Biochemistry

 Volume 41, Number 46 November 19, 2002

Current Topics

Protein Hydration, Thermodynamic Binding, and Preferential Hydration

Serge N. Timasheff

Department of Biochemistry, MS 009, Brandeis University, 415 South Street, Waltham, Massachusetts 02453-2728

Received April 29, 2002

The question of protein hydration, i.e., the interaction of protein molecules with water, and of the involvement of water molecules in protein reactions has been a focus of attention for the good part of a century. Yet the number of water molecules that interact with one protein molecule, W_H (waters of hydration), has been an elusive quantity, notwithstanding measurements by transport (*1*, *2*) and NMR (*3*) techniques. The reason for this lies in the essence of the physical phenomenon of protein hydration. The fact is that there is no rigid shell of water around a protein molecule, but rather there is a fluctuating cloud of water molecules that are thermodynamically affected more or less strongly by the protein molecule. Nevertheless, the description of the overall effect in terms of an effective number of whole water molecules, W_H, has been a useful concept, analogous to the electrostatic double layer representation of the ionic distribution in the cloud around a central ion in the Debye−Hückel theory (*4*), or the surface of shear used in the analysis of transport phenomena (sedimentation, electrophoresis, diffusion) (*5*).

It is characteristic of protein reactions that, during the course of the reaction, the total surface area of the protein in contact with water changes. This must be accompanied by a change in the number of water molecules with which it interacts, i.e., its extent of hydration, $\Delta W_H = W_H(\text{product}) - W_H(\text{reactant})$. This is true of allosteric transitions, protein associations, and the binding of ligands. In general, if a reaction is accompanied by a change in hydration by n effective molecules of water ($n = \Delta W_H$), we may write the reaction as

$$P{\cdot}nH_2O \rightleftharpoons P^* + nH_2O \; (n = \Delta W_H) \qquad (1)$$

The determination of this change in hydration does not lend itself to straightforward approaches,[1] and is nearly impossible in pure water. Attempts have been made to approach this problem by perturbing the protein−water system via the addition of a third component at high concentration (a cosolvent). The number of water molecules measured by this approach, however, is not the sought quantity $n = \Delta W_H$, but it is the change in the number of waters of preferential hydration, ΔW_{PH}. Protein hydration, W_H, and protein preferential hydration, W_{PH}, are two independent concepts, and the two quantities are not related directly. Let us show this by examining the system protein−water−cosolvent in terms of a simple thermodynamic analysis based on multicomponent theory (*6*−*8*).

(A) Water of Hydration, W_H. When a protein molecule is immersed in water (dissolved), its surface by necessity interacts with water molecules.[2] Water of hydration is a manifestation of the sum of all the thermodynamic perturbations of water molecules by a protein molecule. Two basic rules apply: (1) The entire protein surface must make contact with water molecules (there is no vacuum); (2) the protein surface can be regarded as a mosaic of highly diverse loci each of which interacts with water molecules by a mechanism defined by its chemical nature and with its particular free energy, δg_i. These interactions may be attractions or repulsions of various strengths (free energies) which range from strong affinity with complex formation to simple van der Waals contacts, to actually unfavorable contacts that induce

[1] This, of course, does not apply to strongly complexed water molecules.

[2] All the analyses that follow will be for a single isolated protein molecule, which permits one to neglect all interprotein interactions. This can be achieved by extrapolation to zero protein concentration.

10.1021/bi020316e CCC: $22.00
Published on Web 10/26/2002

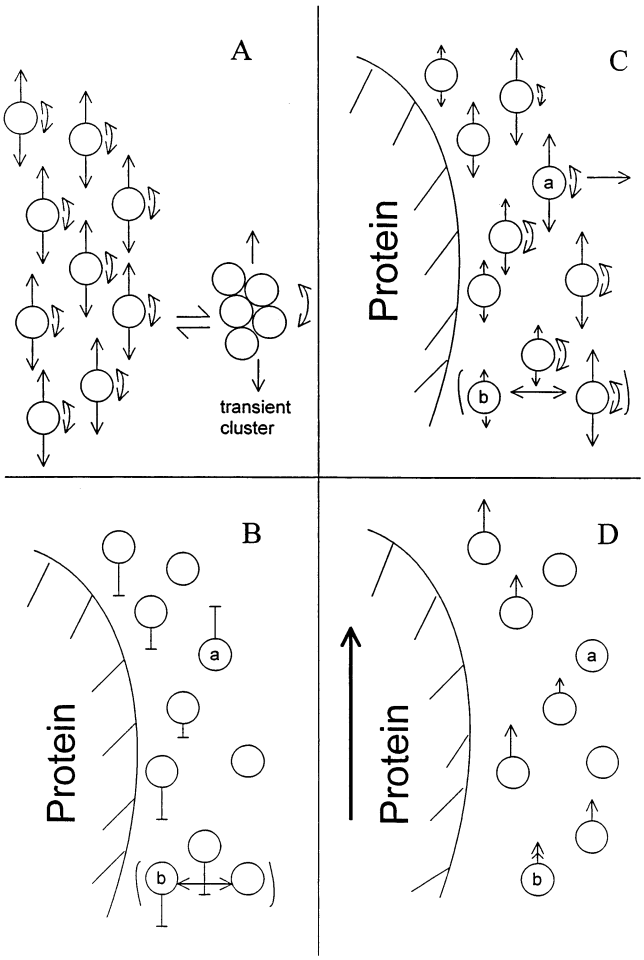

FIGURE 1: Interaction of water molecules with a protein surface within a solvent domain[5] thermodynamically affected by the protein. (A) Free water. (B) An introduced protein molecule causes water molecules to interact with it with different free energies, represented by the vertical bars; downward pointing bars are for negative ΔG (attraction), positive bars for positive ΔG (repulsion); no bars indicate indifference. (C) Corresponding changes in the freedom of translational and rotational motions of the water molecules. Water molecule *a* is repelled from the protein; molecule *b* fluctuates between strong attraction and freedom. (D) Consequences for a transport process. The large vector on the protein indicates its motion induced by the applied field (gravitational in sedimentation, electrokinetic in electrophoresis, chemical potential gradient in diffusion). Molecules with no vector are neutral thermodynamically and are indifferent to the transport of the protein molecule. Molecule *a*, being repelled by the protein, is not transported; molecule *b* migrates at a low rate due to its fluctuation between being attracted and free.

the dynamic water clusters to enter into the least unfavorable configurations (*9*, *10*). As a consequence, some water molecules are immobilized most of the time, and others retain various degrees of freedom of motion and fluctuate between transient contact with protein and being part of bulk water. Finally, there are water molecules that do not make contact with the protein, but whose translational and/or rotational motions are perturbed momentarily by a nearby protein molecule. This is illustrated in Figure 1.

Figure 1A is a picture of a number of idealized free water molecules all with identical unrestrained translational and rotational motions in equilibrium with a transient cluster (pure water). In Figure 1B, a protein molecule has been introduced into the system. The free water molecules interact with it with various values of free energy (δg_i) which may be negative or positive. Some water molecules are not affected, which means thermodynamic indifference ($\delta g_i = 0$). As shown in Figure 1C, these interactions lead to perturbations of the freedoms of translational and rotational motions of the individual water molecules which are restricted to different extents dictated by the values of δg_i.

At any instant, each category of protein−water interaction will contribute an increment of "molecules associated with the protein" when the thermodynamic perturbations are translated into molecules of hydration. This becomes clear when we look at the phenomenon through the transport experiment depicted in Figure 1D. Each arrow on a water molecule represents the time average of its net nonrandom extent of motion in the same direction as that of the protein. This is determined by the strength of its attraction by the protein and, hence, by the ratio of the time that it is "attached" to the protein to the time that it is free, in the hypothetical equilibrium $W_{on} \rightleftharpoons W_{off}$. The fractional contribution to hydration of any water molecule is equal to the ratio of its vector of motion to that of the protein. The sum of the contributions of all the water molecules whose motion is momentarily (and nonrandomly) oriented into the same direction as that of the protein molecule is the measured protein hydration. It is the mass of water that, at any instant, migrates together with the protein (*1*). When divided by the molecular weight of water, this results in the "number" of water molecules, W_H, that interact with the protein. Hence, W_H is an effective number. It is NOT the number of whole water molecules that interact with the protein, as would be in the formation of a shell.

