Protein Folding, Stability, and Solvation Structure in Osmolyte Solutions

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ABSTRACT An understanding of the impact of the crowded conditions in the cytoplasm on its biomolecules is of clear importance to biochemical, medical, and pharmaceutical science. Our previous work on the use of small biochemical compounds to crowd protein solutions indicates that a quantitative description of their nonideal behavior is possible and straightforward. Here, we show the structural origin of the nonideal solution behavior. We discuss the consequences of these findings regarding protein folding stability and solvation in crowded solutions through a structural analysis of the *m*-value or the change in free-energy difference of a macromolecule in solution with respect to the concentration of a third component.

INTRODUCTION

More than a century ago, Ostwald stated that understanding the chemical potentials of biomolecules is a crucial step in understanding life (1). Under highly crowded and nonideal solution conditions, as found in the cytoplasm, chemical potentials or chemical activities are required in place of concentrations to describe equilibria and kinetic processes (2). Over the last century, an increasing number of life scientists have become aware of this necessity in describing protein folding and interactions between biomolecules.

In practice, however, these issues of nonideality remain largely unappreciated (3,4). In addition, molecular crowding in living organisms involves high concentrations not only of macromolecules, but also small molecules. Osmolytes, small organic molecules that can reach intracellular and extracellular concentrations in the molar range in the extreme, are indispensable in the survival of most organisms (5,6). The most extreme example in mammals are kidney medulla cells that have to cope with urea concentrations up to 5.4 molar (7), conditions corresponding to 30% percent urea by mass. Survival of the kidney medulla cells is possible due to protecting osmolytes that counteract the deleterious effects of salt and of the denaturing osmolyte urea (8). Such beneficial effects of high concentrations of protecting osmolytes are crucial to the survival of essentially all taxa, except some halotolerant bacteria and archaebacteria that use salt as an osmoticant instead of organic osmolytes (5).

Given the high concentration of osmolytes in living organisms it is clear that understanding systems in vivo requires a fundamental understanding of protein solvation—the thermodynamic interaction of proteins and the abundant small species in cells, namely water, salt, and osmolytes. Because the reaction of proteins to changes in osmolyte concentration can be quantified by preferential interaction coefficients (9) (i.e., the deviation of the solution around the protein from its bulk properties), description of the bulk solution behavior is an important task. Therefore, this work begins by considering aqueous solutions of osmolytes.

A recurring theme in the description of aqueous solutions with and without protein is the structure of water. Different useful definitions for water structure, or liquid structure in general, are possible (e.g., number of H-bonds, number of neighbors, etc.). Here, we will use the common measurable pair correlation functions and their moment integrals in defining water structure. As discussed by Kirkwood and Buff, the structure of water can be expressed in terms of the average spatial arrangement of molecules in solution, which is given by radial distribution functions (10). Another way of describing the structure of water is in terms of mixtures of differently sized water clusters. Very popular heuristic activity coefficient models (11,12) are based on ''oligomerization'' of solutes and ''binding'' of hydration water to the solutes. Other solution cluster models that are based on Röntgen's approach (13) still seem to have some popularity in biology. But such oversimplified models have long been refuted (14,15) because rigorous cluster theories of solution became available (16,17).

Hill provided a rigorous, partition function-based cluster description of liquids (17), but he pointed out a serious disadvantage of such cluster approaches; namely that the assignment of molecules to any specific kind of molecular cluster is highly arbitrary. There are, however, partition function-based theories that do not rely on an exact structural model of water, yet can provide structural insight. Dill and co-workers provide a two-dimensional water model that allows for an efficient calculation of solution properties via computer simulations (18) and analytical methods (19). It qualitatively captures some properties of water and hydration features in solution. Hydration can also be expressed in terms of the probability of cavity formation in the liquid (20).

We recently presented a rigorous solution theory (21,22) that is based on first principles and provides a quantitative description of experimental data. One of several possible

Submitted May 25, 2005, and accepted for publication August 8, 2005.

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0006-3495/05/11/2988/10 $2.00

doi: 10.1529/biophsyj.105.067330

interpretations of our theory could be formulated in terms of molecular clusters, but again, taking the cluster point of view would unnecessarily lead to trouble. For instance, aqueous glucose activity data are consistent with the model assumption that all the sugar molecules are independent of one another (22), even up to concentrations in excess of 50% by mass. But the idea of independent molecules in such a densely packed solution is highly implausible.

We seek to understand the nonideality of biochemical solutions, protein folding, and stability from a low-order activity series generated from a semigrand canonical partition function where statistically weighted fluctuating numbers of molecules in a volume could be defined as ''clusters''. For this purpose, we use Kirkwood-Buff theory (10) in combination with our recent theory of nonideal solutions (21,22). It will turn out that successful interpretation of the solution properties in terms of the partition function requires a pair correlation description rather than a cluster description of the solution. The development provides fundamental insight into: 1), the molecular origin of the linear dependence of the partition function on osmolyte activity, which is experimentally observed over the whole range of solubility (22), and 2), the dependence of protein stability on the surrounding solution.

