Thermodynamics of interactions of urea and guanidinium salts with protein surface: Relationship between solute effects on protein processes and changes in water-accessible surface area

ELIZABETH S. COURTENAY,^{1,3,4} MICHAEL W. CAPP,² AND
M. THOMAS RECORD, JR.^{1,2}

¹Department of Biochemistry, University of Wisconsin, Madison, Wisconsin, 53706 USA

²Department of Chemistry, University of Wisconsin, Madison, Wisconsin, 53706 USA

³Department of Bacteriology, University of Wisconsin, Madison, Wisconsin, 53706 USA

(RECEIVED May 31, 2001; ACCEPTED September 6, 2001)

Abstract

To interpret effects of urea and guanidinium (GuH^+) salts on processes that involve large changes in protein water-accessible surface area (ASA), and to predict these effects from structural information, a thermodynamic characterization of the interactions of these solutes with different types of protein surface is required. In the present work we quantify the interactions of urea, GuHCl , GuHSCN , and, for comparison, KCl with native bovine serum albumin (BSA) surface, using vapor pressure osmometry (VPO) to obtain preferential interaction coefficients (Γ_{μ_3}) as functions of nondenaturing concentrations of these solutes (0–1 molal). From analysis of Γ_{μ_3} using the local-bulk domain model, we obtain concentration-independent partition coefficients K_p^{nat} that characterize the accumulation of these solutes near native protein (BSA) surface: $K_{\text{P,urea}}^{\text{nat}} = 1.10 \pm 0.04$, $K_{\text{P,SCN}^-}^{\text{nat}} = 2.4 \pm 0.2$, $K_{\text{P,GuH}^+}^{\text{nat}} = 1.60 \pm 0.08$, relative to $K_{\text{P,K}^+}^{\text{nat}} \equiv 1$ and $K_{\text{P,Cl}^-}^{\text{nat}} = 1.0 \pm 0.08$. The relative magnitudes of K_p^{nat} are consistent with the relative effectiveness of these solutes as perturbants of protein processes. From a comparison of partition coefficients for these solutes and native surface (K_p^{nat}) with those determined by us previously for unfolded protein and alanine-based peptide surface K_p^{unf} , we dissect K_p into contributions from polar peptide backbone and other types of protein surface. For globular protein-urea interactions, we find $K_{\text{P,urea}}^{\text{nat}} = K_{\text{P,urea}}^{\text{unf}}$. We propose that this equality arises because polar peptide backbone is the same fraction (0.13) of total ASA for both classes of surface. The analysis presented here quantifies and provides a physical basis for understanding Hofmeister effects of salt ions and the effects of uncharged solutes on protein processes in terms of K_p and the change in protein ASA.

Keywords: Preferential interactions; protein water-accessible surface area; protein folding; Hofmeister Series; urea; guanidinium chloride; guanidinium thiocyanate; peptide backbone

Protein noncovalent processes (including solubility or crystallization, conformational changes, ligand binding, and association) fundamentally involve large changes in water-accessible surface area (ASA) and, hence, large changes in

the interactions of proteins with water and solutes. It is therefore not surprising that most solutes (at molal concentrations) perturb these processes strongly, even though they are not ligands or direct stoichiometric participants. The direction and extent of perturbation vary with the nature and concentration of the solute. Given the explosion in structural information, it is of great scientific and practical interest to obtain a structural interpretation of solute effects on noncovalent processes involving proteins, nucleic acids, or other biopolymers, and to be able to predict these effects

Reprint requests to: M. Thomas Record, Dept. of Biochemistry, 433 Babcock Drive, Madison, WI 53706, USA; e-mail: record@monte.biochem.wisc.edu; fax: (608) 262-3453.

⁴Present address: Department of Biology, MIT, Cambridge, MA 02139.

Article and publication are at <http://www.proteinscience.org/cgi/doi/10.1101/ps.20801>.

from structural input. To this end, we recently derived a general, quantitative relationship between the effects of charged or uncharged solutes on the standard free energy change $\Delta G_{\text{obs}}^{\circ}$ or equilibrium constant K_{obs} of a biopolymer process and the change in biopolymer ASA (ΔASA) that occurs in that process (Courtenay et al. 2000b; see equation 5 below). This derived relationship between thermodynamic observables and ΔASA complements the empirical relationship between heat capacity changes ($\Delta C_{\text{P,obs}}^{\circ}$ and ΔASA of protein processes (Spolar et al. 1989, 1992; Makhadatzte and Privalov 1990; Livingstone et al. 1991; Murphy et al. 1992, 1993; Spolar and Record 1994; Myers et al. 1995). Spolar and Record (1994) used $\Delta C_{\text{P,obs}}^{\circ}$ for a set of protein–protein and protein–ligand interactions, studied both structurally and thermodynamically, to determine whether these processes were rigid-body interactions or involved significant coupled conformational changes (e.g., folding). Analogous comparisons can be made between estimates of ΔASA determined from the effect of urea on the association process and those predicted from the structural data (assuming a rigid body interaction).

The relationship between the effect of a solute on $\Delta G_{\text{obs}}^{\circ}$ or K_{obs} and ΔASA requires knowledge of local-bulk partition coefficients $K_{\text{P},3}$ for the solutes and the biopolymer surface involved in the process. $K_{\text{P},3}$ is defined as the ratio of molal concentrations of the perturbing solute in the local domain near the biopolymer surface and in the bulk solution. In the local-bulk domain model, the bulk domain has the same small solute concentration as the two-component (–biopolymer) solute concentration in dialysis equilibrium with the three-component (+biopolymer) solution (Courtenay et al. 2000a,b):

$$K_{\text{P},3} \equiv m_3^{\text{local}}/m_3^{\text{bulk}} \quad (1)$$

$K_{\text{P},3}$ is independent of solute concentration and is greater than unity for accumulated solutes and less than unity for excluded solutes. The purpose of the present work is to determine model-independent thermodynamic preferential interaction coefficients (Γ_{μ_3} , defined below) that quantify the extent of interaction of protein-destabilizing solutes with native protein surface. Values of $K_{\text{P},3}$ describing the accumulation of these solutes near native BSA surface are then calculated from Γ_{μ_3} using the local-bulk domain model (see below) (Record and Anderson 1995; Courtenay et al. 2000a,b).

The most extensively studied solute effects on any protein process are the effects of urea and guanidinium chloride (GuHCl) on the unfolding of globular proteins and α -helical peptides. The observable in these experiments is the m -value, defined as the negative of the slope of the relatively linear plot of $\Delta G_{\text{obs}}^{\circ}$ versus C_3 , the molar denaturant concentration:

$$m\text{-value} \equiv - (d\Delta G_{\text{obs}}^{\circ}/dC_3) = RT (\text{dln}K_{\text{obs}}/dC_3) \quad (2)$$

Myers et al. (1995) observed that m -values for protein unfolding are proportional to ΔASA , the change in protein ASA on unfolding (empirically corrected for effects of disulfides). Scholtz et al. (1995) and Smith and Scholtz (1996) investigated effects of urea and GuHCl on the stability of a series of chain lengths of alanine-based α -helical peptides, and found that peptide m -values were proportional to chain length. From calculations of the amount and composition of the ASA per residue in the α -helix and completely unfolded states (cf. Fig. 1), we found that the proportionality constant relating m -values to the ΔASA for unfolding these α -helices is approximately four times larger than that characteristic of the globular proteins studied by Myers et al. (1995).

Courtenay et al. (2000b) applied a rigorous thermodynamic analysis to the m -value/ ΔASA ratios, taking all significant sources of nonideality in these multicomponent solutions into account, and thereby showed that $\Delta\Gamma_{\mu_3}$, the difference in denaturant-protein preferential interaction coefficients characteristic of unfolded and folded protein states, is proportional to ΔASA at any specified solute concentration. We found that the dependence of $\Delta\Gamma_{\mu_3}/\Delta\text{ASA}$ on the concentration of urea or GuHCl was quantitatively described by the local-bulk domain model (cf. equation 5 below) and obtained concentration-independent partition coefficients ($K_{\text{P},3}^{\text{unf}}$, listed in Table 1) characterizing the interactions of urea and GuHCl with the protein and peptide surfaces exposed on unfolding.