Let us examine the same situation through a descriptive thermodynamic argument. Each water molecule−protein interaction is characterized by a free energy of interaction, δg_i, as depicted in Figure 1B. These add up to the total free energy of hydration, $\Delta G_H = \sum \delta g_i$. Each individual δg_i corresponds to the probability, P_i, that a given water molecule will be "on the protein" at any instant in time, $P_i = t_{on}/(t_{on} + t_{off})$, which can be equated heuristically to a fraction of a water molecule of hydration, w_i. For example, a molecule that makes contact with protein 50% of the time will make a contribution of 0.5 molecule of hydration ($w_i = 0.5$). A molecule whose freedom of motion is perturbed momentarily by the protein will have a finite value of P_i (even though no contact is made). This may be very small ($P_i = 0.1, 0.01, ...$), but it contributes a fraction of a molecule of hydration. Therefore, total hydration, $W_H = \sum w_i$, is an effective number of water molecules that interact with the protein. Water of hydration must be regarded, then, not as a rigid shell of water around the protein molecules, but as a fluctuating "cloud" of waters that interacts more or less strongly (even unfavorably) with the protein surface. Therefore, any analysis based on the concept that a rigid shell exists is in error. On the other hand, a model that contains a hypothetical shell of water around the protein molecules may be useful in calculations, if it is realized that the model does not correspond to reality and if one remembers that equilibrium thermodynamics are independent of model or pathway.

(B) Colligative Effects of Cosolvents (Osmolytes). A cosolvent is any chemical compound that is added to water at high concentration (0.2−10 M), e.g., an osmolyte, such as sucrose, glycerol, or urea. The term cosolvent stems from the fact that the additives of interest, e.g., osmolytes, can

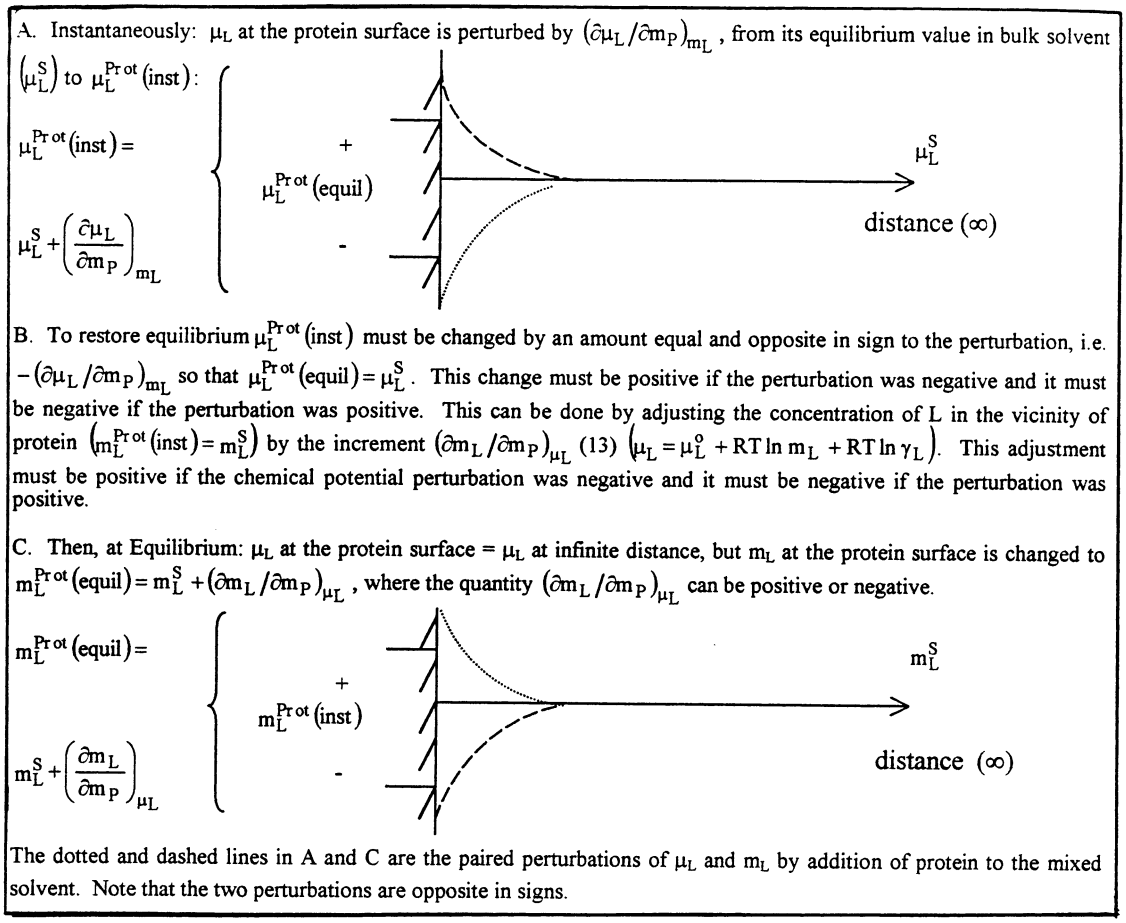

FIGURE 2: Thermodynamic consequences of adding a protein to a water−cosolvent mixture.

occupy up to 40 or 50% of the solvent volume. Hence, they are equivalent to water. In our treatment, we will take water as the principal solvent and measure the binding of cosolvent to protein in an aqueous medium. Symmetrically and equivalently, we could take cosolvent as principal solvent. Then, the reference state would be fully solvated protein in pure cosolvent, and water would be the added ligand. Let us examine thermodynamically the consequences of the addition of a cosolvent to pure water. At constant temperature and pressure, which will be maintained throughout this discussion, the activity of pure water is "one", $a_W = 1$. The chemical potential of pure water is $\mu = \mu^\circ + RT \ln a_W = \mu^\circ$. Addition of any cosolvent to water lowers the activity of water to $a_w^{cs} < 1$ and its chemical potential to $\mu_w^{cs} = \mu^\circ + RT \ln a_w^{cs}$ by the general colligative effect. Then:

$$\Delta\mu_w = \mu_w^{cs} - \mu^\circ = RT \ln a_w^{cs} = RT \ln(p_w/p_w^\circ) = -\bar{V}\pi = -RT(m_{cs}/55.5)\phi^{cs} \quad (2)$$

The colligative lowering of water activity is manifested by the following (*11*): (1) Lowering of the partial vapor pressure of water in the presence of solute (p_w) from the vapor pressure of pure water (p_w°), which, as a consequence, (2) generates an osmotic pressure, π, across a semipermeable membrane and causes the lowering of the freezing point and the raising of the boiling point [$\bar{V}$ is the partial molar volume of water, R is the universal gas constant, and T is the thermodynamic (degrees kelvin) temperature]. The magnitude of the effects is defined by the osmolality, i.e., the product of the cosolvent concentration in molal units (m_{cs}) and its osmotic coefficient, ϕ^{cs}, which is a measure of its departure from ideality. At any value of osmolality, all solutes exercise an identical effect on the activity of water and on the colligative properties.

(C) Perturbation of Cosolvent Chemical Potential by Protein Defines Thermodynamic Binding. Now, let us add the cosolvent to an aqueous solution of protein. What are the thermodynamic consequences? (1) The water activity is lowered by the colligative effect to a_w^{cs}; (2) cosolvent molecules enter into interactions with the protein.

