An analysis of the molecular origin of osmolyte-dependent protein stability

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Abstract

Protein solvation is the key determinant for isothermal, concentration-dependent effects on protein equilibria, such as folding. The required solvation information can be extracted from experimental thermodynamic data using Kirkwood-Buff theory. Here we derive and discuss general properties of proteins and osmolytes that are pertinent to their biochemical behavior. We find that hydration depends very little on osmolyte concentration and type. Strong dependencies on both osmolyte concentration and type are found for osmolyte self-solvation and protein–osmolyte solvation changes upon unfolding. However, solvation in osmolyte solutions does not involve complex concentration dependencies as found in organic molecules that are not used as osmolytes in nature. It is argued that the simple solvation behavior of naturally occurring osmolytes is a prerequisite for their usefulness in osmotic regulation *in vivo*.

Keywords: protein stability; osmolyte; solvation; water structure

The cells of living organisms are exposed to various kinds of stress throughout their lifetime, and one prime strategy of essentially all taxa is to accumulate or release organic osmolytes that can counteract the stress (Hochachka and Somero 2002). These osmolytes have different preferences to interact with the various surface groups of proteins and thus do affect the thermodynamic transitions between protein states that expose different kinds and amounts of surface area (Auton and Bolen 2005). Preferential interaction is a measure of the tendency of solution components and water to localize near proteins. It is a critical factor that determines how proteins and protein folding equilibria respond to changes in solution composition (Casassa and Eisenberg 1964; Timasheff 1993; Schellman

1994; Record Jr. et al. 1998; Parsegian et al. 2000). In addition to local effects of solvation, proteins are also sensitive to the global structure and solvation properties of the bulk solution (Rösigen et al. 2005). We recently discussed general features of the solvation properties of aqueous osmolyte solutions, as well as the preferential solvation changes that accompany protein unfolding in solutions of several organic osmolytes (Rösigen et al. 2005). Here, we investigate in more detail the solvation of each molecular species, protein, osmolyte, and water to characterize better the fundamental mechanism of naturally occurring osmolytes as opposed to other organic cosolvents.

To properly express protein properties in terms of the structure and solvation properties of the solution it is important to define the structure of the solution appropriately. An equilibrium statistical thermodynamic approach as used in this work uses the familiar definition of structure that was given by Kirkwood and Buff (1951). They expressed the structure of the solution in terms of pair distribution functions, which are related to the distance-dependent deviation of the particle density from

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random distribution around each kind of molecule (e.g., protein) in solution. A rigorous connection between integrals over the solution structure and solution thermodynamics is thus established (Kirkwood and Buff 1951).

It took many years until Kirkwood-Buff theory was applied to proteins (Pjura et al. 1995). Within the last few years, however, there has been a rapidly increasing interest in Kirkwood-Buff theory, based on earlier pioneering work by Ben-Naim (1977, 1988). The theory can aid the development of force fields of biologically relevant compounds (Weerasinghe and Smith 2003a,b). Such force fields have already been used for peptide simulations (Abui and Smith 2004). Also, the theory is very useful for relating computer simulations to thermodynamic experiments (Smith 2004a). Kirkwood-Buff theory has been used to develop and to interpret theoretical models for chemical activities and protein preferential interactions (Smith 2004b; Rösgen et al. 2005). Preferential interaction between proteins and other solution components received special attention in recent years (Shimizu 2004; Shimizu and Boon 2004; Shimizu and Smith 2004; Schurr et al. 2005; Shimizu and Matubayasi 2006; Shulgin and Ruckenstein 2006a, 2006b; Smith 2006b). This literature reveals many interesting insights into the separate effects of protein solvation by water and osmolyte on protein stability and preferential interaction. However, there is a lack of comprehensive investigations of naturally occurring osmolytes over a broad range of concentrations. The current work fills this gap by analyzing 19 different osmolytes from dilute solution to the molar concentration range—in most cases up to the solubility limit.

The Kirkwood-Buff analysis of aqueous solutions of osmolytes and proteins, done in this work, reveals interesting insights into the relative solvation behaviors of the species in solution. We show that water has a remarkably constant and small change in self-solvation, independent of the kind and concentration of osmolyte. Also, osmolyte hydration and the change of protein hydration upon unfolding do not depend on the chemical characteristics of the osmolyte. Osmolyte self-solvation and protein solvation by osmolyte are more variable but follow common patterns for all but three (out of 19) osmolytes studied here. Such predictable and simple solvation behavior could be a crucial property that molecules must have to be useful as naturally occurring osmolytes as opposed to arbitrary organic cosolvents.

We begin with discussing the Kirkwood-Buff expressions that are required to extract the solvation information from experimental data. Following that, we consider aqueous osmolyte solutions alone, before focusing on the solvation of proteins. The osmolyte solutions are not only interesting by themselves, their understanding also greatly enhances the insight gained into protein solvation.

