded state unfavorable to transfer (columns 7 and 8).

The results of the calculations of the transfer free energies of native RNase A show marginal free energy changes at low concentrations of osmolytes that become more positive at higher osmolyte concentration. This trend, illustrated in Figure 4, shows that transferring the native state to osmolyte

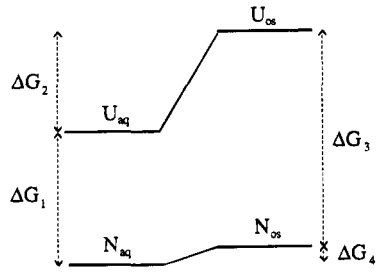


FIGURE 4: Relative free energy diagram for transfer of the native and fully unfolded forms of RNase A from water to either sucrose or sarcosine solutions.

increases the chemical potential, i.e., destabilizes the native state. The reason that osmolytes stabilize proteins against denaturation (Table 3, column 9), as again illustrated in Figure 4, is that they destabilize the unfolded state much more than they destabilize the native state. This same conclusion was drawn by Timasheff, based on thermodynamic measurements that permit evaluation of the change in chemical potentials of native and unfolded protein on transfer to osmolyte (Arakawa et al., 1990a). The fact that these results show the same type of free energy profiles as that of Timasheff provides credibility to the approach and suggests that ignoring activity coefficients and assuming additivity is not sufficiently debilitating to change the overall thermodynamic aspects of osmolyte effects on protein stability.

The dominance of the unfavorable transfer free energy of the peptide backbone over the favorable transfer free energy of the side chains and its importance in protein stabilization is a surprise. There has long been the supposition that proteins should be stabilized by additives because one might expect these compounds to be worse at solvating nonpolar groups than water is (Dill, 1990). The tendency to focus on nonpolar groups as the major factor in stabilization is due primarily to the dominant role nonpolar groups play in protein folding. However, when we compare an unfolding reaction in water with the same unfolding reaction in osmolyte, nature appears to emphasize a different aspect of the protein, viz., the polypeptide backbone becomes the feature of importance and the nonpolar side chains play a much less significant role. In fact, the peptide backbone has been largely ignored as a participant in folding and unfolding processes in various solvent systems, despite the fact that there is a wealth of data suggesting its importance (Nozaki & Tanford, 1971; Robinson & Jencks, 1965a,b). Table 4 gives a compilation of literature data on the transfer free energies of the peptide backbone unit from water to a variety of solvents, and it is seen that peptide backbone transfer is only favorable in the strong denaturants, urea and GdnHCl. Transfer free energies of the peptide backbone unit are evaluated by subtracting the transfer free energy of glycine from that of diglycine (Digly-Gly column), subtracting ΔG_{tr} of glycine from that of triglycine and dividing by 2 (Trigly-Gly column), subtracting ΔG_{tr} of ethyl acetate from that of *N*-acetyltetraglycine ethyl ester and dividing by 3, etc. All of these quantities provide different models for the peptide backbone unit, and while there is disagreement as to what constitutes a good model for free energy evaluation of the peptide backbone, it is clear that the magnitudes of the backbone transfer to all solvent systems other than urea and GdnHCl are quite unfavorable. The peptide backbone unit is the most numerous grouping in a protein, and even a small

Table 4: Peptide Backbone Transfer Free Energy from Water to Solvent

	cal/mol backbone unit				
solvent	Digly-Gly	Trigly-Digly	Trigly-Gly	DKP	ATGEE–EtOAc
6 M urea	−35[a]	−305[a]	−170[a]	−192[g]	−104[h]
	−21[i]	−342[i]	−180[i]		
6 M GdnHCl	−220[b]	−420[b]	−320[b]	−281[g]	−245[h]
	−97[i]	−496[i]	−296[i]		
100% ethanol[c]	1330	820	1075		
60% ethanol[d]	425	65	245		
60% dioxane[e]	585	205	395		
60% glycol[e]	170	115	142		
55% MPD[f]	120				
1 M sucrose[g]				26	
6 M sarcosine[g]				316	

[a] Nozaki & Tanford, 1963. [b] Nozaki & Tanford, 1970. [c] Tanford, 1970. [d] Nozaki & Tanford, 1971. [e] Nozaki & Tanford, 1965. [f] Pittz & Bello, 1971. [g] This paper. [h] Based on data from Robinson & Jencks, 1965a. [i] Lapanje et al., 1978.

unfavorable transfer per peptide backbone unit when multiplied by the number of peptide units in a protein adds considerable unfavorability to unfolding the protein in the presence of that solvent.

