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On the role of surface tension in the stabilization of globular proteins

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Abstract

The stabilization of proteins by a variety of co-solvents can be related to their property of increasing the surface tension of water. It is demonstrated that, during the thermal unfolding of proteins, this increase of the surface tension can be overcome by the increase in the temperature of the solution at the midpoint of the transition, T_m, and the weak binding of co-solvent molecules. Three such co-solvents were studied: trehalose, lysine hydrochloride (LysHCl), and arginine hydrochloride (ArgHCl). Trehalose and LysHCl increase the midpoint of T_m. The increase of the surface tension by addition of trehalose is completely compensated by its decrease due to the increase in T_m. However, for LysHCl, the increase of the surface tension by the co-solvent is partly reduced by its binding to the protein. For trehalose, preferential interaction measurements with RNaseA demonstrate that it is totally excluded from the protein. In contrast, LysHCl gives evidence of binding to RNaseA. ArgHCl also increases the surface tension of water. Nevertheless, T_m of RNaseA decreases on addition of ArgHCl to the solution. Preferential interaction measurements showed very small values of preferential hydration of the native protein, indicating extensive binding of ArgHCl to the protein. During unfolding, the amount of additional ArgHCl binding is sufficiently large to counteract the surface tension effects, and the protein is destabilized. Therefore, although surface tension appears to be a critical factor in the stabilization of proteins, its increase by co-solvent does not ensure increased stabilization. The binding of ligands can reduce significantly, or even overwhelm, its effects.

Keywords: co-solvent stabilization of proteins; preferential binding; preferential hydration; surface tension; thermal unfolding of proteins; trehalose

The stabilization of macromolecules in aqueous solutions by the surface energy needed to create a cavity in the solvent before introducing a macromolecule into it was proposed first by Sinanoglu and Abdulnur (1964, 1965). More recently, Breslow and Guo (1990) have shown that co-solvents can affect the energy required to produce a cavity in the solvent, which is reflected in their effect on surface tension and the solvation energy of the solute. Honig and co-workers (Nicholls et al., 1991; Sharp et al., 1991) have argued that surface tension can be used as a quantitative measure of the hydrophobic stabilization of proteins. These reports are consistent with the reported correlation between the increase in the surface tension of water by sucrose and its stabilization of proteins against thermal denaturation (Lee & Timasheff, 1981). In fact, because surface tension is decreased by increases in temperature, it was found that the transition temperature, T_m, of the proteins in the presence of sucrose occurred at close to constant values of the surface tension, and the proteins unfolded at constant values of the free energy of cavity formation (Lee & Timasheff, 1981).

Later studies on the preferential interaction[2] of proteins with a variety of co-solvents have identified the surface tension ef-

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Abbreviations: RNase A, ribonuclease A; LysHCl, L-lysine hydrochloride; ArgHCl, L-arginine hydrochloride.

[2] The term preferential interaction refers to the net interaction between the protein and the solvent components, water and the co-solvent, as measured by an equilibrium thermodynamic approach, such as dialysis equilibrium. When the interactions are weak, as is the case with co-solvents, such as sugars, glycerol, amino acids, or urea, the measured quantity is the net balance between the weak interactions (binding) of water and co-solvent molecules to the protein over its entire surface. If, on average, the affinity of protein surface loci for the co-solvent is greater than that for water, the dialysis equilibrium experiment will result in an excess of co-solvent at the protein surface over its concentration in the bulk solvent, and the measured binding stoichiometry will be positive, which means that co-solvent is preferentially bound relative to water. In that case, the "preferential binding" will be positive. In the converse case, in which, on average, the affinity of the protein surface loci is greater for water than for co-solvent molecules, there will be an excess of water at the protein surface over its concentration in the

fect as a determining factor for these interactions. In his analysis of surface phenomena, Gibbs (1878) showed that substances that lower the surface tension of water accumulate at the surface, those that raise it are depleted from the surface. At an interface, such as the surface of a protein molecule dissolved in aqueous medium, this will result in either an excess or a deficiency of co-solvent molecules at the water–protein interface, i.e., the protein surface. In a dialysis equilibrium experiment, this will manifest itself in a measured binding stoichiometry that will be positive in the first case and negative in the second. In other words, the preferential interaction, expressed as preferential binding of co-solvent, will assume positive or negative values in the two respective cases. In dialysis equilibrium experiments, it has been found that sugars, amino acids, and many salts are preferentially excluded from the protein surface, i.e., their preferential interactions with proteins are negative (Lee & Timasheff, 1981; Arakawa & Timasheff, 1982a, 1982b, 1983; Kita et al., 1994). All of these co-solvents raise the surface tension of water. The good correlation between the measured negative preferential interactions and the positive surface tension increment in their presence (Arakawa & Timasheff, 1982a, 1982b, 1983, 1984b; Kita et al., 1994) led to the proposal that this is the principal source of the unfavorable free energy of the interactions of these co-solvents with proteins. These observations have suggested that the stabilization of proteins by these co-solvents is due to the increase of the surface tension in their presence.

Several molecules, however, that raise the surface tension of water, gave a poor correlation between the experimentally measured preferential interactions and the values expected from the surface tension increments. Specifically, it was found that the measured preferential interactions of ArgHCl and LysHCl (Kita et al., 1994) with bovine serum albumin did not give a good correlation with the increase in surface tension. A similar lack of correlation has also been found for $MgCl_2$ (Arakawa et al., 1990). Furthermore, in their study of the effects of osmolytes on the thermal stability of RNaseA, Bowlus and Somero (1979) found that all of the intracellular solutes they tested stabilized RNaseA structure, except for ArgHCl, which decreased the transition temperature of the protein. Because ArgHCl and LysHCl have the potential to interact directly with proteins, e.g., by hydrogen bonding to peptide groups, it was proposed that the measured preferential interactions are a summation of the effects of the increase in surface tension and weak binding (Kita et al., 1994).