Solvation expressions

Before discussing the equations for the solvation properties in aqueous solutions of osmolytes and proteins, we briefly review basic features of Kirkwood-Buff theory that we need for the calculations. Overall, the original Kirkwood-Buff theory allows the calculation of thermodynamic properties from structural properties of the solution—the pair correlations between particles (Kirkwood and Buff 1951). Such relations are given, for example, by Equations 1 through 3 below. Here we do the reverse, namely calculate structural properties of the solution (the G_{ij} functions discussed below) from experimental data that were published previously (Chalikian et al. 1997; Rösgen and Hinz 2000; Auton and Bolen 2005; Rösgen et al. 2005). This procedure of calculating G_{ij} from experimental thermodynamic data is also called “inverse Kirkwood-Buff theory” (Ben-Naim 1977). Such an approach is represented by equations of the type starting with Equation 5 below.

The Kirkwood-Buff expressions for solution compressibility κ , partial molar volume $\bar{v}_i$ of component i , chemical potentials μ_i , and equilibrium constants K contain the Kirkwood-Buff integrals G_{ij} , which are a representation of the overall correlation between components i and j , which could be, for example, water and protein. Three useful expressions (from Kirkwood and Buff 1951; Ben-Naim 1988, 1992), which we will use later, are

$$\frac{1}{kT} \left(\frac{\partial \mu_2}{\partial c_2} \right)_{T,p} = \frac{1}{c_2} + \frac{(G_{12} - G_{22})}{1 - (G_{12} - G_{22})c_2}, \quad (1)$$

$$kT\kappa = \bar{v}_1 + \bar{v}_1 c_1 G_{11} + \bar{v}_2 c_2 G_{12}, \quad (2)$$

$$kT\kappa = \bar{v}_2 + \bar{v}_1 c_1 G_{12} + \bar{v}_2 c_2 G_{22}. \quad (3)$$

Component one is water (W) throughout this paper, and component two is either protein (P) or osmolyte (O). An enrichment or relative excess of water around protein corresponds to a positive G_{PW} (positive correlation), whereas a depletion of water around protein corresponds to a negative G_{PW} (negative correlation). The overall correlation G_{PW} is obtained by integrating throughout the solution volume V the deviation of the water distribution from bulk density $g_{PW} - 1 = h_{PW}$ as a function of the distance from the protein

$$G_{PW} = \int_V h_{PW} dV. \quad (4)$$

Therefore, the radial pair correlation functions h_{ij} , which define the structure of the solution, also define

its thermodynamics. According to Equation 4, different structures of the solution h_{ij} could result in the same thermodynamics (Equations 1–3), as long as the different h_{ij} integrate to yield the same Kirkwood-Buff integrals. For this reason, it is not possible to derive the structure in terms of the h_{ij} from the thermodynamics of the solution. However, it is possible to calculate the integral over the structure or correlation between different molecular species given by the Kirkwood-Buff integrals G_{ij} from thermodynamic data (Ben-Naim 1977, 1988). Such an evaluation of the overall solution structure provides very valuable information, because it can give insight into molecular mechanisms governing solvation properties (Rösgen et al. 2005). In this work we provide a detailed analysis of the various G_{ij} of osmolytes and proteins in aqueous solution.

Aqueous osmolyte and aqueous protein

Equations 1 and 3 can be solved for the Kirkwood-Buff integrals as functions of experimentally measurable quantities to obtain the correlation between (or mutual solvation of) components 1 and 2,

$$G_{12} = kT\kappa - \frac{\bar{v}_2}{a_{22}}, \quad (5)$$

where $a_{22} = (\partial\mu_2/\partial\ln c_2)_{p,T}/kT$, and we use the fact that volume fractions sum up to unity ($c_1\bar{v}_1 + c_2\bar{v}_2 = 1$). Component 1 is water here (W), and component 2 can be osmolyte (O) or protein (P). Aqueous solutions have a well-known low compressibility so that for practical purposes in aqueous solution the term $kT\kappa$ can be set to zero (Lide 2004). In dilute solution a_{22} is unity. Therefore, in *ideal dilute* aqueous solution, we have

$$G_{12}^{id} = -\bar{v}_2. \quad (6)$$

Thus, in an aqueous two-component system, the Kirkwood-Buff integral G_{12} (the hydration of component 2) is well approximated by the partial molar volume of component 2, $\bar{v}_2$, corrected by a nonideality factor, a_{22} (Equation 5). For example, in the case of a dilute protein in water, the Kirkwood-Buff integral is reasonably given by the partial volume $\bar{v}_2$ of component 2, which is the protein (Equation 6) in this case.

Analogously, Equations 1 and 3 yield as self-correlations or associations for component 2,

$$G_{22} = kT\kappa - \frac{\bar{v}_2}{a_{22}} + \frac{1 - a_{22}}{a_{22}c_2}. \quad (7)$$

We may write the chemical potential as $\mu_2 = \mu_2^o + kT \ln(c_2\gamma_2)$. Defining $\gamma_{22} = (\partial \ln \gamma_2 / \partial c_2)_{p,T}$, we have

$$G_{22} = kT\kappa - \frac{\bar{v}_2 + \gamma_{22}}{a_{22}}. \quad (8)$$

In general, γ_{22} is not unity, even in the ideal dilute case denoted γ_{22}^{id} . For example, we found expanding to first order (Rösgen et al. 2004a) γ_{22}^{id} equals an effective volume per molecule of component 2. Because of the nature of long-ranged electrostatics, γ_{22}^{id} diverges to infinity in the case of salts. Therefore, we cannot ignore γ_{22}^{id} in general, and we have in ideal dilute aqueous solution

$$G_{22}^{id} = -\bar{v}_2 - \gamma_{22}^{id}. \quad (9)$$

Thus, the correlations between molecules of type 2 are not only determined by the space they occupy relative to the whole solution ($\bar{v}_2$) but also by whether there is a net depletion of the molecules of type 2 near each other (positive γ_{22}) or an enhancement (negative γ_{22}).

