Thermodynamic Characterization of Interactions of Native Bovine Serum Albumin with Highly Excluded (Glycine Betaine) and Moderately Accumulated (Urea) Solutes by a Novel Application of Vapor Pressure Osmometry†

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ABSTRACT: The thermodynamic consequences of interactions of native bovine serum albumin (BSA) with two smaller solutes (glycine betaine or urea) in aqueous solution are characterized by a novel application of vapor pressure osmometry (VPO), which demonstrates the utility of this method of investigating preferential interactions involving solutes that are either accumulated or excluded near the surface of a protein. From VPO measurements of osmolality (water activity) as a function of the solute concentration in the presence and absence of BSA, we determine the dependence of the solute molarity (C_3) on that of BSA (C_2) at fixed temperature (37 °C), pressure (∼1 atm), and osmolality (over the range 0−1.6 molal). After some thermodynamic transformations, these results yield values of ${}^{m}\Gamma^{o}_{\mu_3} \equiv \lim_{m_2\to 0}(\partial m_3/\partial m_2)_{T,P,\mu_3}$, which characterizes the interdependence of solute molalities when temperature, pressure, and the chemical potential of solute 3 are fixed. This form of the preferential interaction coefficient can be interpreted directly in terms of the molecular exclusion or accumulation of the solute (relative to water) near the protein surface. Within experimental uncertainty, ${}^{m}\Gamma^{o}_{\mu_3}$ is proportional to m_3 both for glycine betaine (0−0.9 m) and for urea (0−1.6 m). For glycine betaine $\partial {}^{m}\Gamma^{o}_{\mu_3}/\partial m_3 = -49 \pm 4$, a value consistent with the interpretation that this solute is completely excluded from the hydrated surface of BSA, whereas for urea $\partial {}^{m}\Gamma^{o}_{\mu_3}/\partial m_3 = 6 \pm 1$, which indicates a moderate extent of accumulation at the surface of native BSA. The preferential accumulation of solutes (e.g., urea) that have some binding affinity for a protein can be quantified and interpreted using the two-domain model if the extent of hydration of the protein has been determined using a completely excluded solute (e.g., glycine betaine). Complete exclusion from the local hydration domain surrounding proteins, if general, justifies the use of glycine betaine as a thermodynamic probe of the changes in hydration that accompany protein folding, protein association, and protein−ligand binding interactions.

Cells contain substantial concentrations of both polymeric solutes (proteins, nucleic acids) and low molecular weight solutes. In this environment various kinds of interactions (due, for example, to electrostatic forces, macromolecular crowding, and solvation) cause the chemical potentials of all of the intracellular components, including solvent water, to exhibit large deviations from thermodynamic ideality that depend strongly on solute concentrations. The interactions of a relatively low molecular weight solute with a protein (or other biopolymer) in general have thermodynamic effects that are either more or less favorable than water−protein interactions. As a molecular consequence of these preferential interactions, the ratio of solute to water molecules is either higher (accumulation) or lower (exclusion) in the vicinity of the protein surface, as compared with the solute/water mole ratio characteristic of the remainder of the solution. Because preferential interactions have a profound effect on the concentration-dependent nonideality of a biopolymer, they are of fundamental importance as determinants of the direction and driving force of all cellular processes that entail changes in the amount and/or molecular nature of the biopolymer surface in contact with the solution. Such processes therefore may be regulated as effectively by solute accumulation or exclusion as by the site-binding of ligands.

Timasheff and co-workers (1992, 1993 and references therein), Eisenberg and co-workers (1976, 1994 and references therein), and Schellman (1990 and references therein) have developed a variety of powerful techniques for the experimental investigation and/or theoretical interpretation of preferential interactions. These methods have been applied extensively *in vitro* to aqueous solutions containing two types of biological solutes (or molecules intended to model them). The principal objective of such studies has been the evaluation of preferential interaction coefficients pertaining to the limit of infinite dilution of the larger solute. The sign, magnitude, and concentration dependence of a preferential interaction coefficient can provide essential information about the interactions responsible for biopolymer nonideality (Schellman, 1990; Timasheff, 1992; Record & Anderson, 1995 and references therein). These coefficients also constitute the thermodynamic basis for analyzing and interpreting the effects of varying solution composition on

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the equilibrium distribution of reactants and products in processes involving biopolymers (Wyman, 1964; Record et al., 1978; Anderson & Record, 1993, 1995).

The thermodynamic role of "water as ligand" in various types of biological processes has been widely recognized (e.g., Tanford, 1969; Record et al., 1978; Colombo et al., 1992; Timasheff, 1992; Leikin et al., 1993). "Release" of water of hydrophobic hydration upon burial (removal from contact with solvent) of nonpolar surface provides a large thermodynamic driving force for various processes involving noncovalent interactions of proteins. This "hydrophobic effect" gives rise to the predominantly entropic character of these processes at low temperatures (Spolar & Record, 1994). Changes in solute concentration (typically in the molar range) have often been used to produce changes in the activity of solvent water (osmolality) that have significant effects on equilibria involving biopolymers. "Osmotic stress" studies of this kind (e.g., Sidorova & Rau, 1995; Garner & Rau, 1995), which have been reviewed recently (Parsegian et al., 1995), can be interpreted in terms of the difference in the extent of hydration of reactants and products only if the solute is completely excluded from their interacting surfaces. In general, fulfillment of this requirement has not been demonstrated. Measurements of "osmotic stress" on equilibria do not provide direct information about the preferential interactions of solutes or water with individual reactants or products.

In the present study we use vapor pressure osmometry (VPO) to measure osmolality as a function of solution composition for the specific purpose of evaluating the coefficient that characterizes preferential interactions of a protein with a solute relative to protein−water interactions. We share the view (Timasheff, 1992) that such information is needed to arrive at definitive interpretations of how linked changes in the activity of water and of various kinds of solutes affect the equilibrium extents of processes involving biopolymers. Specifically, we report preferential interaction coefficients as functions of solute molality for aqueous solutions containing bovine serum albumin (BSA) and either glycine betaine or urea. The protein BSA was chosen because of its high stability, solubility, purity, and other favorable characteristics, including the absence of any known tight binding interactions with glycine betaine or urea. Glycine betaine, an important osmoprotectant in many prokaryotic and eukaryotic cells, is in general preferentially excluded from the vicinity of protein surfaces (Arakawa & Timasheff, 1983; Cayley et al., 1992). Because the native structure of a protein has less exposed surface from which glycine betaine is excluded, preferential interactions with this solute contribute to the thermodynamic stability of the native protein. Glycine betaine also has been shown to affect the stability of double-stranded DNA and the relative stability of A-T and G-C base pairs (Rees et al., 1993). Urea, an osmolyte in some living organisms (Lin & Timasheff, 1994), is widely used as a protein denaturant at concentrations higher than those investigated here. The destabilizing effect of urea on protein structure generally is thought to be due to its preferential accumulation in the vicinity of amides and other polar functional groups, more of which are exposed to the solution when the protein is denatured (Simpson & Kauzmann, 1953; Schellman, 1990; Liepinsh & Otting, 1994). Previous studies by various experimental methods indicate that the preferential interactions of most (uncharged) solutes with proteins are intermediate between those exhibited by glycine betaine and urea (Arakawa & Timasheff, 1983; Cayley et al., 1992).

BACKGROUND ON VAPOR PRESSURE OSMOMETRY

Over an aqueous solution the equilibrium vapor pressure of water is lower than the vapor pressure over pure liquid water at the same T and P. The lowering of the water vapor pressure over any solution in which water is the only volatile component can be detected readily by an osmometer such as the Wescor 5500 used in the present study. This instrument operates as a dewpoint hygrometer. Small but accurately detectable changes in the voltage drop across a thermocouple are proportional to ΔT_D, the difference between the temperature of the sample solution (controlled to within 3×10^{-4} K) and the (lower) "dewpoint" temperature of a small volume of pure water that condenses on the thermocouple within the sample chamber after a sudden shock of Peltier cooling. By the Clausius−Clapeyron equation (derived with the usual approximations), ΔT_D can be related to $\ln(P_1^\bullet/P_1)$, where $P_1^\bullet/P_1$ is the ratio of the equilibrium water vapor pressures over pure water and the sample solution:

$$\Delta T_D = (RT^2/\Delta H_{VAP}^\bullet) \ln(P_1^\bullet/P_1) \quad (1)$$

Here T is the controlled sample temperature, fixed at ~37 °C in the Wescor 5500, and $\Delta H_{VAP}^\bullet$ is the (positive) heat of vaporization of pure water at this temperature and the ambient (atmospheric) pressure. Over the operating range of the osmometer $|\Delta T_D|$ is always small enough so that the temperature dependence of $\Delta H_{VAP}^\bullet$ is negligible with respect to other sources of uncertainty. Thus, the constancy of $\Delta H_{VAP}^\bullet$ assumed in deriving eq 1 is justified.