Figure 1 illustrates the key differences in the composition of water-accessible surface calculated for native BSA and the average globular protein (Miller et al. 1987) and for the protein or peptide surface exposed in unfolding. In particular, the surface exposed on unfolding differs significantly from native protein surface because of the virtual absence of any contribution from charged residues, which are water accessible in native and denatured states. Moreover, 48% of the surface exposed on unfolding the alanine-based α -helices is polar peptide backbone, whereas this subclass of polar surface constitutes only 13% of the ΔASA of unfolding of globular proteins (and of the ASA of native globular proteins). Courtenay et al. (2000b) observed that the four-fold greater percentage contribution of polar peptide surface to the ΔASA of unfolding of α -helical peptides as compared to globular proteins matched the four-fold higher m -value: ΔASA ratio observed for the α -helical peptides. This correspondence provides independent evidence that the primary region of interaction of urea or GuH^+ with the protein is the polar peptide backbone, the conclusion obtained in many previous studies of transfers of model peptides from water to solutions of urea (Gill et al. 1961; Nozaki and Tanford 1963; Robinson and Jencks 1965a; Nandi and Robinson 1984; Sijpkens et al. 1993; Wang and Bolen 1997) and GuHCl (Castellino and Barker 1969; Robinson and Jencks

Fig. 1. Decomposition of water-accessible surface areas of native and unfolded proteins and alanine-based peptides and of the surface area exposed on unfolding proteins and alanine-based peptides into contributions from polar peptide backbone, charged, other polar, and nonpolar surface (see Materials and Methods).

1965a; Nozaki and Tanford 1970; Nandi and Robinson 1984).

In the present study we report model-independent preferential interaction coefficients Γ_{μ_3} calculated from osmometric data on interactions of three protein destabilizing solutes (urea, GuHCl, and guanidinium thiocyanate, i.e., GuHSCN) and the reference solute KCl with native BSA.

BSA is a member of the homologous series of native proteins that differ in total ASA but have similar surface compositions (see Fig. 1). In particular, native BSA has the same percentages of nonpolar and polar surface as the average globular protein. Within the class of polar surface, BSA has the same percentage of polar backbone surface (13%), but a larger percentage of charged surface (29% vs. 19%). We

interpret values of Γ_{μ_3} as a function of small solute concentration using the local-domain model to obtain partition coefficients characterizing the accumulation of these solutes near the surface of native BSA relative to their concentrations in the bulk solution. We compare these partition coefficients with those determined by Courtenay et al. (2000b) for the interactions of these solutes with the protein surface exposed on unfolding globular proteins and α -helices. The comparisons presented here between interactions with native and with unfolded protein surfaces are relevant for the mechanism of action of these denaturants, and provide further evidence that urea and GuH^+ unfold proteins primarily by their favorable interaction (local accumulation) with the polar peptide surface that is exposed on unfolding. Our quantitative characterization of interactions of GuH^+ , Cl^- and SCN^- with protein surface is a useful starting point for predicting Hofmeister effects of these and other ions on protein processes from structural information (ΔASA).

Background: preferential interaction coefficients as quantitative measures of solute-protein interactions and solute effects on protein processes

Preferential interaction coefficients quantify the net extent of local accumulation or exclusion of a small solute in the vicinity of biopolymer surface, an effect that arises from differences in biopolymer-solute interactions relative to the interactions of both species with water. Experimentally, they are obtained from analysis of dialysis, osmometric, or other thermodynamic data used to quantify the change in concentration of the small solute as a function of biopolymer concentration while maintaining the chemical potential (μ) of the small solute (and/or of water) constant. For example, $\Gamma_{\mu_3} \equiv (\partial m_3 / \partial m_2)_{T,P,\mu_3}$. Differences in preferential interaction coefficients between products and reactants are the fundamental thermodynamic determinant of the effects of solute concentration on the thermodynamics (ΔG_{obs}^o , K_{obs}) of that process. For example, for uncharged solutes (Wyman 1964; Record et al. 1998; Timasheff 1998):

$$-\left(\frac{1}{RT}\right) \left(\frac{\partial \Delta G_{\text{obs}}^o}{\partial \ln a_3}\right)_{T,P} = \left(\frac{\partial \ln K_{\text{obs}}}{\partial \ln a_3}\right)_{T,P} = \Delta \Gamma_{\mu_3} \quad (3)$$

We previously studied a wide range of stabilizing solutes using VPO and found that Γ_{μ_3} is proportional to m_3^{bulk} at least in the range $m_3^{\text{bulk}} \leq 1$ molal. Comparison of values of $\Gamma_{\mu_3}/m_3^{\text{bulk}}$ for BSA with previous results (Gekko and Morikawa 1981; Gekko and Timasheff 1981; Arakawa and Timasheff 1983, 1984b, 1985; Lin and Timasheff 1994, 1996; Xie and Timasheff 1997) for smaller globular proteins (members of the same homologous series) indicates that $\Gamma_{\mu_3}/m_3^{\text{bulk}}$ is proportional to ASA, as predicted by our development of the local-bulk domain model (Record

and Anderson 1995; Courtenay et al. 2000a,b). Analysis of $\Gamma_{\mu_3}/m_3^{\text{bulk}}$ using this model yields values of $K_{P,3}$ for these solutes, describing their interactions with native protein surface relative to water-protein interactions (see below).

Thermodynamic description of solute effects on protein processes using the local-bulk domain model

In the local-bulk domain model, the protein solution is divided into two domains: a local domain at the protein surface and a bulk domain sufficiently far from the protein surface that the distribution of small solute and water are not affected by the presence of the protein. Courtenay et al. (2000a) showed that this model predicts the proportionality of the purely thermodynamic preferential interaction coefficient Γ_{μ_3} to m_3^{bulk} (at low m_3^{bulk}) and to ASA, in which the proportionality constant is determined by two quantities describing the interactions of solute and water with the protein surface: the local-bulk partition coefficient $K_{P,3}$ (see equation 1) describing the local accumulation or exclusion of the solute, and the biopolymer hydration per $\text{\AA}^2$ (b_1) in the presence of the solute. (Courtenay et al. 2000b):

$$\Gamma_{\mu_3} \equiv \frac{m_3^{\text{bulk}} (K_P - 1) b_1 \text{ASA}}{m_1} \quad (4)$$

in which ASA is the water-accessible surface area of the biopolymer in $\text{\AA}^2$ and $m_1 = 55.5$ moles water/kg. At low m_3^{bulk} or when the solute is preferentially excluded from the protein surface, $b_1 \equiv b_1^o$, the hydration in the absence of small solute.

From equations 3 and 4, the effect of changing solute concentration (on the molar scale) on the observed equilibrium concentration quotient K_{obs} of a process is related to the change in accessible surface area (ΔASA) of the biopolymers (Courtenay et al. 2000b):

$$\left(\frac{\partial \ln K_{\text{obs}}}{\partial C_3}\right)_{T,P} \equiv \frac{n(1 + \epsilon_3^c) b_1^o (K_{P,3} - 1)}{m_1 (1 + \delta_3 C_3)} \Delta\text{ASA} \quad (5)$$

In equation 5, $n = 1$ for nonelectrolyte solutes and $n = 2$ for 1:1 salts; $\epsilon_3^c \equiv (\partial \ln \gamma_3^c / \partial \ln C_3)$ in which γ_3^c is the activity coefficient of the perturbing solute on the molar concentration scale (see Appendix, Table A1), b_1^o is the protein hydration (per $\text{\AA}^2$) in the absence of the small solute and $\delta_3 \equiv (K_P S_1) m_1^{-1} - \bar{V}_3$ in which $S_{1,3}$ is the average number of water molecules displaced from the local domain per solute accumulated, and $\bar{V}_3$ is the partial molar volume of the perturbing solute (see Appendix; Table A1). $K_{P,3}$ and b_1^o are average quantities for the biopolymer surface exposed to or removed from water in the process. For a 1:1 salt, $K_{P,\text{salt}} = 0.5(K_{P,+} + K_{P,-})$ in which $K_{P,+}$ and $K_{P,-}$ are partition coefficients of the individual ions.