Finally, Equations 2 and 5 yield the water–water correlation

$$G_{11} = kT\kappa - \frac{1}{c_1} + \frac{\bar{v}_2}{a_{22}} \cdot \frac{\bar{v}_2 c_2}{1 - \bar{v}_2 c_2}. \quad (10)$$

The volume fraction of component 2 ($\bar{v}_2 c_2$) at infinite dilution is zero. Therefore, under these conditions, the inverse water concentration $1/c_1 = \bar{v}_1^{id}$ remains, and we get an expression that is analogous to Equation 6 as expected

$$G_{11}^{id} = -\frac{1}{c_1}. \quad (11)$$

Note that Equations 5, 7, 8, and 10 are completely general.

Dilute protein in aqueous osmolyte

The aqueous osmolyte in dilute protein solution is essentially like a pure bulk solution that is not significantly altered by the presence of the protein. Thus, we consider the overall pair correlations between the various components, WW, WO, OO, PW, and PO to examine the concentration-dependent solvation behavior of aqueous osmolytes in solutions of dilute protein (Fig. 1). We will discuss these in more detail below. The PP component can be calculated in principle (Smith 2006a), though this

Figure 1. Solvation of saccharides (long dashed lines; trisaccharide raffinose, disaccharides sucrose and maltose, monosaccharides glucose, mannose, fucose, galactose, rhamnose, and xylose), amino acids, and derivatives (short dashed lines; glycine, sarcosine, glycine betaine, alanine, and proline) and polyols (continuous lines; three carbon: glycerol, four carbon: erythritol, six carbon: mannitol and sorbitol) and urea (continuous line) calculated from experimental data (Rösgen et al. 2005). (A) The Kirkwood-Buff integrals of water self-hydration G_{WW} are all close to zero. (B) The osmolyte hydration G_{WO} depends mostly on the size of the osmolyte. (C) The osmolyte self-hydration G_{OO} is highly compound- and concentration-dependent. (D) The protein-independent part, G_{PW} , of the protein hydration change upon unfolding (Equation 16) does not depend much on osmolyte type and concentration. (E) The protein-independent part, G_{PO} , of the protein–osmolyte solvation change upon unfolding (Equation 17) is strongly dependent on concentration and osmolyte type.

is irrelevant for our current purposes. We now discuss the two equations governing explicitly the protein aspects of solvation (Fig. 1, right column) in the same system. Both will be analyzed in section 3 on the basis of experimental data. The use of simulations or theoretical estimations of the Kirkwood-Buff integrals we retain for another work.

It was previously shown (Rösgen et al. 2005) that the change of hydration upon denaturation of dilute protein is

$$\Delta_N^D(G_{PW}) = -\Delta_N^D\bar{v}_P + \phi_O \frac{m}{(\partial\mu_O/\partial\ln c_O)} \quad (12)$$

and the protein–osmolyte solvation change is

$$\Delta_N^D(G_{PO}) = -\Delta_N^D\bar{v}_P - (1 - \phi_O) \frac{m}{(\partial\mu_O/\partial\ln c_O)}, \quad (13)$$

where m is the derivative with respect to osmolyte concentration of the protein folding Gibbs free energy ΔG , $\Delta_N^D\bar{v}_P$ is the change in protein partial molar volume upon denaturation, and the volume fractions ϕ_i are defined as $\phi_i = c_i\bar{v}_i$. We can relate Equations 12 and 13

to the bulk osmolyte–water correlation G_{WO} by the use of Equation 5, which results in

$$\Delta_N^D(G_{PW}) = -\Delta_N^D\bar{v}_P - \frac{m}{RT} G_{WO} c_O \quad (14)$$

$$\Delta_N^D(G_{PW}) = -\Delta_N^D\bar{v}_P + \frac{m}{RT} G_{WO} c_W \frac{\bar{v}_W}{\bar{v}_O} \quad (15)$$

These equations contain two protein-dependent contributions, namely the change in partial molar volume upon denaturation $\Delta_N^D\bar{v}_P$ and the m -value. The denaturation volume change usually does not exceed one percent of the protein volume (Bull and Breese 1973; Rösgen and Hinz 2000; Lin et al. 2002), or about 0.1 L/mol for the well-investigated small proteins. Therefore, $\Delta_N^D\bar{v}_P$ could be a significant contributor to the overall protein hydration change upon unfolding $\Delta_N^D(G_{PW})$ but only when both the osmolyte concentration and the m -value are small. Also in the case of the protein–osmolyte solvation $\Delta_N^D(G_{PO})$, the volume term can become significant if the m -value is small. In any case, $\Delta_N^D\bar{v}_P$ is not expected to be a large contribution to the solvation expressions (Equations

14 and 15). It only becomes significant when the other term is close to zero.

Osmolyte solvation

Osmolytes are small organic molecules that are used in virtually all living organisms to counteract cellular stress. Throughout this paper we will use the term “osmolyte” in a broader sense, including other organic compounds that are closely related to naturally occurring osmolytes.