The output of the Wescor 5500 is expressed in units of mmol/kg (milliosmolality, mOsm), which is related to the water activity a_1 and hence to $P_1^\bullet/P_1$ as follows:

$$\text{mOsm} \equiv -(10^6 \ln a_1)/M_1 \approx (10^6/M_1) \ln(P_1^\bullet/P_1) \quad (2)$$

where M_1 is the molecular weight of water. The first equality is based on the conventional definitions of osmolality and solvent activity: $a_1 \equiv \exp\{(\mu_1 - \mu_1^\bullet)/RT\}$, where $\mu_1^\bullet$ is the chemical potential of pure liquid water at the same temperature and pressure as the solution. The second equality in eq 2 incorporates the usual approximations: that the water vapor over the solution is sufficiently dilute to be considered ideal, and that $P_1^\bullet$ is independent of the total mechanical pressure, P.

According to eqs 1 and 2, the output of the osmometer is directly proportional to the dewpoint depression with respect to the sample temperature. The resolution of the electronic circuitry used to detect the voltage drop across the thermocouple is such that the osmometer can detect temperature differences to within $\sim 3 \times 10^{-4}$ K, which corresponds to a precision of 2 mOsm in the output readings. (We routinely observed this level of precision in immediately sequential readings taken on the same sample.) The relationship between the thermal-voltage response of the thermocouple and the output reading on the osmometer can be calibrated by making measurements on NaCl standards of known osmolalities in the range 0.100−2.0 mol/kg. Provided that

any systematic errors remain sufficiently constant over a series of immediately sequential VPO measurements on samples having different solute concentrations, such errors would have no effect on the accuracy of the preferential interaction coefficients determined by the osmometric method introduced here. As explained below, our approach depends on the accurate detection of *changes* in osmolality caused by changes in the concentrations of solutes.

BACKGROUND ON PREFERENTIAL INTERACTION COEFFICIENTS

Extensive commentaries on the definitions, thermodynamic relationships, and molecular interpretations of preferential interaction coefficients are available. Especially valuable are Eisenberg's monograph (1976) and the concise summaries provided in articles by Schellman (1990) and Timasheff (1992). Here we consider only the simplest and most frequently investigated type of system exhibiting preferential interactions, which is comprised of solvent water (1) and two other components, a dilute biopolymer (2) and a much smaller solute species (3) that is much more abundant in the solution. The interactions of solute 3 and of water with an isolated molecule of solute 2 determine the sign and magnitude of various partial derivatives that reflect the thermodynamic consequences of preferential interactions. These preferential interaction coefficients express the interdependence of the concentrations of two components, subject to constraints on three additional thermodynamic variables. (By the Gibbs Phase Rule, four variables are needed to specify the state of a homogeneous system consisting of three independent components.)

Of primary interest here is the following form of solute−solute preferential interaction coefficient, for which both thermodynamic and molecular interpretations can most readily be constructed:

$$ {}^{m}\Gamma^{o}_{\mu_3}(3,2) \equiv \lim_{m_2 \to 0} (\partial m_3/\partial m_2)_{T,P,\mu_3} \qquad (3)$$

To simplify notation, the specification (3,2) will be omitted, because the analysis presented in this paper is based entirely on solute−solute preferential interaction coefficients. The corresponding solvent−solute preferential interaction coefficient, defined by replacing 3 with 1 everywhere in eq 3, is designated ${}^{m}\Gamma^{o}_{\mu_1}(1,2)$. The exact relationship between ${}^{m}\Gamma_{\mu_3}(3,2)$ and ${}^{m}\Gamma_{\mu_1}(1,2)$, outside the limit $m_2 \to 0$, will be considered elsewhere (Anderson and Record, manuscript in preparation). The superscript "o" in eq 3 and in subsequent expressions implies that the designated term has no significant dependence on m_2 because solute 2 is sufficiently dilute.

The following phenomenological expression for ${}^{m}\Gamma^{o}_{\mu_3}$, initially presented (in a somewhat different form) by Timasheff and co-workers (Timasheff & Inouye, 1968; Inouye & Timasheff, 1972), has been used to interpret preferential interactions in terms of molecular accumulation/exclusion (Timasheff, 1992):

$$ {}^{m}\Gamma^{o}_{\mu_3} = B_{3,2} - B_{1,2} m_3/55.5 \qquad (4)$$

To simplify notation, the superscript "o" is deleted from the $B_{i,j}$, which depend not on m_2 but, in general, on m_3. The positive coefficients $B_{3,2}$ and $B_{1,2}$ are, respectively, the average number of molecules of the uncharged species 3 and 1, within a local domain surrounding an isolated molecule of the uncharged species 2. Any preferential interactions with component 2 experienced by components 3 and 1 cause their mole ratio in the local domain, $B_{3,2}/B_{1,2}$, to differ from their overall ratio in solution $m_3/55.5$. If solute 3 is preferentially accumulated in (excluded from) the local domain, $B_{3,2}/B_{1,2}$ is greater (less) than $m_3/55.5$, and accordingly ${}^{m}\Gamma^{o}_{\mu_3}$ is positive (negative).

The (general) inequality of $B_{3,2}/B_{1,2}$ and $m_3/55.5$ is in some respects analogous to the unequal mole ratios of components 3 and 1 in two solutions separated by a dialysis membrane impermeable only to component 2. On the basis of this analogy, an explicit derivation of eq 4 for uncharged species has been presented and adapted to obtain an analogous expression for charged solutes (Record & Anderson, 1995). For solutions where the two types of solutes are uncharged, the effect of the solvation of component 2 on the magnitude of the solute−solute preferential interaction coefficient can be represented more explicitly by recasting eq 4 as

$$ {}^{m}\Gamma^{o}_{\mu_3} = B_{3,2}\left(1 + S_{1,3}\frac{m_3}{55.5}\right) - B^{max}_{1,2}\frac{m_3}{55.5} \qquad (5)$$

Here $B^{max}_{1,2}$ is the m_3-independent maximum number of solvent molecules in the local domain of an isolated molecule of component 2. This maximum solvation number is attained when $m_3 = 0$ (provided that solvation of component 2 cannot be increased by introducing solute 3 into the solution). The positive coefficient $S_{1,3} \equiv (B^{max}_{1,2} - B_{1,2})/B_{3,2}$ is the cumulative stoichiometry of solvent 1 displaced per solute 3 accumulated in the local domain. As $m_3 \to 0$, $B_{3,2}$ also must vanish, but $B_{1,2} \to B^{max}_{1,2}$ so that $S_{1,3}$ does not diverge.

For strongly excluded solutes, $B_{3,2} \cong 0$ over at least some finite range of m_3, and hence $B^{max}_{1,2}$ can be evaluated directly as the slope of a linear plot of ${}^{m}\Gamma^{o}_{\mu_3}$ vs m_3. For accumulated solutes under typical conditions, $B^{max}_{1,2}$ $m_3/55.5$ cannot be neglected and $B_{3,2}$ varies with m_3. Separate evaluation of $B_{3,2}$ and $B^{max}_{1,2}$ may still be feasible by applying eq 5 to experimental values of ${}^{m}\Gamma^{o}_{\mu_3}$ determined for the accumulated solute, but only if $B_{3,2}$ is not simply proportional to m_3 when $S_{1,3}m_3/55.5 \ll 1$. However, m_3-dependent values of $B_{3,2}$ for an accumulated solute can be most reliably determined via the two-domain model if $B^{max}_{1,2}$ has been evaluated independently for a completely excluded solute. A primary objective of the present study is to demonstrate how $B_{3,2}$ for urea can be quantified after $B^{max}_{1,2}$ has been determined by a two-domain analysis of ${}^{m}\Gamma^{o}_{\mu_3}$ for glycine betaine.

In the context of the two-domain classification, further analysis of the m_3 dependence of $B_{3,2}$ requires additional model assumptions. An explicit parameterization of ${}^{m}\Gamma^{o}_{\mu_3}$ as a function of m_3 has been developed from a detailed model for preferential interactions due to the competitive one-for-one (1:1) "interchange" of two different types of solvent species (Schellman, 1990). Either the primary solvent (1) or the (less abundant) "cosolvent" (3) binds independently to sites of various (thermodynamically nonequivalent) types, located on the solvent-accessible surface of an isolated molecule of some highly dilute solute component (2). The "independent binding" stipulated by this model requires that the occupancy of any site on component 2 by a molecule of component 1 or 3 does not affect the occupancy of any other site. Thus, each of the sites must occupy the same surface

area on component 2, even though the molecules of 3 and 1 need not be equal in size [as indicated in Figure 1 of Schellman (1990)]. In the simplest situation where the 1:1 interchange model could be applied, all N sites are thermodynamically equivalent (have the same affinity for 3 relative to 1), and any site occupied by the principal solvent in the absence of cosolvent is open to occupancy by this component when it is added to the system.