For the process of unfolding proteins or peptides, the observable m -value is related to equation 5 by:

$$\left(\frac{\partial \ln K_{\text{obs}}}{\partial C_3}\right)_{T,P} = -\frac{1}{RT} \left(\frac{\partial \Delta G_{\text{obs}}^{\circ}}{\partial C_3}\right)_{T,P} = \frac{m\text{-value}}{RT} \quad (6)$$

Equation 5 shows that values of $(\partial \ln K_{\text{obs}}/\partial C_3)$, or m -values (cf. equation 6), compared for the same process for different members of a homologous series of biopolymers, are proportional to ΔASA , as observed by Myers et al. (1995) for protein unfolding m -values. From the quantitative comparison of equation 5 with m -value/ ΔASA ratios, Courtenay et al. (2000b) obtained local-bulk partition coefficients characterizing the accumulation of urea and GuHCl near the surface exposed in unfolding globular proteins and α -helical peptides. In the present study, we report local-bulk partition coefficients calculated from Γ_{μ_3} (equation 4) for interactions of urea, GuHCl, and GuHSCN with native protein surface. We compare our results for urea and GuHCl with interactions of those solutes with unfolded protein surface. This comparison provides insight into the thermodynamic basis for the action of these solutes as denaturants, tests the approximation of additivity, and initiates a decomposition of partition coefficients into contributions from the polar peptide backbone and other subclasses of protein surface.

Results and Discussion

Evidence for the accumulation of destabilizing solutes at the surface of native protein from osmolality measurements

Representative VPO data, plotted in Figure 2, show the dependences of solution osmolality on the concentration of three protein-destabilizing solutes: A, urea; B, guanidinium hydrochloride (GuHCl); C, guanidinium thiocyanate (GuHSCN) in the presence (+) and absence (−) of a fixed concentration of BSA (3.16 mM). With and without BSA, osmolality increases with increasing concentration of the small solute but at very different rates. For the GuH^+ salts, the rate of increase of osmolality with GuH^+ concentration in the presence of BSA is sufficiently small that, at concentrations of GuHSCN > 0.2 molal or of GuHCl > 0.6 molal, the osmolality of the −BSA solution significantly exceeds that of the +BSA solution at the same concentration of GuH^+ . The crossovers observed for the GuH^+ salts clearly indicate the existence of strong favorable solute-solute (e.g. BSA- GuH^+) interactions as compared with water-solute interactions. For all three solutes, the difference between the +BSA and −BSA osmolalities is greatest in the absence of the small solute, and decreases with increasing concentration of the small solute. This behavior contrasts strikingly

with that of six protein-stabilizing solutes whose interactions with BSA were investigated by Courtenay et al. (2000a). For all six stabilizing solutes, the difference between +BSA and −BSA osmolalities increases with increasing concentration of the small solute.

Figure 3 shows calculated changes in osmolality in excess of the additive reference value ($\Delta \text{Osm}_{\text{ex}}$) on addition of BSA (final concentration 3×10^{-3} molal) to solutions of urea, GuHCl, and GuHSCN as a function of the initial osmolality of the two-component solution (−BSA). $\Delta \text{Osm}_{\text{ex}}$ is defined as the difference between the observed change in osmolality ($\Delta \text{Osm}_{\text{obs}}$) for the addition of BSA to a particular solution and the osmolality change ($\Delta \text{Osm}_{\text{ref}}$) that would be observed if the contributions of BSA and the small solute to solution osmolality were additive. ($\Delta \text{Osm}_{\text{ref}}$ is the experimentally determined osmolality of 3×10^{-3} molal BSA in the absence of small solute; Courtenay et al. 2000a.) Effects of

Fig. 2. Solution osmolality as a function of solute molality for solutions of protein destabilizing solutes with (filled symbols) and without (open symbols) 3.16 mM BSA. (A): urea, (B): GuHCl, and (C): GuHSCN solutions.

the addition of BSA to solutions of glycine betaine and glycerol, representing the extremes of behavior observed for stabilizing solutes (Courtenay et al. 2000a), are plotted in Figure 3 for comparison. Addition of BSA to a 1 Osm GuHSCN solution results in $\Delta\text{Osm}_{\text{ex}} = -0.14$ Osm, similar in magnitude but opposite in direction to that obtained on addition of BSA to a 1 Osm solution of the highly excluded solute glycine betaine, for which $\Delta\text{Osm}_{\text{ex}} = 0.18$. Addition of BSA to 1 Osm urea results in $\Delta\text{Osm}_{\text{ex}} = -0.02$ Osm, comparable in magnitude but opposite in direction to that obtained for the weakly excluded solute glycerol, for which $\Delta\text{Osm}_{\text{ex}} = 0.03$ (Courtenay et al. 2000a).

Figure 3 clearly shows that the signs and magnitudes of deviation from an additive relationship between the osmotic contributions of BSA and the small solute are solute-specific. These deviations reflect differences between interactions among solutes and interactions of both solutes (small solute and BSA) with water. The large reduction in osmolality on addition of BSA to a GuHSCN solution implies a strong favorable interaction between BSA and GuH^+ and/or SCN^- , resulting in local accumulation of one or both of these ions at the surface of BSA. The lack of strong favorable interactions of GuHSCN and GuHCl with water, relative to self-interactions of these solutes, is evidenced by the negative sign of the concentration-dependent term of $d\text{Osm}/d\text{m}_3$ for the two-component aqueous solutions of these solutes (see Appendix; Table A1). Interpreted in terms

of local accumulation, Figure 3 indicates that GuHCl is less strongly accumulated near BSA than is GuHSCN and, therefore, that Cl^- is less strongly accumulated than SCN^- ; urea is the least accumulated of the three protein-destabilizing solutes. However, it is clear from Figure 3 that the local concentration of all three solutes near BSA surface (local) significantly exceeds that in the reference solution in which the small solute is randomly mixed in both local and bulk domains of the solution.

The large increases in osmolality on the addition of BSA to solutions of the stabilizing solutes imply interactions of BSA with these solutes are unfavorable relative to interactions with water. Indeed Courtenay et al. (2000a) concluded that glycine betaine was almost completely excluded from the water of hydration of BSA. The strong favorable interaction of glycine betaine with water is shown by the large positive betaine concentration-dependence of $d\text{Osm}/d\text{m}_3$ for the two-component (betaine-water) solution (Courtenay et al. 2000a).

Determination of preferential interaction coefficients describing interactions of native BSA with urea, GuHCl, and GuHSCN

The solute-specific nonideal behavior of solution osmolality in Figures 2 and 3 contains information on the strengths of interactions of these small solutes with BSA surface relative to the interactions of BSA and the small solutes with water. To separate solute-specific interactions with BSA surface from other sources of nonideality and from the effects of ideal mixing entropy (Anderson et al., in prep.), and to obtain a thermodynamic quantity capable of describing the effects of these solutes on protein processes, we calculated preferential interaction coefficients (Γ_{μ_3}) from the VPO data as described previously (Courtenay et al. 2000a). Values of Γ_{μ_3} as a function of bulk solute concentration (m_3^{bulk}) are plotted in Figure 4 for urea (panel A), GuHCl (panel B), and GuHSCN (panel C). (Data for KCl, which we find to be a suitable reference salt for decomposition of Γ_{μ_3} into contributions from the individual ions, are given in the Appendix.) For all solutes, values of Γ_{μ_3} were found to be the same as the preferential interaction coefficient that would be measured in an equilibrium dialysis experiment (Γ_{μ_1, μ_3}) calculated as described by Courtenay et al. (2000a). At all concentrations of the protein-destabilizing solutes, Γ_{μ_3} is positive, demonstrating the accumulation of all three solutes in the vicinity of native BSA surface. Figure 4 shows that Γ_{μ_3} is proportional to m_3^{bulk} at sufficiently low concentrations as predicted by the local-bulk domain model (Record and Anderson 1995; Courtenay et al. 2000a,b). As observed in our previous study of interactions of protein-stabilizing solutes with native BSA, the dependence of Γ_{μ_3} on bulk solute concentration is independent of BSA concentration in the experimentally accessible range (2.6–3.7 mM). Values of

Fig. 3. Change in solution osmolality ($\Delta\text{Osm}_{\text{ex}}$) in excess of that expected assuming additivity on addition of BSA (3×10^{-3} molal final concentration) as a function of two-component (small solute-water) solution osmolality. Uncertainties in calculated values of $\Delta\text{Osm}_{\text{ex}}$ are approximately $\pm 15\%$.

