INTERPRETING THE EFFECTS OF SMALL UNCHARGED SOLUTES ON PROTEIN-FOLDING EQUILIBRIA

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Abstract Proteins are designed to function in environments crowded by cosolutes, but most studies of protein equilibria are conducted in dilute solution. While there is no doubt that crowding changes protein equilibria, interpretations of the changes remain controversial. This review combines experimental observations on the effect of small uncharged cosolutes (mostly sugars) on protein stability with a discussion of the thermodynamics of cosolute-induced nonideality and critical assessments of the most commonly applied interpretations. Despite the controversy surrounding the most appropriate manner for interpreting these effects of thermodynamic nonideality arising from the presence of small cosolutes, experimental advantage may still be taken of the ability of the cosolute effect to promote not only protein stabilization but also protein self-association and complex formation between dissimilar reactants. This phenomenon clearly has potential ramifications in the cell, where the crowded environment could well induce the same effects.

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INTRODUCTION

Natural selection has optimized biochemical reactions for a cellular environment that is filled with macromolecules, salts, small solutes, organelles, and the cytoskeleton. However, most in vitro studies are conducted in dilute solution. This mismatch between in vivo and in vitro conditions occurs despite evidence showing that the thermodynamics of biochemical equilibria (e.g., self-association, isomerization, interaction between dissimilar species) can differ markedly between dilute and crowded environments. Addressing the effect of crowding is of great current interest and importance as data from genome and proteome initiatives are combined with equilibrium thermodynamic data in the effort to understand physiology and metabolism at the molecular level (17a).

The difference between in vitro and in vivo environments is easily illustrated when one considers that proteins and nucleic acids alone occupy 30%–40% of the total cellular volume (49), with concentrations as high as 350 mg ml⁻¹ (92). Our purpose is to compare the various models proposed to account for protein stability differences in crowded nonideal solutions compared to dilute ideal solutions.

The cosolutes of primary interest here are osmolytes—a class of small organic molecules that are important in vivo because they stabilize proteins and hence protect organisms against environmental stress (temperature, desiccation, salinity, etc). Osmolytes have been divided into three types (42): free amino acids (and their derivatives), methylamines, and polyols. We focus on polyols because their lack of charge simplifies interpretations.

For over a century, it has been recognized that high concentrations of certain small solutes affect protein stability (10, 29, 90). For example, glycerol and sucrose increase protein stability, whereas urea decreases protein stability (78). Although these two phenomena have been regarded as separate and unrelated, they are just different manifestations of changes in protein-solvent interactions (1, 20, 39, 78).

This chapter is divided into three parts. First, we summarize the data on sugar-induced protein stabilization. Second, we discuss the concepts of ideal and nonideal solutions in terms of classical solution thermodynamics. Third, we review,
compare, and contrast several models currently used to interpret the effects of small, uncharged cosolutes on protein stability.

EXPERIMENTAL OBSERVATIONS

We focus on the elementary protein isomerization reaction between a folded protein (F) and its unfolded state (U). Protein stability is defined as the free energy change associated with the reaction: $\Delta G^\circ = -RT\ln K$, where $K = C_F/C_U$ for a dilute (thermodynamically ideal) solution of protein in solvent. In the presence of a high concentration of osmolyte, the same ratio of molar concentrations, $C_F/C_U$, describes an apparent isomerization constant, $K_{app}$. To assess the effect of osmolyte on protein stability, the osmolyte induced stabilization, $\Delta G^\circ/RT = \ln(K/K_{app})$, is plotted against the osmolyte concentration, $C_3$. Because the plots are often linear (Figure 1), their slopes, $[\delta \Delta G^\circ(F \rightleftharpoons U:H_2O \rightarrow Os)/\delta C_{Os}]$, can be used as the metric for model assessment. Table 1 includes $[\delta \Delta G^\circ(F \rightleftharpoons U:H_2O \rightarrow Os)/\delta C_{Os}]$.

![Figure 1](image)

**Figure 1** The effect of polyol concentration, $C_3$, on the equilibrium between the acid-unfolded and molten globule states of horse heart ferricytochrome c (14). The results are plotted according to the logarithmic form of Equation 19: ◆, glycerol; □, glucose (monosaccharide); ■, trehalose (disaccharide); ○, melezitose (trisaccharide); ●, stachyose (tetrasaccharide).
values for two cytochrome c unfolding reactions: the A-state in equilibrium with the acid-unfolded (U_{pH}) state and the native (N) state in equilibrium with the thermally unfolded (U_T) state. These equilibria have also been studied as a function of polyol size; glycerol can be considered a half saccharide, glucose and galactose are monosaccharides, sucrose and trehalose are disaccharides, melezitose is a trisaccharide, and stachyose is a tetrasaccharide.

The effect of polyols on the A ⇔ U_{pH} reaction has been systematically studied for horse and yeast ferricytochromes c (14, 63). The A-state is an equilibrium model for kinetic folding intermediates. Sometimes called molten globules, these states are compact and possess native-like secondary structures and some native tertiary contacts (36, 43, 82). A-states have been observed for several proteins and are formed by the addition of HCl at low ionic strength to the N state. Initial additions of HCl to the N state unfold the protein (to U_{pH}); upon further addition, the high anion concentration is thought to screen the repulsive positive charges of
CROWDING AND PROTEIN STABILITY

the U,\text{pH} state, allowing the chain to collapse to the A-state (18). Remarkably, the A-state can also form from the acid-unfolded state in the absence of a screening anion. Specifically, isothermal titration of polyol osmolytes into both acid unfolded horse and yeast ferricytochromes c at 1°C causes the proteins to fold into a form closely resembling the A-state (23, 24, 63, 64). NMR and circular dichroism (CD) studies confirm the near-identity of the osmolyte- and salt-induced A-states (14).

The effect of polyols on the \( N \leftrightharpoons U; T \) reaction has been systematically studied for horse ferricytochrome c, ribonuclease A, and chymotrypsinogen (20). The effects of five- and six-carbon polyols on the reversible thermal unfolding of \( \alpha \)-chymotrypsinogen, hen egg white lysozyme, and hen egg white trypsin inhibitor have also been studied (33). For these latter studies, precise values for \( [\Delta \Delta G_{o/b} (F \leftrightharpoons U; H_2O \rightarrow Os)/\delta C_{Os}] \) cannot be extracted because only two polyol concentrations were examined. Nevertheless, the stabilization increases with both polyol size and concentration.

Three general observations can be gleaned from the data in Figure 1 and Table 1. First, polyol osmolytes stabilize proteins. Second, the stabilization is a linear function of osmolyte concentration. Third, \( [\Delta \Delta G_{o/b} (F \leftrightharpoons U; H_2O \rightarrow Os)/\delta C_{Os}] \) increases with increasing polyol size. These observations must be kept in mind when deciding the acceptability of models used to interpret these effects.

COSOLUTES AND THERMODYNAMIC NONIDEALITY

The fundamental information derived from an experiment designed to quantify the effect of a cosolute on protein stability is thermodynamic and hence independent of any model of the phenomenon. It is therefore appropriate to consider the nature of this unequivocal information before introducing the models designed to interpret the experimental results. For that purpose, it is convenient to commence with a discussion of thermodynamic nonideality in the simplest solution comprising a single solute in an inert solvent.

Nonideality in a Single Solute System

Under conditions where each solute molecule acts independently of any other in the solution, the chemical potential of a single solute, component 2 in Scatchard terminology (65), dissolved in inert solvent at fixed temperature \( T \) is related to solution composition by an equation of the form

\[
\mu = \mu^0_2 + RT \ln X_2,
\]

where \( \mu^0_2 \) is the chemical potential of solute in a defined standard state, and \( X_2 \) is the solute concentration, which can be expressed on a mole-fraction, molar, or molal basis. \( R \) is the universal gas constant, and \( T \) is the absolute temperature. However, Equation 1 as it stands does not suffice to describe the chemical potential of the solute because it fails to indicate the conditions under which the parameter
is being defined. In a solution at fixed temperature, there are still two situations to consider: one in which the pressure is held constant (as in a spectrophotometer or light-scattering cuvette), and the other in which the constancy constraint is imposed on the chemical potential of solvent (as in equilibrium dialysis and osmotic pressure experiments). In the former situation, the standard state refers to a solution at the same pressure but a different chemical potential of solvent, whereas maintenance of constant chemical potential of solvent in the latter case gives rise to a standard state in which the pressure differs from that for which \( \mu_2 \) is being defined. The requirement for ideal solution behavior is the absence of any solute–solute or solute–solvent interactions, and hence assumed thermodynamic ideality is only a reasonable approximation in the limit of very dilute solution. At finite concentrations, the expression for chemical potential of solute must be modified to account for physical interactions between solute molecules (and between solute and solvent molecules if the solvent is not inert).

For systems in which the chemical potential of solvent (component 1, \( \mu_1 \)) is held constant, the composition dependence of the chemical potential of the single solute (component 2) is given by

\[
(\mu_2)_{T,\mu_1} = (\mu_2^0)_{T,\mu_1} + RT \ln z_2,
\]

where the molar activity \( z_2 \) and the molar concentration \( C_2 \) are related by the expression

\[
z_2 = \gamma_2 C_2,
\]

where \( \gamma_2 \) is the molar activity coefficient.