Let us examine this in terms of the reciprocal, but equivalent, operation of adding a protein molecule to a water−cosolvent mixture, at a water activity a_w^{cs}. The immediate effects are (*12*) as follows: (1) the protein becomes hydrated; (2) it interacts with the cosolvent, i.e., the chemical potential of the cosolvent (ligand, L) becomes perturbed by the protein, $(\partial\mu_L/\partial m_P)_{m_L}$, and reciprocally that of the protein by the cosolvent, $(\partial\mu_P/\partial m_L)_{m_P} = (\partial\mu_L/\partial m_P)_{m_L}$.[3] This is the thermodynamic definition of interaction (*13*). As explained on Figure 2, these mutual perturbations of the chemical potentials have, as a necessary consequence, the redistribution of solvent components in the vicinity of the protein such that, at any given bulk solvent composition, m_L, the ligand concentration in the vicinity of the protein differs from that of bulk solvent by $(\partial m_L/\partial m_P)_{\mu_L}$ (*6*, *14*). This can

[3] The subscripts W, P, and L refer to water, protein, and ligand (cosolvent), respectively.

be accomplished by addition or removal of cosolvent, removal or addition of water, or both. The quantity $(\partial m_L/\partial m_P)_{\mu_L}$ is thermodynamic binding (*13*). It is the binding measured experimentally at osmotic ≅ dialysis equilibrium, expressed by $\bar{\nu}$ in Scatchard notation (*8*) and by Γ_{23} in Cassassa and Eisenberg (*14*) and Schellman notations (*13, 15*).[4] We will denote it by Γ_{PL}. It is the expression in molecular terms of the mutual thermodynamic pertubations of the protein and cosolvent (*6*):

$$\Gamma_{PL} = (\partial m_L/\partial m_P)_{\mu_L} = \bar{\nu} = \Gamma_{23} = \{-(\partial \mu_L/\partial m_P)_{m_L}/(\partial \mu_L/\partial m_L)_{m_P}\} = -(\partial \mu_P/\partial \mu_L)_{m_P} \quad (3)$$

Figure 2 and eq 3 state that when the interaction parameter, $(\partial \mu_L/\partial m_P)_{m_L}$, is negative, i.e., the interaction is favorable, the measured binding $(\partial m_L/\partial m_P)_{\mu_L}$ is positive (there is an excess of cosolvent in the domain[5] of the protein relative to bulk solvent). Reciprocally, when $(\partial \mu_L/\partial m_P)_{m_L}$ is positive, i.e., the interaction is unfavorable, the measured binding is negative (there is a deficiency of cosolvent in the domain of the protein relative to the bulk solvent).

(D) Thermodynamic Binding Is Water−Cosolvent Exchange. Now, an excess (or deficiency) of ligand (cosolvent) relative to its bulk solvent concentration requires a deficiency (or excess) of water, by the simple physical principle that two objects cannot occupy the same space at the same time. Any molecule of cosolvent present in the system must have replaced water within the particular volume element. In pure water, this gives rise to the colligative effects. However, replacement by cosolvent of a water molecule that is affected thermodynamically by protein must involve some additional free energy contribution, the sign and magnitude of which are determined by whether the interaction at the particular locus is more or less favorable for the cosolvent than for water and by how much. Even the replacement of a water molecule which is only momentarily affected by the protein without making a contact, or of one which is not affected by the protein in a locus where the ligand is affected, will involve a free energy contribution over and above the statistical colligative effect. The water−ligand exchange has been examined by Schellman, who has, in a series of important papers (*13, 15−20*), treated it in terms of the equivalence of the two solvent components (water and cosolvent) (*19*).

In aqueous medium, the reference state of the protein is fully hydrated protein. The binding of a ligand (cosolvent) molecule must involve the replacement of water molecules. Therefore, the measured free energy of binding, ΔG_b°, is an exchange free energy, ΔG_{ex}°. It is the difference between the hypothetical[6] free energies of binding of cosolvent, ΔG_L°, and water, ΔG_W°, respectively, to a dry site on the protein dissolved in the mixed solvent (*21, 22*):

$$\Delta G_{ex}^\circ = \Delta G_L^\circ - \Delta G_W^\circ \quad (4)$$

Schellman (*13, 18, 19*) has shown that the binding constant measured for the reaction, $P{\cdot}nH_2O + L \rightleftharpoons PL + nH_2O$, is an exchange constant:

$$K_{ex} = K_L/K_W = ([PL][H_2O]_n)/([P{\cdot}nH_2O][L]) \quad (5)$$

where K_W and K_L are the equilibrium constants for the hypothetical[6] reactions:

$$\text{Prot (dry)} + nH_2O \rightleftharpoons \text{Prot}{\cdot}nH_2O\ (K_W); \qquad \text{Prot (dry)} + L \rightleftharpoons \text{Prot}{\cdot}L\ (K_L) \quad (6)$$

Treating explicitly the simple model in which one water molecule is replaced by one cosolvent molecule at a totally independent site on the protein, Schellman has derived the dependence of the thermodynamic binding on the exchange constant, K_{ex} (*13, 15, 18, 19*):

$$\left(\frac{\partial m_L}{\partial m_P}\right)_{\mu_L}^{\text{per site}(i)} = \frac{(K'^{(i)}_{ex} - 1/m_W)m_L}{(1 + K'^{(i)}_{ex} m_L)} = \frac{(K^{(i)}_{ex} - 1)X_L}{1 + (K^{(i)}_{ex} - 1)X_L} \quad (7)$$

where K'_{ex} and K_{ex} are exchange constants in molal and mole fraction units,[7] respectively, and X_L is the mole fraction of ligand. The use of cosolvents at high concentration means that the interactions are weak, $K_L \approx K_W$, and K_{ex} hovers around 1, which by eq 7 explains the measurement of negative binding stoichiometries with positive binding constants, K_{ex}. The experimentally measured binding is the sum of interactions at all *n* loci[8] (*12, 13, 18*):

$$\Gamma_{PL} = \left(\frac{\partial m_L}{\partial m_P}\right)_{\mu_L} = \sum_{i=1}^{n}\left(\frac{\partial m_L}{\partial m_P}\right)_{\mu_L}^{i} = -\sum_{i=1}^{n}\left(\frac{\partial \mu_P}{\partial m_L}\right)_{m_P}^{(i)} \Big/ \left(\frac{\partial \mu_L}{\partial m_L}\right)_{m_P} \quad (8)$$

The protein surface is a mosaic of chemically heterogeneous loci,[8] each of which has its individual affinity for water and cosolvent. The sum Γ_{PL} is composed of positive and negative $\left(\frac{\partial m_L}{\partial m_P}\right)_{\mu_L}^{(i)}$ contributions of a variety of magnitudes. There are also neutral loci on the protein surface with $K_{ex}^{(i)} = 1$ and $\left(\frac{\partial m_L}{\partial m_P}\right)_{\mu_L}^{(i)} = 0$.

This pattern of exchange, when summed over the entire protein molecule, is what generates the measured overall positive and negative binding stoichiometries and leads to the terms preferential binding, preferential hydration, and preferential exclusion (*12, 14, 18*). The epithet preferential simply indicates that the protein has a higher affinity for one solvent component than for the other one. As depicted

[4] The subscripts 1, 2, and 3 refer to water, protein, and ligand, respectively, in Scatchard notation (*8*).

[5] Domain means the volume around a protein molecule over which it influences thermodynamically molecules of solvent components. We definitely do not imply the existence of any shell or well-defined compartment around the protein molecule that has physical reality.

[6] The reactions are hypothetical equilibria, since we obviously cannot have a dry site on the protein immersed in the solvent (water or cosolvent).

[7] K_{ex} and $\Delta K'_{ex}$ of eq 7 are apparent (practical) equilibrium constants, which neglect solvent nonideality. Therefore, they vary with solvent composition (*15, 19*).

[8] The term locus does *not* designate a well-defined reactive site on the surface of the protein, such as a binding site for a biological effector. It means simply an element of volume that can accommodate molecules of cosolvent or the corresponding number of water molecules. Loci may fluctuate in number and geometrical disposition on the protein surface, as dictated by any given water−cosolvent system. Furthermore, there may be interacting loci in the solvent volume neighboring the protein, but which do not make contacts with its surface.

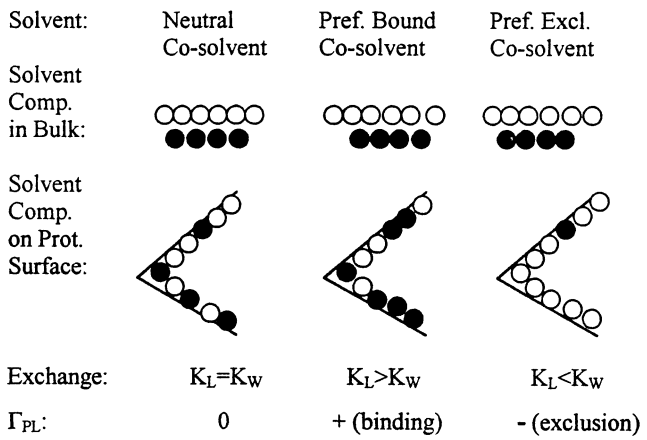

FIGURE 3: What is the binding at the protein surface for cosolvents? It is the difference between the solvent compositions at the protein surface and in the bulk: (○) water, (●) cosolvent.

in Figure 3,[9] this leads to three possible situations: (1) preferential binding; (2) preferential exclusion; (3) thermodynamic indifference, i.e., neutrality. In the case of neutrality (binding = 0), the concentration of ligand "on the protein" is equal to its concentration in the bulk solvent (*17*).