More generally, the principal solvent, usually water, occupying some subset (N_1) of the total number of sites (N) on the surface of component 2 may be (at least effectively) not interchangeable with the cosolvent (or solute) 3, while all the remaining $N_{1,3}$ sites are accessible to both these components (Timasheff, 1992). If component 3 has the same binding affinity (relative to water) at all of the $N_{1,3}$ sites, then the 1:1 interchange model yields the following explicit expression for ${}^m\Gamma^o_{\mu_3}$:

$$ {}^m\Gamma^o_{\mu_3} = N_{1,3}(K - 1/55.5)m_3/(1 + Km_3) - N_1\frac{m_3}{55.5} \quad (6)$$

The notation here differs somewhat from that used in previous work [cf. eqs 7 and 12 of Schellman (1990) and eq 21 of Timasheff (1992)]. The "practical" equilibrium quotient, K, in units of reciprocal molality incorporates the quotient of activity coefficients (on the mole fraction scale) for components 3 and 1 and hence in general has some dependence on the solution composition. According to eq 6, the sign and magnitude of ${}^m\Gamma^o_{\mu_3}$ are determined by the magnitudes of K, N_1, and $N_{1,3}$. At a given m_3, the minimum (most negative possible) value of ${}^m\Gamma^o_{\mu_3}$ is $-(N_{1,3} + N_1)m_3/55.5$, which corresponds to an infinite preferential affinity for water over component 3 (i.e., $K = 0$) at all sites on component 2. If component 3 is strongly excluded, K may be small enough so that the total number of sites accessible to water ($N_{1,3} + N_1$) can be evaluated from the slope of a linear plot of ${}^m\Gamma^o_{\mu_3}$ vs m_3. Even if the affinity of component 3 relative to component 1 at the $N_{1,3}$ sites is so strong that K is much larger than both 1/55.5 and $1/m_3$, component 3 is preferentially accumulated (${}^m\Gamma^o_{\mu_3} > 0$) only if the number of sites for which it has strong affinity is large enough ($N_{1,3} > N_1m_3/55.5$). This situation illustrates that ${}^m\Gamma^o_{\mu_3}$ is a measure of *net* preferential interactions with the *entire* accessible surface of component 2 (Timasheff, 1992).

Equations 5 and 6 are consistent if $B_{3,2} = N_{1,3}Km_3/(1 + Km_3)$, $B^{max}_{1,2} = N_{1,3} + N_1$, and $S_{1,3} = 1$. The existence of some type of exact mathematical correspondence is expected because of the generality of the two-domain description but does not imply the unique applicability of the 1:1 interchange model. In reality $S_{1,3}$ may differ from 1 and depend on m_3, if the interchange is not 1:1 and/or if site occupancy is not strictly independent. Nevertheless, a detailed experimental characterization and theoretical analysis of $B_{3,2}$ and $S_{1,3}$ as functions of m_3 is not needed in the present study for the purpose of demonstrating how $B^{max}_{1,2}$ and $B_{3,2}$ can be evaluated separately by osmometric measurements on appropriate solute−protein solutions.

EVALUATION OF PREFERENTIAL INTERACTION COEFFICIENTS FROM OSMOMETRIC MEASUREMENTS

This section summarizes the thermodynamic expressions needed to relate ${}^m\Gamma^o_{\mu_3}$, as defined in eq 3, to experimental determinations of osmolality as a function of solution composition. Many of the following equations have appeared elsewhere in equivalent or related forms (e.g., Eisenberg, 1976), but not for the specific purpose of establishing rigorous connections between preferential interaction coefficients and data that can be obtained by VPO. The system investigated here, for which osmolality has been determined as a function of the solute molarities, is an aqueous solution containing a protein (BSA) with negligible net charge and a low molecular weight solute (glycine betaine or urea) with no net charge.

In accordance with the Gibbs phase rule for a homogeneous three-component system, the chemical potential of solvent water can be represented as a function of T, P, and the molarities of BSA (C_2) and glycine betaine or urea (C_3), which are the experimental independent variables. Thus, at constant T and P any differential change in μ_1 can be expressed in terms of differentials of C_2 and C_3:

$$ d\mu_1 = (\partial\mu_1/\partial C_2)_{C_3}\, dC_2 + (\partial\mu_1/\partial C_3)_{C_2}\, dC_3 \quad (7)$$

To simplify notation, the subscripts T and P are omitted from the partial derivatives in eq 7 and subsequent equations. These partial derivatives can be related to experimentally accessible quantities by noting that according to eq 2 $d\mu_1$ is directly proportional to dOsm, the differential change in the output from the osmometer:

$$ (\partial\mu_1/\partial C_3)_{C_2} = RT(\partial \ln a_1/\partial C_3)_{C_2} = -55.5^{-1}RT(\partial \mathrm{Osm}/\partial C_3)_{C_2} \equiv {}^c\Omega_3 \quad (8)$$

$$ (\partial\mu_1/\partial C_2)_{C_3} = RT(\partial \ln a_1/\partial C_2)_{C_3} = -55.5^{-1}RT(\partial \mathrm{Osm}/\partial C_2)_{C_3} \equiv {}^c\Omega_2 \quad (9)$$

Here the symbols ${}^c\Omega_i$ designate derivatives that are directly accessible by osmometry.

To express the concentration dependence of μ_1 using the molal scale, which is required for the definition of the preferential interaction coefficient given in eq 3, changes in μ_1 are related to changes in solute molalities by forming the following partial derivatives from eq 7:

$$ (\partial\mu_1/\partial m_2)_{m_3} = (\partial\mu_1/\partial C_2)_{C_3}(\partial C_2/\partial m_2)_{m_3} + (\partial\mu_1/\partial C_3)_{C_2}(\partial C_3/\partial m_2)_{m_3} \quad (10)$$

$$ (\partial\mu_1/\partial m_3)_{m_2} = (\partial\mu_1/\partial C_2)_{C_3}(\partial C_2/\partial m_3)_{m_2} + (\partial\mu_1/\partial C_3)_{C_2}(\partial C_3/\partial m_3)_{m_2} \quad (11)$$

The partial derivatives in eqs 10 and 11 that interconvert the molal and molar concentration scales of the solute species ($i, j = 2$ or 3) can be expressed in terms of their molarities and partial molar volumes $\bar{V}_j$ by the following exact relation:

$$ (\partial C_i/\partial m_j)_{m_{k\neq j}} = (\delta_{ij} - C_i\bar{V}_j)/V_m \quad (12)$$

Here V_m is the volume of solution per kg of solvent and $\delta_{ij} = 1$ if $i = j$, 0 if $i \neq j$. (The derivation of eq 12 incorporates the analog of the Gibbs−Duhem equation for partial molar

volumes and hence does not require that the $\bar{V}_j$ be constant with respect to changes in the composition of the solution.)

With the appropriate forms of eq 12, the quotient of eqs 10 and 11 can be expressed

$$-{}^{m}\Gamma_{\mu_1} = \frac{(1 - C_2\bar{V}_2)(\partial\mu_1/\partial C_2)_{C_3} - C_3\bar{V}_2(\partial\mu_1/\partial C_3)_{C_2}}{-C_2\bar{V}_3(\partial\mu_1/C_2)_{C_3} + (1 - C_3\bar{V}_3)(\partial\mu_1/\partial C_3)_{C_2}} \quad (13)$$

Here the cyclic rule of partial derivatives has been used to introduce ${}^{m}\Gamma_{\mu_1} \equiv (\partial m_3/\partial m_2)_{T,P,\mu_1}$. The analogous derivative, defined with solute concentrations expressed on the molar scale, is

$${}^{c}\Gamma_{\mu_1} \equiv (\partial C_3/\partial C_2)_{\mu_1} = -(\partial\mu_1/\partial C_2)_{C_3}/(\partial\mu_1/\partial C_3)_{C_2} = -{}^{c}\Omega_2/{}^{c}\Omega_3 \quad (14)$$

The thermodynamic variables T, P, and μ_1 can be held fixed during isopiestic distillation, but the preferential interaction coefficient, ${}^{c}\Gamma_{\mu_1}$ (and hence ${}^{m}\Gamma_{\mu_1}$), can be obtained more efficiently by direct osmometric evaluations of ${}^{c}\Omega_2$ and ${}^{c}\Omega_3$ as defined in eqs 8 and 9.