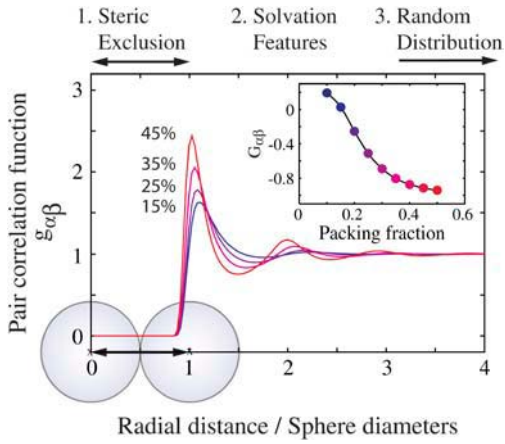

FIGURE 1 Volume fraction dependence of the radial distribution functions $g_{\alpha\beta}$ and Kirkwood $\mathcal{G}$ factors in an example liquid system with Lennard-Jones potential. Functions shown for packing fractions 15, 25, 35, and 45%. (*1*) At distances closer than the sum of the two radii (contact distance), steric exclusion is operative. (*2*) At intermediate distances, there are strongly concentration-dependent solvation features, most notably the first solvation shell just outside the region of steric exclusion. (*3*) At large distances there is no correlation between particles and the pair correlation function approaches unity. The inset shows the Kirkwood-Buff integrals (Eq. 1) as a function of the packing fraction. Note the slope and sign changes with respect to packing fraction.

STRUCTURE OF SOLUTIONS OF AQUEOUS BIOCHEMICAL COMPOUNDS

Pair correlations

MacMillan and Mayer (16) and later, Kirkwood and Buff (10) showed that it is rigorously possible to express the thermodynamic properties of an isotropic solution in terms of the average structure of the solution. The structure required for this discussion is given by radial distribution functions $g_{\alpha\beta}(r)$ between species α and β. The radial distribution functions are a measure of the deviation from the random distribution of particles of type-β around a central particle of type-α as a function of the distance from the central particle (see for example Fig. 1). Particle type-α and type-β could be atoms, as in the site-site theories, or, with a suitable generalization, molecules (e.g., proteins, water, or cosolutes like osmolytes).

In the absence of correlation, $g_{\alpha\beta}(r)$ equals unity. A positive or negative deviation of $g_{\alpha\beta}$ from unity at a certain distance corresponds to an excess or deficit of β at the indicated distance from α and is the positive or negative correlation of α and β at that distance. The overall correlation $\mathcal{G}_{\alpha\beta}$ involving excess or deficit in occupied volume of particles of type-α around type-β (or vice versa) is obtained by integrating the deviations from random distribution, which are given by the zeroth moment of the distribution. These overall correlations as a function of the packing (shown in Fig. 1) are the Kirkwood-Buff integrals defined as

$$\mathcal{G}_{\alpha\beta} = 4\pi \int_0^\infty (g_{\alpha\beta}(r) - 1) r^2 dr. \qquad (1)$$

We consider an aqueous solution of an osmolyte. Osmolytes are ubiquitous, small organic molecules that are utilized by essentially all taxa to cope with environmental, extracellular, or intracellular stress (5,6). After Kirkwood and Buff (10), the dependence of the osmolyte's chemical potential μ_{os} on the osmolyte concentration c_{os} is

$$\frac{1}{RT}\left(\frac{\partial \mu_{os}}{\partial c_{os}}\right)_{T,p} = \frac{1}{c_{os}} + \frac{(\mathcal{G}_{WO} - \mathcal{G}_{OO})}{1 - (\mathcal{G}_{WO} - \mathcal{G}_{OO})c_{os}}, \qquad (2)$$

where the W subscript indicates water and O denotes osmolyte molecules. The solvation behavior strongly depends on the concentration (see Fig. 1), and thus also $\mathcal{G}_{WO}$ and $\mathcal{G}_{OO}$ depend on osmolyte concentration. Therefore, in general, $(\mathcal{G}_{WO} - \mathcal{G}_{OO})$ might be expected to have a complicated concentration dependence. Comparison of Eq. 2 with experimental data will show the extent to which this is true. For this purpose we briefly discuss the chemical potentials of osmolytes based on their experimental behavior.

Chemical activities of osmolytes

Recently, we developed a statistical mechanical theory that captures the nonideal solution behavior over a wide concentration range of cosolutes, including salts and osmolytes in a straightforward and easily applicable manner (21,22). For a number of osmolytes, terms only up to first order in the expansion are sufficient to properly describe their chemical potentials up to their respective solubility limits. In these cases, the chemical potential μ_{os} of the osmolyte is given by Rösgen et al. (22)

$$\mu_{os} = \mu_{os}^{o} + RT\ln\left(\frac{c_{os}}{1 - V_1 c_{os}}\right), \quad (3)$$

where the constant V_1 is the apparent hydrated molar volume of the osmolyte and μ_{os}^{o} is the standard chemical potential. Taking the derivative of the chemical potential (Eq. 3) with respect to osmolyte concentration c_{os} directly results in

$$\frac{1}{RT}\left(\frac{\partial \mu_{os}}{\partial c_{os}}\right)_{T,p} = \frac{1}{c_{os}} + \frac{V_1}{1 - V_1 c_{os}}. \quad (4)$$

Comparison with the Kirkwood-Buff expression (Eq. 2) shows, that to first order, the apparent hydrated volume of the osmolyte V_1 equals $\mathcal{G}_{WO} - \mathcal{G}_{OO}$. This gives a simple interpretation of solution behavior for such osmolytes.

We know from experiment that Eq. 4 applies for many osmolytes and V_1 is a constant in this first-order expression for the chemical potential (22). Therefore, the difference $(\mathcal{G}_{WO} - \mathcal{G}_{OO})$ between osmolyte hydration $\mathcal{G}_{WO}$ and osmolyte self-correlation $\mathcal{G}_{OO}$ must be constant as the concentration is varied, even if osmolyte hydration and self-solvation individually are not constant. That is, hydration $\mathcal{G}_{WO}$ and self-solvation $\mathcal{G}_{OO}$ are concentration dependent, but they change in parallel as a function of concentration for solutions that follow Eq. 3. As a result, the osmolyte molecules behave thermodynamically as if they were independent of each other—even though the individual hydration and solvation correlations between them are nontrivial.