To test more critically these concepts, we undertook a closer examination of the roles of surface tension and weak binding of co-solvents in the stabilization of proteins. Three co-solvents were selected: trehalose, LysHCl, and ArgHCl. Trehalose is a disaccharide (1-α-D-glucopyranosyl-1,1-α-D-glucopyranoside) often used as an osmolyte by organisms during water stress (Yancy et al., 1982; Somero, 1986). It is also a stabilizer in the freezing preservation of macromolecules (Crowe et al., 1990). Due to its similarity to sucrose, it can be expected not to bind to proteins. The other two co-solvents, LysHCl and ArgHCl, were selected because of the increasing deviation of their preferential interactions with proteins from those predicted by the surface tension increment. Their effects on the stability of RNaseA and the correlation with preferential interactions and surface tension increments are described in this paper.

Results

Effects of co-solvents on the thermal stability of RNase A

The effects of trehalose, LysHCl, and ArgHCl on the stability of RNaseA were examined in thermal unfolding experiments by difference absorbance spectroscopy. The melting curves of RNaseA in different concentrations of trehalose at pH 2.8, LysHCl at pH 5.8, and ArgHCl at pH 6.7 are shown in Figure 1A, B, and C, respectively.[3] The melting temperatures (T_m) are listed in Table 1. As shown graphically in Figure 2A, for both trehalose and LysHCl, an increase in co-solvent concentration led to an increase of T_m. This was particularly large with trehalose at pH 2.8, where T_m is 10 °C higher in a 1 M sugar solution than in the dilute buffer. For ArgHCl, the effect was opposite: T_m of RNaseA first decreased with increasing concentration of ArgHCl and then slowly leveled off at high salt concentrations.

Surface tension analysis of the thermal unfolding

The surface tension of the mixed solvents was analyzed at the transition temperatures in terms of the contributions of changes in solvent composition and of temperature to the surface tension of water. If we designate the surface tension at T_m of a protein solution in the absence of co-solvents as σ_o, addition of a co-solvent will raise the surface tension value by an increment, $\Delta\sigma_{sol}$, defined as $\sigma_o + \Delta\sigma_{sol} = \sigma_o + (\partial\sigma/\partial m_3)m_3$. On the other hand, because surface tension decreases as temperature rises (Landt, 1931), any increase in T_m will lower it by an increment $\Delta\sigma_{temp}$ to $\sigma_o + \Delta\sigma_{temp} = \sigma_o + (\partial\sigma/\partial T)\Delta T_m$. If we designate the surface tension at T_m as σ_T, combination of the two effects leads to a σ_T value of

$$\sigma_T = \sigma_o + \Delta\sigma_{sol} + \Delta\sigma_{temp}. \quad (1)$$

In a previous study, it had been found that, for α-chymotrypsin, chymotrypsinogen, and RNaseA in solutions of sucrose, the thermal unfolding transition occurred at a close to constant value of surface tension (Lee & Timasheff, 1981), i.e., $\sigma_T - \sigma_o = 0$ and $\Delta\sigma_{sol} = -\Delta\sigma_{temp}$. The values of $\Delta\sigma_{sol}$ were calculated for treha-

(*Continued from previous page.*)
bulk solvent and the protein will be "preferentially hydrated." Expressed in terms of the dialysis equilibrium stoichiometry of binding of the co-solvent, this stoichiometry will be negative, i.e., there will be "preferential exclusion" of the co-solvent from the protein surface, and the "preferential binding" of the co-solvent will be negative. It is clear, therefore, that "preferential binding," and, more generally, "preferential interaction" can assume either positive or negative values, which simply reflect the relative affinities of water and co-solvent molecules for the protein surface. (For a detailed thermodynamic discussion, see Schellman [1990] or Timasheff [1992, 1993]).

[3] The low pH value for trehalose was selected because melting occurs at a lower temperature. Both the preferential binding and T_m increment for the RNase-trehalose system are identical at pH 2.8 and 5.5 (G.-F. Xie & S.N. Timasheff, unpubl.). LysHCl and ArgHCl studies were performed at close to neutral pH to keep them as monovalent salts, i.e., the form for which the surface tension increments had been measured (Kita et al., 1994).

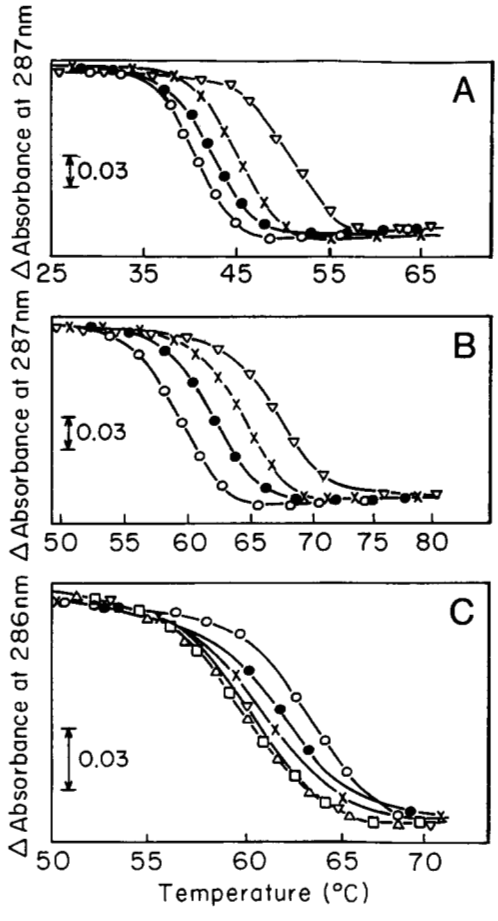

Fig. 1. Thermal transitions of RNaseA in **(A)** 0.04 M glycine, pH 2.8: ○, no trehalose; ●, 0.2 M trehalose; ×, 0.5 M trehalose; and ▽, 1 M trehalose; **(B)** 0.03 M MES, pH 5.8: ○, no LysHCl; ●, 0.5 M LysHCl; ×, 1 M LysHCl; and ▽, 1.5 M LysHCl; **(C)** 0.03 M potassium phosphate, pH 6.7: ○, no ArgHCl; ●, 0.2 M ArgHCl; ×, 0.5 M ArgHCl; ▽, 0.7 M ArgHCl; □, 1.0 M ArgHCl; and △, 1.5 M ArgHCl.