As noted by Hill (28), the standard chemical potential \( (\mu_2^0)_{T,\mu_1} \) refers effectively to a state of the solvent, and the presence of solute in the solution gives rise to osmotic pressure \( \Pi \), which is the difference between the pressures in the solution and the standard state.

On the other hand, the selection of pressure as the second constraint dictates the description of the solute chemical potential in terms of a molal thermodynamic activity \( a_2 \) via the relationship

\[
(\mu_2)_{T,P} = (\mu_2^0)_{T,P} + RT \ln a_2,
\]

where the thermodynamic activity is now expressed in terms of the molal solute concentration \( m_A \) and a molal activity coefficient \( \gamma_2 \) (Equation 5):

\[
a_2 = \gamma_2 m_2.
\]

From an experimental viewpoint, it is convenient to describe the thermodynamic nonideality in terms of a virial expansion in solute concentration—a situation encountered in the familiar osmotic pressure relationship

\[
\Pi/(RT) = [((\mu_2^0)_{T,\mu_1} - (\mu_2)_{T,\mu_1})/(RT)] = C_2 + B_{22}C_2^2 + B_{222}C_2^3 + \cdots.
\]
where $B_{22}$ and $B_{222}$ are the respective second and third osmotic virial coefficients accounting for pairwise and ternary solute self-interactions. Furthermore, thermodynamic arguments (28) show that the molar activity coefficient is described in terms of the same virial coefficients; specifically,

$$\gamma_2 = \exp \left[ 2B_{22}C_2 + \left( \frac{3}{2} \right) B_{222}C_2^2 + \cdots \right].$$

(7)

When the constraints of constant temperature and pressure prevail, the virial expansion is in terms of the solvent chemical potential, and the counterparts of Equations 6 and 7 become

$$\left[ (\mu_1^0)_{T,P} - (\mu_1)_{T,P} \right] / (RT\rho_1) = m_2 + C_{22}m_2^2 + C_{222}m_2^3 + \cdots$$

(8)

and

$$\gamma_2 = \exp \left[ 2C_{22}m_2 + \left( \frac{3}{2} \right) C_{222}m_2^2 + \cdots \right].$$

(9)

where $\rho_1$, the solvent density, is introduced to retain the description of the chemical potential difference as a molar concentration (mol L$^{-1}$) despite the expression of solute concentration in molal (mol kg$^{-1}$) terms. Although the osmotic virial coefficients have strict statistical-mechanical meaning (44), the corresponding $C_i$ coefficients have no such significance except in situations where the solvent and solution are incompressible. Fortunately, such assumed constancy of solvent density and solute partial specific volume ($\bar{\nu}$) is a reasonable approximation for aqueous protein solutions, whereupon the two sets of virial coefficients are related by Equations 10 and 11, in which the partial specific volume of solute is multiplied by $M_2$, its molar mass, to convert it to its molar counterpart:

$$C_{22} = (B_{22} - M_2\bar{\nu}_2)\rho_1,$$

(10)

$$C_{222} = [B_{222} - 2B_{22}M_2\bar{\nu}_2 + (M_2\bar{\nu}_2)^2]\rho_1^2.$$

(11)

Thus any of the virial expansions may be converted to the other concentration scale by coupling Equations 10 and 11 with the corresponding expressions for concentration scale interconversion, namely,

$$C_2 = (m_2\rho_1)/(1 + M_2\bar{\nu}_2\rho_1),$$

(12)

and

$$m_2 = C_2/[1 - (M_2\bar{\nu}_2C_2)\rho_1].$$

(13)

Of particular relevance in the present context is the consequence that the results of experiments involving aqueous protein solutions may invariably be expressed in terms of the conventional osmotic virial coefficients $B_2$. However, account must be taken of the constraints pertaining to the definition of chemical potential and hence the concentration scale in which the thermodynamic activity is being monitored experimentally (2, 38, 86).
Effect of Inert Cosolute Addition

Supplementation of the protein solution with a cosolute (component 3) leads to additional thermodynamic nonideality of the protein solute arising from solute-cosolute interactions. For example, under constraints of constant temperature and solvent chemical potential, Equations 6 and 7 assume the forms

\[
\left( \frac{\mu_2^0 - \langle \mu_2 \rangle_{T, \mu_1}}{RT} \right) = C_2 + B_{222}C_2^2 + B_{222}C_2^3 + \cdots, 
\]

and

\[
\gamma_2 = \exp \left[ 2B_{222}C_2 + \frac{3}{2}B_{222}C_2^2 + B_{233}C_3 + \frac{1}{2}B_{223}C_2^3 + \cdots \right],
\]

which include the contributions from all pairwise and ternary solute-solute and solute-cosolute interactions.

The cosolutes of primary interest here are osmolytes because their lack of charge simplifies the statistical-mechanical interpretation of the magnitudes of the cross-term virial coefficients \( B_{223}, B_{233}, B_{333}, \) etc. Advantage is also taken of the fact that the concentration of cosolute (often as high as 4 M in in vitro studies) greatly exceeds that of protein \( C_3 \gg C_2 \) —a situation that leads to domination of the activity coefficient expressions by the terms involving cosolute concentration. On the grounds that terms containing \( C_2 \) in Equation 15 may be neglected under these circumstances, the expression for the molar activity coefficient becomes

\[
\gamma_2 \approx \exp \left[ B_{233}C_3 + \frac{1}{2}B_{223}C_2^3 \right].
\]

Consequently, the effects of inert cosolutes may be analyzed in terms of the cosolute-concentration dependence of the thermodynamic activity coefficient or some related parameter such as the apparent isomerization constant describing protein unfolding.

Polyols As Inert Cosolutes

Inasmuch as the above thermodynamic considerations presume inertness of the cosolute, the latter should clearly exhibit no chemical reactivity toward either the protein solute or the aqueous solvent. In that regard, Stokes & Robinson (74) have classified polyols as being semi-ideal because they do not interact chemically in very concentrated solution. NMR (19) and magnetic relaxation (75) studies have revealed that hydrogen bonds between saccharides and water resemble water-water hydrogen bonds, but that hydrogen bonding is more extensive in sucrose solutions. Thermodynamic evidence consistent with consideration of polyols as inert solutes comes from the concentration dependence of the activity coefficient inferred from isopiestic measurements (86). From Figure 2, which presents the requisite plot of results (66, 86) for glycerol, sucrose, and urea in accordance with
Figure 2  Results of isopiestic measurements of the concentration dependence of the activity coefficient for sucrose (•), glycerol (□), and urea (■). The data (66) are plotted in accordance with Equation 9.

The logarithmic form of Equation 9, it is evident that the two polyols exhibit the positive deviation from Raoult’s law that characterizes a solute exhibiting neither chemical self-interaction nor interaction with solvent. On the other hand, negative deviations from Raoult’s law are observed for urea, which undergoes reversible self-association via intermolecular hydrogen bonding (67). Inasmuch as the plots for the two polyols are essentially linear, the thermodynamic nonideality of sucrose and glycerol solutions is described adequately by Equation 9 truncated at the second virial coefficient term. Furthermore, the value of 0.29 L mol\(^{-1}\) that is inferred for the osmotic second virial coefficient \(B_{22}\) from the slope, \(2C_{22} = 2(B_{22} - M_2\bar{v}_2)\rho_1\), of the plot for sucrose has also been obtained by frontal gel chromatography (71). As might reasonably be expected, a smaller magnitude (0.085 L mol\(^{-1}\)) for \(B_{22}\) applies to the physical self-interaction of glycerol.

Although sucrose, glycerol, and other small polyols exhibit positive deviations from Raoult’s law, the consequent ability to assign a magnitude to the second virial coefficient for self-interaction does not establish unequivocally the chemical inertness of the solute. If the solute were undergoing self-association, the estimated
osmotic second virial coefficient would be \( B_{22} - K_2 \), where \( K_2 \) is the molar dimerization constant (83, 84). It is therefore always conceivable that the positive slopes observed for glycerol and sucrose in Figure 2 could merely reflect dominance of the magnitude of \( B_{22} \) over that of \( K_2 \)—just as the negative slope for urea reflects a situation where \( K_2 > B_{22} \). It is unfortunate that in studies of protein stabilization by cosolutes, there is no method available for unequivocally establishing cosolute inertness, which can therefore only be surmised on the basis of consistent effects with a range of putatively inert cosolutes.