$\Gamma_{\mu_3}/m_3^{\text{bulk}}$ range from 6 ± 2 for urea to 17 ± 1 for GuHCl and 57 ± 8 for GuHSCN. For comparison, Γ_{μ_3} for KCl is zero within uncertainty at all KCl concentrations investigated (≤ 1 molal; see Appendix). Values of $\Gamma_{\mu_3}/m_3^{\text{bulk}}$ were interpreted using the local/bulk domain model (equation 4) with $\text{ASA} = 2.9 \times 10^4 \text{ \AA}^2$ for native BSA and $b_1^0 = 0.11 \text{ H}_2\text{O}/\text{\AA}^2$ (Courtenay et al. 2000a) to obtain partition coefficients for these solutes near native BSA surface ($K_{P,3}^{\text{nat}}$, equation 1) from linear fittings to the low solute concentration region of the data (≤ 1 molal urea, ≤ 0.5 molal GuHCl, ≤ 0.25 molal GuHSCN; see Fig. 4). These partition coefficients, listed in Table 1, quantify the accumulation of these solutes in the

vicinity of native BSA. KCl is randomly distributed, $K_{P,3}^{\text{nat}} = 1.00 \pm 0.08$. Urea is the least accumulated ($K_{P,\text{urea}}^{\text{nat}} = 1.10 \pm 0.04$), GuHSCN is the most accumulated ($K_{P,\text{GuHSCN}}^{\text{nat}} = 2.0 \pm 0.2$). The observation that GuHCl ($K_{P,\text{GuHCl}}^{\text{nat}} = 1.30 \pm 0.02$) is less accumulated than GuHSCN indicates that Cl^- is less accumulated than SCN^- . This order of favorable interaction of these solutes with native BSA surface correlates with their effectiveness as protein denaturants.

Partition coefficients of cations and anions in BSA solutions: application to quantitative prediction/analysis of Hofmeister salt effects

Partition coefficients for the interactions of the two guanidinium salts with native BSA surface can be decomposed into contributions from GuH^+ and SCN^- ions, by the procedure of Courtenay et al. (2000a), based on the finding (see Appendix, Fig. A1) that $K_{P,\text{KCl}}^{\text{nat}} = 1.00 \pm 0.08$, and the consequent assignment of K^+ as the reference ion (neither accumulated nor excluded; $K_{P,\text{K}^+}^{\text{nat}} \equiv 1$). Because $2K_{P,\text{KCl}}^{\text{nat}} = K_{P,\text{K}^+}^{\text{nat}} + K_{P,\text{Cl}^-}^{\text{nat}}$ (Courtenay et al. 2000a,b), therefore, the local concentration of Cl^- near native BSA surface is also the same as that in the bulk solution within uncertainty, $K_{P,\text{Cl}^-}^{\text{nat}} = 1.00 \pm 0.08$.

Decomposition of the partition coefficient of GuHCl ($K_{P,\text{GuHCl}}^{\text{nat}} = 1.30 \pm 0.02$) yields $K_{P,\text{GuH}^+}^{\text{nat}} = 1.60 \pm 0.08$, which quantifies the large accumulation of GuH^+ cations (relative to K^+) near native BSA. Then from the calculated $K_{P,\text{GuHSCN}}^{\text{nat}} = 2.0 \pm 0.2$, we obtain $K_{P,\text{SCN}^-}^{\text{nat}} = 2.4 \pm 0.2$. Thiocyanate is, therefore, more strongly accumulated near native BSA surface than is GuH^+ , which in turn is more strongly accumulated than urea ($K_{P,\text{urea}}^{\text{nat}} = 1.10 \pm 0.04$). The net accumulation near native BSA of GuHSCN ($K_{P,\text{GuHSCN}}^{\text{nat}} = 2.0$) is substantially greater than that of GuHCl ($K_{P,\text{GuHCl}}^{\text{nat}} = 1.3$), because SCN^- is strongly accumulated, whereas Cl^- is not.

For the average protein surface exposed on unfolding, Courtenay et al. (2000b) calculated $K_{P,\text{GuHCl}}^{\text{unf}} = 1.16 \pm 0.02$, and from this we estimate $K_{P,\text{GuH}^+}^{\text{unf}} = 1.32 \pm 0.08$, assuming that chloride is randomly distributed around the surface exposed on unfolding ($K_{P,\text{Cl}^-}^{\text{unf}} = 1.0$). We propose that the smaller net accumulation of GuHCl (and by implication, of GuH^+) near the surface exposed on unfolding compared to native BSA surface arises from the very different contributions of charged groups to the composition of these surfaces (see Fig. 1) as discussed below.

From previous VPO studies of stabilizing solute interactions with native BSA, we obtained the partition coefficient K_P^{nat} for potassium glutamate (Courtenay et al. 2000a). From $K_{P,\text{KGlut}}^{\text{nat}} = 0.65 \pm 0.07$ and $K_{P,\text{K}^+}^{\text{nat}} = 1.0$, it follows that $K_{P,\text{Glu}^-}^{\text{nat}} = 0.3 \pm 0.1$, corresponding to strong local exclusion of glutamate from the BSA surface and consistent with the placement of glutamate (Leirimo et al. 1987; Ha et

Fig. 4. Preferential interaction coefficients (Γ_{μ_3}) for native BSA–destabilizing solute interactions as a function of bulk solute molality (m_3^{bulk}) calculated as described by Courtenay et al. (2000a). Panels A–C show all data for urea (at 2.70, 3.16, and 3.61 mM BSA), GuHCl (at 2.90 and 3.16 mM BSA), and GuHSCN (at 2.56, 3.16, and 3.75 mM BSA). Error bars represent the propagated experimental uncertainty. No dependence of Γ_{μ_3} on BSA concentration was observed.

al. 1992) with fluoride and sulfate (von Hippel and Schleich 1969a,b, and references therein; Arakawa and Timasheff 1991) at one end of the Hofmeister series as anions that drive processes in which the amount of protein surface exposed to water is reduced.

Partition coefficients of individual ions obtained from the present analysis are compared in Figure 5, which initiates the quantitative comparison of the extents of accumulation or exclusion of different uncharged and ionic solute species from both folded and unfolded protein surface. From the partition coefficients of individual ions, a quantitative framework for the analysis or prediction of Hofmeister salt effects is emerging.

Quantitative studies have been performed in other laboratories on proteins (Arakawa and Timasheff 1982, 1984a,b) and on model compounds that chemically mimic aspects of protein surface (Robinson and Jencks 1965b; von Hippel et al. 1973) to elucidate the dominant interactions that define the overall energetics of protein stabilization or destabilization by Hofmeister salts (Baldwin 1996). Although none of these studies directly yield local-bulk partition coefficients K_P to compare with our results for SCN^- , both the von Hippel et al. (1973) and Robinson and Jencks (1965b) data allow us to estimate values for K_{P,SCN^-} for amide surface (based on the relative interactions of Cl^- and SCN^- salts) that are at least as large as that measured for BSA. A more quantitative approach to the analysis of solubility data using preferential interaction coefficients is in progress in this laboratory to compare these amide backbone results with our results for native and unfolded protein surface.

Proposed division of solute partition coefficients into contributions from interactions with polar peptide backbone, charged groups, and other polar and nonpolar protein surfaces

Table 1 presents the partition coefficients determined in this work for native BSA interactions with urea, GuHCl , and GuHSCN and compares the urea and GuHCl $K_{P,3}^{\text{nat}}$ with partition coefficients $K_{P,3}^{\text{unf}}$ determined previously for interactions of these solutes with the surfaces exposed on unfolding globular proteins and α -helical peptides (Courtenay et al. 2000b). $K_{P,3}$ for interactions of GuHCl and urea with the different kinds of folded and unfolded protein and peptide surface can be decomposed into contributions from interactions of these solutes with polar peptide backbone, charged surface, and other types of protein surface. From this decomposition, a quantitative framework for the structural prediction of effects of these solutes on protein processes, as well as the molecular interpretation of experimentally determined effects of these solutes, is emerging.