Use of DKP as a model for the peptide backbone arises from the need for a model which is representative of internal peptide backbone units. Table 4 shows that the peptide backbone transfer free energy changes in 6 M GdnHCl and urea are quite similar (within 10−15%) to those shown in the Trigly-Gly column. The agreement is within the normal range of errors, suggesting that DKP is a reasonable model for evaluating the peptide backbone transfer free energy.

Nozaki and Tanford recognized the significance of the unfavorable peptide backbone transfer free energy to non-urea or non-GdnHCl solutions and its contribution to protein conformation under certain circumstances (Nozaki & Tanford, 1971). The strong preference for the peptide backbone unit for water over mixed organic solvents led these authors to state: "This result undoubtedly explains why proteins dissolved in *high concentrations* of organic solvents (e.g., 2-chloroethanol) tend to assume highly helical conformations" (Nozaki & Tanford, 1971). The α-helical structure is one in which the peptide backbone unit would be minimally exposed to solvent while the side chains (which interact favorably with the organic solvent) are optimally exposed to solvent. The driving force for helical formation in 2-chloroethanol is due to the strong propensity to remove the peptide backbone unit from unfavorable interaction with solvent while permitting favorable side chain−solvent interactions to occur. Since alcohols and other organic solvents and solutes are currently used in protein conformational studies, in modeling of intermediate states of protein folding, and in attempts to understand helix−coil transitions in peptides, it is useful to recognize that the magnitude and character of the forces affecting polypeptide structures greatly depend on the solvation free energies of the component parts of the peptide or protein. These forces can drive the ensemble of microstates of the protein or polypeptide into species bearing little relationship to those that predominate in water.

The stabilities of RNase A listed in the last column of Table 3 suggest extraordinary stabilization (e.g., see 4 and 6 M sarcosine). The quantities in this column overestimate

the stabilization afforded by osmolyte, primarily because we have used the extended polypeptide chain as a model of the unfolded state. The unfavorable transfer free energies of the native and unfolded states show that the osmolytes are poorer than water as solvents for the protein. In response, the action of a poorer solvent on denatured protein will cause it to become more compact in order to reduce exposure of the polypeptide backbone to solvent. In short, the *actual* denatured state in the presence of osmolyte should be more compact than the denatured state in water, and the denatured states in either water or osmolyte cannot be considered an extended polypeptide chain since both are poor solvents (Dill & Shortle, 1991). The result of the osmolyte forcing the *actual* denatured ensemble to a more compact form than we have modeled would result in smaller stabilities than those listed in the last column of Table 3.

The value of the quantities we have obtained using Scheme 1 and eq 3 lies not in their absolute magnitude; their importance comes in identifying the major players involved in protein stabilization. Our results reproduce the main features of the free energy profile (Figure 4) determined for real systems (Arakawa et al., 1990a; Timasheff, 1993), indicating that the approach we have used reproduces the essential aspects of the stabilization phenomenon. The major player in this process is the unfavorable transfer of the polypeptide backbone, which is responsible for the much larger destabilization of the denatured state in comparison with the destabilized native state. The free energy contributions of the side chains actually favor the denatured state, so their role in the process of stabilization is one of attenuating the effect of the polypeptide backbone contributions. The principles evident in osmolyte stabilization of proteins emphasize different aspects of protein structure–solvent relationships than we normally consider. Except for urea and GdnHCl, it is clear from the preponderance of solvents which have been studied thus far that the unfavorable transfer of the polypeptide backbone from water to solvent systems is a common aspect of all such systems, and this feature must be incorporated into any discussion of the stability of the protein in the presence of that solvent.

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