MOLECULAR INTERPRETATIONS OF THE THERMODYNAMIC NONIDEALITY

Consider the effect of cosolute concentration on the equilibrium constant for the interconversion of a protein between the folded (F) and the unfolded (U) states, \( F \leftrightarrow U \), that is being monitored spectrophotometrically. Because the experiment is conducted under conditions of constant pressure, the dimensionless isomerization constant \( K \) should be expressed in terms of molal species activities as

\[
K = \frac{a_U}{a_F} = \frac{z_U}{z_F},
\]

(17)

where U and F are the two forms of the protein solute (component 2). However, for incompressible solutions, \( K \) is also given by the corresponding ratio of molar activities. In such experiments, the cosolute concentration (0.1–1.0 M) greatly exceeds that of the relatively dilute (1–10 \( \mu \)M) protein solution (\( C_3 \gg C_2 = C_F + C_U \)), and hence nonideality emanates solely from solute-cosolute virial terms. From Equations 7 and 16 it follows that

\[
K = \left( \frac{C_U}{C_F} \right) \exp[(B_{U3} - B_{F3})C_3 + \cdots].
\]

(18)

Upon identifying the ratio \( C_U/C_F \) as the measured (apparent) equilibrium constant \( K_{app} \), we obtain the relationship

\[
K_{app} = K \exp[(B_{F3} - B_{U3})C_3 + \cdots].
\]

(19)

Thus, subject to the proviso that the activity coefficients are described adequately by pairwise solute-cosolute interactions, the logarithm of the measured isomerization constant exhibits a linear dependence upon cosolute concentration, and the slope defines the difference between the second virial coefficients for cosolute and the two protein states. Such behavior is shown in Figure 1, which summarizes the effects of a range of small polyols (glycerol \( \rightarrow \) tetrasaccharide) on the equilibrium between the acid unfolded- and A-states of horse cytochrome c (14): Clearly \( (B_{F3} - B_{U3}) \) becomes progressively more negative with increasing cosolute size.

This thermodynamic analysis of cosolute effects on protein-folding equilibria is certainly unequivocal, but the magnitude of \( (B_{F3} - B_{U3}) \) per se provides no molecular insight into the source of the cosolute-dependence of \( K_{app} \). A range of models
has therefore been devised to account for the cosolute-dependent nonideality in terms of acceptable molecular interpretations. Although resort to model-dependent interpretations of thermodynamic data represents a departure from the classical protocol, the approach is justified if the model provides a helpful explanation of the thermodynamic behavior of the system under consideration (17).

Molecular information about the source of the linear dependence of \( \ln K_{\text{app}} \) upon cosolute concentration (Figure 1) has been sought by two major avenues of reasoning. In one, the nonideality is rationalized on the statistical-mechanical basis of excluded volume (44) wherein displacement of the equilibrium is viewed as an entropic consequence of molecular crowding (26, 27, 72). This category of models also includes the preferential solvation treatment of thermodynamic nonideality (2, 3, 9, 35, 38, 59, 79) as well as those based on scaled particle theory (5, 37, 45, 61). Other models are based on Wyman linkage functions (89), whereby the dependence of the equilibrium constant upon cosolute concentration is viewed in terms of linked chemical equilibria (68, 77, 80).

**Excluded Volume Treatment of Thermodynamic Nonideality**

This consideration of models begins with the interpretation of the second virial coefficient on the statistical-mechanical basis of excluded volume (44), whereby the nonideality emanates from the space-filling effects of the cosolute. In this model, the solute and cosolute are usually modeled as rigid, impenetrable spheres (26, 27, 72, 86), whereupon the second virial coefficient for the physical (excluded volume) interaction of a solute with radius \( r_2 \) and a cosolute with radius \( r_3 \) is simply the covolume. That is

\[
B_{23} = \frac{4}{3} \pi N (r_2 + r_3)^3,
\]

where \( N \) is Avogadro’s number. \( B_{23} \) reflects the volume from which the centers of the solute and cosolute are mutually excluded (Figure 3). Inasmuch as the portrayal of either a protein or a small polyol as a rigid impenetrable sphere represents a radical departure from physical reality, \( r_2 \) and \( r_3 \) must be regarded as effective thermodynamic radii, and their magnitudes should be sought from thermodynamic data. Justification for ascribing an effective thermodynamic radius to polyols such as sucrose and glycerol is provided by the positive linear dependence of the logarithm of the activity coefficient upon cosolute concentration (Figure 2). Interpretation of the consequent second virial coefficient for self-interaction on the basis of the relationship,

\[
B_{33} = \frac{16}{3} \pi N r_3^3,
\]

leads to effective thermodynamic radii of 2.0 and 3.1 Å for glycerol and sucrose, respectively (86).

Application of a comparable approach to obtain \( B_{23} \) and hence determine the effective thermodynamic radius of a protein is illustrated in Figure 4, which summarizes results from sedimentation equilibrium studies of the effect of sucrose
concentration on the buoyant molecular weight of bovine thyroglobulin. In such experiments, the apparent buoyant molecular weight (derivative of the logarithm of protein concentration with respect to the square of radial distance) is given by (30)

\[ M_2(1 - \phi_2' \rho_d) = M_2(1 - \bar{\nu}_2 \rho_1) B_{23} M_{23} C_3 + \cdots, \]  

(22)

where \( \phi_2' \) is the apparent partial specific volume of the protein solute obtained by coupling the density of the dialyzed protein solution with the density \( \rho_d \) of the sucrose-supplemented diffusate against which it has been dialyzed (7); \( \rho_1 \) remains the density of unsupplemented solvent. Substitution of the consequent value of
Illustrative use of sedimentation equilibrium data for evaluating the second virial coefficient $B_{23}$ describing the excluded volume interaction between bovine thyroglobulin and sucrose. The data (30) are plotted according to Equation 22.

$1500 \pm 300$ L mol$^{-1}$ for $B_{23}$ into Equation 20 yields a value of $84 \pm 5$ Å for $(r_2 + r_3)$ and hence an effective thermodynamic radius of $81$ Å for thyroglobulin. The effective thermodynamic radii of the few proteins determined (30, 32, 73) indicate that the effective hydrodynamic radius (Stokes radius) is likely to be a reasonable estimate of $r_2$ in instances where it has not been measured.

Although the few illustrative applications of the excluded volume approach (15, 26, 27, 71, 72, 81a, 83, 86) have tended to consider the protein solute in terms of spherical geometry, this approximation is not an absolute requirement (57). Covolume expressions are also available to describe the physical interactions of a spherical cosolute and a solute that is better modeled as a prolate or oblate ellipsoid of revolution (51, 52). Furthermore, from the viewpoint of determining the covolume of a polyol and a fully unfolded protein, depiction of the latter as a random-flight polymer (31, 48) is preferable to its consideration as a rigid impenetrable sphere or ellipsoid. In that regard, Zhou & Hall (91) have treated the unfolded protein as a string of spherical beads for their determination of $B_{23}$, but the validity of their expression for the covolume has been questioned (48).

Application of the excluded volume approach to the study of reversible protein isomerization is illustrated in Figure 5a, which refers to the stabilizing effect of sucrose against the acid-unfolding of ribonuclease (72). For this system, the inclusion of the polyol clearly displaces the spectrophotometrically detected transition
Figure 5 Use of molecular crowding by small inert cosolutes to study reversible protein unfolding (72). (a) pH-dependence of the spectrally observed transition between native and acid-extracted states of ribonuclease in the absence of cosolute and in the presence of 0.5 M sucrose. (b) Effect of glycerol concentration on the isomerization equilibrium between native and thermally unfolded states of ribonuclease, the results being plotted according to the logarithmic form of Equation 19.
between native and unfolded states to more acidic pH. Combination of any value of $K_{app}$ with that of $K$, inferred from the spectral change at the same pH in the absence of sucrose, gives rise to an estimate of $0.56 \pm 0.15$ for the ratio $K_{app}/K$ in the presence of 0.5 M sucrose. Combination of the consequent value (Equation 19) of $-1.2 \pm 0.5$ L mol$^{-1}$ for $(B_{F3} - B_{U3})$ with that of 25.1 L mol$^{-1}$ calculated from respective radii of 3.1 and 18.4 Å for sucrose (Figure 2) and native ribonuclease (13) leads to an effective thermodynamic radius of 18.7 $\pm 0.1$ Å for the acid-stabilized isomeric state of ribonuclease—a value commensurate with the hydrodynamic radius deduced viscometrically (6).

The quantitative agreement observed between experiment and prediction in Figure 5 reflects not only the chemical inertness of the cosolute but also the conformation of the acid-expansion of ribonuclease with representation of the native and unfolded enzyme states as rigid impenetrable spheres. Extension of the approach to glycerol-effected stabilization of the same enzyme against thermal unfolding (Figure 5b) exhibits qualitative conformity with the predictions of Equation 19. However, the inferred effective thermodynamic radius of 18.6 Å for the fully unfolded ribonuclease is a gross underestimate of the corresponding Stokes radius (72). This discrepancy, which finds quantitative parallel in the studies of cytochrome c stabilization reported in Figure 1, signifies the invalidity of assumptions inherent in the application of Equation 19 to the system.