The principles of water−cosolvent exchange (*13, 18, 21, 22*) and solvent component equivalence (*19*) lead to a reciprocity principle: The excess of either solvent component in the vicinity of the protein can be expressed in terms of the deficiency of the other component. The thermodynamic binding of ligand (whether positive or negative) can be expressed equivalently in terms of the binding of water, $\Gamma_{21} = \Gamma_{PW}$, as (*23*)

$$\Gamma_{21} = \Gamma_{PW} = -(m_W/m_L)\Gamma_{PL} = -(m_W/m_L)(\partial m_L/\partial m_P)_{\mu_L} \quad (9)$$

When the thermodynamic binding of ligand is negative, Γ_{PW} is preferential hydration (preferential binding of water); equivalently, a positive value of ligand binding is preferential exclusion of water, $m_L\Gamma_{PW} = -m_W\Gamma_{PL}$.

(E) Symmetry between Binding and Exclusion. The principles of exchange and reciprocity lead to the realization that thermodynamic binding is a symmetrical function about the point at which contacts of the protein with cosolvent and water are strictly statistical. As pointed out by Schellman (*17, 18*), this defines the point of thermodynamic indifference. Hence, a cosolvent solution of that particular composition may be regarded as neutral with respect to the protein, since the protein is indifferent to whether the contacts are with molecules of the cosolvent or of water. It must be emphasized that contact per se does not mean binding in a thermodynamic sense (*17*). The symmetry of thermodynamic binding is described in Figure 4. Symmetrically distributed on the two sides of the point of thermodynamic indifference are equivalent zones of interaction, which differ only in sign. On the left-hand side lies preferential binding of cosolvent of increasing strength, hence, preferential exclusion of water (see eq 9). On the other side lies preferential exclusion of cosolvent of increasing strength and a corresponding increasing preferential hydration. The principle of symmetry means that the perturbation of the chemical potential of cosolvent by protein can attain negative or positive values that are identical in absolute magnitude. Hence, preferentially bound and preferentially excluded cosolvents can exercise equal in magnitude and opposite in sign perturbing effects on protein properties, e.g., their modulation of protein folding (*24*).

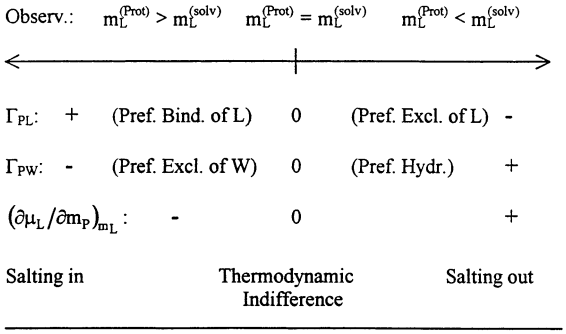

FIGURE 4: Symmetry of thermodynamic binding.

Preferential binding, $(\partial m_L/\partial m_P)_{\mu_L}$, and the chemical potential perturbation, $(\partial\mu_L/\partial m_P)_{m_L}$, refer to a given solvent composition, m_L. The effect of the cosolvent relative to pure water as reference state is expressed by $\Delta\mu_{P,tr}$, the free energy of transferring the protein from water to a solvent of composition m_L. It is related to preferential binding by (*12, 16, 25−28*)

$$\Delta\mu_{P,tr} = \int_0^{m_L}(\partial\mu_P/\partial m_L)_{m_P}\,dm_L = \Delta G_b^\circ = \Delta G_{pb}^\circ = \Delta G_{p.excl}^\circ \quad (10)$$

$\Delta\mu_{P,tr}$ can attain positive and negative values of equal absolute magnitude. The transfer free energy defines also the free energy of binding, ΔG_b°. A negative value of $\Delta\mu_{P,tr} = \Delta G_b^\circ$ means that the state of the protein in the cosolvent solution is more favorable thermodynamically than in pure water. This is manifested by the preferential binding of the cosolvent to the protein, so that $\Delta\mu_{P,tr}$ is, more exactly, the free energy of preferential binding, ΔG_{pb}°. A positive value of $\Delta\mu_{P,tr} = \Delta G_b^\circ$ means that the state of the protein is less favorable thermodynamically in the cosolvent solution than in water. This is manifested by the preferential exclusion of the cosolvent, and $\Delta\mu_{P,tr}$ is the free energy of preferential exclusion, $\Delta G_{p.excl}^\circ$, which is the amount of free energy that must be expended in order to remove statistically distributed ligand molecules from the protein domain and replace them with water (*12, 28*). The principle of symmetry applies also to the free energy of binding, ΔG_b°, with a distribution of positive and negative values on either side of the point of neutrality, $\Delta G_b^\circ = 0$. Consideration of eq 10 in terms of

[9] In this and subsequent schematic representations of the physical process (Figures 5 and 6), water and cosolvent (osmolyte) molecules have been drawn as equal in size and replacing each other on a one-to-one basis. The reason for this representation is that it conveys in a simple and lucid manner a description of "what is happening" and helps in thinking about the general process. In the real situation, cosolvent molecules, being larger, displace several water molecules, some of which do not make contact with the protein surface. This is implied in the exchange equation (eq 5 and accompanying discussion). The same simple model has been used by Schellman (*18, 19*) to explain in an easy-to-grasp way how negative binding can arise from positive values of the exchange constant (eq 7). Other models, which take into account replacement of several water molecules by one ligand molecule or cooperativity between molecules, will lead to more complicated binding equations. The fundamental thermodynamic principles (see Figure 2) and the thermodynamic equations (e.g., eq 3 and others that follow) are independent of model or pathway and are rigorously valid for the real situation.

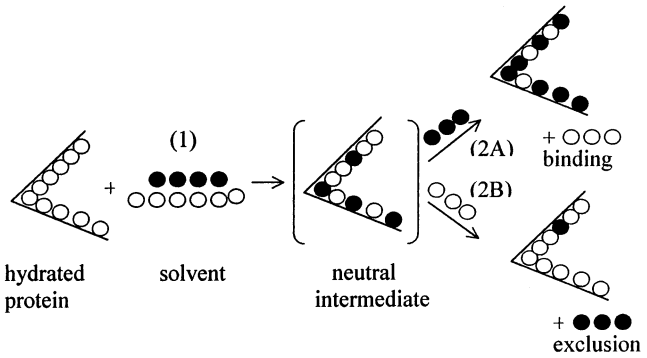

FIGURE 5: Equivalence of preferential binding and preferential exclusion. Step 1: Binding of cosolvent at the same composition as in bulk solvent, thermodynamically indifferent, $\Delta G^\circ_L - \Delta G^\circ_W = 0$. Step 2: Replacement of solvent component molecules by each other. 2A: Thermodynamically favorable, $\Delta G^\circ_{pb} = -$. 2B: Thermodynamically unfavorable, $\Delta G^\circ_{p.excl} = +$.

symmetry leads to the definition of inertness of a cosolvent toward a protein. An inert cosolvent is one for which $\Delta\mu_{P,tr} = \Delta G^\circ_b = 0$ at all concentrations of the cosolvent, i.e., total thermodynamic indifference (*17*, *26*). This requires $(\partial\mu_P/\partial m_L)_{m_P} = 0$ and $(\partial m_L/\partial m_P)_{\mu_L} = 0$ at all cosolvent concentrations. In this situation, the cosolvent−protein contacts are strictly statistical at all solvent compositions.