Table 1. *Surface tension analysis of the thermal unfolding of RNaseA at T_m*

Solvent (M)	m_3 (mol/kg)	T_m (°C)	$\Delta\sigma_{temp}$ (dyne/cm)	$\Delta\sigma_{sol}$ (dyne/cm)	σ_T (dyne/cm)	σ_b (dyne/cm)	$(\partial\sigma_b/\partial m_3)^{exp}$ (dyne/cm/mol)
Trehalose, pH 2.8							
0	0	40.9	0	0	69.39	0	
0.2	0.21	42.6	−0.22	0.28	69.45	−0.06	−0.13
0.5	0.56	45.4	−0.70	0.75	69.44	−0.05	−0.01
1.0	1.26	50.9	−1.61	1.69	69.47	−0.08	−0.04
LysHCl, pH 5.8							
0	0	59.5	0	0	66.27	0	
0.5	0.53	62.4	−0.51	1.24	66.99	−0.73	−1.46
1.0	1.15	65.0	−0.98	2.67	67.96	−1.69	−1.61
1.5	1.86	67.7	−1.46	4.33	69.14	−2.87	−1.66
ArgHCl, pH 6.7							
0	0	63.9	0	0	65.49	0	
0.2	0.21	62.3	0.28	0.46	66.23	−0.74	−3.10
0.5	0.54	60.9	0.53	1.09	67.11	−1.62	−2.42
0.7	0.78	60.3	0.64	1.50	67.63	−2.14	−1.84
1.0	1.17	60.1	0.68	2.05	68.22	−2.73	−1.26
1.5	1.92	60.1	0.68	2.81	68.98	−3.49	−1.01

lose, LysHCl, and ArgHCl from surface tension increments measured at 20 °C (Kita et al., 1994). The application of these increments to the data at T_m was based on the fact that such increments for sucrose and a number of other small organic molecules, e.g., acetic acid, are known not to change with temperature (International Critical Tables, 1928). Similarly, $\Delta\sigma_{temp}$ for water is not affected by small solutes (International Critical Tables, 1928). The resulting values of $\Delta\sigma_{sol}$, $\Delta\sigma_{temp}$, and σ_T are plotted in Figure 2B, and all the parameters are listed in columns 4, 5, and 6 of Table 1. It is clear that, in the trehalose solution at T_m, the increase in surface tension due to the presence of the co-solvent was compensated exactly by its decrease due to the rise of the transition temperature. This is shown by the constancy of σ_T at all co-solvent concentrations. This, together with the earlier observations with sucrose (Lee & Timasheff, 1981), has led to the assumption that, in the absence of other interactions between the protein and the solvent system, the thermal unfolding occurs at a constant value of the surface free energy at the protein–solvent interface. The increase of σ_T with co-solvent concentration for the LysHCl and ArgHCl solutions was used, therefore, as a test of this assumption. What is the

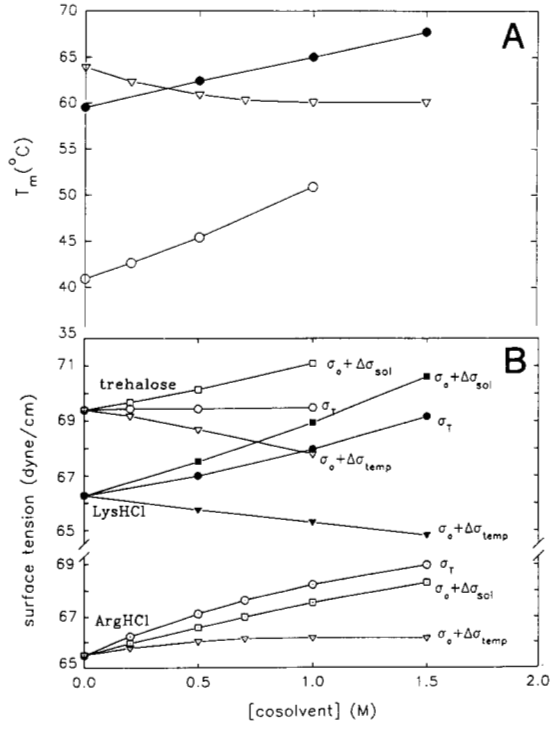

Fig. 2. A: Midpoint of thermal unfolding (T_m) of RNase A: ○, in trehalose, pH 2.8; ●, in LysHCl, pH 5.8; ▽, in ArgHCl, pH 6.7. **B:** Surface tensions of solutions of trehalose, LysHCl, and ArgHCl during the thermal unfolding of RNaseA. Open symbols are for trehalose, filled symbols are for LysHCl, and open symbols with dot are for ArgHCl. Squares, surface tension increases due to addition of co-solvents; triangles, surface tension variation due to change in T_m; circles, the surface tensions of the solutions at T_m in the presence of co-solvents.

Table 2. *Preferential interaction parameters of RNaseA in trehalose, LysHCl, and ArgHCl solutions at 20 °C*

Solvent (M)	ϕ_2° (mL/g)	$\phi_2'^\circ$ (mL/g)	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial m_3/\partial m_2)^{exp}_{T,\mu_1,\mu_3}$ (mol/mol)	$(\partial m_3/\partial m_2)^{a}_{T,\mu_1,\mu_3}$ [a] (mol/mol)	$(\partial \mu_2/\partial m_3)^{exp}_{T,P,m_2}$ (cal/mol^2)
Trehalose, pH 2.8							
0	0.710 ± 0.001 [b]	0.706 ± 0.001 [b]					
0.2	0.713 ± 0.001	0.722 ± 0.001	−0.036	0.499	−1.43	−1.64	4,140
0.5	0.711 ± 0.001	0.741 ± 0.001	−0.103	0.538	−4.12	−4.15	4,720
0.7	0.710 ± 0.001	0.750 ± 0.003	−0.146	0.521	−5.86	−5.85	4,760
LysHCl, pH 5.8							
0	0.686 ± 0.001	0.689 ± 0.002 [b]					
0.5	0.690 ± 0.001	0.711 ± 0.001	−0.071	0.729	−5.33	−4.52	9,700
1.0	0.693 ± 0.001	0.723 ± 0.002	−0.121	0.576	−9.08	−9.31	8,020
1.5	0.696 ± 0.002	0.723 ± 0.001	−0.129	0.378	−9.69	−12.98	6,140
ArgHCl, pH 6.7							
0	0.701 ± 0.001 [b]	0.707 ± 0.001 [b]					
0.2	0.698 ± 0.001	0.708 ± 0.002	−0.013	0.294	−0.83	−1.74	3,760
0.5	0.702 ± 0.001	0.716 ± 0.003	−0.028	0.249	−1.83	−4.39	2,820
1.0	0.700 ± 0.001	0.717 ± 0.001	−0.045	0.182	−2.90	−8.08	1,810