Preferential Protein Solvation Model

The stabilization of protein structure by high concentrations of inert cosolutes has frequently been analyzed in terms of preferential solvent occupancy of the protein domain at the expense of small cosolutes (1–3, 20, 35, 38, 39, 78–80). In this analysis, the solvation parameter, $\zeta_3$, defined as (9, 59)

$$\zeta_3 = (M_3/M_2)(\partial m_3/\partial m_2)_{\mu_1=\mu_3},$$  \hspace{1cm} (23)

is negative in the excluded volume situation (Figure 3) wherein $B_{23}$ is positive. Indeed, $\zeta_3$ is sometimes converted to a solvation parameter $\delta$,

$$\delta = (\partial g_1/\partial g_2)_{\mu_1=\mu_3} = -\zeta_3/(m_3M_3),$$  \hspace{1cm} (24)

which corresponds to the mass of solvent $(g_1)$ in the protein domain per unit mass of protein. However, it should be recognized that the protein domain defined in this manner includes the solvent contained in the covolume shell (hatched area in Figure 3) surrounding the protein (2). Indeed, the equivalence of the preferential solvation and excluded volume treatments of thermodynamic nonideality is established by the demonstration (85) that

$$-\zeta_3/(m_3M_3) = (B_{23} - M_2\bar{v}_2)\rho_1/M_2 + \cdots.$$  \hspace{1cm} (25)

The solvation parameter thus represents a measure of the difference between the apparent volume in the protein region that is inaccessible to cosolute $(B_{23})$
and the corresponding volume that is also inaccessible to solvent ($M_2 \bar{v}_2$). Because the point of closest approach for the centers of protein and cosolute molecules is determined by the sum of their radii, the extent of solvation is a manifestation of the size of the cosolute rather than of the protein, which may well exhibit the same radius ($r_2$) in concentrated solutions of (say) glycerol and sucrose but is described as being more solvated in the presence of the disaccharide. The concept of protein solvation is frequently misconstrued as signifying a cosolute-dependent change in the magnitude of $r_2$ instead of $(r_2 + r_3)$.

Use of the preferential solvation model to account for displacement of a protein unfolding equilibrium ($F \rightleftharpoons U$) by small inert cosolutes (3, 38, 78, 79) entails introduction of the Wyman (89) linkage function

$$\frac{\partial \ln K_{app}}{\partial \ln a_3}_{T,P,m_2} = \left( \frac{\partial m_3}{\partial m_U} \right)_{T,P,\mu_3} - \left( \frac{\partial m_3}{\partial m_F} \right)_{T,P,\mu_3}.$$ (26)

The finding that the apparent equilibrium constant decreases with increasing cosolute concentration (as in Figure 5b) signifies a larger negative value of $\zeta_3$ for protein in the unfolded state. Stabilization is thus attributed to a cosolute-mediated decrease in the extent of protein solvation; or, in other words, cosolute-mediated stabilization of the protein in the more compact, native state.

### Scaled Particle Theory

In keeping with the excluded volume model, scaled particle theory is also a statistical-mechanical treatment that considers solution nonideality arising from the presence of uncharged cosolutes. This alternative approach leads to expressions for the chemical potentials of particles that depend upon the particle size and number density (37, 46, 61). Calculations of interaction potentials have yielded exact values for the second and third virial coefficients for hard spheres, and magnitudes of the fourth and fifth virial coefficients have acceptably small errors. An obvious advantage of scaled particle theory is thus the relative ease with which analysis of thermodynamic nonideality may be extended to incorporate a range of solute or cosolute concentrations for which description of the nonideality in terms of nearest-neighbor interactions ceases to be an adequate approximation. The theory has also been extended to accommodate thermodynamic nonideality arising from solutes with nonspherical geometry (5, 21). Scaled particle theory was developed initially on the basis that the solvent could be regarded as a continuum (47, 62), but it has been modified subsequently (4) to allow for space-filling effects of the solvent as well as the cosolute. Here, we focus on the modified theory (4). Although this adaptation seemingly has merit on the physical grounds that a water molecule ($r_1 \approx 1.4 \text{ Å}$) is only marginally smaller than glycerol ($r_3 \approx 2.0 \text{ Å}$), it should be noted that the link with thermodynamics becomes more tenuous in that the fundamental description of experimental data for the effect of cosolute on protein unfolding (Equation 19) contains no solvent term. Consequently, the radii assigned to solute and cosolute cease to be effective thermodynamic parameters.
Nonideal effects are considered by scaled particle theory as follows. We need to know the fraction of the solution chemical potential that is due to solvating a hard particle B, considered for simplicity to be a sphere. This is done by calculating the amount of work necessary to create a cavity the size of a solute particle. Nonideal effects are described as the excess chemical potential $\mu_{E1}$ derived from dissolving a test molecule in pure solvent versus a crowded mixture,

$$
\mu_{E1} \left( \frac{r_3}{r_1}, \frac{r_2}{r_3}, \Phi \right) = \mu_1 \left( \frac{r_3}{r_1}, \frac{r_2}{r_3}, 0 \right) - \mu_1 \left( \frac{r_3}{r_1}, \frac{r_2}{r_3}, \Phi \right),
$$

(27)

where $r_i$ is the radius of each component, and $\mu_1$ is the chemical potential of water.

The fraction of solution volume occupied by a species (i.e., its volume occupancy), $\Phi_i$, is

$$
\Phi_i = \frac{4}{3} \pi r_i^3 d_i,
$$

(28)

where $d$ is the number density and the rest of term is the cosolute volume. The excess chemical potential is the work, $g$, required to create a cavity the size of the protein molecule in crowded solution minus the work required to create the same cavity in pure solvent.

$$
\mu_{E1} \left( \frac{r_3}{r_1}, \frac{r_2}{r_3}, \Phi \right) = g_1 \left( \frac{r_3}{r_1}, \frac{r_2}{r_3}, \Phi \right) - g_1 \left( \frac{r_3}{r_1}, \frac{r_2}{r_3}, 0 \right).
$$

(29)

Scaled particle theory describes the reversible work, $g(r_i)$, necessary to create a spherical cavity of radius $r_2$ for a single protein molecule in a solution containing different hard-sphere species,

$$
g(r_2, \Phi) = -\ln(1 - S_3) + \left[ \frac{6 S_2}{(1 - S_3)} \right] r_2 + \left[ \frac{12 S_1}{(1 - S_3)} + \frac{18 S_2^2}{(1 - S_3)^2} \right] r_2^2 \\
+ \left[ \frac{8 S_0}{(1 - S_3)} + \frac{24 S_1 S_2}{(1 - S_3)^2} + \frac{24 S_2^3}{(1 - S_3)^3} \right] r_2^3,
$$

(30)

where, for a hard sphere solution containing only solvent and cosolute molecules, the terms in $S$ are:

$$
S_0 = \frac{1}{8 r_3^2} \left[ \left( \frac{r_1}{r_3} \right)^3 \Phi_1 + \Phi_3 \right]; \quad S_1 = \frac{1}{4 r_3^2} \left[ \left( \frac{r_1}{r_3} \right)^2 \Phi_1 + \Phi_3 \right]; \\
S_2 = \frac{1}{2 r_3^2} \left[ \left( \frac{r_1}{r_3} \right) \Phi_1 + \Phi_3 \right]; \quad S_3 = \Phi_1 + \Phi_3.
$$

(31)

The last term in $r_3^3$ in Equation 30 represents the hard sphere pressure, which is generally neglected because, for real fluids, it is negligible compared to the other terms. For model fluids, however, the hard sphere pressure is usually thousands of atmospheres, whereas the true pressure is only one atmosphere, indicating that the attractive interactions between and among solvent and solute molecules largely
cancel the hard sphere repulsions. Consequently, the term in $r^2$, the surface work, dominates. Although scaled particle theory does not explicitly take into account the chemical interactions between solvent molecules, they are included implicitly because the solvent density is taken from experiment, and it is primarily the soft interactions (hydrogen bonding, van der Waal’s interactions, etc) that determine the solution density.

The volume occupancy of water molecules, $\Phi_{1,0}$, is 0.368. That is, in pure water the water molecules occupy 37% of the total solution volume. Addition of cosolutes decreases $\Phi_1$. This decrease can be estimated by assuming a spherical cosolute and using density data from the literature. Initial estimates of cosolute radii can be obtained from a bond-counting approach. The radii are then fine-tuned using an iterative approach that makes the hard sphere pressure of the crowder solution equal to that for pure water, as suggested by Guttman et al. (25).

Berg derived a method for estimating $\Phi_1$ when density data are unavailable:

$$\Phi_1 \left( \frac{r_3}{r_1}, \Phi \right) = \Phi_{1,0} \left[ 1 - \Phi_3 - \Phi f \left( \frac{r_3}{r_1} \right) \right]$$

(32)

where

$$f \left( \frac{r_3}{r_1} \right) = \frac{3(1 - \Phi_{1,0})}{\left( \frac{r_3}{r_1} \right)^2 (1 + 2\Phi_{1,0})^{-2}} \left[ \frac{r_3}{r_1} (1 + 2\Phi_{1,0}) + (1 - \Phi_{1,0}) \right]$$

$$\times \left[ \frac{1 + (1 - \Phi_{1,0})}{3 \Phi_{1,0}} \right]$$

(33)

For protein folding, the difference in the work of cavity formation in the presence and absence of cosolute for each protein conformation is calculated. At a given cosolute concentration, the change in free energy upon folding ($\Delta G^f$) is then determined by knowing the sizes of the solution components and their respective volume occupancies.