In a simple, binary, aqueous osmolyte solution we have as the two components water (component 1) and osmolyte (component 2). We used Equations 5, 8, and 10 to calculate from experimental data given by Rösigen et al. (2005) the solvation integrals of various osmolytes, that is, G_{WO} , G_{OO} , and G_{WW} . The results are shown in Figure 1.

Water self-solvation

The water–water correlation G_{WW} (Fig. 1A) barely deviates from zero in comparison with G_{WO} and G_{OO} . As already known from Equation 11, all compounds have a G_{WW} of $-1/c_W = -18$ mL/mol in the limit of zero molar osmolyte. With increasing concentration of osmolyte, G_{WW} increases. In the case of the amino acids, this increase is small and goes to a maximum of ~ -10 mL/mol. The increase is stronger for polyols and urea (up to $+10$ mL/mol) and largest for the saccharides, which reach $+40$ mL/mol. In view of the large range of G_{WO} and G_{OO} values (Fig. 1B,C) even the most extreme change in G_{WW} found for the saccharides is negligibly small.

On the basis of Equation 10, we can identify three contributions to the water self-solvation, namely the compressibility κ , the inverse water molarity, and a more complex term. The last term has by far the strongest dependence on osmolyte concentration c_O , because it has a numerator that is directly proportional to c_O . The denominator contains two contributions that have opposite concentration dependencies for protecting osmolytes. The term $1 - \bar{v}_O c_O$, which equals the volume fraction of water $\bar{v}_W c_W$, clearly decreases from unity toward zero as a function of c_O . The slope of the chemical potential with respect to the log of the concentration, a_{OO} , is constant and nearly unity in the case of urea but a_{OO} strongly increases in the case of protecting osmolytes, thereby compensating to some degree for the decrease in $1 - \bar{v}_O c_O$. This results in the overall weak dependence of G_{WW} on c_O .

Osmolyte hydration

At low concentration, the osmolyte hydration G_{WO} depends on the size of the osmolyte given by its partial

molar volume $\bar{v}_O$ (Equation 6). Accordingly, we find the osmolytes in Figure 1B sorted by size. The trisaccharide raffinose replaces ~ 0.4 L of water per mole of raffinose in dilute aqueous solution. This corresponds to ~ 0.4 L/mol $\times 55$ mol/L = 22 water molecules. The amount of water molecules replaced by the disaccharides ranges from 11 to 6, depending on the concentration. In comparison, the amino acids, polyols, urea, and monosaccharides have more similar molecular weight and cluster in a range of G_{WO} between -0.1 and 0 L/mol. They “replace” >6 water molecules. Interestingly, the slope of G_{WO} as a function of osmolyte concentration also depends on osmolyte size. Note that the use of the expression “osmolyte size” for $\bar{v}_O$ is not valid for compounds such as salts, which have a very high hydration density. This increase in density due to electrostriction can even overcompensate the contribution of the van der Waals volume to $\bar{v}_O$, so that the overall magnitude of the partial molar volume of a salt can be negative.

For a discussion of the functional dependence of G_{WO} on osmolyte concentration we use Equation 5 and ignore the compressibility contribution, which is small (Lide 2004). The partial molar volumes $\bar{v}_O$ of the osmolytes are either constant or depend very little on concentration (Rösigen et al. 2004a). We can therefore focus on a_{OO} , which starts at unity at infinite dilution. Except for urea and glycine, a_{OO} is larger than unity and it can be as large as 3.5–4 in the case of some protecting osmolytes like sucrose, sorbitol, and glycine betaine (Rösigen et al. 2004a,b). G_{WO} is therefore expected to start at a value of $-\bar{v}_O$ at 0 M osmolyte and for most osmolytes change to less negative values as the concentration increases. At 0 M, water G_{WO} would ultimately approach $-\bar{v}_W$ for all osmolytes, because of the symmetry of Equation 5 ($kT\kappa - \bar{v}_O/a_{OO} = G_{WO} = G_{OW} = kT\kappa - \bar{v}_W/a_{WW}$). In the case of urea, a_{OO} remains close to unity over the range of solubility, and in the case of glycine it drops slightly below unity as the concentration is increased. Accordingly, G_{WO} remains at $-\bar{v}_O$ or becomes slightly more negative, respectively. In addition, we see the steeper slope in G_{WO} for larger osmolytes (Fig. 1B). This effect originates in the scaling of a_{OO} with the osmolyte size (Rösigen et al. 2004a), which directly exerts its effect of G_{WO} through equation 5.

The tendency of G_{WO} to approach zero as the osmolyte concentration increases can also be rationalized in a more intuitive way—with the caveat that such a posteriori rationalization of osmolyte behavior may or may not be valid for other kinds of compounds. The initial negative value of G_{WO} means that there is a deficit of water around osmolyte molecules. The primary reason for this deficit is the mutual exclusion of water and osmolyte, because the molecules cannot overlap (see, e.g., Figure 1 in Rösigen et al. 2005). Since the water deficit is a deficit relative to

the amount of water in the bulk solution, the negative value of G_{WO} is expected to approach zero as the bulk water concentration decreases, that is, as the osmolyte concentration increases. In other words, the deficit of water around osmolyte becomes less and less remarkable as the total amount of water in solution decreases. This trivial effect is more pronounced in the case of the larger osmolytes, because c_W decreases more rapidly with osmolyte molarity.