About half of the investigated osmolytes (22) follows this first-order behavior. The others are properly described by second-order terms. Among these osmolytes urea is special, because it behaves nearly ideally (22)

$$\frac{1}{RT}\left(\frac{\partial \mu_{os}}{\partial c_{os}}\right)_{T,p} \approx \frac{1}{c_{os}}. \quad (5)$$

Comparison of Eq. 5 with Eq. 2 shows that urea hydration and urea self-solvation are about equal, $\mathcal{G}_{WO} \approx \mathcal{G}_{OO}$, independently of urea concentration. So, ideal behavior could be considered a special case of first-order behavior, where the relation $\mathcal{G}_{WO} = \mathcal{G}_{OO} + const.$ holds over the whole range of solubility, as explained above.

PROTEIN STABILITY AND THE STRUCTURE OF THE SOLUTION

Protein solvation

We have seen that a combination of experimental data with the Kirkwood-Buff theory and with our theory of solution yields information about structural features of osmolyte solutions. Now, we turn to three-component solutions and consider the preferential interaction of proteins with water and osmolytes.

If the protein is dilute, its chemical potential μ_{prot} depends on the osmolyte concentration c_{os} through the relation (23,24)

$$\frac{1}{RT}\left(\frac{\partial \mu_{prot}}{\partial c_{os}}\right)_{T,p} = \frac{\mathcal{G}_{PW} - \mathcal{G}_{PO}}{1 - c_{os}(\mathcal{G}_{WO} - \mathcal{G}_{OO})}, \quad (6)$$

which is similar to the expression derived by Ben Naim using the mol fraction scale (25). We shall provide in a later publication a detailed discussion of a derivation of Eq. 6 for the general case that includes high protein concentration.

Equation 6 has two contributions. Firstly, the denominator $1 - c_{os}(\mathcal{G}_{WO} - \mathcal{G}_{OO})$, which contains only information on the bulk solution structure (cf. Eq. 2)

$$\frac{1}{1 - c_{os}(\mathcal{G}_{WO} - \mathcal{G}_{OO})} = \frac{1}{RT}\left(\frac{\partial \mu_{os}}{\partial c_{os}}\right)_{T,p}, \quad (7)$$

namely the Kirkwood-Buff integrals for osmolyte self-solvation $\mathcal{G}_{OO}$ and osmolyte hydration $\mathcal{G}_{WO}$. These integrals are the same as for the case of a two-component aqueous osmolyte solution (cf. Eq. 2). The denominator does not contain any protein-related expressions. The second contribution is given by the numerator, which contains the Kirkwood-Buff integrals for the hydration $\mathcal{G}_{PW}$ and osmolyte solvation $\mathcal{G}_{PO}$ of the protein. If the difference $\mathcal{G}_{PW} - \mathcal{G}_{PO}$ between protein solvation by water and osmolyte is multiplied by osmolyte concentration c_{os}, it equals the preferential interaction parameter $-\Gamma_{\mu_3} = c_{os}(\mathcal{G}_{PW} - \mathcal{G}_{PO})$ (25,26). For an overview of different kinds and definitions of preferential interaction parameters see, e.g., Anderson et al. (27). Whether or not a cosolute is stabilizing (with respect to either the native or the denatured state) depends on the protein's preference to have positive correlations either with water or with osmolyte. This preference determines the sign of the solvation expression $\mathcal{G}_{PW} - \mathcal{G}_{PO}$, or, equivalently, the sign of the preferential interaction parameter Γ_{μ_3}. The denominator in Eq. 6 does not determine the sign, because it is always positive. However, it does modulate (up or down) the sensitivity of the protein chemical potential with respect to the concentration of the osmolyte.

Recently, a theoretical Kirkwood-Buff-based protein solvation model was developed to describe protein stability (28). Also, other models have been used to separate the effects of hydration from those of osmolyte solvation of proteins: the exchange model (29), the osmotic stress model (30), the local domain model (31), and a model that might be called constant solvation model (32). However, obtaining information on protein solvation does not require model-dependent assumptions. This is because inverse Kirkwood-Buff theory (25,33) allows for a numerical determination of the Kirkwood-Buff integrals $\mathcal{G}_{\alpha\beta}$ (the correlations between solution components) from experimental data. In this way, Shimizu (26) calculated numbers for protein hydration change upon native (N) to denatured (D) conversion $\Delta_N^D(\mathcal{G}_{PW})$ as well as protein-denaturant solvation changes $\Delta_N^D(\mathcal{G}_{PO})$.

Recent molecular dynamics simulations on the preferential solvation of RNaseA and RNaseT1 in aqueous urea and glycerol give important insight into the molecular details of protein solvation thermodynamics (34). However, for

tractability reasons, the limiting assumption of ideal solution conditions had to be used in the data evaluation. As we will show below, this is a serious limitation in the case of several stabilizing osmolytes, because deviation of those osmolytes from ideal behavior has a major impact on the solvation of proteins. In the case of the denaturant urea, the simulation results (34) are valid over a larger concentration range, because urea behaves thermodynamically in a nearly ideal manner (22).

We now derive general, system-independent concepts about the impact of the structure of nonideal solutions on protein stability. This detailed structural analysis of the contributions to protein stability also allows us to address a question that remains an issue (26); viz. whether water can be thought of as a protein denaturant.