GuHCl exhibits significantly different partition coefficients for interactions with different types of protein and peptide surface. Table 1 shows that $K_{P,\text{GuHCl}}$ is largest for the surface exposed on unfolding alanine-based α -helical peptides, next largest for native BSA surface and smallest for the surface exposed on unfolding the average globular protein. The compositions of these three surfaces differ most strongly in the contributions of polar peptide backbone and of charged groups (see Fig. 1). Polar peptide backbone represents 48% of the surface exposed on unfolding the α -helical peptides (Courtenay et al. 2000b), but is only 13% of

Fig. 5. Quantitative solute series tabulating local-bulk partition coefficients K_P for the interactions of cations, anions, and uncharged or zwitterionic solutes with native protein surface and with the surface exposed on unfolding proteins or alanine-based peptides. Ion partition coefficients are calculated based on the assignment $K_{P,K^+} \equiv 1$ and the assumption that $K_{P,\text{Cl}^-} = 1$ has the same value for the surface exposed on unfolding as for native BSA surface ($K_{P,\text{KCl}}^{\text{nat}} = 1.00 \pm 0.08$).

Table 1. Partition coefficients for urea and guanidinium salts

Protein surface	$K_{P,urea}$	$K_{P,GuHCl}$	$K_{P,GuHSCN}$
Native BSA	1.10 ± 0.04	1.30 ± 0.02 $K_{P,-} = 1.00 \pm 0.08$ $K_{P,+} = 1.60 \pm 0.08$	2.0 ± 0.2 $K_{P,-} = 2.4 \pm 0.2$ $K_{P,+} = 1.60 \pm 0.08$
Exposed on unfolding globular proteins ^a	1.12 ± 0.01	1.16 ± 0.02 $K_{P,+} = 1.32 \pm 0.08^b$	—
Exposed on unfolding alanine-based α -helices ^a	1.48 ± 0.05	1.61 ± 0.07 $K_{P,+} = 2.20 \pm 0.1^b$	—

^a From Courtenay et al. (2000b).^b Assuming $K_{P,Cl^-} = 1.00 \pm 0.08$, as for native BSA surface.

the surface of native BSA and of the surface exposed on unfolding typical globular proteins. Charged groups represent 29% of the surface of native BSA, but less than 4% of the surface exposed on unfolding proteins or α -helical peptides. Courtenay et al. (2000b) proposed that the four-fold difference in the contribution of polar peptide backbone to the composition of the surface exposed in unfolding peptides versus proteins was the primary origin of the differences in K_P^{unf} values for GuHCl and for urea observed for these two types of surface. Here we quantify this effect and estimate the contributions of interactions with charged surface and other nonpolar/polar surfaces.

We assume that the observed $K_{P,3}$ of a solute component is a sum of contributions from different types of biopolymer surface. With only three different surface compositions to analyze, we assume for the ionic solute GuHCl that

$$K_{P,GuHCl} = f_{ppb} K_{P,GuHCl}^{ppb} + f_{ch} K_{P,GuHCl}^{ch} + (1 - f_{ppb} - f_{ch}) K_{P,GuHCl}^{other}$$

in which f_{ppb} and f_{ch} are fractions of polar peptide backbone and of charged surface, K_P^{ppb} and K_P^{ch} are the corresponding solute partition coefficients, and K_P^{other} is the average partition coefficient for all other types of protein surface. From the data of Table 1 and Figure 1, we solve three equations in three unknowns to obtain $K_{P,GuHCl}^{ppb} = 2.3 \pm 0.2$, $K_{P,GuHCl}^{ch} = 1.5 \pm 0.4$, and $K_{P,GuHCl}^{other} = 1.0 \pm 0.1$ (Table 2). GuHCl (and by inference, GuH^+) is very strongly accumulated near polar peptide backbone surface, moderately ac-

cumulated near charged surface (at least in the absence of added salt), and randomly distributed, on average, near other polar and nonpolar surface.

For urea, the similarity in K_P values for native BSA surface and the surface exposed on unfolding a globular protein indicates that, for this uncharged solute, the effects of differences in f_{ch} are small. Because the contribution of polar peptide backbone surface is the same for these two classes of surface, the urea data yield only two independent equations, and we therefore analyze these data as

$$K_{P,urea} = f_{ppb} K_{P,urea}^{ppb} + (1 - f_{ppb}) K_{P,urea}^{other}$$

which yields $K_{P,urea}^{ppb} = 2.0 \pm 0.1$ and $K_{P,urea}^{other} = 1.0 \pm 0.1$. Therefore, urea (like GuH^+) is strongly accumulated near polar peptide backbone but randomly distributed, on average, in the vicinity of all other protein surface.

To extend this analysis, it is necessary to investigate interactions of a wider range of accumulated and excluded solutes and Hofmeister salts with these three types of protein surfaces or suitably designed model systems. Interactions of excluded solutes with unfolded surface may prove to be more difficult to quantify, because at least some of these solutes are expected to reduce the ΔASA of unfolding in a concentration-dependent manner (Qu et al. 1998).

Conclusions and Summary

The present study, combined with our previous work describing BSA-stabilizing solute interactions, shows the usefulness of VPO as a general experimental technique for obtaining preferential interaction coefficients and from them, local-bulk partition coefficients characterizing the entire spectrum of weak interactions between small solutes and protein surface in aqueous solution. The sign and magnitude of the differences in preferential interaction coefficients between products and reactants determine the direction and size of the effect of adding a solute on a biopolymer process. Knowledge of local-bulk partition coefficients for different solutes and different types of protein surface will allow solute effects on biopolymer processes to be interpreted and eventually predicted from structural information.

Table 2. Calculated partition coefficients quantifying the extent of accumulation of urea and GuHCl near different types of protein surface

Type	$K_{P,urea}$	$K_{P,GuHCl}$
Polar peptide backbone	2.0 ± 0.1	2.3 ± 0.2
Charged groups	$1.0^a \pm 0.1$	1.5 ± 0.4
Other polar, nonpolar	$1.0^a \pm 0.1$	1.0 ± 0.1

^a Fit together.

We propose that the Hofmeister rankings of cations and anions, as well as the observed effects of neutral solutes on protein processes, have a common origin in the extent of accumulation or exclusion of the solute from the protein surface as quantified by its local bulk partition coefficient K_p . Our previously published results for protein stabilizing solutes (Courtenay et al. 2000a) are presented in Figure 5 for comparison with the effects of individual ions (discussed above) and with the uncharged destabilizing solute, urea. We have shown here that the overall partitioning of urea near protein surface can be described by very significant accumulation near backbone amide groups and an average random distribution near other types of protein ASA.

Until now, the only thermodynamic quantity used to assess whether a protein–protein or protein–ligand interaction is rigid body or involves coupled conformational changes in ASA (e.g., folding) has been the heat capacity change (ΔC_p°), determined from the temperature dependence of $\Delta H_{\text{obs}}^\circ$ (first derivative) or of $\Delta G_{\text{obs}}^\circ$ or $\ln K_{\text{obs}}^\circ$ (second derivative) for the process of interest (cf. Spolar and Record 1994, and references therein). Large negative temperature- and salt concentration-independent values of ΔC_p° for processes are a thermodynamic signature of burial of nonpolar surface area in the interface and in coupled conformational changes; quantitative interpretation of ΔC_p° can be used to distinguish rigid body processes from those involving coupled folding or other large-scale coupled conformational changes. Complementary information on changes in ASA can be obtained using equation 5 from the derivative of $\ln K_{\text{obs}}^\circ$ with respect to urea concentration. Protein folding studies have been used to calibrate ΔC_p° – ΔASA (Livingstone et al. 1991) and urea m -value– ΔASA relationships (Myers et al. 1995). These two techniques may be of great use in combination because their effects are dominated by changes in different types of surface area: ΔC_p° is most sensitive to changes in nonpolar ASA, whereas the urea effect on a process is most sensitive to changes in polar peptide backbone ASA.