Osmolyte self-solvation

Large effects occur in the self-solvation of the osmolytes G_{OO} as shown in Figure 1C. The saccharides (long dashed lines) are again sorted by size. The self-solvation of the trisaccharide raffinose is ~ 1 L/mol, the disaccharides G_{OO} vary from ~ 0.6 L/mol to -0.3 L/mol as a function of concentration, and the monosaccharides have a comparably constant self-solvation around -0.2 L/mol. We also note the polyols are sorted by size. In the case of the amino acids it is instructive to look at a series of methyl group additions from glycine to sarcosine (N-methyl glycine) to glycine betaine (trimethyl glycine). Glycine itself is very unusual, since G_{OO} is positive, that is, its attractive interactions as a small zwitterion overpower the hard-core repulsion that is more dominant for the other amino acids and the saccharides. As methyl groups are added to the glycine amino group, G_{OO} first reduces to around -200 mL/mol upon addition of one methyl group (for sarcosine = methylglycine), and on addition of a total of three methyl groups to a range from -450 to -350 mL/mol (glycine betaine = trimethyl-glycine).

Although the equations for osmolyte hydration G_{WO} (Equation 5) and self-solvation G_{OO} (Equation 8) are very similar, they demonstrate different solvation behaviors (Fig. 1B,C). Among the osmolytes, however, their *qualitative* behavior is quite similar. Note, for example, that the saccharides are still sorted by size and display similarly sloped curves. The amino acids still cluster together with the monosaccharides, with the exception of glycine betaine and glycine. Urea also stands out having a comparably high self-solvation G_{OO} .

The reason for the qualitative similarity between self-solvation behavior and hydration for most osmolytes is that both $\bar{v}_O$ and γ_{OO} have a dependence on the osmolyte size. For example, in the case of the saccharides, γ_{OO} contributes uniformly ~ 0.1 L/mol for each saccharide unit at low osmolyte concentration. Since the presence of γ_{OO} is the only difference between G_{WO} (Equation 5) and G_{OO} (Equation 8), it is therefore not surprising that osmolyte hydration and self-solvation behave in a qualitatively similar fashion. Most of the osmolytes show the same kind of pattern. Only in the

three cases, in which γ_{OO} takes either unusually positive (glycine betaine) or negative (glycine and urea) values, is a qualitative and quantitative deviation between hydration and self-solvation observed (Fig. 1B,C). The ultimate limit of G_{OO} in pure osmolyte is $-1/c_{O,\max}$, where $c_{O,\max}$ is the molarity of the neat osmolyte (compare with discussion of Equations 10 and 11, and take species 1 as osmolyte).

The general trend of G_{OO} toward the moderately sized values of $-1/c_{O,\max}$ as the osmolyte concentration increases can thus be rationalized in a more intuitive way (again with the caveat that this rationalization may or may not be valid for compounds other than osmolytes). For glycine the same ideas apply as seen in the section on the osmolyte hydration G_{WO} . Finding glycine molecules in proximity to other glycine molecules might be surprising at low concentrations (positive G_{OO}), but it is not remarkable at all in a densely packed glycine solution near the solubility limit (G_{OO} close to zero). Also in the case of the osmolytes that have an initially negative self-solvation G_{OO} , the slope of G_{OO} with osmolyte concentration is readily understandable. Even in systems that are entirely governed by repulsive volume exclusion (e.g., hard sphere systems), the system is forced to optimize its packing if the concentration is high. Such packing effects are reflected in an increasing G_{OO} .

Protein solvation

In the previous sections we discussed the experimental bulk solvation behavior in osmolyte solution, which is shown on the left-hand side of Figure 1. Now we turn to the solvation of the dilute protein component (right side of Fig. 1), which is given by Equations 14 and 15. The concentration dependence of the expressions, Equations 14 and 15, comes from the protein-independent parts of the equations, which are given by $G_{WO}c_O$ and $G_{WO}c_W\bar{v}_W/\bar{v}_O$, respectively, as explained in the following paragraph. These contributions are shown in Figure 1D and E. All curves are universally valid for dilute protein in aqueous osmolyte, and therefore it is possible to derive some general features. We consider this first for protein hydration and protein–osmolyte solvation. Then we extend the discussion to temperature-dependent hydration in the absence of osmolyte. We will consider protein unfolding, and all changes upon unfolding are marked with the symbol Δ_N^D .