Structural basis for the *m*-value

The protein stability, or Gibbs free energy of unfolding ($\Delta_N^D G = -RT\ln K$), can be expressed in terms of differences of chemical potentials. The derivative, m, of $\Delta_N^D G$ with respect to osmolyte concentration is directly obtained from Eq. 6 by taking the difference between the native and the denatured state (indicated as Δ_N^D)

$$-\left(\frac{\partial \ln K}{\partial c_{os}}\right)_{T,p} = \frac{m}{RT} = \frac{\Delta_N^D(\mathcal{G}_{PW} - \mathcal{G}_{PO})}{1 - c_{os}(\mathcal{G}_{WO} - \mathcal{G}_{OO})}. \quad (8)$$

With urea as a denaturant there is substantial experimental evidence from the use of the linear extrapolation method that the m-value of protein unfolding is constant and negative in sign, and that it does not depend on the concentration of this (destabilizing) osmolyte (35–40). The m-values for protecting (stabilizing) osmolytes are found to be positive in sign, and are commonly assumed to be constant. There is some experimental evidence that this is a good assumption at least for trimethylamine-*N*-oxide (41) and glycine betaine (42).

The denominator of Eq. 8 only contains information on the bulk osmolyte, as seen from Eq. 7, and can be evaluated using analytical expressions available in the literature (22). Upon combining Eqs. 7 and 8, the observation of a constant m-value is seen to have direct implications on the solvation preference of the native state compared to that of the denatured state

$$\Delta_N^D(\mathcal{G}_{PW} - \mathcal{G}_{PO}) = m \Big/ \left(\frac{\partial \mu_{os}}{\partial \ln c_{os}}\right)_{T,p}. \quad (9)$$

The solvation preference relative to that at 0M osmolyte is then

$$\frac{\Delta_N^D(\mathcal{G}_{PW} - \mathcal{G}_{PO})}{\Delta_N^D(\mathcal{G}_{PW} - \mathcal{G}_{PO})_{c_{os}=0}} = RT \Big/ \left(\frac{\partial \mu_{os}}{\partial \ln c_{os}}\right)_{T,p}, \quad (10)$$

where $(\partial \mu_{os}/\partial \ln c_{os})_{T,p,c_{os}=0} = RT$. The derivative in Eq. 10 for the first-order and ideal cases are given by Eqs. 4 and 5. The second-order case is discussed in the Appendix. Fig. 2 shows for several osmolytes the change in solvation preference of proteins upon denaturation $\Delta_N^D(\mathcal{G}_{PW} - \mathcal{G}_{PO})$ as a function of osmolyte concentration (Eq. 10). The curves are grouped according to a trend found earlier in a different context (22): in the case of all stabilizing osmolytes, except glycine, the slope is exceedingly steep (the concentration dependence is larger) in comparison with the case of the denaturant urea. Consequently, the change of solvation preference upon urea denaturation, $\Delta_N^D(\mathcal{G}_{PW} - \mathcal{G}_{PO})$, is relatively independent of concentration (Fig. 2). In the presence of protecting osmolytes, however, the protein changes its solvation preferences severalfold as the osmolyte concentration is increased. More specifically, the decreasing value of $\Delta_N^D(\mathcal{G}_{PW} - \mathcal{G}_{PO})$ indicates that the protein transition becomes more indifferent with regard to distinguishing between water and protecting osmolyte. If $\Delta_N^D(\mathcal{G}_{PW} - \mathcal{G}_{PO})$ is zero, there is no difference in solvation preference between the native and the denatured state.

This behavior was recently observed experimentally by Felizky and Record in the case of the protecting osmolyte glycine betaine (42). The partition coefficient of glycine betaine between the surface of LacI HTC protein and the bulk solution significantly increases (approaching unity) with concentration, which means that the preferential interaction approaches zero. The partition coefficient of urea, however, is essentially independent of concentration (42). Recent direct evaluation of the solvation from experimental data shows that both urea and guanidine hydrochloride (GdnHCl) denaturants are nearly concentration independent with respect to $\Delta_N^D(\mathcal{G}_{PW} - \mathcal{G}_{PO})$ (26).

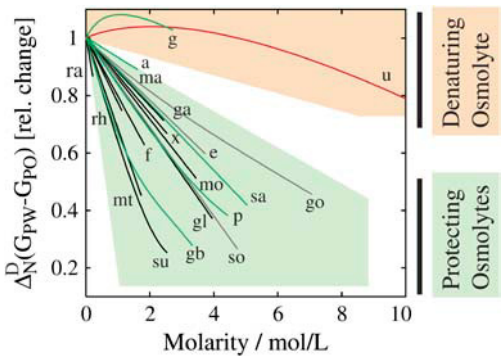

FIGURE 2 Change in solvation preference $\Delta_N^D(\mathcal{G}_{PW} - \mathcal{G}_{PO})$: dependence on osmolyte concentration (normalized to solvation preference at 0M). The curves were calculated from Eq. 8 using osmolyte activity coefficient data (22) assuming a constant m-value. In the case of the denaturing osmolyte urea the solvation preference deviates plus or minus a few percent. In the case of the protecting osmolytes (except glycine), it changes two- to fourfold. The curves are labeled at the right end: u = urea. Polyols are: go = glycerol, e = erythritol, ma = mannitol, so = sorbitol. Amino acids are: g = glycine, a = alanine, p = proline, sa = sarcosine, gb = glycine betaine. Saccharides are: x = xylose, f = fucose, gl = glucose, ga = galactose, rh = rhamnose, mo = mannose, mt = maltose, su = sucrose, ra = raffinose.