We end this section with a comparison of scaled particle theory and excluded volume theory in terms of the expected cosolute size dependence of protein stabilization (Figure 6). First, we consider the effect of cosolute size at constant volume occupancy (Figure 6a). Both theories predict that the stabilization nearly vanishes for large cosolutes because the cosolute concentration approaches zero. That is, there are very few cosolute molecules in solution. However, the theories predict very different behavior for small crowders. Excluded volume theory predicts that the stabilization will approach infinity as the size of the cosolute approaches zero, but scaled particle theory predicts that the stabilizing effect vanishes as the size of the cosolute approaches the size of water. For excluded volume theory, the stabilizing effect approaches infinity because the cosolute concentration approaches infinity. For scaled particle theory, the stabilizing effect smoothly vanishes because the cosolute and water become equivalent. This smoothly vanishing stabilization is satisfying, but the satisfaction comes at a price—the introduction of an additional adjustable parameter (the water radius). As discussed above, this factor causes
Figure 6  Effects of crowder size on excluded volume theory (○) and scaled particle theory (●). The stabilizing effect, $\Delta \Delta G_U$, is plotted against the size of the crowding molecule at (a) constant volume occupancy, $\Phi$, and (b) constant crowder concentration. Equation 19 was used to produce panel a and Equations 29–33 were used to produce panel b. Radii of 1.375 Å, 22.0 Å, and 31.0 Å were used for water, the folded state, and the unfolded state, respectively.
scaled particle theory to lose touch with fundamental solution thermodynamics. Interestingly, scaled particle theory predicts a maximum stabilization for cosolutes with a radius about 1.8 times that of water. This maximum reflects the packing possibilities of cosolute and water, which are not a part of excluded volume theory. If water is treated as a structureless background in scaled particle theory (47, 62), the effect of cosolute size on stabilization mimics the curve for excluded volume theory (data not shown).

Figure 6b shows the effect of cosolute radius at constant cosolute concentration. For excluded volume theory, the stabilization is an approximately linear function of cosolute radius over a small range of radii, but the stabilization does not vanish when the cosolute radius approaches zero. For scaled particle theory, the stabilization does vanish as the cosolute radius approaches the radius of water, but the stabilization begins to increase steeply at the largest cosolute radii. This steep increase is driven by the steep rise in the volume occupancy of the cosolute. If water is treated as a structureless background in scaled particle theory (47, 62), the effect of cosolute size on stabilization mimics the curve for excluded volume theory out to a radius of \(\sim 10\ \text{Å}\), but then increases sharply (data not shown). The deviation between the scaled particle theories and excluded volume theory at high solute concentration is due to the neglect of higher order virial coefficients in the excluded volume treatment.

Unfortunately, sufficient experimental data are not available to differentiate the theories. Furthermore, each theory has advantages and disadvantages in terms of the definition of cosolute radius. The radii used for excluded volume theory are fundamental thermodynamic quantities, but they need not reflect the size as defined by, say, a crystal structure. For scaled particle theory, the concept of the hard sphere radius is easy to grasp, but the hard sphere radius is not a well-defined thermodynamic quantity. Specifically, scaled particle theory does not take explicit account of binding interactions between the species in solution.

**Wyman Linkage Functions**

Wyman linkage functions relate thermodynamic equilibria to the binding of small molecule effectors (e.g., a ligand, cofactor, etc). The fundamental expression for a reaction \(F \rightleftharpoons U\) is

\[
\left( \frac{\partial \ln K}{\partial \ln a_3} \right)_{T,P,a_1} = \bar{n}_3^U - \bar{n}_3^F = \Delta \bar{n}_3,
\]  

(34)

where \(K\) is the equilibrium constant, \(a_3\) is the activity of the modulating ligand, \(\bar{n}\) is a thermodynamic measure of the number of ligands binding the reacting molecule, and \(T\) and \(P\) are the temperature and pressure, which are held constant. Because water is not a reactant, the water activity is taken to be constant—a nonrigorous assumption in the sense that it requires \(\mu_1\) instead of \(P\) as the second constraint on the partial differential in Equation 34. Albeit inexact for that reason, Equation 34 provides an expression that signifies that the effector (cosolute in the present context) controls the reaction because its interactions with the products
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and reactants are different. Thus, the equilibrium is linked to effector binding such that a change in any one parameter implies a change in all the others.

This model holds for ligands that bind with considerable affinity ($K > 10^2$ M$^{-1}$). Water need not be considered if it is not a significant participant in the equilibrium:

$$P + L \rightleftharpoons P \bullet L \quad K = \frac{C_{P \bullet L}}{C_P C_L},$$

(35)

where $P$ is the protein, $L$ is the ligand and $P \bullet L$ is the protein-ligand complex. However, in the context of protein stabilization by small neutral solutes, the role of water as a reactant must be considered. It is the interaction of cosolute and water with the endstates of the protein (i.e., F and U) that determines whether the protein is stabilized or destabilized.

Tanford modified Wyman’s expression to incorporate hydration changes when cosolute is present (77). For this three-component system, Tanford treats the activities of water and cosolute ($a_1$ and $a_3$, respectively) as independent components, while constraining the system via the Gibbs-Duhem equation such that $a_1$ depends on $a_3$. In this way, the modified linkage function considers two different ligands instead of just one. For example, we might consider that one sugar molecule could replace several water molecules at a specific site:

$$P_i \bullet (H_2O)_m + L \rightleftharpoons P_i \bullet L + mH_2O$$

(36)

where $P_i$ is the protein site undergoing exchange, $L$ is the cosolvent, and $mH_2O$ is the number of water molecules involved in the exchange reaction. In the case of osmolytes and denaturants, ligand binding is weak and significant concentrations (millimolar to molar) are required to exert a thermodynamic effect. This weak binding implies that water competes effectively with weak ligands, and its contribution must be explicitly represented (68). The modified expression is:

$$\left(\frac{\partial \ln K}{\partial \ln a_3}\right)_{T,P} = \Delta \tilde{n}_3 - \frac{m_3}{m_1}\Delta \tilde{n}_1,$$

(37)

where $\Delta \tilde{n}_3$ and $\Delta \tilde{n}_1$ are the changes in bound ligand and water, respectively. The absence of $\Delta \tilde{n}_1$ in the original Wyman linkage equation does not imply that water binding is absent, but that water binding is weak compared to effector binding. It is for this reason that water can be ignored in the case of strong ligand binding at low ligand concentration.

Preferential Interactions and Surface Tension

Timasheff has suggested that the ability of sugars to exert cohesive forces on water may explain their preferential exclusion from protein surfaces. The cohesive effect is reflected as surface tension increases as more sugar is added (39). Increases in surface tension result in the depletion of solute at the air-solution interface (22). This surface tension effect is meant to be analogous to the depletion of a cosolute near a solvated protein—the preferential hydration effect. It follows that, if a solute increases the surface tension of water, more work is required to increase the
The surface area of the cavity occupied by the protein in the solution. The qualitative correlation between preferential hydration effects and surface tension increases led to the hypothesis that surface tension is the primary source of unfavorable interaction free energy between proteins and cosolutes. Osmolyte-induced protein stabilization is therefore attributed to the increase in solution surface tension upon adding osmolyte (2, 33, 34, 39). The surface tension ($\sigma$), a macroscopic parameter, is defined as:

$$\sigma = \left( \frac{\partial G^S}{\partial S} \right)_{T,m_1}$$

(38)

and is a measure of the change in the surface free energy of water, $\partial G^S$, due to an incremental surface area increase, $\partial S$, at the protein-solution interface at constant temperature and solution composition (34, 39).

The increased surface tension of osmolyte solutions, compared to pure water, means that more free energy is needed to create the same-sized cavity in a solution containing cosolute compared to pure water. In this analysis, however, the energy of cavity formation is only superficially addressed and, moreover, surface tension does not have a microscopic counterpart. Therefore, any molecular-level interpretation cannot be approached with statistical-mechanical rigor. The approach has two additional caveats. First, surface tension, measured at a “flat, homogeneous air-water interface” is probably a poor model of the interfacial tension between protein and solvent (60) because a protein surface is neither flat nor homogeneous. Second, the cavity is occupied by protein, not vapor, and air is a poor model for a protein surface.

Solvent Exchange Equilibria

Schellman’s solvent exchange model, derived rigorously from statistical-mechanical and thermodynamic viewpoints, applies to systems having weak interactions with macromolecules as well as systems in concentrated solutions (68–70). Like Tanford, Schellman also modified the Wyman linkage relation in terms of weak binding equilibria (68). The primary difference is that Schellman’s model assumes that ligand and solvent undergo a 1:1 exchange reaction at a specific site on the protein surface. This can be written as $P_i\cdot H_2O + L \rightleftharpoons P_i\cdot L + H_2O;$

$$K = \frac{C_{H_2O}C_{P_i\cdot L}}{C_{P_i\cdot H_2O}C_L} = \frac{a_1C_{P_i\cdot L}}{C_{P_i\cdot H_2O}a_3}.$$  

(39)

This model assumes that the protein surface possesses contact points for water and/or cosolvent that are equivalent and independent. The nature and number of sites available for solvent/cosolvent binding depends on the protein conformation. However, water is distributed over the entire surface of the protein, and site occupancy is a time-average phenomenon (a definition of affinity) that varies from one protein molecule to another. Because solvent and cosolute are effective competitors for binding at the protein surface owing to their mutually weak affinities, activity coefficients must be employed (68). The assumption that one
sugar molecule displaces one water molecule is simplistic, especially given the size of a sucrose molecule (4.0 Å calculated (16) radius) relative to the size of water (1.38 Å radius). Nevertheless, this analysis is mathematically rigorous and predicts the essential elements of protein stabilization and destabilization.