As indicated above, we first discuss that part of the protein solvation expressions (Equations 14 and 15) that (1) is independent of the protein and (2) is the major source of the concentration dependence of the solvation of the protein. We therefore rearrange Equations 14 and 15 so that concentration-dependent and protein-independent terms are on the right side as

$$(\Delta_N^D(G_{PW}) + \Delta_N^D \bar{v}_P) \frac{RT}{m} = -G_{WO} c_O \quad (16)$$

$$(\Delta_N^D(G_{PO}) + \Delta_N^D \bar{v}_P) \frac{RT}{m} = G_{WO} c_W \frac{\bar{v}_W}{\bar{v}_O} \quad (17)$$

The m -value is the slope of the protein folding stability with osmolyte concentration. Normally, m is assumed to be a constant for osmolytes without net charge. This is clear for urea (Greene Jr. and Pace 1974; Santoro and Bolen 1988; Makhatadze 1999; Courtenay et al. 2000; Timasheff and Xie 2003; Ferreon and Bolen 2004) and the small number of proteins investigated there is evidence that it is also constant for stabilizing osmolytes (Mello and Barrick 2003; Felitsky and Record Jr. 2004; Holthauzen and Bolen 2007). Therefore, m will only influence the amplitude but not the shape of the curves Equations 16 and 17. In addition to discussing the protein-independent solvation characteristics (Equations 16 and 17), experimental m -values for (un)folding of reduced and carboxy-amidated ribonuclease T1 (RCAM RNase T1) will be used for illustration purposes on how the solvation character behaves experimentally.

Protein hydration

The part of protein hydration in the presence of osmolytes that depends on the bulk solution properties (Equation 16) is given in Figure 1D. All curves have positive values and do not change much between different osmolytes. This nearly uniform behavior of the osmolytes makes sense in view of the direct connection between the protein hydration and G_{WO} (Equations 14 and 5) and the approximately uniform behavior of G_{WO} for most of the osmolytes. To discuss the protein hydration character, we have to take into account that the expression for the protein-independent part of hydration (Equation 16) plotted in Figure 1D has an m -value that is a positive or negative coefficient. That means the hydration change upon unfolding is always negative for stabilizing osmolytes (positive m -value) and positive for the denaturing osmolyte urea (negative m -value). Only a $\Delta_N^D \bar{v}_P$ that has the opposite sign compared with the m -value can cause $\Delta_N^D(G_{PW})$ to switch sign at some concentration. This effect is limited to low osmolyte concentrations, because $\Delta_N^D \bar{v}_P$ is usually small, as discussed above.

To illustrate the effect of the m -value, we use the collection of m -values of RCAM T1 stabilization and destabilization in several osmolytes (Auton and Bolen 2005). The protein solvation is shown in Figure 2, with the change of protein hydration upon unfolding $\Delta_N^D(G_{PW})$ given in Figure 2A. These hydration values are very close to zero for the stabilizing osmolytes. A volume change $\Delta_N^D \bar{v}_P$ in the observed range of several dozen milliliters

Figure 2. Solvation of RCAM RNase T1 in osmolyte solutions. The values are offset by the small contribution of the denaturational volume change $\Delta_N^D \bar{v}_P$. Destabilizing regions are shaded in gray. (A) Hydration change upon unfolding $\Delta_N^D(G_{PW})$. (B) Osmolyte solvation change upon unfolding $\Delta_N^D(G_{PO})$. The curves were calculated using Equations 12 and 13. The m -values of RCAM RNase T1 (Auton and Bolen 2005) in units of kJ/molM are 7.73 (urea), 1.84 (glycine betaine), 2.59 (proline), 6.48 (sucrose), 5.77 (sorbitol), and 6.77 (sarcosine).

per mole for proteins of this size (Rösgen and Hinz 2000; Seemann et al. 2001; Lin et al. 2002) could make the hydration change upon unfolding switch sign, especially in the case of glycine betaine and proline. In the regime of low osmolyte concentration any positive $\Delta_N^D \bar{v}_P$ will invert the sign of $\Delta_N^D(G_{PW})$ for the case of the protecting osmolytes, whereas a negative $\Delta_N^D \bar{v}_P$ will do the same for the denaturant urea. Besides such possible effects, the general observation for RCAM ribonuclease T1 is that the denaturation hydration change is close to zero for all investigated osmolytes.

Returning to the general protein-independent hydration properties (Fig. 1D), we see different degrees of curvature in $\Delta_N^D(G_{PW})$. Above, we saw that the magnitude and slope of the bulk osmolyte hydration G_{WO} (see Fig. 1B) depends on the size of the osmolyte. The slope in G_{WO} translates into the curvature in the protein hydration because of the factor c_O in Equation 14. Accordingly, the curvature of the hydration plots in Figure 1D increases with increasing osmolyte size.

Protein–osmolyte solvation

The protein-independent part of the change in protein–osmolyte solvation upon unfolding is given in Figure 1E. The curves start at -1 and remain negative. Equation 17 shows that the 0 M concentration values are normalized to unity, because the initial value of G_{WO} is $-\bar{v}_O$ (Equation 6), and G_{WO} is then normalized by $\bar{v}_O$ (Equation 17).

Under dilute conditions, the volume fraction of water $c_W \bar{v}_W$ is unity and does not play a role in Equation 17 at infinite dilution.

The sign of the m -value determines whether the denaturation change in protein–osmolyte solvation, $\Delta_N^D(G_{PO})$ (Equation 15), has a positive or negative sign. Therefore, stabilizing osmolytes (positive m -value) generally become more excluded from the protein upon unfolding, whereas destabilizing osmolytes (negative m -value) become more enriched. A potential exception to this rule can be deduced in the following way by inspecting Figure 1E and Equation 15. An exceptionally large $\Delta_N^D \bar{v}_P$ of the same sign as the m -value could, in principle, lead to a switch in the sign of $\Delta_N^D(G_{PO})$ for a few stabilizing osmolytes, such as sorbitol, which approaches zero furthest in Figure 1E. However, such a switch caused by a $\Delta_N^D \bar{v}_P$ that is overpowering the m -value term (Equation 15) is very unlikely to occur because of the generally small magnitude of $\Delta_N^D \bar{v}_P$.