[a] After correcting for the curvature of the protein-water interface by a factor of 0.53.
[b] The difference between ϕ_2° and $\phi_2'^\circ$ was corrected in calculations of preferential interaction parameters.

source of this departure of the surface tension at T_m from a constant value? A plausible explanation is the contribution of a favorable free energy of interaction of the protein with the co-solvent at the protein surface, i.e., weak binding of the co-solvent at some loci in the protein surface which, in effect, is penetration of the cavity surface by the co-solvent (Breslow & Guo, 1990). This can be expressed as $\Delta G_b = \Delta\mu_2^b$, where ΔG_b is the free energy of binding and $\Delta\mu_2^b$ is the contribution of the binding to the transfer free energy of the protein from water to the solvent system. At a surface (e.g., the protein-water interface), this surface interaction can be expressed in terms of an increment to the surface tension due to the binding, $\sigma_b = \Delta G_b/s = \Delta\mu_2/s$, where s is the protein surface area. The basic assumption that T_m occurs at a constant free energy of interaction at the protein-solvent interface, as has been observed for the sugars, requires that the increase in surface free energy by addition of the co-solvent must be balanced by the sum of its decreases due to the rise in temperature and to the binding of co-solvent to the protein. Because at T_m the protein exists as a mixture of equal concentrations of native and denatured species, $\Delta G_b/s$ must be an average of the binding contributions of the two states ($\Delta G_b^{N,D}/s^{N,D}$) (see Appendix). Then,

$$\sigma_T - \sigma_o = \left(\frac{\partial\sigma}{\partial m_3}\right)m_3 + \left(\frac{\partial\sigma}{\partial T}\right)\Delta T + \frac{\Delta G_b^{N,D}}{s^{N,D}} = 0. \qquad (2)$$

The values of $\Delta G_b^{N,D}/s^{N,D}$, expressed as σ_b needed to satisfy Equation 2 for the three systems are listed in Table 1. As shown in Figure 2B and Table 1, trehalose does not bind to RNaseA, because its values of σ_b is zero. In contrast, for LysHCl, σ_b is negative, which means that, although it stabilizes RNaseA, it does bind to the protein. For ArgHCl, the positive values of $\Delta\sigma_{temp}$ indicate that the binding of ArgHCl is sufficiently strong to make impossible its balancing out by solely the co-solvent-induced increase in surface tension. As a consequence, maintenance of a constant total free energy of interaction at the surface permits the protein to unfold at a lower temperature.[4] The corresponding variation of the binding free energy with concentration, expressed in terms of a surface tension increment, $(\partial\sigma_b/\partial m_3)^{exp}$, is listed for all three co-solvents in the last column of Table 1.

Preferential interactions: LysHCl and ArgHCl bind to native RNaseA

To test the binding of these co-solvents to RNaseA deduced from the surface tension analysis of denaturation, the preferential interactions of RNaseA with these three co-solvents were measured. The partial specific volumes, ϕ_2° and $\phi_2'^\circ$, measured at isomolal and isopotential conditions, respectively, of RNaseA at 20 °C in trehalose, LysHCl, and ArgHCl solutions, are given in Table 2. The needed thermodynamic parameters of all three co-solvents are listed in Table 3. The preferential binding parameters, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, are negative at all co-solvent concentrations for trehalose, LysHCl, and ArgHCl. Therefore, the native RNase A is preferentially hydrated. The lack of dependence of the preferential hydration, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$, on g_3 (Table 2, column 5) shows that trehalose does not bind to any sites on RNaseA. On the other hand, the decrease of $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ of RNaseA in LysHCl and ArgHCl solutions with an increase in co-solvent concentration does indicate that these amino acid salts bind weakly to the protein. The binding of LysHCl and ArgHCl is also reflected in the variation of the chemical potential of RNaseA in these co-solvents, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$, calculated by Equation 12, as shown in the last column of Table 2. For trehalose, it is positive and close to invariant. For the two amino acid

[4] It must be noted that, in the context of the analysis that follows, co-solvent interactions were measured with the native protein only. The extent of the interactions with the unfolded (denatured) state was inferred from calculations (see Appendix).

Table 3. *Thermodynamic parameters of trehalose, LysHCl, and ArgHCl in aqueous solutions at 20 °C*

Solvent (M)	g_3 (g/g)	$\bar{v}_3$ (mL/g)	$\partial \ln \gamma_3/\partial m_3$ (mol^{-1})	$(\partial\mu_3/\partial m_3)_{T,P,m_2}$ (cal/mol^2)	$\partial\sigma/\partial m_3$ (dyne/cm·mol)
Trehalose, pH 2.8					
0	0				
0.2	0.0714	0.609	0.177[a]	2,893	1.34[b]
0.5	0.1912	0.613	0.177[a]	1,145	1.34[b]
0.7	0.2810	0.615	0.177[a]	810	1.34[b]
1.0	0.4341	0.618	0.177[a]	562	1.34[b]
LysHCl, pH 5.8					
0	0				
0.5	0.0976	0.703[c]	−0.309[d]	1,820	2.32[b]
1.0	0.2100	0.714[c]	−0.111[d]	884	2.32[b]
1.5	0.3419	0.726[c]	0.010[d]	637	2.32[b]
ArgHCl, pH 6.7					
0	0				
0.2	0.0433	0.671[b]	−0.971[d]	4,536	2.23[b]
0.5	0.1132	0.681[b]	−0.537[d]	1,542	1.91[b]
0.7	0.1640	0.685[b]	−0.432[d]	992	1.71[b]
1.0	0.2455	0.690[b]	−0.323[d]	623	1.42[b]
1.5	0.4039	0.696[b]	−0.172[d]	407	1.00[b]

[a] Calculated from unpublished measurements of γ_3.
[b] Data from Kita et al. (1994).
[c] Data from Arakawa and Timasheff (1984a).
[d] Data calculated from Bonner (1982).

salts, however, the preferential interaction parameter decreases linearly according to $(\partial\mu_2/\partial m_3)_{T,P,m_2} = 11{,}100 - 2{,}660m_3$ for LysHCl and $(\partial\mu_2/\partial m_3)_{T,P,m_2} = 4{,}055 - 1{,}980m_3$ for ArgHCl. This means that the interaction between the protein and the co-solvent becomes decreasingly unfavorable as the co-solvent concentration increases, which is expected from the law of mass action if binding of the co-solvent to protein sites takes place. It is clear that these results are fully consistent with the analysis of the relation between T_m and surface tension.