Electrostatic effects and specific interaction

GdnHCl is a good example for demonstrating that in the case of salts the general solvation properties of proteins derived here have to be complemented by additional information. Often a strongly concentration-dependent *m*-value is observed in the low GdnHCl concentration region, though the *m*-value of protein denaturation by GdnHCl is usually constant at elevated concentration (40,37,43–45). The major cause of this nonlinearity seems to be salt-dependent changes of protein protonation (43). Therefore, in the case of salts, the details of protein electrostatics and salt-dependent pK_a values might have to be taken into account (46) in addition to the solvation features discussed here.

In general, salt effects on the Kirkwood-Buff integrals $\mathcal{G}_{\text{Protein Salt}}$ in dilute protein solution could be subdivided into three contributions that correspond to three salt concentration regimes. 1), At extremely low salt concentration high-affinity specific binding can positively contribute to $\mathcal{G}_{\text{Protein Salt}}$ up to a number that equals the number of binding sites. For, in this dilute regime, each bound ion contributes a value of $+1$ to $\mathcal{G}_{\text{Protein Salt}}$. 2), At low-to-intermediate salt concentration long-range Debye-Hückel (47) electrostatic effects (48) have to be considered. Because proteins are poly-ions, their chemical potential depends on the presence of screening charges (49). In addition, the pK_a values of the protein's ionizable groups vary as a function of low salt concentration, which makes an additional contribution to the energetics of the protein (50). Such long-range electrostatic effects are screened at elevated ionic strength. 3), At high salt concentration indirect electrostatic effects and solvation effects (electrostriction/hydration) become important (51).

As an example for a salt-macromolecule solvation that exhibits strong effects in the low-salt region (contributions 1 and/or 2) we qualitatively discuss now RNA-magnesium interaction. In contrast to the comparably small contribution of electrostatics to the overall effect of GdnHCl on protein stability, the strong stabilization of RNA by magnesium ions is largely electrostatic in nature (52). Compared to even the most effective protecting osmolytes, Mg^{2+} is extremely stabilizing—already at concentrations well below the millimolar region. The preferential interaction parameter Γ_{μ_3} (for RNA plus Mg^{2+} and water) is not linear and even switches to a negative slope at higher concentration. This holds for both the folded and the unfolded state as well as their difference $\Delta_F^U\Gamma_{\mu_3}$ (52). Considering Eqs. 6 and 8 it would follow that an extreme change occurs in solvation preference of the RNA ($\mathcal{G}_{\text{RnaWater}} - \mathcal{G}_{\text{RnaSalt}}$) and of its folding equilibrium $\Delta_F^U(\mathcal{G}_{\text{RnaWater}} - \mathcal{G}_{\text{RnaSalt}})$. Due to the small concentration range over which the change occurs this effect is only insignificantly modulated by the decreasing electrostatic Debye-Hückel activity coefficient (47) of the salt $MgCl_2$ (denominator in Eqs. 6 and 8). We, therefore, see that the pattern observed for proteins—namely that stabilizing compounds solvate the macromolecule in a strongly concentration-dependent manner—also can be seen in the case of RNA.

A full quantitative Kirkwood-Buff description of protein solvation and protein chemical potentials in saline protein solutions will be very important for future understanding of biological systems. The usefulness of a solvation description of hydrophobic chain polymers in aqueous salt solution has been demonstrated recently (53). (Incidentally, in contrast to salt, osmolytes seem to have but a small effect on hydrophobic interaction (54,55).) Within our framework, such salt solvation behavior of organic solutes can be easily described if electroneutrality (56,57) is taken into account. We will address this kind of system in a later publication on n-component solutions. This task goes beyond the scope of the current work. Kirkwood-Buff theory has been applied to Debye-Hückel salts (56) and to salt mixtures (57). Our previous theoretical work on the activity of salt solutions showed which extensions are required in the case of chemical potentials in two-component systems (21). Expressions for the general three-component system involving protein will appear separately.

Deconvolution of solvation changes upon protein folding

The change in solvation preference upon unfolding, $\Delta_N^D(\mathcal{G}_{PW} - \mathcal{G}_{PO})$ that we discussed above, can, in principle, be derived from classical considerations (9,29). Application of the inverse Kirkwood-Buff theory yields additional information on hydration $\Delta_N^D(\mathcal{G}_{PW})$ and osmolyte solvation changes $\Delta_N^D(\mathcal{G}_{PO})$ separately. Equation 8 together with the relation $\Delta_N^D\bar{V}_{\text{prot}} = -\phi_{os}\Delta_N^D(\mathcal{G}_{PO}) - (1-\phi_{os})\Delta_N^D(\mathcal{G}_{PW})$ (25) (for dilute protein in the essentially incompressible aqueous solution) yields as the hydration change

$$\begin{aligned}\Delta_N^D(\mathcal{G}_{PW}) &= -\Delta_N^D\bar{V}_{\text{prot}} + \phi_{os}\frac{m}{(\partial\mu_{os}/\partial\ln c_{os})}\\ &= -\Delta_N^D\bar{V}_{\text{prot}} + \frac{m}{RT}\phi_{os}\times\begin{cases}1 & \text{, ideal}\\ (1-c_{os}V_1) & \text{, first order,}\end{cases}\end{aligned} \tag{11}$$

and for the osmolyte solvation change

$$\begin{aligned}\Delta_N^D(\mathcal{G}_{PO}) &= -\Delta_N^D\bar{V}_{\text{prot}} - (1-\phi_{os})\frac{m}{(\partial\mu_{os}/\partial\ln c_{os})} = -\Delta_N^D\bar{V}_{\text{prot}}\\ &\quad - \frac{m}{RT}(1-\phi_{os})\times\begin{cases}1 & \text{, ideal}\\ (1-c_{os}V_1) & \text{, first order,}\end{cases}\end{aligned} \tag{12}$$

where ϕ_{os} is the volume fraction of osmolyte. The term $(\partial\mu_{os}/\partial\ln c_{os})$ equals RT for the ideal case, and it equals $RT/(1-c_{os}V_1)$ for the first-order case (22), as given by Eq. 4. The constant, V_1, has the same meaning as given above (Eq. 4).