Equations 13 and 14 together yield the general relationship between ${}^{c}\Gamma_{\mu_1}$ and ${}^{m}\Gamma_{\mu_1}$:

$${}^{m}\Gamma_{\mu_1} = [(1 - C_2\bar{V}_2){}^{c}\Gamma_{\mu_1} + C_3\bar{V}_2]/[(1 - C_3\bar{V}_3) + C_2\bar{V}_3{}^{c}\Gamma_{\mu_1}] \quad (15)$$

As $C_2 \rightarrow 0$, this equation takes the simpler form

$${}^{m}\Gamma^{o}_{\mu_1} = ({}^{c}\Gamma^{o}_{\mu_1} + C_3^{o}\bar{V}_2^{o})/(1 - C_3^{o}\bar{V}_3^{o}) \quad (16)$$

Here the superscript "o" is placed on all symbols because in general each of these thermodynamic functions depends on the concentration of solute 2. In accord with the general usage in this context (cf. eq 3), "o" denotes high dilution of component 2 but does *not* specify any restriction on the concentration of solute 3. Equation 16 indicates that the difference between ${}^{m}\Gamma^{o}_{\mu_1}$ and ${}^{c}\Gamma^{o}_{\mu_1}$ can remain substantial even as $C_2 \rightarrow 0$, whenever C_3^{o} and/or $\bar{V}_2^{o}$ are large enough in comparison to the magnitude of ${}^{c}\Gamma^{o}_{\mu_1}$. To represent ${}^{m}\Gamma^{o}_{\mu_1}$ as a function of the molality that corresponds to C_3^{o} in eq 16, the limit $m_2 \rightarrow 0$ can be applied to the relationships $m_3 = C_3V_m$ and $V_m = 55.5\,\bar{V}_1 + m_2\bar{V}_2 + m_3\bar{V}_3$.

According to eq 16, evaluation of ${}^{m}\Gamma^{o}_{\mu_1}$ as a function of C_3^{o}, or the corresponding molality requires knowledge of the partial molar volumes of the solute components. In calculating the results reported here partial molar volumes of 98, 45, and 4.9×10^5 mL/mol were assigned to glycine betaine, urea, and BSA, respectively (Durchschlag, 1986). Although solute partial molar volumes $\bar{V}_i$ ($i = 2$ or 3) cannot be strictly independent of solution composition, slight if any concentration dependence is expected for the types of solutes and the ranges of solute concentrations investigated here (Durchschlag, 1986). For BSA, values of $\bar{V}_2$ reported for various solution compositions (even at 6 M guanidine hydrochloride) and for temperatures over the range 4−30 °C all fall within 5% (from 4.8×10^4 to 5.0×10^4 mL/mol; Durchschlag, 1986). For glycine betaine in pure water, $\bar{V}_3$ is almost invariant from 0.7 to 2.0 M (97.9 vs 97.8 mL/mol) at 20 °C (Arakawa & Timasheff, 1983). Although data are not available on the temperature dependence of $\bar{V}_3$ for glycine betaine, for most amino acids this dependence is very small (Durchschlag, 1986). Thus we have taken $\bar{V}_3 = 98$ mL/mol for glycine betaine in aqueous solution. The partial molar volumes of urea available in the literature (Stokes, 1967) also are relatively constant (variable by less than 5%) over a wide range of urea concentrations (0−10 molal). In the temperature range of interest, the reported temperature-dependence of $\bar{V}_3$ for urea is small: 44.6 mL/mol at 30 °C and 45 mL/mol at 40 °C (Stokes, 1967). Accordingly, $\bar{V}_3$ for urea is assigned the value 45 mL/mol at 37 °C. Slight changes in $\bar{V}_3$ ($\leq$10%) would not affect the values of ${}^{m}\Gamma^{o}_{\mu_1}$ obtained by applying eq 16 to the osmometric data obtained in the present study.

The following relationship connects the form of the preferential interaction coefficient defined in eq 3 and the form given in eq 16 that is evaluated from osmometric measurements:

$${}^{m}\Gamma^{o}_{\mu_3} = {}^{m}\Gamma^{o}_{\mu_1} + 1/[m_3(\partial\mu_3/\partial m_3)^{o}] \quad (17)$$

This equation is derived in the Appendix, which also considers its limiting form as $m_3 \rightarrow 0$. By the Gibbs−Duhem equation $m_3(\partial\mu_3/\partial m_3)^{o} = -55.5(\partial\mu_1/\partial m_3)^{o}$, which is related simply to the derivative ${}^{c}\Omega_3^{o}$ that can be evaluated from osmometric measurements on a solution containing only solute 3.

EXPERIMENTAL PROCEDURES

Enzyme grade (99.5%) urea was obtained from Gibco BRL (Gaithersburg, MD) and anhydrous glycine betaine was obtained from Sigma (St. Louis, MO). Series of glycine betaine solutions (from 50 mM to 4 M) and urea solutions (from 50 mM to 8 M) in ultrapure water were prepared. Bovine serum albumin (BSA, fraction V, molecular weight of 66 100 g/mol) was obtained from Sigma. A solution of BSA (~250 mg/mL) was made in a Hepes buffer and extensively dialyzed with tubing (Spectrum Industries) having a 12 000−14 000 g/mol molecular weight cut-off (MWCO) against 10 mM Hepes, pH 7.5, 2 mM EDTA, and 20 mM NaCl, then 10 mM NaCl, followed by three equilibrations against ultrapure water (purified by Barnstead E-Pure). The BSA solution was concentrated in a vacuum evaporator, and the protein concentration was determined to ±2% precision by UV absorbance measurements on a Cary 210 spectrophotometer using an $E_{280}^{1\%}$ of 5.5. Stock solutions containing 250 and 100 mg/mL BSA were made in ultrapure water. The viscosity of BSA solutions more concentrated than 250 mg/mL was judged to preclude accurate, reproducible pipetting.

Samples containing fixed concentrations of BSA (80 or 200 mg/mL BSA) and different concentrations of glycine betaine (0−0.8 M) or urea (0−1.6 M) were made up volumetrically by appropriate standard additions from stock solutions of glycine betaine or urea. From the standpoint of experimental design, for a given level of accuracy molarities can be determined more readily than molalities, especially when, as in the present study, osmometric measurements are conducted on a series of progressively diluted samples. For each of the concentrations of glycine

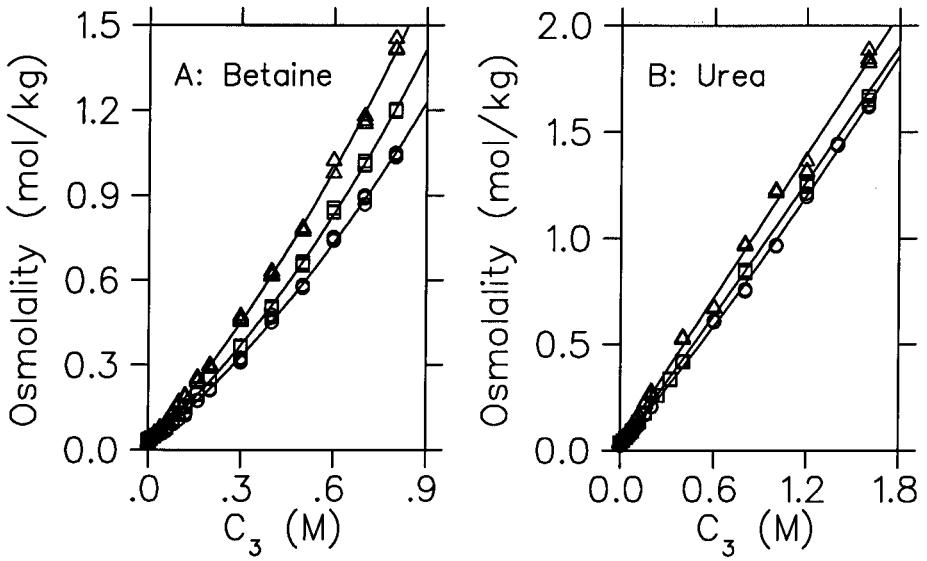

FIGURE 1: Representative plots of measured osmolalities of glycine betaine−BSA (A) and urea−BSA (B) aqueous solutions as functions of C_3 at various C_2, i.e., 0 (○), 80 (□) and 200 (△) mg/mL BSA. At each C_3 and C_2, measurements are triplicate. Solid lines are the best fitted quadratic curves.

betaine or urea that were investigated at fixed concentrations of BSA (80 and 200 mg/mL), control samples containing no BSA also were prepared. At each increment of solute concentration the osmolalities of three aliquots of each sample were measured in succession using a Wescor 5500 vaporphase osmometer (Logan, UT), whose principles of operation are summarized above in the section giving background on osmometry. After each of the standard additions whereby the molar concentration of glycine betaine or urea was increased while the molarity of BSA was maintained, samples containing the same concentration of the solute but no BSA were measured (also in triplicate) to minimize the effect of instrument drift. After every four to six samples the instrument was recalibrated against NaCl standards supplied by Wescor. The thermocouple head and sample chamber were checked for contamination according to the manufacturer's recommended procedure, and they were cleaned as needed.