Discussion

Nature of the protein surface and interaction with solvent components

The present analysis is based on variations of the interfacial tension at the protein surface. As such, it contains assumptions about the protein-solvent interface. Classically, surface tension is measured at a flat homogeneous water-air interface, as are its variations with temperature and solvent composition. The protein surface is neither flat nor chemically homogeneous. As will be discussed later, the effect of its curvature on the surface tension can be accounted for by geometric considerations (Nicholls et al., 1991; Sharp et al., 1991). Chemically, the surface that a protein presents to solvent can be regarded as a mosaic of loci that vary in polarity, hydrophobicity, and electrostatic charge. On the micro scale, this leads to a complex pattern of interactions between solvent components and regions on the protein surface. Even in very weak binding systems, such as co-solvents, some co-solvent molecules will have an affinity for individual loci on the surface (e.g., urea for an exposed peptide bond). This manifests itself in binding. Because binding is an exchange phenomenon where the ligand must displace water molecules from the given site (Schellman, 1987, 1990; Timasheff, 1992, 1993), the binding free energy is the difference between the free energies of interaction of the ligand and water with the particular site (locus) on the protein surface (Timasheff & Kronman, 1959). Where the protein is indifferent to being in contact with water or ligand, there is thermodynamic neutrality. Where the ligand has no affinity at all, there is exclusion.

Now, the presence of a protein molecule in the solvent requires that first a cavity be formed in the latter. This requies energy. The free energy of cavity formation is given by the surface tension properly modified for curvature, namely by the protein-solvent interfacial tension. Being a function only of the generation of a surface or an interface, the surface free energy of the cavity will be uniform over the entire surface of the cavity, within the approximation of uniform curvature. The area of this surface is equal to the surface area of the protein molecule. Introduction of a co-solvent that raises the surface tension of water will, then, lead to an excess of water being uniformly present over the entire interface (Gibbs, 1878; Lee & Timasheff, 1981; Kita et al., 1994). In a dialysis equilibrium experiment, this will be measured as exclusion of the co-solvent. Penetration of this layer of excess water by a ligand molecule displaces some water molecules and manifests itself as weak binding. Combination of the two opposite effects, summed over the entire protein surface, results in the macroscopic preferential binding, or exclusion, measured in a dialysis equilibrium experiment.

On denaturation, a globular protein molecule becomes more asymmetric and its surface of contact with the solvent becomes larger. As a consequence, the total free energy of interaction with a solvent system also becomes larger per protein molecule, and the extent of exclusion due to the increase in surface ten-

sion by addition of a co-solvent also becomes larger per molecule. This involves the assumption that the protein surfaces in the native and denatured states are similar in nature with respect to the nonspecific exclusion due to the surface tension effect. The change in the chemical composition of the surface mosaic, however, engenders changes in local affinity and binding ability of the co-solvents. Hence, the local binding pattern will change with denaturation and the preferential binding measured will also change. This, as shown below, is what controls the denaturation equilibrium at any given solvent composition.

Why does denaturation occur at a constant surface free energy?

As stated above, denaturation involves expansion of the protein-containing cavity. This takes work for which the energy must be furnished by the expanding protein. In thermal unfolding studies, it is frequently assumed that the bulk denatured form (structure) of a protein is not greatly affected by addition of a co-solvent, i.e., the degrees of expansion are close to the same. This expansion will require more work if the surface tension is raised by the co-solvent. Intrinsically, expansion of a protein from state A to state B (N → D) releases the same amount of energy whether the environment is water[5] or water mixed with a co-solvent. If the resistance of cavity expansion is greater in the presence of a co-solvent than in its absence, there may not be enough energy offered by protein expansion to expand the cavity to the same extent as in water.[5] This can be balanced by an increase in temperature, which lowers the surface tension and the resistance to the expansion of the protein.[6] Compensation of the increase of the free energy of cavity formation due to the effect of a co-solvent on the cohesive forces of water can also occur through penetration of co-solvent molecules to loci on the protein surface and interaction (binding) with these loci.

Separation of the measured preferential exclusion into contributions from the surface tension effect and weak binding

Preferential interaction values measured by dialysis equilibrium consist of contributions from the weak binding of the co-solvent and its exclusion due to the surface tension increment:

$$\left(\frac{\partial m_3}{\partial m_2}\right)^{\mathrm{Exp}}_{T,\mu_1,\mu_3} = \left(\frac{\partial m_3}{\partial m_2}\right)^{\mathrm{bind}}_{T,\mu_1,\mu_3} + \left(\frac{\partial m_3}{\partial m_2}\right)^{\sigma}_{T,\mu_1,\mu_3}. \qquad (3)$$

The second term of Equation 3 can be calculated with the Gibbs adsorption isotherm (Gibbs, 1878; Lee & Timasheff, 1981),

$$\left(\frac{\partial m_3}{\partial m_2}\right)^{\sigma}_{T,\mu_1,\mu_3} = \frac{sa_3}{RT}\left(\frac{\partial \sigma}{\partial a_3}\right)_T, \qquad (4)$$

where s is the molar surface area of the protein, and σ is the surface tension of the solvent. The molar surface area for RNaseA is 2.8×10^{11} cm^2/mol, as calculated from the surface area to volume ratio (s/V) determined by small-angle X-ray scattering (Krigbaum & Godwin, 1968; Pessen et al., 1973; Lee & Timasheff, 1981), $s = (s/V)M_2\bar{v}_2$, where $\bar{v}_2$ is the partial specific volume of the protein. Combination of Equations 4 and 11 gives the chemical potential variation of the protein induced by the change in surface tension on addition of the co-solvent, $(\partial\mu_2/\partial m_3)^{\sigma}$, as

$$\left(\frac{\partial \mu_2}{\partial m_3}\right)^{\sigma}_{T,P,m_2} = s\left(\frac{\partial \sigma}{\partial m_3}\right)_{T,P,m_2}. \qquad (5)$$