We discuss two general examples for the application of Eqs. 11 and 12, namely the denaturant urea, and those protecting osmolytes that follow first-order behavior. Urea chemical activity has been shown to very nearly behave ideally in the molar scale (22). Therefore, Eqs. 11 and 12 (ideal) can be applied to urea-induced unfolding of proteins as seen below. Before discussing urea, we first consider protecting osmolytes.

The hydration change upon unfolding $\Delta_N^D(\mathcal{G}_{PW})$ of a protein in the presence of a protecting osmolyte has the two contributions shown in Eq. 11 (first order): 1), an offset by the change in partial molar volume of the protein $-\Delta_N^D\bar{V}_{prot}$, and 2), a strongly concentration-dependent term $\phi_{os}(1 - c_{os}V_1)m/RT$ that is proportional to the m-value. This second term has a bell shape, starting at zero, increasing to a maximum and finally decreasing toward zero as $c_{os}V_1$ approaches unity. As a consequence, the overall contribution of hydration to the stabilization of proteins by protecting osmolytes is small at both very low and high osmolyte concentration. At intermediate concentrations of osmolyte, however, hydration becomes important.

In comparison to the hydration, the osmolyte solvation change $\Delta_N^D(\mathcal{G}_{PO})$ behaves in a very different manner (Eq. 12, first order). It also has the offset $-\Delta_N^D\bar{V}_{prot}$, but the strongly concentration-dependent term, $-(1 - \phi_{os})(1 - c_{os}V_1)m/RT$ in this case, starts at its maximal absolute value m/RT and approaches zero monotonically. Overall, both $\Delta_N^D(\mathcal{G}_{PW})$ and $\Delta_N^D(\mathcal{G}_{PO})$ approach the value $-\Delta_N^D\bar{V}_{prot}$ at high osmolyte concentrations and their difference $\Delta_N^D(\mathcal{G}_{PW}) - \Delta_N^D(\mathcal{G}_{PO})$ converges to zero, i.e., the numerator of Eq. 8 goes to zero. Simultaneously, the denominator of Eq. 8 approaches zero, because the first-order activity coefficient diverges to infinity. It follows then that the stabilizing effect of the osmolyte that is given by the m-value does not diminish. Accordingly, the osmolyte activity coefficient may be considered the major contributor to the m-value at extremely high osmolyte concentrations.

The two preceding paragraphs show that the mechanism of protecting osmolyte action changes with concentration. The solvation difference, $\Delta_N^D(\mathcal{G}_{PW}) - \Delta_N^D(\mathcal{G}_{PO})$, always determines whether the osmolyte is stabilizing or destabilizing. With respect to protecting osmolytes, the relative importance of which factor is most stabilizing changes with osmolyte concentration. At low osmolyte concentration only the solvation of the protein by osmolyte $\Delta_N^D(\mathcal{G}_{PO})$ is of importance. As the concentration is increased, protein hydration—reflected in $\Delta_N^D(\mathcal{G}_{PW})$—gains importance. And at very high protecting osmolyte concentration both $\Delta_N^D(\mathcal{G}_{PW})$ and $\Delta_N^D(\mathcal{G}_{PO})$ become small and the major contribution to the stabilizing effect is the chemical activity of the osmolyte.

Protein solvation in the presence of urea is given by Eqs. 11 and 12 (ideal). Both protein hydration and protein osmolyte solvation change are linearly dependent on the urea volume fraction ϕ_{os}, or the urea molarity. $\Delta_N^D(\mathcal{G}_{PW})$ and $\Delta_N^D(\mathcal{G}_{PO})$ change in parallel and are separated by a constant offset: $\Delta_N^D(\mathcal{G}_{PO}) = \Delta_N^D(\mathcal{G}_{PW}) + m/RT$.

In the presence of osmolytes, does water unfold proteins?

It has been suggested that, under protein denaturing conditions in multicomponent solutions, unfolding is caused by a change in water structure (58,15,59,60) or even that water is the unfolding agent (61). Also, the experimental observation of more (rotationally) mobile water in urea solution (62) and the computational observation of (translationally) less mobile water in urea solution (60) has led to the idea that water could be instrumental in the process of denaturation. Within an equilibrium description of the solution we cannot comment on the kinetic process of denaturation. Below, we show a weakness in the argument that it is the altered water mobility and the changed water structure in solutions of denaturant that shifts the equilibrium from native to denatured protein.

Notice that the water structure integral $\mathcal{G}_{WW}$ does not occur in either of the equations on cosolute chemical activity (Eq. 2), protein chemical activity (Eq. 6), or protein stability (Eq. 8). That is, the structure of water does not have a direct influence on the stability of proteins. If water structure has an influence it would be an indirect effect on protein energetics. This is because the Kirkwood-Buff integrals $\mathcal{G}_{\alpha\beta}$ are coupled through all the correlation functions $g_{\alpha\beta}(r)$ as well as thermodynamically via the Gibbs-Duhem relation (10). So, it is possible that a change in water structure $\mathcal{G}_{WW}$ could indirectly affect one of the Kirkwood-Buff integrals that are relevant to the protein. However, water-water correlations are well known to be quite resistant to solute concentration changes over a wide range of concentrations and types of solutes (57,63,64).