Preferential binding is a weak gradual solvent replacement process, dispersed over the entire protein solvent interface, and ΔG°_b is a continuous function of ligand concentration defined by the given protein−cosolvent pair. Schellman has shown that (*13*, *18*)

$$\Delta G^\circ_b = -RT\sum_{i=1}^{n}\ln\left(A_W + K^{(i)}_{ex}A_L\right) \qquad (11)$$

where A_W and A_L are the mole fraction activities of water and cosolvent at each solvent composition. ΔG°_b is a complex function of cosolvent concentration: it is a summation over all loci on the protein surface, each characterized by its particular value of the exchange constant, $K^{(i)}_{ex}$, positive, negative, or zero, and which reach saturation at different cosolvent concentrations. The variation of the interaction parameters can assume complicated nonlinear forms, which may pass through maxima and minima and changes of sign with variation in cosolvent concentration (*12*). These may be caused by the solvent composition variations of A_W and A_L (*15*, *19*).

The thermodynamic equivalence and symmetry of binding and exclusion are illustrated in Figure 5, where the protein−cosolvent interactions are treated as a hypothetical two-step process. In a first step, a hydrated protein surface element is immersed into the mixed solvent in a hypothetical thermodynamically indifferent process: Part of the water molecules become replaced statistically by cosolvent molecules, making the solvent composition in the protein domain[5] identical with that of bulk solvent, $K_{ex} = 1$. Protein−cosolvent interaction specificity is introduced in the second step: in nonneutral events, some molecules of one solvent component are removed and replaced by the other solvent component. In step 2A, water molecules are replaced by cosolvent molecules ($K_{ex} > 1$). Water being the principal solvent, this is a thermodynamically favorable process, with a negative free energy of binding, ΔG°_{pb}. In step 2B, some of the cosolvent molecules are removed from the protein surface and replaced by water molecules ($K_{ex} < 1$). This step has a positive free energy change, which is the free energy of exclusion $\Delta G^\circ_{p.excl}$. Had we taken the cosolvent as principal solvent and water as ligand, the signs of the free energy changes would have been inverted (exclusion of water in step 2A and binding of water in step 2B).

(F) Is the Water of Preferential Hydration, W_{PH}, Related to the Water of Hydration, W_H? The mixed solvents most pertinent to biological systems are aqueous solutions of osmolytes, e.g., glycerol, sucrose, glycine, betaine, sorbitol. These are preferentially excluded from proteins (*12* and references cited therein), and $(\partial m_L/\partial m_P)_{\mu_L} = \Gamma_{PL}$ is negative. By eq 9, Γ_{PW} is positive; this preferential hydration can be expressed in terms of moles of water preferentially bound per mole of protein, $W_{PH} \equiv \Gamma_{PW}$.

Now, what is the relation between W_{PH} and W_H, i.e., between moles of water of preferential hydration and moles of water of hydration? Careful reflection over the chemical origins of W_H and W_{PH} leads to the conclusion that there is NO direct relationship between these quantities because they refer to chemically different processes. W_H is a measure of water interactions with a protein due to protein surface−water forces in a pure binary protein−water system. In the protein−solvent interaction equation (eq 4), W_H is a function of ΔG°_W (K_W of eq 6) alone, as ΔG°_L does not exist. It changes little with changes in solvent composition.[10] W_{PH}, on the other hand, requires the presence of a third component, the cosolvent, in the absence of which W_{PH} cannot exist, while W_H is always present. For any given cosolvent, the magnitude of Γ_{PL} and, hence, of $\Gamma_{PW} = W_{PH}$ (eq 9) is a direct function of ΔG°_{ex}, the magnitude of which, by eq 4, is determined by the value of the corresponding ΔG°_L because ΔG°_W remains essentially constant. Hence, the magnitudes of W_H and W_{PH} are determined each by one of the two independent terms on the right-hand side of eq 4. Furthermore, the chemical interaction that occurs on addition of the osmolyte (cosolvent) and generates W_{PH} is the mutual perturbation of the chemical potentials of the osmolyte and the protein by each other, $(\partial\mu_L/\partial m_P)_{m_L} = (\partial\mu_P/\partial m_L)_{m_P}$ measured as $\Gamma_{PL} = -(m_L/m_W)\Gamma_{PW}$. Then, by eqs 3 and 9:

$$W_{PH} = \Gamma_{PW} = \{(m_W/m_L)(\partial\mu_L/\partial m_P)_{m_L}\}/\{(\partial\mu_L/\partial m_L)_{m_P}\} \qquad (12)$$

Equation 12 states that the magnitude of W_{PH}, i.e., the preferential hydration, is established by the extent of the perturbation of the chemical potential of the ligand (cosolvent, osmolyte) by the protein (*12*). Symmetrically with eq 10, we can define the free energy of preferential binding or exclusion of the osmolyte as the transfer free energy of the osmolyte from pure water to a water−protein mixture of a given composition, m_P:

$$\Delta\mu_{L,tr} = \int_0^{m_P}(\partial\mu_L/\partial m_P)_{m_L}\,dm_P = \Delta G^\circ_{pb} = \Delta G^\circ_{p.excl} \qquad (13)$$

Combination of eqs 12 and 13 leads to

$$W_{PH} = \Gamma_{PW} = \{(55.56/RT)(\partial\Delta\mu_{L,tr}/\partial m_P)\}/\{1 + m_L[\partial \ln\gamma_L/\partial m_L]_{m_P}\} \qquad (14)$$

Therefore, at identical osmolalities of different cosolvents, the sign and magnitude of W_{PH} are dictated by the particular protein−cosolvent pair, i.e., by $\Delta G^{\circ}_{pb} = \Delta G^{\circ}_{p.excl}$. For example, for RNase A, at identical osmolalities of ligands, W_{PH} is positive for trehalose, glycine, betaine, and glycerol, but its numerical values differ for the various osmolytes (*12* and references cited therein). It is clear that $\boldsymbol{W_{PH}}$ **is** ***NOT*** a measure of protein−water interaction, but only an expression in terms of water molecules of the strength of the protein−cosolvent interaction in an aqueous medium. While W_{PH} and W_H are independent phenomena, and neither concept requires contact with the protein, they both occur within the volume element neighboring to a protein molecule. Logic tells us that they should overlap and that the same water molecules may be counted physically in both W_{PH} and W_H. Yet, there is no rigorous way of translating knowledge of one into direct information on the other. Nevertheless, it seems reasonable to assume, as a first working hypothesis, that the equality of values of $\Gamma_{PW} = W_{PH}$ and W_H points to a strong overlap of the water molecules involved (*29*). The physical situation is depicted schematically in Figure 6, in which the same protein surface with constant hydration, W_H (shown in diagram A), is immersed, in turn, into differently interacting cosolvents of composition B. In part C, scheme 1 is the case of neutrality: some waters of hydration are replaced statistically by ligand, as the protein is indifferent to contact with water or ligand. In schemes 2 and 3, ligand molecules replace nonstatistically hydration water molecules (C2) and some bulk solvent water molecules (C3) because the protein has a stronger to much stronger affinity for ligand than for water, resulting in preferential binding of increasing magnitude. In schemes 4−6, few ligand molecules can penetrate in the vicinity of the protein because the protein has a stronger affinity for water than for ligand, or there is even repulsion of the ligand that leads to perturbation of solvent composition beyond the water of hydration (diagram C6). As a consequence, what in the binary system (diagram A), were bulk water molecules unaffected by the protein, may become affected by the protein−ligand interaction in the ternary system and be identified as waters of preferential hydration (second vertical row in diagram C6)?

This gradation of interactions between the protein and aqueous cosolvents (osmolytes) permits us to align them on a single scale, shown in Figure 7. We note that, although the hydration of the given protein (W_H) remains constant, the preferential hydration (W_{PH}) varies from highly negative values at the top of the scale to increasing positive values at the bottom of the scale, after passing through zero at the point of neutrality, i.e., of thermodynamic indifference.