RESULTS AND DISCUSSION

Characterization of Glycine Betaine− and Urea−BSA Preferential Interactions. Osmolalities of glycine betaine−BSA and urea−BSA aqueous solutions at various fixed BSA concentrations ($C_2 = 0$, 80, and 200 mg/mL) were measured as functions of C_3, the concentration of the small solute (glycine betaine or urea). The dependence of osmolality on C_3 is plotted for glycine betaine in Figure 1A and for urea in Figure 1B. The presence of BSA in a solution at any concentration of glycine betaine or urea significantly increases its osmolality, and this enhancement becomes progressively larger with increasing solute concentration, as indicated by the upward curvature at high C_3 in both panels A and B of Figure 1. For urea−BSA ternary solutions, the increase in total osmolality due to BSA is substantially less, even at urea concentrations higher than those investigated for glycine betaine. In every case, ${}^c\Omega_3$ as defined in eq 8 is negative, and its magnitude decreases slightly with increasing solute concentration. Thus, increasing the concentration of either solute has a favorable (decreasing) effect on the chemical potential of water. This effect is further (and monotonically) enhanced by the presence of BSA.

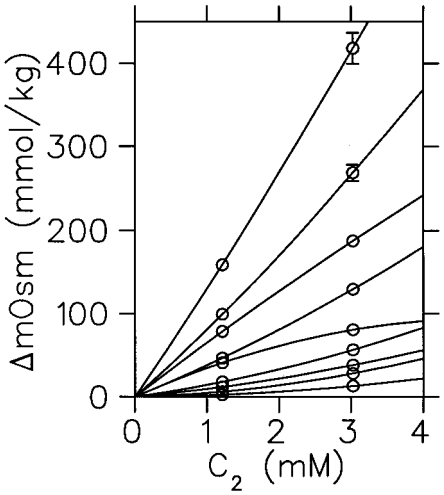

FIGURE 2: Representative plots of differences in measured osmolality, obtained by subtracting osmolality of glycine betaine binary aqueous solution from osmolality of glycine betaine−BSA ternary solution, as functions of C_2 at various glycine betaine concentrations (from top to bottom: $C_3 = 0.8$, 0.6, 0.4, 0.3, 0.2, 0.1, 0.06, 0.02, and 0 mM). Solid lines are the best fitted quadratic curves.

Measurements of osmolality as a function of C_3 at constant C_2 (0, 80, or 200 mg/mL BSA) were fitted to polynomials. Quadratic functions yield adequate fittings of all of the data sets, as shown in Figure 1 (solid lines). Comparing residuals of each quadratic fitting with those obtained by including higher order polynomial terms indicates that the latter are not needed (except at very low C_3, where some minor deviations may be due to systematic errors that arise outside the normal calibration range of the osmometer). The derivative ${}^c\Omega_3$ was evaluated by differentiating the quadratic fittings of osmolality vs C_3.

Effects of BSA on the C_3 dependence of osmolality were analyzed by subtracting the osmolality of the BSA-free binary solution from that of the glycine betaine−BSA ternary solution at the corresponding C_3. Differences in osmolality (ΔOsm) increase as C_2 increases, with slight curvature at the lower C_3 values, as shown in Figure 2 at selected glycine betaine concentrations. Series of ΔOsm data were fitted to quadratic functions of C_2 with zero intercept, shown as the

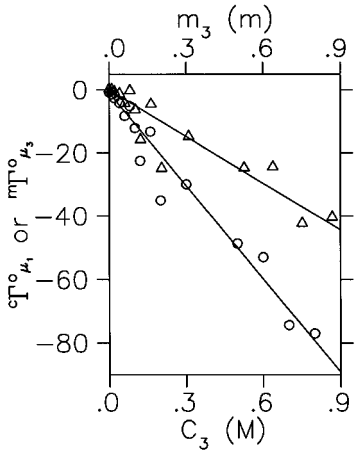

FIGURE 3: Representative plots of preferential interaction coefficients of glycine betaine with BSA as functions of glycine betaine concentrations: ${}^{c}\Gamma^{o}_{\mu_1}$ vs C_3 (○) and ${}^{m}\Gamma^{o}_{\mu_3}$ vs m_3 (△). Solid lines are the best fitted linear functions, with constraints at zero glycine betaine concentration (details in text).

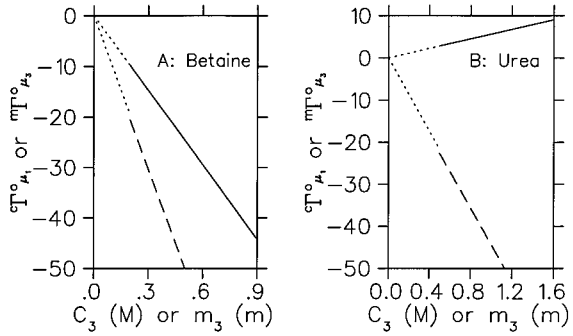

FIGURE 4: Representations of calculated preferential interaction coefficients as functions of solute concentrations. (A) Preferential interaction coefficients of glycine betaine with BSA as functions of glycine betaine concentrations: ${}^{c}\Gamma^{o}_{\mu_1}$ vs C_3 (dashed line) and ${}^{m}\Gamma^{o}_{\mu_3}$ vs m_3 (solid line). (B) Preferential interaction coefficients of urea with BSA as functions of urea concentrations: ${}^{c}\Gamma^{o}_{\mu_1}$ vs C_3 (dashed line) and ${}^{m}\Gamma^{o}_{\mu_3}$ vs m_3 (solid line). The dotted lines at low C_3 for both glycine betaine and urea represent extrapolations (see text).

solid smooth curves in Figure 2. Values of ${}^{c}\Omega_2$ (as defined in eq 9) were estimated at $C_2 = 0$ using the linear coefficients of plots of ΔOsm vs C_2 at various values of C_3. The magnitudes of the positive slopes observed in each of the ΔOsm vs C_2 plots are measures of the effectiveness of BSA at decreasing the chemical potential of water.

The preferential interaction coefficient, ${}^{c}\Gamma^{o}_{\mu_1}$, for glycine betaine−BSA interactions in the limit of zero protein concentration, as evaluated by applying the limit $C_2 \rightarrow 0$ to eq 14, is plotted as a function of C_3 in Figure 3. (The superscript "o" on C_3 is omitted to simplify notation.) The value of ${}^{c}\Gamma^{o}_{\mu_1}$ at $C_3 = 0$ was deduced from the theoretical considerations given in the Appendix. The experimentally determined values of ${}^{c}\Gamma^{o}_{\mu_1}$ are negative over the entire concentration range of glycine betaine (0−0.8 M), and, within experimental uncertainty, ${}^{c}\Gamma^{o}_{\mu_1}$ decreases linearly with increasing C_3, from −1 (the theoretical value fixed in fitting the data) in the absence of glycine betaine to −81 ± 4 at 0.8 M glycine betaine. The linear regression yields ${}^{c}\Gamma^{o}_{\mu_1} = -1 - 98\ (\pm\ 4)C_3$.

The conversion from ${}^{c}\Gamma^{o}_{\mu_1}$ to ${}^{m}\Gamma^{o}_{\mu_3}$, was performed according to eqs 16 and 17. Values of ${}^{m}\Gamma^{o}_{\mu_3}$, for glycine betaine−BSA interactions are plotted vs the molality corresponding to C_3 in Figure 3. The best fitted line is represented by ${}^{m}\Gamma^{o}_{\mu_3} = -49\ (\pm\ 4)m_3$. (The superscript "o" on m_3 is omitted to simplify notation.) Errors reported in all the linear regressions are the standard errors of fitting of the experimental data to given functions. Although the concentration dependences of ${}^{m}\Gamma^{o}_{\mu_3}$ and ${}^{c}\Gamma^{o}_{\mu_1}$ both are linear, values of ${}^{m}\Gamma^{o}_{\mu_3}$ are consistently less negative because of the positive term $C_3^o \bar{V}_2^o$ in eq 16 and decrease from the theoretical limit of 0 to −42 ± 3 over the range of glycine betaine concentrations investigated (0−0.8 M). In contrast, the difference between ${}^{m}\Gamma^{o}_{\mu_3}$ and ${}^{m}\Gamma^{o}_{\mu_1}$, given by eq 17, never exceeds 1 and hence is negligible under the conditions investigated here.