On purely thermodynamic grounds we can give a second reason why water is unlikely to be the denaturing agent in aqueous solutions of denaturant. Because of Eq. 11 (ideal), the protein hydration at zero molar denaturant is $\Delta_N^D(\mathcal{G}_{PW}) = -\Delta_N^D\bar{V}_{prot}$. Therefore, based on the experimental observation of generally negative denaturational volume changes $\Delta_N^D\bar{V}_{prot}$ at room temperature (65), the denatured state must be considered to be more hydrated than the native state. This is valid at least at low urea concentrations. Already by the law of mass action it is clear that a decrease in water concentration or chemical activity must shift the equilibrium to the less hydrated native state. An efficient way of decreasing the water activity is by addition of urea. If the determinant of protein stability were the water, we, therefore, would expect urea to stabilize proteins at least at low urea concentrations. This is clearly not the case. So, there must be another contribution, which overcomes the stabilizing effect of protein hydration $\Delta_N^D(\mathcal{G}_{PW})$ due to decreased water activity. This contribution of opposite sign is the preferential solvation of the protein by the osmolyte urea $\Delta_N^D(\mathcal{G}_{PO})$ given by Eq. 12 (ideal). The sign of the m-value (Eq. 8) determines whether a compound stabilizes or destabilizes proteins. Within this equation, the change of solvation preference of the protein upon unfolding $\Delta_N^D(\mathcal{G}_{PW} - \mathcal{G}_{PO})$ is the key factor. In the case of denaturing agents it is composed of a stabilizing (native state promoting) contribution by water $\Delta_N^D(\mathcal{G}_{PW})$ and a destabilizing (denatured state promoting) contribution by the cosolvent $\Delta_N^D(\mathcal{G}_{PO})$.

Only in cases in which the native state is more hydrated than the denatured state, would both hydration and urea solvation of the protein drive the unfolding reaction forward.

This happens at elevated urea concentration, where the second term in Eq. 11 overpowers the first term. In this case, water switches from stabilizing the protein to being destabilizing. There has never been, however, any experimental indication that urea switches from being a denaturant to a stabilizer as a function of concentration (the experimentally determined *m*-value is constant with respect to urea concentration).

Evidence of a lack of a switch in the nature of urea is not surprising if we take into account that water alone does not determine the denaturing capacity of urea. Rather, according to Eq. 8, we have to consider the difference between protein hydration and solvation by urea. The constant *m*-value for urea (35,36,40) shows that any changes in hydration and urea solvation occur in parallel, which is consistent with the constant difference between Eqs. 11 and 12 (ideal). Urea replaces water molecules around the protein without substantially disturbing the solution structure in terms of the local density around the protein. This is consistent with the experimental observation that water structure is quite resistant to the addition of urea (63,64). There is no indication of any change in water structure upon addition of urea that goes beyond the dilution of the water by urea molecules that neatly fit into the water framework (64).

CONCLUSIONS

Using Kirkwood-Buff theory and our previously described theory of solution we have presented a structural approach to the thermodynamics of concentrated aqueous solutions of biochemical compounds. Combination of solution theory with experimental results has allowed for a rationalization of the finding that aqueous solutions often can successfully be described in terms of clusters of molecules, despite the cluster approach being highly improbable for physical reasons. This paradox was resolved using radial distribution functions as a measure of the structure of the solution and the solvation properties of its components. We found that a parallel change of osmolyte hydration and self-solvation gives rise to solution behavior giving the illusion of a solution composed of water-osmolyte oligomers and osmolyte monomers.

Going a step further and including protein as a third component in the consideration allows for predictions of protein solvation behavior in the presence of high concentrations of osmolytes. Protecting (stabilizing) osmolytes turn out to have a much more concentration-dependent protein solvation behavior than the denaturing osmolyte urea (see Fig. 2). Interestingly, the structure of water ($\mathcal{G}_{WW}$) does not directly occur either in the structure-based activity coefficient expression for the osmolyte, or in the structure-based expression for protein stability. That is, in denaturing solutions, changes in the stability of proteins do not come directly from changes in water structure. What little impact urea exerts on water structure makes even indirect influence of water structure on protein stability changes unlikely. Also, given that the *m*-value for urea-induced protein unfolding is a constant, we show that the Kirkwood-Buff integrals for protein hydration and protein solvation by urea change in parallel (Eqs. 11 and 12, ideal), indicating that in addition to the bulk solution regime, water structure in the presence of urea at the protein surface is largely unperturbed, a conclusion drawn also from previous computer simulations (66).

TABLE 1 Molar activity coefficient parameters $g_{2,c}$, V_1, and V_2 used for calculating the curves shown in Fig. 2