In our opinion, urea is the best choice of solute to probe large-scale changes in ASA in protein processes for the following reasons: (1) It has the same partition coefficient for both the average native protein surface and the average surface exposed on unfolding a protein ($K_{p,\text{urea}} = 1.11 \pm 0.04$). (The 4% uncertainty in $K_{p,\text{urea}}$ results in a ~35% uncertainty in ΔASA calculated using equation 5 from the effect of urea on a protein process. This is comparable to the typical uncertainty in ΔASA estimated from ΔC_p° ; both methods are useful primarily at a semiquantitative level to determine whether a process is rigid body or involves large-scale conformational changes.) (2) Low concentrations of urea (< 2 molal) are not expected to perturb the structure of either the initial or final states of protein processes as much as a highly excluded solute. (Excluded solutes have been observed to compact nonnative (unfolded) protein structures; Qu et al. 1998). (3) As a nonelectrolyte, urea does not

show any significant net interaction with charged surfaces (see above). In our opinion, GuHCl is less suitable than urea because GuH^+ is accumulated near (negatively) charged protein surface, and because changes in GuHCl concentration change the salt concentration and, therefore, may exert a variety of electrolyte effects on processes involving changes in net charge or charge density.

Materials and methods

Urea (MW 60.06 g/mol) and guanidinium chloride (GuHCl; MW 95.13 g/mol) were obtained from Gibco BRL (Life Technologies, UltraPure > 99%). Guanidinium thiocyanate (GuHSCN; MW 118.2 g/mol) was obtained from Sigma (Mol Bio grade > 99%). These compounds were used without further purification. Bovine serum albumin (BSA; MW 66,411) was obtained from Sigma and purified as described previously (Courtenay et al. 2000a). The partial molar volume ($\bar{V}_3$) of GuHSCN was obtained by densimetry. Partial molar volumes of urea and GuHCl were determined previously by the same method (Courtenay et al. 2000b). These partial molar volumes are given in Table A1 of the Appendix. Determination of solution osmolality as a function of accumulated solute molality was performed as described in Courtenay et al. (2000a) using a Wescor VAPRO 5520 vapor pressure osmometer. At least 24 solutions (each measured in triplicate) were used to determine the dependence of the two-component (small solute–water) solution osmolality on solute concentration. The dependence of three-component solution osmolality on accumulated solute concentration was determined using at least 12 solutions for a particular BSA concentration. At least two BSA concentrations were investigated for each solute. All solutions were prepared using the gravimetric technique described by Courtenay et al. (2000a).

The dependence of solution osmolality on solute molality for two- and three-component solutions were analyzed to determine Γ_{μ_1} , Γ_{μ_3} , and $\Gamma_{\mu_1+\mu_3}$ as described by Courtenay et al. (2000a). Uncertainties in Γ_{μ_3} and the calculated partition coefficients for native BSA–destabilizing solute interactions were propagated from the standard deviation of the individual triplicate osmometry readings for each three-component solution and the fitting uncertainty given from weighted nonlinear fitting (using SPSS SigmaPlot) of the two-component osmolality versus solute molality data. Γ_{μ_3} was calculated using either of the two independent approximations described previously (Courtenay et al. 2000a). When Γ_{μ_1} was used (in combination with BSA-only and three-component VPO data) to determine Γ_{μ_3} (Approximation I), individual point determinations of Γ_{μ_1} with their corresponding propagated uncertainties were used instead of a weighted fitting of Γ_{μ_1} versus small solute molality.

The excess change in solution osmolality on addition of BSA to a small solute solution presented in Figure 3 is calculated from $\Delta \text{Osm}_{\text{ex}} = \Delta \text{Osm}_{\text{obs}} - \Delta \text{Osm}_{\text{ref}}$ in which $\Delta \text{Osm}_{\text{ref}}$ is determined from the osmolality of a 3×10^{-3} molal BSA solution in the absence of small solute (see Courtenay et al. 2000a). $\Delta \text{Osm}_{\text{obs}}$ is the observed change in osmolality on the addition of 3×10^{-3} molal BSA, calculated as described previously (Courtenay et al. 2000a) from the dependence of osmolality on small solute molality in the absence of BSA (see Appendix, Table A1) and the dependence in the presence of 3×10^{-3} molal BSA calculated from Γ_{μ_3} (see Fig. 4). The dependence of Γ_{μ_3} on destabilizing solute concentration is determined by the two-domain model (see equation 4).

Protein and α -helical water-accessible surface area calculations were calculated as described previously (Livingstone et al. 1991;

Fig. A1. KCl-BSA interactions. Panel A contains representative VPO data showing the dependence of solution osmolality on KCl molality in the presence (filled circles) and absence (open circles) of 3.88 mM BSA. Panel B plots Γ_{μ_3} as a function of bulk KCl molality (at 3.07, 3.51, and 3.88 mM BSA), calculated as described previously (Courtenay et al. 2000a). For this system, the two approximations (see Courtenay et al. 2000a) give equivalent determinations of Γ_{μ_3} for the entire concentration range presented. No BSA concentration dependence of Γ_{μ_3} as a function of bulk KCl concentration is observed.

Courtenay et al. 2000a,b) or by using another program developed in this lab that gives equivalent results (Tsodikov et al., in prep.). PDB file IBO0 of human serum albumin (Sugio et al. 1999) was used to estimate the ASA of BSA as described previously (Courtenay et al. 2000a). α -helical and extended chain conformations of the sequence AKAAEAAKAAEA in the context of the peptide Ac-YAEAAKAAEAAKAAEAAKAF-NH₂ (Scholtz et al. 1995; Smith and Scholtz 1996) were constructed using Insight II (Biosym/MSI). Charged surfaces for all calculations presented in Figure 1 were calculated by summing the ASA of nitrogen and oxygen atoms in the following residues: aspartate and glutamate carboxylate groups, arginine guanidine groups, lysine amino groups, all histidine imidazole ring nitrogens, and the N- and C-termini. Polar peptide backbone ASA was calculated by summing the exposure of amide nitrogen and oxygen atoms.

Acknowledgments

We thank Drs. Robert Baldwin, Ruth Saecker, and Serge Timasheff for their comments; Dr. Oleg Tsodikov for assistance with calculations of surface areas and water-solute exchange coeffi-

cients; Dr. Charles Anderson, Dan Felitsky, and Jiang Hong for helpful discussions; Sheila Aiello for assistance in preparation of the manuscript. We acknowledge support from National Institutes of Health grant GM47022 and partial support of E.S. Courtenay from Biotechnology Training Grant 5 T32 GMO8349.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

Appendix

KCl-BSA interactions

Panel A of Figure A1 presents representative VPO data for solution osmolality as a function of KCl molality with and without 3.88 mM BSA. The constant difference in osmolality between these two series (+BSA and -BSA) qualitatively shows the absence of preferential interactions in this system. Interpreted as in Figure 3, the effect of the addition of BSA on solution osmolality is the same at any KCl concentration as it is in the absence of KCl.

Panel B of Figure A1 plots the preferential interaction coefficient Γ_{μ_3} calculated as described previously from the osmometric data (Courtenay et al. 2000a). We find that $\Gamma_{\mu_3} = 0$ within ex-

Fig. A2. Plots of Γ_{μ_3} for interactions of BSA with protein-destabilizing GuH⁺ salts as a function of bulk solute molality (m_3^{bulk}). Panels A and B show data for GuHCl and GuHSCN in which Γ_{μ_3} has been calculated using either Approximation I, $\mu_{22} = \mu_{22}^0$ (circles), or Approximation II, $\mu_{33} = \mu_{33}^0$ (squares) (Courtenay et al. 2000a). The triangle represents the preferential interaction coefficient for GuHCl-BSA interactions determined by equilibrium dialysis at $m_3^{\text{bulk}} = 1$ molal (Arakawa and Timasheff 1984a).

Table A1.