The BSA dependence of osmolality at fixed C_3 also was examined by interpolating quadratic functions fitted to the osmolality vs C_3 data (cf. Figure 1). Compared with the first approach, which requires measurements of osmolality at the same C_3 but different C_2, interpolation by fitting curves at constant C_3 may give rise to a greater uncertainty but theoretically can yield osmolality at any solute concentration within the experimental range. The resulting differences in osmolality between the solutions with and without BSA were plotted as functions of C_2 and fitted using quadratic functions with zero intercept. The resulting values of the derivative ${}^{c}\Omega_2^o$ were used, together with values of ${}^{c}\Omega_3^o$ at corresponding values of C_3, to calculate ${}^{c}\Gamma_{\mu_1}$ in the limit $C_2 \rightarrow 0$, according to eq 14. Figure 4 shows the averaged preferential interaction coefficients as functions of solute concentration (${}^{c}\Gamma^{o}_{\mu_1}$ vs C_3 and ${}^{m}\Gamma^{o}_{\mu_3}$ vs m_3) for glycine betaine (A) and urea (B). The data at low C_3 (below 0.2 M for glycine betaine and 0.5 M for urea) were judged to be affected by systematic as well as random sources of experimental uncertainty and therefore are not shown. We find within experimental error that both ${}^{c}\Gamma^{o}_{\mu_1}$ and ${}^{m}\Gamma^{o}_{\mu_3}$ for glycine betaine and for urea are linear functions of C_3 and m_3. The best fitted lines in Figure 4A are ${}^{c}\Gamma^{o}_{\mu_1} = -1 - 98\ (\pm\ 4)C_3$ and ${}^{m}\Gamma^{o}_{\mu_3} = -49\ (\pm\ 4)m_3$ for glycine betaine−BSA interactions. The best fitted lines in Figure 4B are ${}^{c}\Gamma^{o}_{\mu_1} = -1 - 43\ (\pm\ 1)C_3$ and ${}^{m}\Gamma^{o}_{\mu_3} = 6\ (\pm\ 1)m_3$ for urea−BSA interactions. The dotted lines are extrapolations from higher C_3 or m_3 to zero solute concentration. Comparison of the line for glycine betaine in Figure 4A with the fitted lines in Figure 3 indicates acceptable agreement between the two approaches used to estimate ${}^{c}\Omega_2^o$.

For the preferential interactions of urea with BSA, Figure 4B shows that ${}^{c}\Gamma^{o}_{\mu_1}$ and ${}^{m}\Gamma^{o}_{\mu_3}$ ($\approx {}^{m}\Gamma^{o}_{\mu_1}$) have opposite signs. The negative value of ${}^{c}\Gamma^{o}_{\mu_1}$ includes a substantial contribution dependent on the partial molar volume of BSA. According to eq 16 the term $C_3^o \bar{V}_2^o$ remains significant even in the limit of zero biopolymer concentration, as a mathematical consequence of the differential operations whereby ${}^{c}\Gamma^{o}_{\mu_1}$ and ${}^{m}\Gamma^{o}_{\mu_1}$ are interconverted. Only if $C_3 \rightarrow 0$ are these coefficients equal. This striking effect of partial molar volume on the signs and magnitudes of preferential interaction coefficients expressed on different concentration scales indicates the importance of choosing the form (${}^{m}\Gamma^{o}_{\mu_1}$ rather than ${}^{c}\Gamma^{o}_{\mu_1}$) that leads to the most straightforward physical interpretation of preferential interactions. In contrast, the difference between ${}^{m}\Gamma^{o}_{\mu_1}$ and ${}^{m}\Gamma^{o}_{\mu_3}$ is always insignificant in

the systems and under the conditions investigated here, because the strengths and/or numbers of solute−protein interactions are large enough so that the magnitudes of these preferential interaction coefficients are much larger than unity even at the lowest solute concentrations investigated.

Two-Domain Analysis of Glycine Betaine−BSA and Urea−BSA Interactions. Glycine betaine is highly excluded in BSA solutions at all m_3 in the range 0−1.6 M, in agreement with the implications of a study by Arakawa and Timasheff (1983) of BSA interactions with glycine betaine at three concentrations (0.7, 1.4, and 2.0 M). From a two-domain interpretation of the experimental result, ${}^{m}\Gamma^{o}_{\mu_3} = -49\ (\pm 4)m_3$, we infer that glycine betaine behaves as if completely excluded from the local domain of water surrounding BSA, and that this local domain is a monolayer on the protein surface. This inference is based on the following considerations. For a completely excluded solute the term $B_{3,2}$ in eq 5 is by definition zero, so that ${}^{m}\Gamma^{o}_{\mu_3}$ is proportional to m_3 with a proportionality constant of $-B^{max}_{1,2}/55.5$. If glycine betaine is completely excluded from BSA, the slope of the ${}^{m}\Gamma^{o}_{\mu_3}$ vs m_3 plot (-49 ± 4) indicates that the hydration of BSA, $B^{max}_{1,2}$, is 2700 ± 200 mol of H_2O/mol of protein (on a weight basis, $B^{max}_{1,2} = 0.74 \pm 0.06$ g of H_2O/g of protein). If exclusion of glycine betaine were incomplete, the true hydration would exceed this estimate, which is above the average but within the range of values for protein hydration calculated from hydrodynamic measurements: 0.12−1.04 g of H_2O/g of protein, with an average value of 0.53 ± 0.26 g of H_2O/g of protein (Squire & Himmel, 1979). Previous studies of protein hydration based on the preferential interactions of glycerol with proteins (Gekko & Timasheff, 1981) yield smaller hydration numbers than those deduced from hydrodynamic measurements, probably because glycerol is not completely excluded. Osmometric measurements indicate that glycerol is accumulated near BSA to a greater extent than glycine betaine (Capp et al., unpublished results).

To test the hypothesis of complete exclusion of glycine betaine and to provide a molecular picture of the local domain surrounding BSA, the thermodynamic extent of hydration of BSA is now compared with a prediction of its water-accessible surface area. The water-accessible surface area of a native protein (A in Å^2) increases as a power function of the protein molecular weight (M_r in g/mol) (Janin, 1976; Teller, 1976; Miller et al., 1987):

$$A = aM_r^{\,b} \tag{18}$$

Different studies have obtained slightly different values of the empirical constants a and b [$a = 11.4$ Å^2 and $b = 0.67$ (Janin, 1976; Teller, 1976; W.Z., unpublished results), and $a = 6.3$ Å^2 and $b = 0.73$ (Miller et al., 1987)]. Water-accessible surface areas estimated using the different sets of values of a and b agree to within 5% (for the usual range of M_r). From eq 18 and the two sets of values of a and b, we estimate the water-accessible surface area of the native BSA to be $2.0\ (\pm 0.1) \times 10^4$ Å^2. If the 2700 ± 200 H_2O molecules of hydration (i.e., $B^{max}_{1,2}$) constitute a surface layer, the surface density of water molecules on BSA is 0.14 ± 0.01 H_2O/Å^2.

To assess how our estimate of water surface density compares with the predictions of different structural models for hydration, we first assume a hexagonal closest packing of water molecules on the protein surface and a van der Waals radius of 1.4 Å for water. For this model the maximum surface density of water molecules in the first layer around the protein is approximately 0.15 H_2O per Å^2 of protein surface area. Alternatively, if the effective cross-sectional area of water is 9 Å^2 (Gill et al., 1985), as inferred from the density of bulk water (∼1 g/mL) and its molecular weight, then the predicted water surface density is 0.11 H_2O/Å^2. Our experimental value (0.14 ± 0.01 H_2O/Å^2) falls between these two estimates. Thus if the extent of hydration determined from the glycine betaine−BSA preferential interaction coefficients (0.74 ± 0.06 g of H_2O/g of protein) is approximately equal to that of a monolayer of H_2O at the protein surface, then the density of this water of hydration is somewhat greater (∼25%) than that of bulk water. This finding is consistent with the inference that water of hydration adjacent to both charged and nonpolar surfaces is more dense than bulk water, as judged by the negative partial molar volume changes associated with ionization of carboxylic acids in water [reviewed by Edsall and McKenzie (1978)] and with the transfer of hydrophobic liquids to water [reviewed by Edsall and McKenzie (1983)].

The foregoing considerations indicate that the local domain in the two-domain model consists of a monolayer of water of hydration enclosing the protein surface. Glycine betaine may be excluded from polar surface areas on BSA because water (with its multiple donor−acceptor hydrogen bonding capability) interacts more strongly with such surfaces. Glycine betaine may be excluded also from nonpolar surfaces because contacts of its zwitterionic dipole with such surfaces are thermodynamically even more unfavorable than contacts with the water dipoles. The complete exclusion of glycine betaine from protein surfaces is in accord with the proposed osmoprotective mechanism of glycine betaine in *Escherichia coli* K-12 cells, where glycine betaine appears to be more excluded than other neutral osmolytes (Cayley et al., 1992). Most natural osmolytes and amino acids are found to be excluded from proteins to some degree (Liu & Bolen, 1995; Arakawa & Timasheff, 1983).