A more likely scenario in which the volume change upon denaturation could make a major contribution to the solvation is that the m -value is small. This is the case for several osmolytes solvating RCAM RNase T1, as shown in Figure 2B. The small m -value of proline and glycine betaine brings the hydration change close to zero (Fig. 2A), as discussed above. The same holds true for solvation of the protein by the osmolyte (Fig. 2B). Therefore, $\Delta_N^D(G_{PO}) + \Delta_N^D \bar{v}_P$ approaches zero so closely in the case of some of the protecting osmolytes that even a small negative $\Delta_N^D \bar{v}_P$ could bring at least part of the $\Delta_N^D(G_{PO})$ into the positive, destabilizing range (shaded areas in Fig. 2). The hydration change (Fig. 2A) would be concomitantly shifted up further into the stabilizing range. Consequently, the protein unfolding transition would involve an increase in both preferential solvation by the osmolyte and preferential hydration.

The protein–osmolyte solvation (Fig. 1E) spreads over a much wider range than the protein hydration (Fig. 1D). This is in spite of both containing the bulk osmolyte hydration G_{WO} as a factor (Equations 14 and 15). The reason for the different behavior is that in the case of protein hydration the factor of c_O increases while the absolute value of G_{WO} decreases. Thus, the two contributions oppose one another. In contrast with that, the two corresponding terms in the case of protein–osmolyte solvation (G_{WO} and c_W , Equation 15) enhance each other. This enhancement is larger for larger osmolytes, because both G_{WO} and c_W change more rapidly with osmolyte concentration if the osmolyte is larger. As a consequence the curves in Figure 1E have a rank order with respect to the slope, sorted according to the osmolyte size.

The strong concentration dependence of $\Delta_N^D(G_{PO})$ can be rationalized in terms of packing effects in the case

of excluded osmolytes. As the volume fraction of the protein-excluded osmolyte increases, the molecules are forced to optimize their packing (in addition to hydrogen bonding and other interactions). This leads to less exclusion of the osmolytes at higher concentration. Those osmolytes that are preferentially interacting with the protein surface will be enriched relative to the bulk—especially at low concentrations. As the bulk concentration increases the enrichment is worth less and less relative to the higher and higher bulk concentration. Thus, here also $\Delta_N^D(G_{PO})$ will have a trend toward zero as the osmolyte concentration increases.

Protein hydration in the absence of osmolyte

In the previous sections we discussed concentration-dependent solvation of aqueous osmolytes and proteins at constant temperature. Now we investigate the temperature dependence of the denaturation hydration change $\Delta_N^D(G_{PW})$ for dilute aqueous proteins. This property is directly connected to the change in partial molar volume upon unfolding $\Delta_N^D \bar{v}_P$ (Equations 6, 12, 14).

The partial molar volume v_P of the protein lysozyme is ~ 10 L/mol (Rösgen and Hinz 2000). That means, the protein replaces 556 water molecules ($N_W = c_W G_{PW} = -556$). Accordingly, from a thermodynamic point of view the hydration of lysozyme is negative and this water exclusion largely determines its volumetric properties (Equations 2 and 3), as well as its chemical potential (Equation 1). In general, there are two contributions to the hydration of the protein: (1) the steric exclusion of water from the protein core and (2) the solvation of the protein extending from the protein–water interface some distance into the solution. The steric exclusion has several contributions, including the van der Waals volume of the protein, cavities (in the native state), and the thermal volume increase due to vibrations, while the solvation beyond these effects is manifested by alterations in solution density around the protein (Chalikian and Breslauer 1996; Chalikian 2003). Because native and denatured proteins have the same van der Waals volume, the volume change upon denaturation will be dominated by these latter solvation properties plus some steric exclusion effects, for example, cavities or packing defects in the native state that disappear upon denaturation. In the case of a dilute protein in water ($c_P \approx 0$) the volume change upon unfolding is

$$\Delta_N^D \bar{v}_P = kT \Delta_N^D \kappa - \Delta_N^D G_{PW} \approx -\Delta_N^D G_{PW} \quad (18)$$

according to Equation 3.

Figure 3 shows the change in hydration number upon denaturation $\Delta_N^D N_W = c_W \Delta_N^D(G_{PW})$ (compare with Equation 18) for several proteins calculated from experimental

Figure 3. Hydration change upon unfolding of several proteins as a function of temperature. Equation 18 was used to calculate $\Delta_N^D N_W = c_W \Delta_N^D(G_{PW})$ from published volume changes $\Delta_N^D \bar{v}_P$. The data were taken from Rösigen and Hinz (2000) and Chalikian et al. (1997). All data refer to acidic conditions except for α -Lactalbumin, where the pH was neutral.