Solute	2-component solutions			3-component solutions	
	$(\partial \text{Osm}/\partial m_3)_{T,P}$	$\bar{V}_3$ (L/mol) ^a	$1 + \epsilon_3^c$ ^b	$S_{1,3}$ ^c	$\Gamma_{\mu_1} = \left(\frac{\partial m_3}{\partial m_2} \right)_{T,P,\mu_1}$
Urea	0.981 (±0.007) −0.07 (±0.01) m_3 $m_3 \leq 2.5$ molal	$(4.423 \pm 0.004) \times 10^{-2}$ $+ (1.3 \pm 0.2) \times 10^{-4} m_3$ $− (4 \pm 1) \times 10^{-6} m_3^2$ $m_3 \leq 12$ molal	(0.9176 ± 0.0008) $+ (7 \pm 1) \times 10^{-3} C_3$ $+ (5.5 \pm 0.5) \times 10^{-3} C_3^2$ $− (9.5 \pm 0.7) \times 10^{-4} C_3^3$ $+ (8.2 \pm 0.3) \times 10^{-5} C_3^4$ $C_3 \leq 10$ molar	2.7	$−13.7 \pm 0.3$ $+ (7 \pm 2) m_3^\Delta$ $m_3^\Delta \leq 1$ molal
GuHCl	1.731 (±0.007) −0.33 (±0.01) m_3 $m_3 \leq 1.5$ molal	$(6.83 \pm 0.02) \times 10^{-2}$ $+ (1.0 \pm 0.2) \times 10^{-3} m_3$ $− (9 \pm 2) \times 10^{-5} m_3^2$ $m_3 \leq 4.5$ molal	(0.7990 ± 0.0002) $− (1.40 \pm 0.04) \times 10^{-2} C_3$ $+ (8.4 \pm 0.3) \times 10^{-3} C_3^2$ $− (2.79 \pm 0.05) \times 10^{-3} C_3^3$ $+ (5.88 \pm 0.05) \times 10^{-4} C_3^4$ $C_3 \leq 6$ molar	GuH ⁺ = 2.7 Cl [−] = 1.7	$−6.4 \pm 0.2$ $+ (12 \pm 2) m_3^\Delta$ $m_3^\Delta \leq 1$ molal
GuHSCN	1.783 (±0.008) −0.52 (±0.02) m_3 $m_3 \leq 1.6$ molal	$(9.098 \pm 0.006) \times 10^{-2}$ $+ (5.7 \pm 0.5) \times 10^{-4} m_3$ $− (5.2 \pm 0.8) \times 10^{-5} m_3^2$ $m_3 \leq 6.5$ molal	(0.9210 ± 0.0001) $− (0.402 \pm 0.001) C_3$ $+ (0.122 \pm 0.004) C_3^2$ $+ (3.15 \pm 0.04) \times 10^{-2} C_3^3$ $+ (1.59 \pm 0.02) \times 10^{-2} C_3^4$ $C_3 \leq 1.5$ molar	GuH ⁺ = 2.7 SCN [−] = 2.3	$−6.6 \pm 0.3$ $+ (55 \pm 4) m_3^\Delta$ $m_3^\Delta \leq 1$ molal

^a Partial molar volume data for urea and GuHCl were reported by Courtenay et al. (2000b).

^b $1 + \epsilon_3^c$ dependences on solute concentration were determined as previously described (Courtenay et al. 2000b) using VPO data from this laboratory in combination with isopiestic distillation data from Scatchard et al. (1938) for urea and from Schrier and Schrier (1977) for GuHCl.

^c Solute-water exchange coefficients were estimated as previously described (Courtenay et al. 2000b) using van der Waals radii determined as described by Tsodikov (1994).

perimental uncertainty for all concentrations of BSA and KCl examined. Interpreted using the local-bulk domain model, $K_{P,KCl} = 1.00 \pm 0.08$; local and bulk concentrations of KCl in a solution of native BSA are equal within experimental uncertainty.

Comparison of independent methods of calculating Γ_{μ_3} at high concentrations of guanidinium salts

To calculate Γ_{μ_3} from VPO data, two independent approximations are used to estimate the derivatives of chemical potentials of the small and large solutes with respect to their own concentration in the three-component solution (Courtenay et al. 2000a; Anderson et al., in prep.). In Approximation I, the BSA chemical potential (μ_2) dependence on BSA molality (μ_{22}) is assumed to be the same with or without another solute present. In Approximation II, the small solute chemical potential (μ_3) dependence on small solute molality (μ_{33}) is assumed to be the same with or without BSA present. For the six protein-stabilizing solutes (osmolytes) examined previously, both approximations yielded the same result for Γ_{μ_3} as a function of small solute concentration (Courtenay et al. 2000a). At solute concentrations > 0.5 molal, the two approximations do not give equivalent determinations of Γ_{μ_3} for the GuH⁺ salts investigated. Figure A2 shows values of Γ_{μ_3} calculated using either Approximation I ($\mu_{22} \equiv \mu_{22}^{(3)}$) (circles) or Approximation II ($\mu_{33} \equiv \mu_{33}^{(2)}$) (squares) for GuHCl (panel A) and GuHSCN (panel B). This behavior indicates that for these ionic solutes with one or two strongly accumulated ions, either μ_{22} or μ_{33} (or both) is not adequately approximated by its value in the corresponding two-component solution (i.e., $\mu_{22}^{(3)}$ or $\mu_{33}^{(2)}$).

Two lines of evidence indicate that Approximation I is more accurate for the GuH⁺ salts. First, Approximation I predicts a dependence of Γ_{μ_3} on m_3^{bulk} , which agrees with the near-linear functional form of the local-bulk domain model (plotted in Fig. A2). Second, it also predicts a value of Γ_{μ_3} at 1 molal GuHCl, which agrees very well with an independent determination of $\Gamma_{\mu_1\mu_3}$ for GuHCl-BSA interactions obtained by Arakawa and Timasheff (1984b) using equilibrium dialysis and densimetry. The failure of Approximation II for highly accumulated solutes like GuHSCN in part may result from the difference between the bulk and total concentration of the solute. To test this, we performed an iterative calculation using bulk small solute concentrations (m_3^{bulk}) estimated from values of Γ_{μ_3} instead of using the total concentration of the small solute to calculate $(\partial \text{Osm}/\partial m_3)_{T,P}$ from the two-component (water-small solute) solution data. Use of m_3^{bulk} to calculate μ_{33} leads to a better agreement between the two approximations but does not account for the majority of the effect or for the local maximum predicted by Approximation II for GuHSCN in Figure A2. Use of a 20% larger exchange coefficient $S_{1,3}$ (cf. equation 5) is also not sufficient to account for this curvature.

References

- Arakawa, T. and Timasheff, S.N. 1982. Preferential interactions of proteins with salts in concentrated solutions. *Biochemistry* **21**: 6545–6552.
- . 1983. Preferential interactions of proteins with solvent components in aqueous amino acid solutions. *Arch. Biochem. Biophys.* **224**: 169–177.
- . 1984a. Protein stabilization and destabilization by guanidinium salts. *Biochemistry* **23**: 5924–5929.
- . 1984b. The mechanism of action of Na glutamate, lysine HCl, and