Values of $(\partial\mu_2/\partial m_3)^{\sigma}_{T,P,m_2}$ were calculated with Equation 5, with the application of the correction for the curvature of a protein-surface interface, because surface tension is measured for flat water-air interfaces (Choi et al., 1970; Tanford, 1979; Nicholls et al., 1991; Sharp et al., 1991). This is done by multiplying the calculated value of $(\partial\mu_2/\partial m_3)^{\sigma}_{T,P,m_2}$ by the ratio, R, of the experimental to the calculated value determined for systems that show no evidence of binding. In previous studies, it has been shown that R has values between 0.5 and 0.7 (Arakawa & Timasheff, 1982a, 1983, 1984b), which are similar to those calculated by Honig and co-workers from geometric considerations (Nicholls et al., 1991; Sharp et al., 1991). Therefore, for consistency, all the preferential interaction parameters calculated with the Gibbs adsorption isotherm were corrected by this factor. For trehalose, R remains constant, within experimental error, at $R = 0.53$, which is identical with the sucrose value found earlier (Lee & Timasheff, 1981). These calculated values of $(\partial m_3/\partial m_2)^{\sigma}_{T,\mu_1,\mu_3}$ are compared with the experimental ones in columns 6 and 7 of Table 2. The progressively increasing positive departure of the experimental values of $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ from $(\partial m_3/\partial m_2)^{\sigma}_{T,\mu_1,\mu_3}$ for LysHCl and especially ArgHCl, give the increasing binding of these amino acid salts to the protein.

Changes in binding during unfolding

At any solvent composition, the relation between the change in preferential binding of a co-solvent to a protein and its effect on the stability of the protein is defined by the linkage equation (Wyman, 1964):

$$\left(\frac{\partial \ln K}{\partial \ln a_3}\right)_{T,P,m_2} = \left(\frac{\partial m_3}{\partial m_2}\right)^{D}_{T,P,\mu_3} - \left(\frac{\partial m_3}{\partial m_2}\right)^{N}_{T,P,\mu_3} = \Delta\nu_3 \qquad (6)$$

where $\Delta\nu_3$ is the difference in preferential binding to the two end states of the unfolding reaction, K is the unfolding equilibrium constant at any given temperature, a_3 is the activity of the co-solvent, and N and D refer to the folded (native) and unfolded (denatured) states of the protein, respectively. Analysis of the transition data according to Equation 6 is given in Figure 3. The values of $\Delta\nu_3$ are listed in Table 4. The negative values for trehalose and LysHCl give a measure of the stabilization, which is seen to be considerably greater for trehalose than for LysHCl. Comparison of the $\Delta\nu_3$ values with the $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ val-

[5] In practical terms, water means dilute buffer, because co-solvent is present at high concentration.

[6] In this context, it should be emphasized that the present analysis applies only to the increment in T_m needed to lower the surface tension in the co-solvent system to its value in water. It does not treat the fundamental processes in protein unfolding, which are assumed to be the same whether co-solvent is present or not. Because the T_m increments are not greater than 10° (see Table 1), changes in entropic contributions to the free energy of unfolding, such as conformational entropy ($T\Delta S$), will vary by not more than 3% between water and the co-solvent system.

Table 4. *Analysis of the preferential interaction parameters of the native and the denatured RNaseA in solution at T_m*

Solvent (M)	$\Delta\nu_3$	T_m (°C)	$(\partial m_3/\partial m_2)^{b,N}_{T,\mu_1,\mu_3}$	$(\partial m_3/\partial m_2)^{b,D}_{T,\mu_1,\mu_3}$	$(\partial m_3/\partial m_2)^{N}_{T,\mu_1,\mu_3}$ [a]	$(\partial m_3/\partial m_2)^{D}_{T,\mu_1,\mu_3}$ [a]
			(mol/mol)			
Trehalose						
0.2	−0.85	42.6	0.25	0.12	−1.28	−2.14
0.5	−1.39	45.4	−0.20	0.25	−4.02	−5.41
0.7	−2.59	47.2	0.20	0.21	−5.17	−7.74
LysHCl						
0.5	−0.70	62.4	2.49	3.69	−1.46	−2.16
1.0	−1.32	65.0	5.64	8.21	−2.43	−3.75
1.5	−1.82	67.7	8.10	11.65	−3.01	−4.81
ArgHCl						
0.2	0.35	62.3	2.09	3.17	0.56	0.91
0.5	0.60	60.9	4.83	7.28	0.97	1.57
0.7	0.39	60.3	5.69	8.67	0.31	0.70
1.0	0.09	60.1	6.09	9.61	−1.02	−0.93
1.5	0.05	60.1	7.76	11.51	0.10	0.15

[a] Preferential binding parameters of the last two columns were calculated from:

$$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3} = \left(\frac{\partial m_3}{\partial m_2}\right)^{\sigma}_{T,\mu_1,\mu_3} + \left(\frac{\partial m_3}{\partial m_2}\right)^{b}_{T,\mu_1,\mu_3}$$

by Equations A5 and A6 (see Appendix).

ues listed in Table 2 shows that the relative increase of the preferential exclusion with protein unfolding is much smaller for LysHCl than for trehalose (e.g., at 0.5 M, preferential exclusion increases by 34% for trehalose and by 13% for LysHCl). Therefore, LysHCl must bind significantly to the unfolded protein. For the ArgHCl solutions, unfolding is accompanied by a small increase in the preferential binding of ArgHCl, as shown by the values of $\Delta\nu_3$. These results lead to the same general conclusion as the surface tension analysis of the unfolding, namely, that weak binding of LysHCl and ArgHCl compensates for the exclusion of co-solvents caused by the surface tension increment, the compensation being larger for ArgHCl than for LysHCl.

The increment of surface newly exposed to contact with solvent on protein denaturation, Δs, was calculated from the variation of K with surface tension, because (Lee & Timasheff, 1981)

$$\left(\frac{\partial \ln K}{\partial \sigma}\right)_T = -\frac{\Delta s}{RT}. \tag{7}$$

Application to the data of Figure 1 for the unfolding of RNase A in trehalose solution gave $\Delta s = 1.35 \times 10^{11}$ cm^2/mol.