(G) Nonthermodynamic Expression of Preferential Interactions: Site Occupancy. The definition of thermodynamic binding as the sum of all the water−cosolvent exchanges over the entire protein surface leads to the following question: what are the total numbers of water and cosolvent molecules that interact with the protein? These molecules may be located at protein loci that are favorable to water or to cosolvent or are neutral ($K^{(i)}_{ex}$ <1, >1, or =1). Formally, it is possible to decompose $(\partial m_L/\partial m_P)_{\mu_L}$ into total numbers

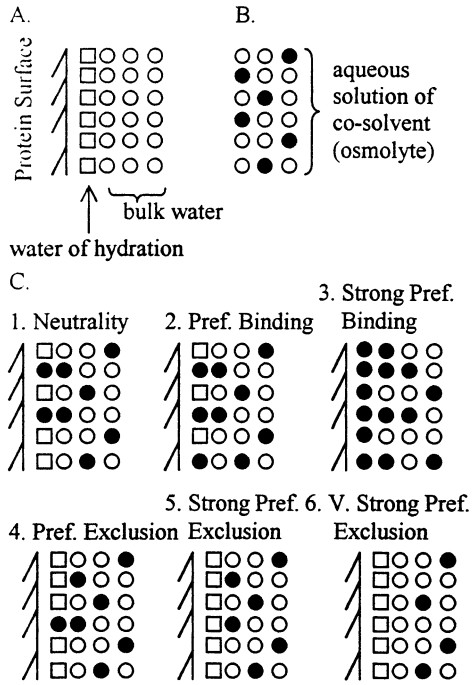

FIGURE 6: Molecular distributions of water and osmolytes in preferential interactions. The difference between protein hydration and preferential hydration. (A) The reference state: protein in pure water: (□) waters of hydration; (○) bulk solvent waters. (B) Mixed solvent in which the protein will be dissolved: (○) water; (●) cosolvent (L). (C) Replacement in the protein solution of water molecules (□,○) by ligand molecules (●). The consequences may be: 1. Neutrality: Concentration of L identical throughout the system, from protein surface to infinity; $K_{ex} = 1$; $\Delta G_b = 0$; $(\partial\mu_L/\partial m_P) = 0$. 2. Pref. Binding: Concentration of L close to protein > than in bulk solvent. (Replacement of some waters of hydration by L, with $K_{ex} > 1$; $\Delta G_b = -$; $(\partial\mu_L/\partial m_P) = -$.) 3. Strong Pref. Binding: Concentration of L close to protein ≫ than in bulk solvent. (Replacement of waters of hydration plus some "next layer" waters by L, with $K_{ex} \gg 1$; $\Delta G_b = --$; $(\partial\mu_L/\partial m_P) = --$.) 4. Pref. Exclusion: Concentration of L close to the protein is less than in the bulk solvent. (Replacement of some waters of hydration by L, with $K_{ex} < 1$; $\Delta G_b = +$; $(\partial\mu_L/\partial m_P) = +$.) 5. Strong Pref. Exclusion: There is no replacement of waters of hydration by L: $K_{ex} \ll 1$; $\Delta G_b = ++$; $(\partial\mu_L/\partial m_P) = ++$. 6. V. Strong Pref. Exclusion: There is no replacement of waters of hydration by L, which is excluded from the protein to an extent greater than W_H, i.e., from some "bulk water": $K_{ex} \ll 1$, $\Delta G_b = +++$; $(\partial\mu_L/\partial m_P) = +++$.

of water and cosolvent molecules that interact with the protein. There exist several derivations of the pertinent relation (*19, 29*−*35*). While formally correct, these derivations are intuitive and not thermodynamically rigorous. Let B_L and B_W express the effective numbers of ligand and water molecules that make contact with the protein, respectively (*12*). The derived relation is

$$(\partial m_L/\partial m_P)_{\mu_L} = \Gamma_{PL} = B_L - (m_L/m_W)B_W \qquad (15)$$

As will be shown now, B_L and B_W are not thermodynamic quantities (*15, 26*) nor do they have a defined physical meaning (*30*). B_L and B_W are <u>not</u> actual numbers of ligand and water molecules in contact with the protein, but they are <u>effective numbers</u> which represent the summations over all the interactions of various strengths between the protein and the solvent component molecules, ranging from complexation to momentary perturbation. The total mutual

[10] Added cosolvents may affect protein hydration, W_H, by, e.g., lowering the dielectric constant of the medium by an extent that is sufficient to affect significantly the ionization of charged groups.

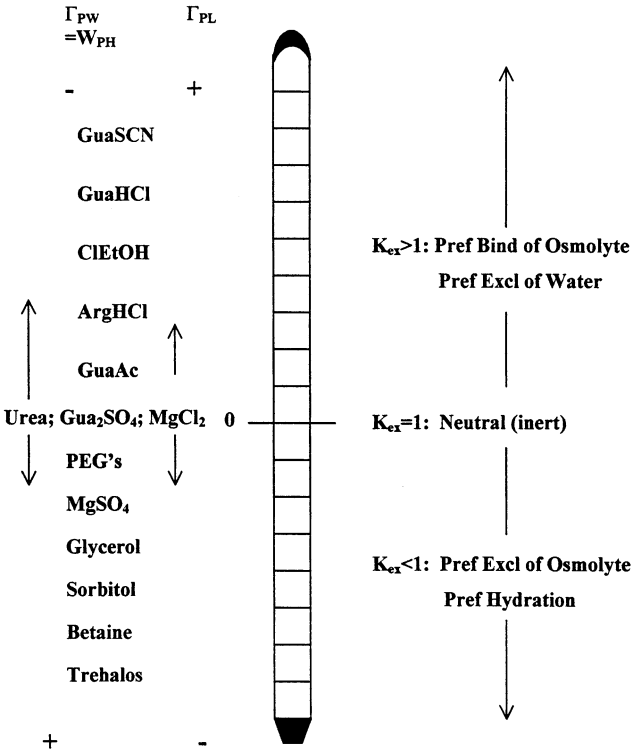

FIGURE 7: Preferentially interacting osmolytes. For any given protein, K_W is independent of the solvent system, and W_H remains constant. Therefore, at identical osmolality of cosolvents, their location on this scale is defined solely by K_L because $K_{ex} = K_L/K_W$.

thermodynamic perturbations of the protein and cosolvent are the sum of perturbations of all cosolvent molecules affected by the protein (eq 8). Application of eq 4, i.e., the formal definition of the exchange free energy at any locus *i*, $\Delta G^{\circ(i)}_{ex} = \Delta G^{\circ(i)}_{L} - \Delta G^{\circ(i)}_{W}$, gives (*12*)

$$(\partial\mu_p/\partial m_L)^{(i)}_{m_p} = \left(\frac{\partial\Delta G^{\circ}_{ex}}{\partial m_L}\right)^{(i)}_{m_p} = \left(\frac{\partial\Delta G^{\circ}_{L}}{\partial m_L}\right)^{(i)}_{m_p} - \left(\frac{\partial\Delta G^{\circ}_{W}}{\partial m_L}\right)^{(i)}_{m_p} \quad (16)$$

The two terms on the right-hand side of eq 16 lead formally to the effective numbers of cosolvent (B_L) and water (B_W) molecules that interact with protein and give rise to eq 15. The operation of eq 16, while formally correct, refers to physically impossible situations, and, therefore, it has no physical meaning, as pointed out by Tanford in his analysis of the same question in terms of equilibrium constants (*30*). Therefore, B_L and B_W have no defined physical meaning (*30*) and are not thermodynamic quantities (*15, 26*). They are summations at any instant of the fluctuating contributions at all the *n* sites: $B_L = \sum_{i=1}^{n} B_L^{(i)}$ and $B_W = \sum_{i=1}^{n} B_W^{(i)}$, where $B_L^{(i)}$ and $B_W^{(i)}$ may vary between 0 and 1. They are only effective parameters that may be useful in a qualitative description of the experimental data, but they do not describe the real molecular situation.

(H) Is B_W Related to W_H? The cautious use of B_W and B_L may be of help in a descriptive elucidation of the molecular picture and in attempts to answer questions, such as: Is a given preferentially excluded cosolvent totally prevented from making contacts with the protein surface? Can B_W be numerically equated with W_H in some particular case? What are the relative exclusions of various osmolytes, expressed as "numbers of water molecules on the protein surface"? The usual approaches (*12, 29*) have been to compare values of B_W deduced by setting B_L equal to zero with values of W_H derived independently from, e.g., transport experiments (*1*) or NMR (*3*). At times, the equality of such calculated B_W with W_H has been interpreted as total exclusion of cosolvent from the protein surface (*29, 36*) with the tacit implication that this is the maximal exclusion possible. This, however, presents problems. First, it implies that only waters of hydration may be replaced nonneutraly by osmolyte molecules. This is incorrect because the nature of the water molecules replaced in the protein−osmolyte mutual chemical potential perturbation is immaterial to the process. Second, B_W and B_L are not unique pairs of numbers for a given value of Γ_{PL}; they cannot be measured independently. The only measured quantity is Γ_{PL}, and the assignment of any value to B_L fixes B_W, and vice versa [$B_L = \Gamma_{PL} + (m_L/55.56)B_W$]. As a consequence, any pair of B_L and B_W values derived for a given value of Γ_{PL} carries an uncertainty of $\pm n$ in B_L and $\pm n(55.56/m_L)$ in B_W, where *n* can be any number (*12, 28, 37*).