Compared with other methods, the use of VPO measurements to determine the concentration dependence of osmolality readily yields values of ${}^{m}\Gamma^{o}_{\mu_3}$ as a function of m_3. Earlier studies have reported discrete values of ${}^{m}\Gamma^{o}_{\mu_3}$ at a few solute concentrations. At 0.7 M glycine betaine, our measurements (37 °C) indicate that ${}^{m}\Gamma^{o}_{\mu_3}$ is -37 ± 3, whereas Arakawa and Timasheff (1983) report a value of −26.9 (no error given) for $(\partial m_3/\partial m_2)^{o}_{T,\mu_1,\mu_3}$ at 20 °C. This difference could be influenced by differences in the solution conditions (e.g., temperature and buffer) under which the preferential interaction coefficient was determined in these two studies.

As in the case of glycine betaine, values of ${}^{c}\Gamma^{o}_{\mu_1}$ and ${}^{m}\Gamma^{o}_{\mu_3}$ for urea also exhibit linear dependences on C_3 and m_3 within experimental uncertainties, shown in Figure 4B. However, the positive sign of ${}^{m}\Gamma^{o}_{\mu_3}$ indicates that the $B_{3,2}$ term (in eq 5) must be large enough to ensure net preferential accumulation of urea. From Figure 4B ${}^{m}\Gamma^{o}_{\mu_3}/m_3 = 6 \pm 1$. If $B^{max}_{1,2}/55.5 = 49 \pm 4$, as obtained from betaine−BSA preferential interactions (${}^{m}\Gamma^{o}_{\mu_3}/m_3 = -49 \pm 4$), then for urea−BSA interactions $B_{3,2}/m_3 = 54 \pm 5$ (if $S_{1,3}m_3 \ll 55.5$ in eq 5), in the urea concentration range investigated (up to

1.6 M). Clearly, in this situation independent knowledge of the hydration term, $B_{1,2}^{max}$, is essential for a correct evaluation of $B_{3,2}$. On the basis of a more detailed model, $B_{3,2}$ can be expressed in terms of additional parameters, for example, by introducing the number of interchangeable sites, $N_{1,3}$, and the average binding constant, K, as in eq 6. However, because the plot of ${}^{m}\Gamma^{o}_{\mu_3}$ vs m_3 is linear in the range of urea concentrations investigated here, individual values for N_1 and K cannot be resolved. With the value $B_{1,2}^{max} = N_1 + N_{1,3} = 2700 \pm 200$ mol of H_2O/mol of BSA determined from betaine−BSA interactions, the product of $N_{1,3}K$ is estimated to be 54 ± 5 m^{-1}.

To estimate $N_{1,3}$ and K individually, we investigate the correlation of $N_{1,3}$ with polar surface areas of BSA. The preferential accumulation of urea near protein surfaces generally is thought to result from favorable interactions with solvent-accessible amides and possibly other polar groups (Robinson & Jencks, 1965; Nozaki & Tanford, 1970; Liepinsh & Otting, 1994). Previous studies using various methods to quantify urea−protein and urea−peptide interactions at urea concentrations up to 8 M have reported estimates of site binding constants (defined in various but similar ways) ranging from 0.04 to 0.3 m^{-1} (Makhatadze & Privalov, 1992; Sijpkes et al., 1993; Liepinsh & Otting, 1994; Scholtz et al., 1995). Assuming that over the concentration range 0−1.6 M $B_{3,2}$ is determined by urea−amide group interactions, which are among the strongest urea−protein interactions, we can dissect values of K by equating $N_{1,3}$ with the number of amide groups accessible on the surface of native BSA. This number (n_{acc}) can be estimated by quantifying the water-accessible polar surface areas of the native ($A_{p,N}$) and denatured ($A_{p,D}$) BSA with procedures described by Livingstone et al. (1991). Once the total number of amide groups (n_{tot}) is known, and if the numbers of urea-accessible amide groups in both native and denatured states are assumed to be proportional to the polar surface areas (i.e., $n_{acc}/n_{tot} = A_{p,N}/A_{p,D}$), then $n_{acc} = N_{1,3} \approx 220$ (W.Z., unpublished results). The corresponding value of K derived from our experimental determination of $N_{1,3}K$ (54 ± 5 m^{-1}) is 0.25 m^{-1}, which is comparable to values that have been deduced from NMR measurements (Liepinsh & Otting, 1994).

The foregoing analysis of urea−BSA preferential interactions demonstrates that the quantitative interpretation of solute accumulation in terms of the two-domain model according to eq 5 requires knowing the hydration term $B_{1,2}^{max}$ (as well as the solute−solvent exchange stoichiometry $S_{1,3}$ at higher m_3). Therefore, studies of solute−biopolymer interactions should be accompanied by an independent determination of $B_{1,2}^{max}$ for the biopolymer, which can be accomplished by a two-domain analysis using glycine betaine or another completely excluded solute.

The positive values of ${}^{m}\Gamma^{o}_{\mu_3}$ over the range of 0−1.6 m indicate a preferential accumulation of urea in the vicinity of BSA, which is consistent with the mechanism that has been proposed for the denaturation of proteins induced by sufficiently high concentrations of urea (Simpson & Kauzmann, 1953; Schellman, 1990; Liepinsh & Otting, 1994). The magnitude of ${}^{m}\Gamma^{o}_{\mu_3}$ (6 ± 1 in 1 m urea) is comparable to corresponding values for urea interacting with other proteins, as reported by Lin and Timasheff (1994). However, a direct comparison is complicated by the expected dependence of ${}^{m}\Gamma^{o}_{\mu_3}$ on the sizes and characteristics of the surface areas of proteins that have different proportions of nonpolar, polar, and charged groups. In a subsequent paper (Zhang et al., manuscript in preparation) we examine how well information about protein surface areas can be correlated with the magnitudes of preferential interaction coefficients that characterize the interactions of different types of osmolyte solutes with biological macromolecules.

CONCLUSIONS

This study introduces the use of vapor pressure osmometry as the sole source of experimental input needed to evaluate preferential interaction coefficients of the type ${}^{c}\Gamma^{o}_{\mu_1}$. Under conditions where ${}^{c}\Gamma_{\mu_1}$ is independent of C_2, values of ${}^{c}\Gamma^{o}_{\mu_1}$ are obtained directly from series of VPO measurements of osmolality as a function of C_3 at different fixed values of C_2. With the appropriate thermodynamic relationships ${}^{c}\Gamma^{o}_{\mu_1}$ is transformed to ${}^{m}\Gamma^{o}_{\mu_3}$, whose value as a function of the solute concentration can be interpreted more directly on the basis of the two-domain model. The values of ${}^{m}\Gamma^{o}_{\mu_3}$ determined in this study are interpreted to indicate complete preferential exclusion of glycine betaine from BSA and moderate preferential accumulation of urea near BSA. Because glycine betaine behaves as if completely excluded from BSA, it appears to be well suited for studies that use the approach introduced here to quantify the hydration of individual proteins as well as changes in hydration that accompany processes involving proteins. The results and calculations reported here for glycine betaine and BSA indicate that the local domain surrounding BSA consists of one monolayer of water of hydration. Our analysis of urea−BSA interactions demonstrates why characterizing the accumulation of solutes in terms of the two-domain model generally requires an independent determination of the hydration coefficient $B_{1,2}^{max}$ (in eq 5).

The signs, magnitudes, and m_3 dependences of ${}^{m}\Gamma^{o}_{\mu_3}$ for glycine betaine−BSA and urea−BSA interactions are qualitatively consistent with previous work (Arakawa & Timasheff, 1983; Lin & Timasheff, 1994). If consideration is given to both accuracy and efficiency, the use of VPO to investigate preferential interactions compares favorably with established methods, whose strengths and limitations have been discussed recently (Eisenberg, 1994). As with most experimental methods, the accuracy of VPO investigations improves with the magnitude of the effects being measured. At a given C_2, the C_3 dependence of the osmolality is significantly greater for glycine betaine than for urea, and the change in osmolality with C_2 is greater at a given C_3 when glycine betaine is the solute. Moreover, fitting the m_3 dependence of ${}^{m}\Gamma^{o}_{\mu_3}$ for glycine betaine and BSA requires only one parameter ($B_{1,2}^{max}$), whereas an additional coefficient ($B_{3,2}$) is required to characterize the preferential interactions of urea with BSA. In this laboratory, work is progressing on the further development of strategies for the acquisition and analysis of osmometric data that can be used to quantify preferential interactions of solutes, charged and uncharged, and of water with proteins and nucleic acids.