	$g_{2,c}$ mol/l	$1/V_1$ mol/l	$2/V_2$ mol/l	c_{max} mol/l	Highest c mol/l	Root mean square deviation 10^{-3}	Data reference	Density reference
Xylose	–	7.6	–	10.16	2.6	1.93	(70)	(70)
Glucose	–	6.28	–	8.670	4	17.4	(71,72)	(73)
Fucose	–	4.89	–	9.05	1.9	16.5	(74)	(75)
Galactose	790	8.27	c_{max}	8.99	2.5	3.3	(72)	(76)
Rhamnose	–	4.4	–	8.07^{H}	1.2	24.8	(74)	(73)
Mannose	–	7.04	–	8.54	3.5	18.4	(72)	(73)*
Maltose	–	3.135	–	4.27^{H}	1.8	14.1	(70)	(73)
Raffinose	–	1.523	–	2.46^{H}	0.22	1.48	(77)	(78)*
Sucrose	70.4	2.466	c_{max}	4.617	2.6	8.05	(79,80)	(73)
Glycerol	19	4.8	c_{max}	13.69	7.1	5.77	(79)	(73)
Mannitol	–	7.35	–	8.173	1.1	2.51	(80)	(73)
meso-Erythritol	–	9.30	–	11.88	3.8	1.59	(81)	(73)*
Sorbitol	–	6.475	–	8.17	4.8	34.8	(82,83,71)	(73)*
Urea	21.6	20.3	c_{max}	22.03	10.1	1.23	(85,84,79)	(73)
Glycine	3.765	3.260	c_{max}	21.41	2.8	2.24	(77,86)	(87)
Alanine	–	14.40	–	16.07	1.7	5.43	(88,89)	(90)
Proline	120.5	5.38	c_{max}	12.52	4.5	13.4	(91)	A
Sarcosine	–	8.68	–	16.29	5.1	32.1	(91)	A
Betaine	16.88	1.97	4.94	10.72	3.4	13.8	(91)	A

The parameters were obtained from a fit of the given experimental data as described previously (22). H, density refers to hydrated crystalline solid; A, internal density data from Dr. Matthew Auton, University of Texas Medical Branch, Galveston.
*Crystal density and Eq. 17 used.

Protecting osmolytes that follow the commonly observed first-order behavior, were analyzed separately with regard to their protein hydration and protein osmolyte solvation behavior (Eqs. 11 and 12, first order). The protein hydration change upon unfolding is found to be concentration dependent in a strongly nonlinear manner. The maximum contribution of hydration to protein stabilization by protecting osmolytes is determined to occur at intermediate osmolyte concentrations. The protein solvation by osmolyte contributes in a monotonically decreasing fashion as a function of osmolyte concentration.

Our semigrand partition function approach, in conjunction with the Kirkwood-Buff framework, allows for a straightforward extraction of average structural information from thermodynamic data and thermodynamic information from the structure of the solution. Yet, we have not had to rely on any model assumptions. This rigorous, combined structural and thermodynamic description of multicomponent biological solutions provides a valuable tool for understanding the origin and impact of crowding effects in biochemistry.

Finally, *m*-values for urea-induced protein denaturation are determined by the linear extrapolation method, an empirical method with only some degree of theoretical foundation (67,68). What has been established about *m*-values, again empirically, is that they are proportional to the surface area that is newly exposed on unfolding, and to the heat capacity difference between the native and denatured states (69). In the case of protecting osmolyte-induced folding of proteins the *m*-values are opposite in sign from that of urea-induced denaturation, and are proportional to the surface area that is newly buried on folding. What this work offers is a rigorous theoretical foundation from which *m*-values can be understood in terms of the solvation effects of protecting and nonprotecting osmolytes on the denatured and native states of the protein and the solution properties of these ubiquitous agents that play such essential roles in the survival of many living systems.

APPENDIX

Fig. 2 shows protein solvation behavior in a protein-independent manner as given by Eq. 10; which can be written

$$RT\Big/\left(\frac{\partial\mu_{os}}{\partial\ln c_{os}}\right)_{p,T}=\left(\frac{\partial\ln c_{os}}{\partial\ln a_{os}}\right)_{p,T}. \tag{13}$$

We have already calculated the derivative $(\partial\mu_{os}/\partial\ln c_{os})$ for the case of first-order (Eq. 4) and ideal behavior (Eq. 5). Incidentally, different concentration scales can be used to define ideal behavior. In our case the molar scale is most appropriate (22). For seven out of the 19 compounds listed in Table 1, second-order equations are required for evaluating Eq. 10. The second-order expression for c_{os} is (22)

$$c_{os}=\frac{a_{os}+2a_{os}^2/g_{2,c}}{1+V_1a_{os}+V_2a_{os}^2/g_{2,c}}, \tag{14}$$

where V_1 and V_2 are first- and second-order volume and $g_{2,c}$ is an interaction parameter. We therefore have

$$\left(\frac{\partial\ln c_{os}}{\partial\ln a_{os}}\right)_{p,T}=-\frac{1}{1+2a_{os}/g_{2,c}}+\frac{2+V_1a_{os}}{1+V_1a_{os}+V_2a_{os}^2/g_{2,c}}, \tag{15}$$

where the activity of the osmolyte is (22)

$$a_{os}=\frac{g_{2,c}}{2}\frac{1-cV_1}{2-cV_2}\left[-1+\sqrt{1+\frac{4c}{g_{2,c}}\frac{2-cV_2}{(1-cV_1)^2}}\right]. \tag{16}$$

The parameters V_1, V_2, and $g_{2,c}$ are given in Table 1. In all of the cited references, molal activity coefficients are given. These coefficients were converted to the molar scale using solution density data (22). In cases in which solution density data are not available, the equation

$$c_{os}=\frac{m_{os}\rho_w}{1+m_{os}\rho_w/c_{max}}, \tag{17}$$

can be used as a good approximation (22), where m_{os} is the osmolyte molality, ρ_w the density of pure water, and c_{max} the molarity of the pure (crystalline or liquid) osmolyte.

The computations for Fig. 1 were performed by Kip Dyer, University of Houston.

This work was supported in part by a training fellowship from the W. M. Keck Foundation to the Gulf Coast Consortia through the Keck Center for Computational and Structural Biology, NIH (NIGMS 49760 to D.W.B. and NIGMS 37675 to B.M.P.) and the Robert A. Welch Foundation (grants E-1028 to B.M.P. and H-1444 to D.W.B).

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