Knowledge of these increments ($\Delta\nu_3$ and Δs) together with the data of Table 2 permits the calculation of $(\partial m_3/\partial m_2)^{b,N}$ and $(\partial m_3/\partial m_2)^{b,D}$, i.e., the binding of the co-solvents to the protein in the native and denatured states at T_m. Results of such calculations are given in Table 4. (Details of the calculation are presented in the Appendix.) It is evident that, at T_m, the binding of trehalose is negligible to both the native and the denatured protein. On the other hand, both amino acid salts are bound to both states of the protein, i.e., they are in contact with nonthermodynamically neutral loci on the protein surface, the binding being greater to the denatured protein. Summation of these values with those calculated[7] for the exclusion due to the surface tension increment, $(\partial m_3/\partial m_2)^{\sigma,D}$ and $(\partial m_3/\partial m_2)^{\sigma,N}$, gave the expected preferential interactions of all three co-solvent

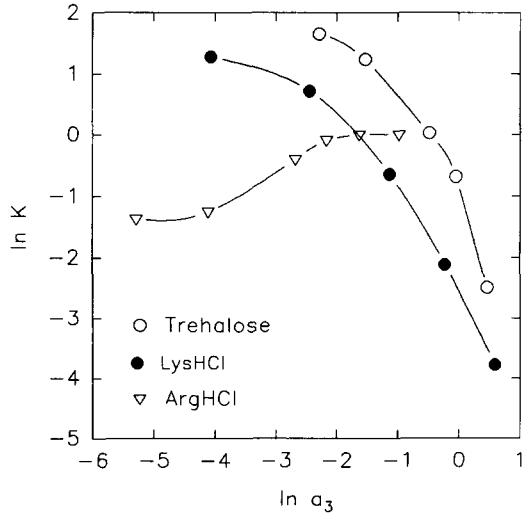

Fig. 3. Dependence of the unfolding equilibrium constant of RNaseA on co-solvent activity: O, in trehalose solutions; ●, in LysHCl solutions; and ▽, in ArgHCl solutions.

[7] As discussed above, this calculation assumes that the additional exposed surface is similar in nature to that of the native protein with respect to its preferential hydration behavior due to the surface tension increment. It can be regarded as a generic surface that is identical in nature in Equations 7 and A3, and has the effective value calculated by Equation 7.

molecules with the native and unfolded RNaseA species at each corresponding T_m, which for the two amino acid salts is a decrease of the degree of preferential exclusion. The values listed in the last two columns of Table 4 show that the large extent of binding of the positively charged amino acids makes $(\partial m_3/\partial m_2)^N$ and $(\partial m_3/\partial m_2)^D$ less negative for LysHCl than for trehalose, and even positive in the case of ArgHCl. It is these slightly more positive values of $(\partial m_3/\partial m_2)^D$ than of $(\partial m_3/\partial m_2)^N$ that give rise to the destabilizing action of ArgHCl.

In conclusion, the results of these studies, based on a very simple model, support the prediction (Sinanoglu & Abdulnur, 1964, 1965; Nicholls et al., 1991; Sharp et al., 1991) that surface tension can play an important role in the stabilization of proteins. On the other hand, weak binding of solvent components to proteins can reduce significantly or even reverse this effect, similar to the reduction of the energy of cavity formation by solvation of a low molecular weight solute (Breslow & Guo, 1990).

Materials and methods

Bovine pancreas ribonuclease A (lot 128F-0462) was obtained from Sigma. α,α-Trehalose dihydrate (lot 19458 and 19635-A) was purchased from Phanstiehl Laboratories, Inc. LysHCl and ArgHCl were obtained from Sigma. RNaseA was further purified on a sulfoethyl-Sephadex C25 column, according to Crestfield et al. (1962). The protein was deionized exhaustively by dialyzing against doubly distilled water or passing through a mixed-bed ion exchange resin (Amberlite MB-1) and finally lyophilized (Gekko & Timasheff, 1981; Arakawa & Timasheff, 1982a).

Thermal denaturation

For the thermal transition equilibrium, the change in absorbance with temperature was followed on a Gilford Response II UV/Vis spectrophotometer with a temperature increment of 0.1 or 0.5 °C and a scan rate of 0.25 °C/min. The wavelength monitored was 287 nm.

Density measurements

The densities of the solvents and the protein solutions were determined on a Precision Density Meter DMA-02 (Anton Paar, Gratz) (Lee & Timasheff, 1974; Lee et al., 1979; Gekko & Timasheff, 1981). The apparent partial specific volume, ϕ, was then calculated from the solution density by (Kielley & Harrington, 1960; Casassa & Eisenberg, 1961, 1964)

$$\phi = \frac{1}{\rho_0} \times [1 - (\rho - \rho_0)/c], \tag{8}$$

where ρ is the density of the solution in grams per milliliter, ρ_o is that of the solvent, and c is the protein concentration in grams per milliliter. The densities of protein solutions were measured at 20 °C at conditions such that the molality of the solvent composition and the chemical potential were kept, in turn, identical in the solvent and in the protein solution. According to the notation of Scatchard (1946) and Stockmayer (1950), water was designated as component 1, protein as component 2, and the additive as component 3. The apparent partial specific volumes of the protein, ϕ_2 at the isomolal conditions, or ϕ_2' at the isopotential conditions, were measured as a function of protein concentration, and extrapolated to zero protein concentration to obtain ϕ_2° and $\phi_2'^\circ$, respectively. Because there was little dependence of the apparent partial specific volumes on protein concentrations, the values of ϕ_2° and $\phi_2'^\circ$ were taken as the average of measurements at different protein concentrations.