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representatives of the Wescor Corporation who provided useful information about the operational principles and capabilities of their osmometer.

APPENDIX

Transformation of Solute−Solute Preferential Interaction Coefficients. The rigorous relationship between ${}^{m}\Gamma_{\mu_1}$ and ${}^{m}\Gamma_{\mu_3}$, derived without any restrictions on the solute concentrations, will be presented elsewhere, together with other generalized relationships between alternative forms of solute−solute and solute−solvent preferential interaction coefficients (Anderson and Record, manuscript in preparation). For the purpose of analyzing the data reported in the present study, consideration of ${}^{m}\Gamma_{\mu_1}$ and ${}^{m}\Gamma_{\mu_3}$ in the limit $m_2 \rightarrow 0$ suffices. That ${}^{m}\Gamma^{o}_{\mu_1} \cong {}^{m}\Gamma^{o}_{\mu_3}$ has often been stated (cf. Eisenberg, 1976), but the following derivation provides a general explicit expression for the difference between these coefficients and hence indicates why it can be neglected for the systems investigated here (though not for all possible systems of biological interest) *except* in the limit $m_3 \rightarrow 0$ considered in the following section.

The derivation of eq 17 is initiated by applying the cyclic transformation to ${}^{m}\Gamma^{o}_{\mu_1}$, defined by analogy to ${}^{m}\Gamma^{o}_{\mu_3}$ in eq 3:

$$ {}^{m}\Gamma^{o}_{\mu_1} \equiv \lim_{m_2 \rightarrow 0} (\partial m_3/\partial m_2)_{T,P,\mu_1} = -(\partial\mu_1/\partial m_2)^{o}_{T,P,m_3}/(\partial\mu_1/\partial m_3)^{o}_{T,P,m_2} \quad \text{(A-1)} $$

The partial derivatives in both numerator and denominator of eq A-1 can be recast by introducing appropriate forms of the Gibbs−Duhem equation:

$$ {}^{m}\Gamma^{o}_{\mu_1} = -[m_3(\partial\mu_3/\partial m_2)^{o}_{T,P,m_3} + 1]/m_3(\partial\mu_3/\partial m_3)^{o}_{T,P,m_2} = {}^{m}\Gamma^{o}_{\mu_3} - 1/m_3(\partial\mu_3/\partial m_3)^{o}_{T,P,m_2} \quad \text{(A-2)} $$

In the second equality another cyclic transformation has been introduced.

Equation A-2 demonstrates that the inequality of ${}^{m}\Gamma^{o}_{\mu_3}$ and ${}^{m}\Gamma^{o}_{\mu_1}$ can be traced ultimately to the contribution to μ_2 that arises from ideal entropy of mixing: $\lim_{m_2 \rightarrow 0} m_2 \ln m_2 = 1$. Assessing the difference between these forms of the preferential interaction coefficient requires some information about the concentration dependence of μ_3. When solute 3 has no net charge, $\mu_3 \equiv \mu_3^{o}(P,T) + RT \ln \gamma_3 m_3$, where γ_3 is the molal-scale activity coefficient. Hence $\lim_{m_3 \rightarrow 0} m_3(\partial\mu_3/\partial m_3)^{o}_{T,P,m_2} = 1$, because the nonideality of any (real) solute cannot diverge as its concentration approaches zero. Accordingly, $\lim_{m_3 \rightarrow 0}({}^{m}\Gamma^{o}_{\mu_3} - {}^{m}\Gamma^{o}_{\mu_1}) = 1$. This limiting value constitutes a maximum if $\ln \gamma_3$ increases monotonically with m_3, as is typical of uncharged solutes. Whether the difference between ${}^{m}\Gamma^{o}_{\mu_1}$ and ${}^{m}\Gamma^{o}_{\mu_3}$ is significant compared with the magnitudes of the individual coefficients is determined not by the high dilution of component 2 but rather by characteristics of the interactions of the solutes with each other and with water.

Solute−Solute Preferential Interaction Coefficients in the Limit $m_3 \rightarrow 0$. At low concentrations of solute 3, ${}^{m}\Gamma^{o}_{\mu_3}$ and ${}^{m}\Gamma^{o}_{\mu_1}$ become difficult to evaluate by any experimental method. As indicated in the discussion of Figures 3 and 4, the analysis and interpretation of the m_3 dependences of ${}^{m}\Gamma^{o}_{\mu_3}$ and ${}^{m}\Gamma^{o}_{\mu_1}$ is facilitated by knowing their limits as $m_3 \rightarrow 0$. These can be deduced theoretically from eq 17 and the following considerations. By successive applications of the cyclic transformation and Euler reciprocity, the preferential interaction coefficient defined in eq 3 can be represented in terms of the chemical potentials of the solute components:

$$ {}^{m}\Gamma^{o}_{\mu_3} \equiv \lim_{m_2 \rightarrow 0} (\partial m_3/\partial m_2)_{T,P,\mu_3} = -(\partial\mu_3/\partial m_2)^{o}_{T,P,m_3}/(\partial\mu_3/\partial m_3)^{o}_{T,P,m_2} = -(\partial\mu_2/\partial\mu_3)^{o}_{T,P,m_2} \quad \text{(A-3)} $$

By representing the solute chemical potentials μ_2 and μ_3 in the form appropriate for a solute i with no net charge ($\mu_i \equiv \mu_i^{o}(P,T) + RT \ln \gamma_i m_i$), the last equality in eq A-3 can be written more explicitly as

$$ {}^{m}\Gamma^{o}_{\mu_3} = m_3(\partial \ln\gamma_2/\partial m_3)^{o}_{T,P,m_2}/(1 + m_3(\partial \ln\gamma_3/\partial m_3)^{o}_{T,P,m_2}) \quad \text{(A-4)} $$

As $m_3 \rightarrow 0$ the effect of solute 3 on its own nonideality and on the nonideality of solute 2 (itself highly dilute) must vanish, because no force due to interactions between (finite) solute particles can persist at infinite separation. Consequently, at low enough m_3, $\ln \gamma_2 \sim m_3^{\sigma_2}$ and $\ln \gamma_3 \sim m_3^{\sigma_3}$, where σ_2 and σ_3 are the smallest positive exponents needed to represent the m_3 dependences of $\ln \gamma_2$ and $\ln \gamma_3$, respectively. Introducing these asymptotic forms into eq A-4 demonstrates that ${}^{m}\Gamma^{o}_{\mu_3}$ must vanish as $m_3 \rightarrow 0$.

For uncharged polymers and polyampholytes with a minimal net charge, such as BSA, $\ln \gamma_2$ generally is found to be proportional to m_3, at least when this concentration is low enough. Hence, according to eq A-4 ${}^{m}\Gamma^{o}_{\mu_3}$ also becomes proportional to m_3 [because $m_3(\partial \ln\gamma_3/\partial m_3)^{o}_{T,P,m_2} \ll 1$]. A distinctly different asymptotic m_3 dependence of ${}^{m}\Gamma^{o}_{\mu_3}$ appears to be manifested in solutions containing highly charged rod-like polyions such as DNA, and ${}^{m}\Gamma^{o}_{\mu_3}$ appears to approach a characteristic nonzero constant (determined chiefly by the mean axial charge density on the polyion) at sufficiently low salt concentrations, which, however, must always greatly exceed the polyion concentration. This apparent limiting behavior can be accounted for by either the counterion condensation model (Manning 1969) or the PB cell model (Anderson & Record, 1980) for an infinite cylindrical polyion. For any *finite* rodlike polyion, ${}^{m}\Gamma^{o}_{\mu_3}$ must approach zero at sufficiently low m_3, even though it still may appear to be approaching a nonzero limit at the lowest m_3 where measurements can be made.

As $m_3 \rightarrow 0$, the limiting value of ${}^{m}\Gamma^{o}_{\mu_1}$, unlike that of ${}^{m}\Gamma^{o}_{\mu_3}$, cannot be deduced directly from elementary physical considerations, because no expression analogous to eq A-4 exists for ${}^{m}\Gamma^{o}_{\mu_1}$ (with the conventional definitions of activity coefficients and solute molalities). Nevertheless, eqs A-2 and A-4, together with the low-m_3 asymptotic forms of $\ln \gamma_2$ and $\ln \gamma_3$ given above, yield unequivocally the result $\lim_{m_3 \rightarrow 0} {}^{m}\Gamma^{o}_{\mu_1} = -1$, which according to eq 16 also equals $\lim_{C_3 \rightarrow 0} {}^{c}\Gamma^{o}_{\mu_1}$.

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