Therefore, just as is the case for W_{PH}, there is no fundamental principle that permits us to equate B_W with W_H. To illustrate the preceding discussion, let us look at the thermodynamic interactions of RNase A with several osmolytes. The hydration calculated by the Kuntz NMR procedure (*3*) and from vapor pressure measurements of Bull and Breese (*38*) has a value of 0.330−0.360 g of water per gram of protein, which gives 250−275 H_2O/RNase (*39, 40*). Let us compare this value of W_H with some experimentally measured values of W_{PH}. For the series of sugars glucose (*41*), lactose (*41*), sucrose (*36*), and trehalose (*42*), the values of W_{PH} are 130, 170, 350, and 400 molecules of water per molecule of protein. Glucose and lactose appear to penetrate to the protein surface. Sucrose and trehalose are more than fully excluded if maximal exclusion, i.e., maximal preferential hydration, is equated with W_H. For sorbitol (*40*), Γ_{PW} varies from 440 H_2O/RNase at 0.6 M to 240 H_2O/RNase at 3 M, which suggests that it is fully excluded with a large "second layer contribution" at low concentration. A similar situation pertains to 2-methyl-2,4-pentanediol (MPD), for which preferential hydration attains a value of 800 molecules of water per molecule of RNase (*43*). In their critical analysis of preferential hydration, Courtenay et al. (*29*) have identified glycine betaine as approaching full exclusion ($B_L = 0$) from bovine serum albumin on the basis of the near-equality between W_{PH} and W_H. Nevertheless, they caution about the practice of identifying changes in W_{PH} with changes in water of hydration.

The large variations of $W_{PH} = \Gamma_{PW}$ for different proteins and cosolvents are a consequence of the variety of causes of the exclusion (repulsion) of the cosolvent from the surface of the protein. These include surface free energy (surface tension) perturbation (*12, 36, 41*), repulsion from charged loci (*43*), steric exclusion (*44, 45*), and solvophobicity (*39, 46, 47*). Bolen and co-workers (*48, 49*) have shown that a variety of osmolytes are specifically preferentially excluded from some amino acid residues and strongly so from peptide linkages, which led them to identify the major biological protein-stabilizing mechanism of osmophobicity (*50*).

(I) Modulation of Protein Reactions by Osmolytes. How does an osmolyte modulate (promote or inhibit) a reaction which involves a change in W_H, e.g., the uptake of some waters of hydration, $\Delta W_H = n$:P + nH$_2$O ⇌ P*·nH$_2$O? Let us consider the protein transition, P ⇌ P*, as the opening of a crevasse that exposes to contact with solvent a previously buried surface, while the solvation of the remainder of the molecule remains identical in the two end states of the reaction. Let us decompose this process into two hypothetical steps: first, a solvent-independent protein transition (in a hypothetical dry state). This must be identical for all solvents. In the second step, let us solvate the newly exposed surface with mixed solvents of various compositions (such as those depicted in Figure 6). Then, we have

$$P \overset{K_{prot}}{\rightleftharpoons} [P^*(dry)] \overset{K_{solv}}{\rightleftharpoons} P^*(solvated);\ K_{prot}K_{solv} = K_{exp} \quad (17)$$

$$\Delta G^\circ_{exp} = \Delta G^\circ_{prot} + \Delta G^\circ(solv) = \Delta G^\circ_{prot} + \Delta G^\circ_W + \Delta G^\circ_{ex} \quad (18)$$

where K_{exp} is the experimentally measured equilibrium constant in a given solvent and ΔG°_{exp} is the corresponding free energy change; ΔG°_{prot} and K_{prot} are the free energy change and equilibrium constant of the intrinsic protein reaction (in the hypothetical dry state); they remain constant in all solvent systems; ΔG°(solv) and K_{solv} are the free energy change and equilibrium constant of solvating the newly exposed surface. Applying reasoning similar to that of Figure 5, in a mixed solvent let us first solvate the newly exposed surface with water, ΔG°_W, then replace water molecules by cosolvent molecules in an exchange process, each cosolvent having its own characteristic value of the exchange free energy, ΔG°_{ex}. Then, $\Delta G^\circ(solv) = \Delta G^\circ_W + \Delta G^\circ_{ex}$. By eq 18, the difference between the measured free energy changes in two different cosolvents, i.e., the difference in their ability to modulate a reaction, is then given by

$$\delta\Delta G^\circ_{exp} = \Delta G^\circ_{ex}(\text{solvent 1}) - \Delta G^\circ_{ex}(\text{solvent 2}) \quad (19)$$

Equation 18 also states that for a cosolvent to modulate a reaction, i.e., for ΔG°_{exp} to be different from its value in water ($\Delta G^\circ_{prot} + \Delta G^\circ_W$), ΔG°_{ex} must have a nonzero value; i.e., there must be either preferential binding or preferential exclusion. Such a cosolvent must necessarily modulate the protein reaction by the change in the free energy of preferential interaction, $\delta\Delta G^\circ_b$, during the course of the protein transition as has been discussed in detail previously (*12, 28*). A thermodynamically neutral cosolvent, $\Delta G^\circ_{ex} = 0$, whose sole effect is the lowering of water activity can have no effect on the reaction: it must be inert, except in some very special cases (*28, 51*).

(J) What Is the Water Detected by the Cosolvent Perturbation of a Reaction? While cautious estimation of the degree of exclusion of osmolytes by comparison of W_{PH} with W_H is a valid procedure (*29*), the identification of W_{PH} (or B_W) with W_H and of perturbations of W_{PH} with perturbations of W_H is precarious and replete with uncertainties. The direct determination of the number of stoichiometric water molecules, n, in a reaction (eq 1) is extremely difficult and frequently impossible. Attempts have been made to probe the involvement of water in a reaction (*36, 37, 52*−*54*) by perturbing the equilibrium with cosolvents and plotting the results in terms of the Wyman linkage equations:

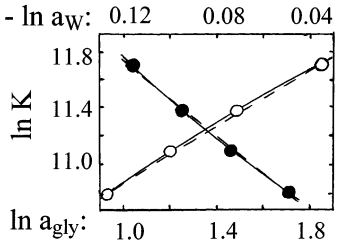

FIGURE 8: Wyman plots of the effect of glycerol, between 2 and 4 M, on the addition of one $\alpha\beta$-tubulin dimer to a growing microtubule (*37*): (○) eq 20 (ln K vs ln a_{gly}); (●) eq 21 (ln K vs ln a_W). Dashed line: best straight line through the points; full line: best curve joining the points (the slight curvature is symptomatic of the departure of both solvent components during the course of the reaction).

$$(\text{d log } K/\text{d log } a_L) = \Gamma^{Prod}_{PL} - \Gamma^{React}_{PL} = \delta\Gamma_{PL} = \delta B_L - (m_L/m_W)\delta B_W \quad (20)$$

$$(\text{d log } K/\text{d log } a_W) = \Gamma^{Prod}_{PW} - \Gamma^{React}_{PW} = \delta\Gamma_{PW} = \delta B_W - (m_W/m_L)\delta B_L \quad (21)$$

where a_L and a_W are the activities of cosolvent and water, respectively. The further identification of the slope of eq 21 with the number of stoichiometric water molecules, n, i.e., setting $\delta\Gamma_{PW} = n = \delta B_W = \delta W_H$, as has been done at times (*53, 54*), can lead to serious errors. First, $\delta\Gamma_{PW}$ is the change in preferential hydration (δW_{PH}), which involves both water and cosolvent molecules, while n is the total change in the number of waters of hydration (δW_H). Second, even if δB_L is assumed to be equal to zero (eq 21), the resulting value of δB_W can be greater or smaller than n, just as B_W can be greater or smaller than W_H (see section H). Furthermore, the molecular events in the desolvation during the reduction of a protein surface are complicated (*51*). The desolvation contains contributions from (1) the departing stoichiometric water, n; (2) any additional departing waters of preferential hydration; and (3) any departing cosolvent molecules. There is no way to resolve these. If the formation of the interprotein contact involves the departure of cosolvent (δB_L) as well as water molecules (δB_W), then, by eq 21, each departing cosolvent molecule would mask $55.56/m_L$ departing water molecules. Therefore, by the last equality of eq 21, the measured slope, $\delta\Gamma_{PW}$, can give only the net number of departing water molecules. However, the actual total numbers of departing solvent component molecules (both water and cosolvent) cannot be determined by this approach.