**Combining Scaled Particle Theory and Solvent Exchange**

Whereas the models discussed thus far consider osmolyte-induced effects in terms of either weak binding or excluded volume models, the formulation of Saunders et al (63) is an effort to unite both concepts. The osmolyte-induced difference in standard free energy change for protein unfolding ($\Delta G^o$) is considered to be divisible into two additive contributions: the difference in standard free energy change arising from hard-sphere exclusion effects ($\Delta G^o_H$) and that emanating from binding (soft) interactions ($\Delta G^o_S$). The latter parameter merely accounts for any discrepancy between the actual standard free energy change ($\Delta G^o$) and the value of $\Delta G^o_H$ that has been calculated (63) by means of the Berg adaptation (4) of scaled particle theory.

Consideration of water as a solute component in the Berg adaptation of scaled particle theory (4) means that the standard state refers to the unfolding reaction in an ideal gas phase, whereupon it is convenient to define a secondary standard state that takes solvation into account. Protein solvation for a given (folded) state of a protein is envisaged in terms of two steps (Figure 7). In the first step, the gas-phase protein is solvated in either water or osmolyte solution, both of which are interpreted in terms of hard-sphere interactions. Then follows a step involving soft interactions, which include phenomena such as electrostatic, van der Waals, and hydrophobic interactions. The water-to-osmolyte transfer free energy changes [e.g., $\Delta G^o_{tr}^{H_2O \rightarrow Os}$] may likewise be divided into hard and soft components; for present purposes, however, they are redundant because their contributions to the energetics have already been incorporated into the $\Delta G^o_H$ and $\Delta G^o_S$ values for the first and second steps. An analogous pattern of reactions is considered to describe solvation of the unfolded protein state from the gas phase.

In terms of a solvated standard state, the standard free energy changes for protein unfolding in the aqueous and osmolyte environments defined as, $\Delta G^o_{H_2O}(F \rightleftharpoons U)$ and $\Delta G^o_{Os}(F \rightleftharpoons U)$ are related to the corresponding parameter for reaction in the gas phase, $\Delta G^o(F \rightleftharpoons U)$, by the expressions

$$
\Delta G^o_{H_2O}(F \rightleftharpoons U) = \Delta G^o(F \rightleftharpoons U) + \left[\Delta G^o_{H_2O}(F \rightleftharpoons U)\right]_{H_2O} + \left[\Delta G^o_S(F \rightleftharpoons U)\right]_{H_2O},
$$

(40)

and

$$
\Delta G^o_{Os}(F \rightleftharpoons U) = \Delta G^o(F \rightleftharpoons U) + \left[\Delta G^o_{Os}(F \rightleftharpoons U)\right]_{Os} + \left[\Delta G^o_S(F \rightleftharpoons U)\right]_{Os},
$$

(41)

where, as noted above, the free energy changes arising from solvation in the aqueous
and osmolyte environments are divided into excluded volume (hard) and chemical (soft) contributions. The required osmolyte-induced difference in standard free energies for the unfolding transition (see Table 1) then becomes

$$\Delta \Delta G^\circ(F \rightleftharpoons U : H_2O \rightarrow Os) = \Delta G^\circ_{Os}(F \rightleftharpoons U) - \Delta G^\circ_{H_2O}(F \rightleftharpoons U). \quad (42)$$

The dependence of $\Delta \Delta G^\circ(F \rightleftharpoons U: H_2O \rightarrow Os)$ on osmolyte concentration, $C_3$, is determined by calculating $\Delta G^\circ_{H_2O}(F)$ and $\Delta G^\circ_{H_2O}(U)$ at a variety of osmolyte concentrations. Estimation of these hard-particle contributions involves calculating protein radii in the folded and unfolded states based on solvent-exposed surface areas from analysis of crystallographic data (50). Surface area for the unfolded states shown in Table 1 were calculated using data from Cremer et al (12), Chalikian et al (8), and Myers et al (50). Realistic magnitudes of $\Delta G^\circ_{Os} (F)$ and $\Delta G^\circ_{Os} (U)$ clearly rely upon reasonable estimates of the $\Delta G^\circ_{H_2O}$ values because of their interdependence.

Analysis of hard and soft effects has been applied to the data in Table 1 (63). In each case, the hard interactions dominate—a finding that implicates steric repulsions as the major driving force for stabilization of folded protein states. The analysis also predicts that larger sugars have a bigger effect than smaller ones because of their greater volume occupancy. An obvious feature of those results is the different extent to which excluded volume effects account for the $\Delta \Delta G^\circ$ values for the pH and thermal transitions—a finding in keeping with earlier studies of ribonuclease (72). In terms of the present model, this attenuation of the stabilizing effect of osmolytes on thermal unfolding implies the existence of more favorable osmolyte-protein interactions or less favorable water-protein interactions in the thermally unfolded protein state. Presumably, the water-protein interactions would be more important because most of the osmolytes (apart from glycerol) have minimal affinity for proteins (78). For the transition between the A-state and acid-unfolded state of cytochrome c, however, soft interactions seemingly enhance the stabilizing effect slightly—a finding that implicates either preferential osmolyte interaction with the A-state or less favorable water interaction with the acid-unfolded state. These possibilities are discussed further elsewhere (63).

Although this rationalization of the osmolyte-induced stabilization of folded protein states is seemingly reasonable, it has several weaknesses.

1. The folded and unfolded states are modeled as impenetrable spheres, an approximation that is particularly suspect in the case of the thermally denatured state, for which a random-flight polymer is probably a better model (31)—as mentioned during discussion of the assessment of excluded volume contributions to thermodynamic nonideality. Some justification for the present approach is provided by the use of solvent-exposed surface areas to calculate the radius of the effective sphere. Although the magnitudes of the hard and soft contributions depend on the choice of surface areas, unrealistic values of the surface area (and hence radius) are required to change the sign of the contributions (63).
2. Changes in endstates in osmolyte solution are ignored. To that end, crystal structures are obtained under crowded conditions but seem to be consistent with dilute solution data in most instances. However, detailed and systematic studies of both endstates in the presence of high osmolyte concentrations are needed.

3. Use of the Berg adaptation of scaled particle theory (4) blurs the connection between radii and thermodynamic measurements. Although evaluation of the second virial coefficient for a given protein state by (say) sedimentation equilibrium provides a valid estimate of the effective thermodynamic radius (86) for the prediction of excluded volume effects by McMillan-Mayer (44) or conventional scaled particle (37) theories, there is no method for obtaining an unequivocal estimate of the corresponding parameter in the Berg adaptation of scaled particle theory (4) because of its inclusion of a solvent contribution to the nonideality (77a).

4. Apportioning of $\Delta G^\circ$ between hard and soft contributions is totally reliant upon correct calculation of the former because the magnitude of the soft contribution is attributed to the disparity between the measured thermodynamic parameter and the standard free energy change assigned to hard particle interactions (77a).

These deficiencies draw attention to weaknesses in this particular interpretation of osmolyte-induced protein stabilization. However, a major virtue of this approach is that it highlights the improbability of any interpretation of the thermodynamic nonideality in terms of a single phenomenon providing an accurate description of the molecular situation.

Transfer Free Energies

The analysis of transfer free energies, which is based on the horizontal arrows in Figure 7, can be understood in terms of Timasheff’s preferential interaction model (80). Pioneered by Bolen’s group (42), this analysis provides both a thermodynamic treatment of protein stability in osmolytes and chemical insight into the interactions responsible for stabilization. The goal is to determine the strength of the interactions between the protein’s chemical groups and the other solution components (solvent, osmolyte). The difference between the energetics of such interactions for the F and U states then drives protein stabilization.

Proteins may be regarded as heteropolymers made from a variety of chemical moieties (amino acid residues), each with its own affinity for water and osmolyte. A basic division is made between the polypeptide backbone and the sidechains, which are further categorized with respect to type. The nature of the interactions of solvent (and osmolyte-supplemented solvent) with the backbone and each type of sidechain can be examined by first determining the free energy transfer from solvent to osmolyte solution ($\Delta G^\text{transfer}_{\text{aa}}$) for each amino acid. Such advantage of the properties of small molecules has been taken to probe osmolyte-protein interactions on the
basis of experience with ribonuclease as the test protein. Calculation of the overall
transfer free energy for the F or the U state does, of course, assume additivity of
the individual transfer free energies \( \Delta G_{tr}^{\alpha} \) (76).

The transfer free energy from water to osmolyte solution for each amino acid
is calculated from solubility data. Beyond the limit of solubility, the crystalline
(solid) amino acid phase is in equilibrium with amino acid in the saturated solution
phase—a situation that reflects the identity of chemical potentials (free energies)
in the two phases. Thus,

\[
(\mu_{\text{aa}})_{\text{solid}} = (\mu_{\text{aa}})_{\text{solution}}.
\]

(43)

Furthermore, there is an additional mandatory condition,

\[
[(\mu_{\text{aa}})_{\text{solid}}]_1 = [(\mu_{\text{aa}})_{\text{solid}}]_3,
\]

(44)

that equates the chemical potentials of solid amino acid phase in solvent (sub-
script 1) and osmolyte solution (subscript 3) because the chemical potential of a
solid is independent of the phase with which it is in equilibrium.