- piperazine-N, N'-bis (2-ethanesulfonic acid) in the stabilization of tubulin and microtubule formation. *J. Biol. Chem.* **259**: 4979–4986.
- . 1985. The stabilization of proteins by osmolytes. *Biophys. J.* **47**: 411–414.
- . 1991. The interactions of proteins with salts, amino acids, and sugars at high concentration. In *Advances in Comparative and Environmental Physiology*, 9 (ed. R. Gilles), pp. 226–244. Springer-Verlag, Berlin.
- Baldwin, R.L. 1996. How Hofmeister ion interactions affect protein stability. *Biophys. J.* **71**: 2056–2063.
- Castellino, F.J. and Barker, R. 1969. The effect of guanidinium, carbamoylguanidinium, and guanylguanidinium salts on the solubility of benzoyl-L-tyrosine ethyl ester and acetyltetraglycine ethyl ester in water. *Biochemistry* **8**: 3439–3442.
- Courtenay, E.S., Capp, M.W., Anderson, C.F., and Record, M.T., Jr. 2000a. Vapor pressure osmometry studies of osmolyte-protein interactions: Implications for the action of osmoprotectants in vivo and for the interpretation of “osmotic stress” experiments in vitro. *Biochemistry* **39**: 4455–4471.
- Courtenay, E.S., Capp, M.W., Saecker, R.M., and Record, M.T., Jr. 2000b. Thermodynamic analysis of interactions between denaturants and protein surface exposed on unfolding: interpretation of urea and guanidinium chloride *m*-values and their correlation with changes in accessible surface area (ASA) using preferential interaction coefficients and the local-bulk domain model. *Proteins* **41**: 72–85.
- Gekko, K. and Morikawa, T. 1981. Preferential hydration of bovine serum albumin in polyhydric alcohol-water mixtures. *J. Biochem.* **90**: 39–50.
- Gekko, K. and Timasheff, S.N. 1981. Mechanism of protein stabilization by glycerol: Preferential hydration in glycerol-water mixtures. *Biochemistry* **20**: 4667–4676.
- Gill, S. J., Hutson, J., Clopton, J.R., and Downing, M. 1961. Solubility of diketopiperazine in aqueous solutions of urea. *J. Phys. Chem.* **65**: 1432–1435.
- Ha, J.-H., Capp, M.W., Hohenwarter, M.D., Baskerville, M., and Record, M.T., Jr. 1992. Thermodynamic stoichiometries of participation of water, cations and anions in specific and nonspecific binding of *lac* repressor to DNA: Possible thermodynamic origins of the “glutamate effect” on protein-DNA interactions. *J. Mol. Biol.* **228**: 252–264.
- Leirmo, S., Harrison, C., Cayley, D.S., Burgess, R.R., and Record, M.T., Jr. 1987. Replacement of potassium chloride by potassium glutamate dramatically enhances protein-DNA interactions in vitro. *Biochemistry* **26**: 2095–2101.
- Lin, T. and Timasheff, S.N. 1994. Why do some organisms use a urea-methylamine mixture as osmolyte? Thermodynamic compensation of urea and trimethylamine *N*-oxide interactions with protein. *Biochemistry* **33**: 12695–12701.
- . 1996. On the role of surface tension in the stabilization of globular proteins. *Protein Sci.* **5**: 372–381.
- Livingstone, J.R., Spolar, R.S., and Record, M.T., Jr. 1991. Contribution to the thermodynamics of protein folding from the reduction in water-accessible nonpolar surface area. *Biochemistry* **30**: 4237–4244.
- Makhadatzke, G. and Privalov, P. 1990. Heat capacity of proteins II. Partial molar heat capacity of the unfolded polypeptide chain of proteins: Protein unfolding effect. *J. Mol. Biol.* **213**: 375–384.
- Miller, S., Janin, J., Lesk, A.M., and Chothia, C. 1987. Interior and surface of monomeric proteins. *J. Mol. Biol.* **196**: 641–656.
- Murphy, K.P., Bakhuni, V., Xie, D., and Freire, E. 1992. Molecular basis of cooperativity in protein folding III. Structural identification of cooperative folding units and folding intermediates. *J. Mol. Biol.* **227**: 293–306.
- Murphy, K.P., Xie, D., Garcia, K.C., Amzel, L.M., and Freire, E. 1993. Structural energetics of peptide recognition: Angiotensin II-antibody binding. *Proteins* **15**: 113–120.
- Myers, J.K., Pace, C.N., and Scholtz, J.M. 1995. Denaturant *m*-values and heat capacity changes: Relation to changes in accessible surface areas of protein unfolding. *Protein Sci.* **4**: 2138–2148.
- Nandi, P.K. and Robinson, D.R. 1984. Effects of urea and guanidine hydrochloride on peptide and nonpolar groups. *Biochemistry* **23**: 6661–6668.
- Nozaki, Y. and Tanford, C. 1963. The solubility of amino acids and related compounds in aqueous urea solutions. *J. Biol. Chem.* **12**: 4074–4081.
- . 1970. The solubility of amino acids, diglycine, and triglycine in aqueous guanidine hydrochloride solutions. *J. Biol. Chem.* **245**: 1648–1652.
- Qu, Y., Bolen, C.L., and Bolen, D.W. 1998. Osmolyte-driven contraction of a random coil protein. *Proc. Natl. Acad. Sci.* **95**: 9268–9273.
- Record, M.T., Jr. and Anderson, C.F. 1995. Interpretation of preferential interaction coefficients of nonelectrolytes and of electrolyte ions in terms of a two-domain model. *Biophys. J.* **68**: 786–794.
- Record, M.T., Jr., Zhang, W., and Anderson, C.F. 1998. Analysis of effects of salts and uncharged solutes on protein and nucleic acid equilibria and processes: A practical guide to recognizing and interpreting polyelectrolyte effects, Hofmeister effects, and osmotic effects of salts. *Adv. Protein Chem.* **51**: 281–353.
- Robinson, D.W. and Jencks, W.P. 1965a. The effect of compounds of the urea-guanidinium class on the activity coefficient of acetyltetraglycine ethyl ester and related compounds. *J. Am. Chem. Soc.* **87**: 2462–2470.
- . 1965b. The effect of concentrated salt solutions on the activity coefficient of acetyltetraglycine ethyl ester. *J. Am. Chem. Soc.* **87**: 2470–2479.
- Scatchard, G., Hamer, W.J., and Wood, S.E. 1938. Isotonic solutions. I. The chemical potential of water in aqueous solutions of sodium chloride, potassium chloride, sulfuric acid, sucrose, urea, and glycerol at 25°C. *J. Am. Chem. Soc.* **60**: 3061–3070.
- Scholtz, J.M., Barrick, D., York, E.J., Stewart, J.M., and Baldwin, R.L. 1995. Urea unfolding of peptide helices as a model for interpreting protein unfolding. *Proc. Natl. Acad. Sci.* **92**: 185–189.
- Schrier, M.Y. and Schrier, E.E. 1977. Osmotic and activity coefficients of aqueous guanidine hydrochloride solutions at 25°C. *J. Chem. Eng. Data* **22**: 73–74.
- Sijpkens, A.H., van de Kleut, G.J., and Gill, S.C. 1993. Urea-diketopiperazine interactions: A model for urea induced denaturation of proteins. *Biophys. Chem.* **46**: 171–177.
- Smith, J.S. and Scholtz, J.M. 1996. Guanidine hydrochloride unfolding of peptide helices: Separation of denaturant and salt effects. *Biochemistry* **35**: 7292–7297.
- Spolar, R.S., Ha, J.-H., and Record, M.T., Jr. 1989. Hydrophobic effect in protein folding and other noncovalent processes involving proteins. *Proc. Natl. Acad. Sci.* **89**: 8382–8385.
- Spolar, R.S. and Record, M.T., Jr. 1994. Coupling of local folding to site-specific binding of proteins to DNA. *Science* **263**: 777–784.
- Spolar, R.S., Livingstone, J.R., and Record, M.T., Jr. 1992. Use of liquid-hydrocarbon and amide transfer data to estimate contributions to thermodynamic functions of protein folding from the removal of nonpolar and polar surface from water. *Biochemistry* **31**: 3947–3955.
- Sugio, S., Kashima, A., Mochizuki, S., Noda, M., Kobayashi, K. 1999. Crystal structure of human serum albumin at 2.5 Å resolution. *Protein Eng.* **12**: 439–446.
- Timasheff, S.N. 1998. Control of protein stability and reactions by weakly interacting cosolvents: The simplicity of the complicated. *Adv. Prot. Chem.* **51**: 355–433.
- Tsodikov, O.V. 1994. M.S. Thesis. Geometrical properties of protein atomic surface. Moscow Institute of Physics and Technology, Moscow.
- von Hippel, P.H. and Schleich, T. 1969a. Ion effects on the solution structure of biological macromolecules. *Accounts Chem. Research* **2**: 257–265.
- . 1969b. The effects of neutral salts on the structure and conformational stability of macromolecules in solution. In *Biological Macromolecules, 2 Structure and Stability of Biological Macromolecules* (eds. S.N. Timasheff and G. Fasman), pp. 417–573. Marcel Dekker, New York.
- von Hippel, P.H., Peticolas, V., Schack, L., and Karlson, L. 1973. Model studies on the effects of neutral salts on the conformational stability of biological macromolecules. I. Ion binding to polyacrylamide and polystyrene columns. *Biochemistry* **12**: 1256–1263.
- Wang, A. and Bolen, D.W. 1997. A naturally occurring protective system in urea-rich cells: Mechanism of osmolyte protection of proteins against urea denaturation. *Biochemistry* **36**: 9101–9108.
- Wyman, J. 1964. Linked functions and reciprocal effects in hemoglobin: A second look. *Adv. Protein Chem.* **19**: 223–286.
- Xie, G. and Timasheff, S.N. 1997. The thermodynamic mechanism of protein stabilization by trehalose. *Biophys. Chem.* **64**: 25–43.