Preferential interactions

The preferential binding parameter, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ was obtained from (Cohen & Eisenberg, 1968)

$$\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3} = \rho_0 \frac{(\phi_2^0 - \phi_2'^\circ)}{(1 - \rho_0 \bar{v}_3)}, \tag{9}$$

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

$$\left(\frac{\partial g_1}{\partial g_2}\right)_{T,\mu_1,\mu_3} = -\left(\frac{1}{g_3}\right)\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3}. \tag{10}$$

Preferential interactions reflect the mutual perturbations of the chemical potentials of the co-solvent and protein by each other, as (Kirkwood & Goldberg, 1950; Casassa & Eisenberg, 1964):

$$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3} = -\frac{(\partial \mu_2/\partial m_3)_{T,P,m_2}}{(\partial \mu_3/\partial m_3)_{T,P,m_2}}, \tag{11}$$

where m_i is the molal concentration of component i and P is pressure. Rearrangement gives the preferential interaction parameter

$$\left(\frac{\partial \mu_2}{\partial m_3}\right)_{T,P,m_2} = -\left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3}\left(\frac{\partial \mu_3}{\partial m_3}\right)_{T,P,m_2}$$
$$= -\left(\frac{\partial g_3}{\partial g_2}\right)_{T,P,\mu_3}\left(\frac{RTM_2}{M_3}\right)\left(\frac{1}{m_3} + \frac{\partial \ln \gamma_3}{\partial m_3}\right), \tag{12}$$

where M_i is the molecular weight of component i, R is the universal gas constant, and γ_3 is the activity coefficient of the co-solvent.

Protein concentration

Protein concentration was measured on a Perkin-Elmer Lambda 3B UV/Vis spectrophotometer. Extinction coefficients of RNaseA at 277 nm used were 0.706 L/(g-cm) at pH 2.8, and 0.711 L/(g/cm) at pH 5.8 and pH 6.7. Values of the extinction coefficients of RNaseA in LysHCl at pH 5.8 were found to be: 0.723 L/(g-cm) in 0.5 M, 0.727 L/(g-cm) in 1.0 M, and 0.729 L/(g-cm) in 1.5 M. In the solutions of ArgHCl, they were: 0.720 L/(g-cm) in 0.2 M, 0.721 L/(g-cm) in 0.5 M, and 0.722 L/(g-cm) in 1.0 M at pH 6.7.

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Appendix

Calculation of co-solvent binding to the native and the denatured forms of the protein from the combined surface tension analysis

At constant temperature, the transfer free energy of a protein from water to a co-solvent system consists of two contributions: (1) positive, due to the surface tension increment ($\Delta\mu_2^\sigma$), and (2) negative, due to the weak binding of the co-solvent to the protein ($\Delta\mu_2^b$):

$$\Delta\mu_2 = \Delta\mu_2^\sigma + \Delta\mu_2^b. \tag{A1}$$

In a system that is undergoing a transition, both states of the protein contribute to the total measured transfer free energy:

$$(\Delta\mu_2^{b,N} + \Delta\mu_2^{b,D}) = s^N\sigma^{b,N} + s^D\sigma^{b,D}, \tag{A2}$$

where the native protein is indicated by superscript N and the denatured protein by superscript D, and σ^b is the binding free energy per unit surface area. The corresponding preferential binding parameters are obtained by taking the derivative of Equation A2 with respect to m_3 and applying Equation 11, to the result. This gives

$$\left(\frac{\partial\mu_3}{\partial m_3}\right)_{T,P,m_2}\left[\left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3}^{b,D} + \left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3}^{b,N}\right] = -\left(s^N\left(\frac{\partial\sigma^{b,N}}{\partial m_3}\right) + s^D\left(\frac{\partial\sigma^{b,D}}{\partial m_3}\right)\right). \tag{A3}$$

At T_m, the two species are in equal distribution. Therefore, $(\partial\sigma^b/\partial m_3)^{exp} = [(\partial\sigma^{b,N}/\partial m_3) + (\partial\sigma^{b,D}/\partial m_3)]/2$. Now, $s^D = s^N + \Delta s$, where Δs is the additional surface area generated on protein denaturation[7] and, Equation A3 becomes

$$\left(\frac{\partial\mu_3}{\partial m_3}\right)_{T,P,m_2}\left[\left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3}^{b,D} + \left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3}^{b,N}\right] = -(2s^N + \Delta s)\left(\frac{\partial\sigma^b}{\partial m_3}\right)^{Exp} + \frac{\Delta s}{2}\left(\left(\frac{\partial\sigma^{b,D}}{\partial m_3}\right) - \left(\frac{\partial\sigma^{b,N}}{\partial m_3}\right)\right). \tag{A4}$$

Neglecting the term

$$\frac{\Delta s}{2}\left(\left(\frac{\partial \sigma^{b,\mathrm{D}}}{\partial m_3}\right) - \left(\frac{\partial \sigma^{b,\mathrm{N}}}{\partial m_3}\right)\right),$$

which is relatively small ($\leq 5\%$), we obtain at T_m

$$\left(\frac{\partial \mu_3}{\partial m_3}\right)_{T,P,m_2}\left[\left(\frac{\partial m_3}{\partial m_2}\right)^{b,\mathrm{D}}_{T,P,\mu_3} + \left(\frac{\partial m_3}{\partial m_2}\right)^{b,\mathrm{N}}_{T,P,\mu_3}\right]$$

$$= -(s^{\mathrm{N}} + s^{\mathrm{D}})\left(\frac{\partial \sigma_b}{\partial m_3}\right)^{exp}. \qquad \text{(A5)}$$

In turn, the value of $\Delta\nu_3$ obtained from the Wyman linkage equation can be decomposed into contributions from the surface tension effect and binding:

$$\Delta\nu_3 = \left(\frac{\partial m_3}{\partial m_2}\right)^{\sigma,\mathrm{D}}_{T,P,\mu_3} - \left(\frac{\partial m_3}{\partial m_2}\right)^{\sigma,\mathrm{N}}_{T,P,\mu_3} + \left(\frac{\partial m_3}{\partial m_2}\right)^{b,\mathrm{D}}_{T,P,\mu_3} - \left(\frac{\partial m_3}{\partial m_2}\right)^{b,\mathrm{N}}_{T,P,\mu_3}$$

(A6)

Simultaneous solution of Equations A5 and A6, where the $(\partial\sigma_b/\partial m_3)^{exp}$ values are taken from column 8 of Table 1 and $(\partial m_3/\partial m_2)^{\sigma,\mathrm{D}}$ and $(\partial m_3/\partial m_2)^{\sigma,\mathrm{N}}$ are calculated by the Gibbs adsorption isotherm (Gibbs, 1878) at T_m (Equation 4), gives the stoichiometries of the binding of co-solvent to the protein in the native and denatured states at T_m.