On the grounds that the difference in chemical potential and hence free energy
for each amino acid in the aqueous and osmolyte solutions must be zero, it follows
that

\[
\Delta G_{aa}^{\text{o,tr}} = RT \ln \left[ \frac{(z_{\text{aa}})_1}{(z_{\text{aa}})_3} \right],
\]

(45)

where \((\gamma_{\text{aa}})_1\) and \((\gamma_{\text{aa}})_3\) are the respective activity coefficients for saturated solu-
tions of a given amino acid in water and osmolyte solution. Introduction of Equation 3
for the relationship between molar activities and concentrations allows Equation 46
to be written as

\[
\Delta G_{aa}^{\text{o,tr}} = RT \ln \left( \frac{(C_{\text{aa}})_1}{(C_{\text{aa}})_3} \right) + RT \ln \left( \frac{\gamma_{\text{aa}})_1}{(\gamma_{\text{aa}})_3} \right),
\]

(47)

where \((\gamma_{\text{aa}})_1\) and \((\gamma_{\text{aa}})_3\) are the respective activity coefficients for saturated solu-
tions of a given amino acid in water and osmolyte solution. Unfortunately, calcula-
tions of these transfer free energies for the amino acids (cited as \( \Delta G_{tr}^{\alpha} \)) can
only be approximate because a scarcity of information on the magnitudes of ac-
tivity coefficients has necessitated neglect of the final term in Equation 47. At first
sight, such assumed identity of activity coefficients in the two environments seem-
ingly contradicts the earlier concept that protein stabilization is a consequence
of osmolyte-induced thermodynamic nonideality. However, effects of nonidea-
ality are now also manifested in the different magnitudes of \((C_{\text{aa}})_1\) and \((C_{\text{aa}})_3\) for
saturated solutions of a given amino acid in solvent and osmolyte-supplemented
environments.
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The same procedure is used with cyclic glycylglycine (diketopiperazine) as the model compound for determining the transfer free energy for the peptide backbone. Because of the two peptide bonds in cyclic glycylglycine, the values so determined are halved to obtain the transfer free energy for a single peptide backbone unit ($\Delta g_{\text{tr}}^{\text{pep}}$). Sidechain contributions to the transfer free energy of a protein are obtained by subtracting $\Delta G_{\text{gly}}^{\text{tr}}$, the transfer free energy for glycine, from $\Delta G_{\text{aa}}^{\text{tr}}$ for the remainder of the amino acids to yield the residue parameter, designated $\Delta g_{\text{tr}}^{\text{aa}}$.

The next stage of the calculation entails the appropriate combination of $\Delta g_{\text{tr}}^{\text{pep}}$ and $\Delta g_{\text{tr}}^{\text{aa}}$ values to estimate $\Delta G^{\text{tr}}$ for the transfer of the F and U protein states from water to osmolyte solution. For the folded (native) state of ribonuclease, the value of $\Delta G^{\text{tr}} (\text{F: } H_2 O \rightarrow \text{Os})$ has been calculated from the relationship

$$\Delta G^{\text{tr}} (\text{F: } H_2 O \rightarrow \text{Os}) = \sum n_i \cdot \alpha_i \cdot \Delta g_{\text{tr}}^{\text{pep}},$$

where $\Delta g_{\text{tr}}^{\text{pep}}$ is the transfer free energy of component i (peptide backbone or amino acid sidechain), $n_i$ is the number of such units in the protein, and $\alpha_i$ is the corresponding fractional solvent-accessible surface area for species i. Values of $\alpha_i$ for the F state can be calculated from surface area analysis of X-ray crystal structures, but the corresponding values for the unfolded state are more equivocal. For initial calculations of the transfer free energy for the U state of ribonuclease, a value of unity for $\alpha_i$ was applied globally on the grounds that all of the protein components are solvent-exposed in a random coil. However, limits were set subsequently (58) for solvent exposure in a typical unfolded state as outlined by Creamer et al (12), whereupon the counterpart of Equation 48 became

$$\Delta G^{\text{tr}} (\text{U: } H_2 O \rightarrow \text{Os}) = \sum n_i \cdot (\alpha_{\text{tr}}^{\text{up}})_{\text{ub}} \cdot \Delta g_{\text{tr}}^{\text{pep}},$$

and

$$\Delta G^{\text{tr}} (\text{U: } H_2 O \rightarrow \text{Os}) = \sum n_i \cdot (\alpha_{\text{tr}}^{\text{up}})_{\text{ub}} \cdot \Delta g_{\text{tr}}^{\text{pep}},$$

where $(\alpha_{\text{tr}}^{\text{up}})_{\text{lb}}$ and $(\alpha_{\text{tr}}^{\text{up}})_{\text{ub}}$ represent the respective lower and upper bounds placed on solvent exposure in the unfolded state. On folding, nonpolar sidechains are usually buried, whereas polar and charged sidechains tend to remain exposed.

Although a given sidechain may have either a favorable or an unfavorable $\Delta g_{\text{tr}}^{\text{pep}}$, the collective effect of sidechains is to favor their transfer from water to osmolyte. On the other hand, $\Delta g_{\text{tr}}^{\text{pep}}$ opposes the transfer from water to osmolyte, and the contribution from this source to $\Delta G^{\text{tr}}$ outweighs the collective sidechain contribution. $\Delta G^{\text{tr}}$ is thus positive, which signifies that the transfer of both F and U states from water to osmolyte is unfavorable. However, because $\Delta G^{\text{tr}} (\text{F: } H_2 O \rightarrow \text{Os})$ is less positive than $\Delta G^{\text{tr}} (\text{U: } H_2 O \rightarrow \text{Os})$, the presence of osmolyte favors the folded protein state (39).

In summary, a given sidechain may have either a favorable or an unfavorable $\Delta g_{\text{tr}}^{\text{pep}}$, but collectively, the sidechains favor transfer from water to osmolyte. The backbone units oppose transfer from water to osmolyte, and the $\Delta G^{\text{tr}}$ for all the
backbone units dominates $\Delta G^t$ for the collective sidechains, resulting in compaction of the protein in the presence of osmolyte. These results are in qualitative agreement with those of Arakawa et al (1).

There is, however, a potential caveat. With the exception of tryptophan, phenylalanine, tyrosine, and histidine, the amino acid and backbone transfer free energies (water to sucrose) are less than $\sim|50|$ cal mol$^{-1}$. As stated above, these transfer free energies are calculated from water and sucrose solubilities by assuming that the activity coefficients of the amino acids are the same in water and sucrose solution. A 10% difference in activity coefficients could reverse the sign of the calculated transfer free energies; even a 5% difference will change the conclusions. For several amino acids, there is more amino acid (by weight) than water. Under such conditions, a 5% difference in the activity coefficients between osmolyte solution and water seems conservative.

Because the analysis is based on measured transfer free energies, binding and excluded volume interactions of water and osmolyte with the backbone and sidechains are handled implicitly. However, small molecules are used to model the F and U states. Consequently, the excluded volume effect is considered only indirectly and incompletely from the surface area analysis of the whole protein. This is best illustrated in the context of the previous model (63), which divides free energies into hard and soft contributions. In modeling the F and U states, Bolen and colleagues consider the fraction (as solvent accessible surface areas) of all the amino acid sidechains and backbones that are available to interact with the solvent. The transfer free energies for the contributing components are then scaled down accordingly, but, because the excluded volume component also contributes to $\Delta G^H$ (as $\Delta G_{H}$), $\Delta G^H$ is also scaled down, resulting in some loss of steric interactions. To preserve the excluded volume, we might consider scaling only the soft term, i.e.,

$$
\Delta G'' = \sum n_i \cdot \alpha_i \cdot \Delta g''_{i,S}.
$$

(51)

Therefore, an amino acid that may be completely buried will still contribute its excluded volume component. Unfortunately, even this analysis is deficient because small molecule models (amino acids) cannot embody the excluded volume effect of large molecules (proteins). We might view this in terms of scaled particle theory. Recall that the energy of cavity formation is proportional to cavity size. It is energetically less costly to solvate many single amino acids in random spaces away from each other than it is to solvate them as a cluster (4). However, amino acids that are constrained in a string cannot be solvated the same way and, consequently, the solution must make a larger cavity. For this reason, it is difficult to assess excluded volume effects in the context of transfer free energies for small molecule models.

**Osmotic Stress Analysis**

Osmosis is the process by which a pure solvent (water) flows into a solution separated from it by a membrane permeable only to solvent. This occurs because the chemical potential of pure water ($\mu_i$) is higher than that of the solution. The
flow of pure solvent occurs until the chemical potential of solvent is the same in both phases. The osmotic pressure, \( \Pi \), then becomes the force generated by this flow of solvent.