$\Delta_N^D \bar{v}_P$ values. All five proteins have a change in hydration number that decreases with increasing temperature. This is because the denatured state expands more with temperature than does the native state (Rösigen and Hinz 2000; Lin et al. 2002), and this effect is directly coupled to hydration through Equation 18. Interestingly, only two or three of the given proteins have a positive $\Delta_N^D N_W$ (i.e., a negative $\Delta_N^D \bar{v}_P$). This is in contrast with previous claims that the volume change upon unfolding is generally negative for proteins at room temperature (Royer 2002). Because the data that were listed in the review (Royer 2002) were extrapolated from high pressure to atmospheric pressure, this discrepancy may originate from small uncertainties in the compressibility difference between native and denatured state as a function of pressure.

It is clear from Figure 3 that the hydration might increase or decrease upon denaturation, depending on the kind of protein and the temperature. This is interesting in light of our discussion of the effect of protein solvation on protein stability. It shows that hydration will support the destabilizing effect of urea at high temperatures (negative solvation contribution from volume change brings the urea curve in Fig. 2A more into the destabilizing region), whereas at low temperatures hydration tends to oppose denaturation at low urea concentrations (positive solvation contribution from volume change shifts the urea curve in Fig. 2A upward). For stabilizing osmolytes the opposite holds: At high temperature hydration opposes the stabilizing effect, whereas it tends to enhance the stabilization by certain osmolytes at low temperature.

Overall, the effect of hydration on cosolute-dependent protein stability is not very big compared with other solution processes, such as protonation changes (pH), which exhibit very similar volume changes (Höiland 1986; Taulier and Chalikian 2003).

Conclusions

The pair correlations of proteins with water and osmolytes are the determining structural factors for the proteins' response to the presence of osmolytes. Only very recently, however, has effort been devoted to calculate thermodynamic solvation of proteins from experimental data (Shimizu 2004; Shimizu et al. 2006; Shimizu and Boon 2004; Shimizu and Smith 2004; Smith 2004a; Rösigen et al. 2005; Schurr et al. 2005; Shulgin and Ruckenstein 2005; Shimizu and Matubayasi 2006). In contrast with studies that target but a small set of osmolytes, we previously gave an overview of general biochemically relevant features of dozens of different kinds of osmolytes (Rösigen et al. 2005). In the current work, we extended our previous approach to a more comprehensive overview of the correlated solvation effects of water and osmolyte, as well as their impact on protein stability. Several important findings resulted from this study.

1. Hydration is more sensitive to osmolyte size than osmolyte type (excluded volume effect of hydration). We found that in terms of thermodynamic solvation (i.e., Kirkwood-Buff integrals G_{ij}) hydration effects are of minor importance for the unfolding energetics of proteins in different osmolyte solutions and for the energetics of the bulk osmolyte. Hydration generally makes a significant but nonspecific effect that mostly depends on the size of the osmolyte (Fig. 1B,D) with regard to magnitude and curvature of the solvation contribution. This holds for osmolyte hydration G_{WO} and protein hydration change $\Delta_N^D(G_{PW})$, respectively. Both are remarkably invariant with variable osmolyte type, and only the larger saccharides are an exception for G_{WO} because of their volume.
2. Water self-correlations are largely unaffected by osmolyte concentration and type. The energetic contribution of water self-hydration G_{WW} turns out to be insignificant to both the energetics of osmolytes and proteins in osmolyte solutions. G_{WW} is both very small in absolute magnitude and with regard to its slope as a function of osmolyte (Fig. 1A). This is consistent with a recent finding that the water–water correlation in the vicinity of the “chaotrope” urea and the “cosmotrope” TMAO (trimethyl-amine-N-oxide) are very similar with regard to both angles and distances between water molecules (Gallagher and Sharp 2003).
3. Differences between osmolytes stem mostly from osmolyte self-solvation. The major determinant for the thermodynamic behavior of osmolytes in water is the osmolyte correlation with itself. G_{OO} has by far the largest variability with respect to both magnitude and slope among the Kirkwood-Buff integrals of water–water, water–osmolyte, and osmolyte–osmolyte interaction (Fig. 1A–C).

4. Energetically, neither bulk water nor protein hydration is the main player in osmolyte concentration-dependent effects on protein stability and osmolyte energetics. As in the case of G_{OO} , the interaction $\Delta_N^D(G_{PO})$ between the two organic substances, protein and osmolyte, also displays a large variability with osmolyte concentration and type. In comparison, it is again the interaction with water $\Delta_N^D(G_{PW})$ that is quite insensitive to osmolyte type, with little sensitivity to osmolyte concentration. For ligand binding reactions different principles might hold, because of the potentially smaller m -values. This is likely, especially in protein–osmolyte phenomena in which the effect of osmolyte does not depend on osmolyte type (Sidorova and Rau 1996).
5. Very predictable and monotonic solvation properties might be prerequisite for a molecule to be useful as a biological osmolyte. The very simple activity coefficient (Rösgen et al. 2004a,b) and solvation behavior (Rösgen et al. 2005; this study) seems to be a general property of osmolytes. It might be argued that a simple monotonic solvation as a function of concentration is a necessary property of a compound to be useful as an osmolyte in vivo. Compounds such as alcohols, which have very complex and nonmonotonic solvation curves (Ben-Naim 1977; Matteoli and Lepori 1984), seem to be inappropriate for in vivo purposes, and even toxic. This is understandable, since predictably fine-regulating the chemical activities of cellular components has to rely on constant solvation behavior without sudden switches.

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