Let us illustrate the meaning of the Wyman slopes of eqs 20 and 21 in terms of the analysis of the effect of glycerol on the microtubule elongation reaction by addition of $\alpha\beta$-tubulin dimers (*37*). The plots, presented in Figure 8, show enrichment of the protein domain by one glycerol molecule, which reflects the net departure of 13 ± 1.5 water molecules. This corresponds to a diminution of surface area of only 122 Å^2 per protomer added, which is unrealistically small for three-dimensional assembly of an organelle. This means that there was an indeterminate number of departing cosolvent molecules, δB_L, which masked $(m_W/m_L)\delta B_L$ departing water molecules δB_W. This renders impossible any conclusion on

ΔW_H. This view has been supported by Courtney et al. (*29*), who have concluded that "any interaction between macromolecular surfaces that involves release of water of hydration will also release small solute molecules present in the water of hydration, and must be analyzed using preferential interaction coefficients".

(K) A Corollary Comment. X-ray crystallographers see often around a protein molecule a halo of lower electron density than that of the bulk solvent (we must recall that protein crystals are ~65% solvent). This is interpreted as a layer of water around the protein molecule. It is then frequently assumed that this layer of water is the water of hydration, W_H. If we apply the above discussion to these observations, we must realize that such a unique conclusion is unwarranted. The solvent in the protein crystals is usually a concentrated salt (Na_2SO_4) or other preferentially excluded cosolvent (PEG, MPD, etc.), and the layer of different electron density seen is the water of preferential hydration, W_{PH} (*55*). Therefore, such X-ray crystallographic data can give no more information on the water of hydration than preferential binding measurements. (Note: this does not apply to tightly bound water molecules which the crystallographic data show in constant orientation with defined space coordinates.) Water of hydration can be measured only in experiments in which the system is binary, protein and water (in practice, dilute buffer). This is a difficult task.

REFERENCES

1. Oncley, J. L. (1943) in *Proteins, Amino Acids and Peptides* (Cohn, E. J., and Edsall, J. T., Eds.) Chapter 22, Reinhold, New York.
2. Tanford, C. (1961) *Physical Chemistry of Macromolecules*, Chapter 6, Wiley, New York.
3. Kuntz, I. D., Jr. (1971) *J. Am. Chem. Soc. 93*, 514−516.
4. Debye, P., and Hückel, E. (1923) *Phys. Z. 24*, 185−206.
5. Smoluchowski, M. V. (1918) *Z. Phys. Chem. (Leipzig) 92*, 129−168.
6. Kirkwood, J. G., and Goldberg, R. J. (1950) *J. Chem. Phys. 18*, 54−57.
7. Stockmayer, W. H. (1950) *J. Chem. Phys. 18*, 58−61.
8. Scatchard, G. (1946) *J. Am. Chem. Soc. 68*, 2315−2319.
9. Kauzmann, W. (1959) *Adv. Protein Chem. 14*, 1−63.
10. Tanford, C. (1970) *Adv. Protein Chem. 24*, 1−95.
11. Glasstone, S. (1947) *Thermodynamics for Chemists*, pp 292−295, Van Nostrand, New York.
12. Timasheff, S. N. (1998) *Adv. Protein Chem. 51*, 355−432.
13. Schellman, J. A. (1978) *Biopolymers 17*, 1305−1322.
14. Casassa, E. F., and Eisenberg, H. (1964) *Adv. Protein Chem. 19*, 287−395.
15. Schellman, J. A. (1993) *Biophys. Chem. 45*, 273−279.
16. Schellman, J. A. (1975) *Biopolymers 14*, 999−1018.
17. Schellman, J. A. (1987) *Annu. Rev. Biophys. Biophys. Chem. 16*, 115−137.
18. Schellman, J. A. (1987) *Biopolymers 26*, 549−559.
19. Schellman, J. A. (1990) *Biophys. Chem. 37*, 121−140.
20. Schellman, J. A. (1994) *Biopolymers 34*, 1015−1026.
21. Katz, S. (1950) Doctoral Dissertation, Harvard University, Cambridge, MA.
22. Kronman, M. J., and Timasheff, S. N. (1959) *J. Phys. Chem. 63*, 629−633.
23. Timasheff, S. N. (1963) in *Electromagnetic Scattering* (Kerker, M., Ed.) pp 337−355, Pergamon, New York.
24. Qu, Y., Bolen, C. L., and Bolen, D. W. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 9268−9273.
25. Vlachy, V., and Lapanje, S. (1978) *Biopolymers 17*, 2041−2043.
26. Timasheff, S. N. (1992) *Biochemistry 31*, 9857−9864.
27. Timasheff, S. N. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 67−97.
28. Timasheff, S. N. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 7363−7367.
29. Courtenay, E. S., Capp, M. W., Anderson, C. F., and Record, M. T., Jr. (2000) *Biochemistry 39*, 4455−4471.
30. Tanford, C. (1969) *J. Mol. Biol. 39*, 539−544.
31. Aune, K. C., Goldsmith, L. C., and Timasheff, S. N. (1971) *Biochemistry 10*, 1617−1622.
32. Inoue, H., and Timasheff, S. N. (1972) *Biopolymers 11*, 737−743.
33. Kupke, D. W. (1973) in *Physical Principles and Techniques of Protein Chemistry* (Leach, S. J., Ed.) Part C, p 1, Academic Press, New York.
34. Reisler, E., Haik, Y., and Eisenberg, H. (1977) *Biochemistry 16*, 197−203.
35. Record, T. M., Jr., and Anderson, C. F. (1995) *Biophys. J. 68*, 786−794.
36. Lee, J. C., and Timasheff, S. N. (1981) *J. Biol. Chem. 256*, 7193−7201.
37. Lee, J. C., and Timasheff, S. N. (1977) *Biochemistry 16*, 1754−1764.
38. Bull, H. B., and Breese, K. (1974) *Arch. Biochem. Biophys. 161*, 665−670.
39. Gekko, K., and Timasheff, S. N. (1981) *Biochemistry 20*, 4667−4676.
40. Xie, G., and Timasheff, S. N. (1997) *Protein Sci. 6*, 211−221.
41. Arakawa, T., and Timasheff, S. N. (1982) *Biochemistry 21*, 6536−6544.
42. Xie, G., and Timasheff, S. N. (1997) *Biophys. Chem. 64*, 25−43.
43. Pittz, E. P., and Timasheff, S. N. (1978) *Biochemistry 17*, 615−623.
44. Arakawa, T., and Timasheff, S. N. (1985) *Biochemistry 24*, 6756−6762.
45. Bhat, R., and Timasheff, S. N. (1992) *Protein Sci. 1*, 1133−1143.
46. Gekko, K., and Morikawa, T. (1981) *J. Biochem. (Tokyo) 90*, 51−60.
47. Gekko, K., and Koga, S. (1984) *Biochim. Biophys. Acta 786*, 151−160.
48. Liu, Y., and Bolen, D. W. (1995) *Biochemistry 34*, 12884−12891.
49. Wang, A., and Bolen, D. W. (1997) *Biochemistry 36*, 9101−9108.
50. Bolen, D. W., and Baskakov, I. V. (2001) *J. Mol. Biol. 310*, 955−963.
51. Timasheff, S. N. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 9721−9726.
52. Lee, J. C., and Lee, L. L. Y (1979) *Biochemistry 18*, 5518−5526.
53. Colombo, M. F., Rau, D. C., and Parsegian, V. A. (1992) *Science 256*, 655−659.
54. Rand, R. P., Fuller, N. L., Butko, P., Francis, G., and Nicholls, P. (1993) *Biochemistry 32*, 5925−5929.
55. Timasheff, S. N., and Arakawa, T. (1988) *J. Cryst. Growth 90*, 39−46.

BI020316E