In osmotic stress analysis (53–55), an osmolyte is used to adjust the osmotic pressure (or equivalently, the water activity, \( a_1 \)) of a solution containing a notional membrane that is impermeable to osmolyte and that separates the macromolecule(s) from the osmolyte solution. The introduction of cosolute outside the notional membrane lowers the water activity, introducing a gradient so that water molecules leave the protein surface. Osmotic stress is the osmotic work required to restore the equilibrium by removing water from the protein-solute interface (transfer free energy). This analysis is based on the equation

\[
\delta \mu_{tr}^{1-2} = \delta \Delta G^{o, tr} = \Delta V_1 \Pi^{m_3},
\]

where \( \delta \mu_{tr}^{1-2} \) and \( \delta \Delta G^{o, tr} \) are the osmotic work and the transfer free energy, respectively. \( \Delta V_1 \) is the volume of water involved in a reaction, and \( \Pi \) is the osmotic pressure attributed to the cosolute concentration, \( m_3 \). The use of a membrane implies that the solution contains two phases, and the flow of solvent reestablishes the equivalency of the chemical potentials of both phases (81).

The membrane in osmotic stress studies is, as stated above, notional. Macromolecules are considered to be confined within a membrane permeable only to water. The domain of the protein, from which osmolytes are excluded, is more highly hydrated than is the bulk solution. Yet, the absence of a membrane makes this a one-phase system. Inasmuch as Parsegian and coworkers (53, 54) have not identified the positioning of the hypothetical membrane, it is of interest to note that its placement at a distance \( r_3 \) from the protein surface would meet the requirement for an osmolyte-free phase; \( \Delta V_1 \) would then become the change in covolume (57). The validity of the osmotic stress concept has certainly been questioned (11, 57, 81, 87).

Timasheff and colleagues have shown that cosolute exclusion requires a positive free energy change (2, 80, 81). The addition of cosolute to a protein solution (in the absence of a membrane) changes the chemical potential of water but need not affect the reaction itself. To assume that no free energy change is required (hence, an inert cosolute) violates the laws of thermodynamics (81). The driving force for osmotic stress is not the change in water activity but the change in the free energy of exclusion and therefore a change in preferential interactions (81).

Osmotic stress analysis purports to measure the number of water molecules involved in a reaction (40, 53, 54). In the context of the present discussion, it attempts to describe osmolyte-induced protein stabilization in terms of the difference in the number of water molecules bound to the end states (i.e., the F and U states).

This concept arises from the Tanford modification of the Wyman linkage function (77, 78, 81)

\[
\delta \Delta G^{o, tr} = \Delta V_1 \Pi^{m_3} = \bar{v}_1 \delta \left( \frac{\partial m_1}{\partial m_3} \right) \Pi^{m_3} = \bar{v}_1 \left[ \Delta \bar{\eta}_1 - \left( \frac{m_1}{m_3} \right) \Delta \bar{\eta}_3 \right],
\]

where \( \bar{v}_1 \) is the number of water molecules involved in the reaction.
where $\tilde{\nu}_1$ is the partial specific volume of water. Note that $\delta \Delta G^o_{tr}$ is a thermodynamic, macroscopic quantity that measures the total interaction of the entire protein surface with the solvent components. It is measured by, for example, dialysis equilibrium and sedimentation equilibrium techniques. The above equations describe molecular interactions with respect to binding at discrete sites on the protein surface. Interpretation of these parameters is imposed by binding models and therefore these parameters are nonthermodynamic quantities (78).

The equations above demonstrate that counting waters is a misinterpretation of $\Delta \tilde{V}_1$, which is not strictly the change in water volume (57). Instead, $\Delta \tilde{V}_1$ is the difference between the changes in water volume and the changes in cosolute volume expressed in terms of the water volume being displaced by cosolute. Cosolutes can penetrate, to some degree, the hydration layer around the protein (80, 81). Furthermore, $\Delta \tilde{n}_1$ and $\Delta \tilde{n}_3$ are not true stoichiometries. They are, rather, descriptions of the perturbations of water and cosolvent by protein, which involves the entire protein surface not specific sites (81). Timasheff states that, although the theoretical basis of this analysis is incorrect, the results are correct in terms of preferential interactions (81).

In summary, osmotic stress implies that osmotic pressure drives the reaction. The driving force arises from the positive free energy change associated with changes in preferential interactions. However, a cosolute cannot be both inert (free energy change of zero) and excluded (positive free energy change). As discussed above, the interpretation of $\Delta \tilde{V}_1$ as the literal accounting of water molecules is incorrect. Finally, this model is inconsistent with protein stabilization data because it suggests that osmolyte size determines the number of water molecules released upon folding (14, 63).

CONCLUDING REMARKS

Protein stabilization by small inert cosolutes (osmolytes) is a well-established phenomenon from the thermodynamic viewpoint. However, the manner in which the stabilization should be interpreted remains the subject of controversy. Models based on the excluded and preferential solvation concepts of thermodynamic nonideality, on preferential solvent interactions, and on osmotic stress analysis may all be invoked to account for the same experimental observations. On that score, the fact that the detailed mechanism of stabilization remains equivocal is hardly surprising inasmuch as the observations being interpreted are thermodynamic and therefore independent of any mechanistic pathway. As noted by Eisenberg (17), thermodynamic rigor ceases the moment that resort is made to any molecular interpretation of the second virial coefficient $B_{23}$, but such departure from thermodynamic rigor is justified if adoption of the molecular interpretation leads to a greater understanding of the phenomenon. At first sight, the use of osmotic stress analysis or of the preferential solvent-binding models to determine the number of water molecules displaced by osmolyte was seemingly an example of one such
application meeting that proviso. However, the downfall of these literal mechanistic interpretations of the second virial coefficient’s magnitude followed subjection of the procedural bases to closer scrutiny.

Most of the studies described in this review have been presented in light of a search for a molecular understanding of the thermodynamic phenomenon, whereby the smaller (folded) state is favored by supplementing a protein solution with a high concentration of a small polyol. However, mention has also been made of the use of high sucrose concentrations to probe isomerization equilibria. Whereas the reversible acid expansion of ribonuclease (Figure 5) demonstrably reflects the equilibrium coexistence of native (F) and acid-unfolded (U) enzyme states, the corresponding acid-expansion of bovine serum albumin seems to reflect the progressive expansion of a single protein state (72). In similar vein, the sucrose-induced displacement of an isomerization equilibrium toward the smaller isomeric state has been used to establish the isomerization responsible for the allosteric behavior of pyruvate kinase (27).

Advantage has also been taken of the ability of osmolytes to favor protein self-association (71) and complex formation in equilibrium interactions between dissimilar reactants (57). These observations, as well as those on protein isomerization, have obvious potential ramifications in cellular systems, where the crowded molecular environment could well induce the same thermodynamic nonideality effects. The relevance of equilibrium constants determined in vitro under conditions approaching thermodynamic ideality to the same reaction under physiological conditions clearly requires further examination.

As emphasized in the section on the combination of scaled particle theory and solvent exchange, the extent of osmolyte-induced displacement of an isomerization equilibrium (or any other type of equilibrium interaction) does not always equate with the effect predicted on the basis of excluded volume considerations alone. For the thermal unfolding of cytochrome \( c \), this finding was attributed to preferential interaction of the polyol cosolutes with the unfolded U state or preferential solvent interaction with the folded F state—either phenomenon being in opposition to the excluded volume effect. Such interplays of preferential chemical interaction and excluded volume effects prevent meaningful predictions of the consequence of thermodynamic nonideality in the highly concentrated physiological environment. For example, although considerations of thermodynamic nonideality alone lead to the expectation that complex formation should tend to be favored under physiological conditions, the binding constant for the interaction of desipramine with its elicited monoclonal antibody in vivo is tenfold smaller than the in vitro value determined in dilute solution (56). In that regard, even if the necessity to divide the polyol-mediated effect upon cytochrome \( c \) unfolding (Figure 1) into hard and soft interactions were merely to reflect incorrect estimation of the excluded volume contribution to \( \Delta \Delta G^0 \), the exercise would still have served an extremely useful purpose by drawing attention to the need for consideration of the extent to which a cosolute is inert. In a physiological environment, there is clearly an opportunity for compounds unrelated to the interaction of interest to act in concert
as well as in competition with the excluded volume effect arising from the high extent of volume occupancy by the unrelated solutes.

In summary, significant progress has been made in our efforts to understand the effects of cosolutes on protein equilibria, but much remains to be accomplished. Perhaps the most important task is the acquisition of more high-quality data that can be used to test and then refine the models presented here. However, we must always keep in mind the potential pitfalls inherent in using molecular models to interpret bulk thermodynamic data.

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Figure 7  The solvation process for a single protein conformational state is broken into two steps (63). In the first step (red arrows), the protein is removed from the gas phase and introduced into a water or water/osmolyte solution. The solvent and solute are represented as hard spheres, and only solvent-solvent and solvent-solute interactions and steric repulsions between the protein and solvent are “turned on”. In the second step, solvent-protein and solute-protein binding interactions (green arrows) are turned on. The transfer of a protein conformation between pure water and water/osmolyte mixture is also divided into two steps, $\Delta G_S^{tr}$ (F:H$_2$O→Os) and $\Delta G_H^{tr}$ (F:H$_2$O→Os) (black arrows).