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Water as Ligand: Preferential Binding and Exclusion of Denaturants in Protein Unfolding†

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The denaturation of proteins by agents such as urea or guanidine hydrochloride (Gdn·HCl)[1] usually requires high concentrations, 3–8 M, of denaturant. At these concentrations, denaturants destabilize the folded state of a typical globular protein by about 10–20 kcal/mol (Pace, 1975). This free energy contribution ($\delta\Delta G$) is the result of additional interactions between the protein and the denaturant when the protein becomes unfolded. The primary data are normally obtained in the form of transition curves such as the one shown in Figure 1A (Thomson et al., 1989). Such curves have been usually analyzed by several approaches: (i) The Wyman (1964) plot, log K_D vs log denaturant concentration, where K_D is the equilibrium constant for the N ⇌ D reaction; the slope has been at times equated to the number, Δn, of additional denaturant molecules binding to the protein on denaturation [for review, see Tanford (1970) and Pace (1975, 1986)]; (ii) The linear extrapolation, ΔG vs denaturant concentration (Greene & Pace, 1974), which gives a characteristic slope, m, that is directly related to the parameter Δn of the Wyman plot (Pace, 1975); (iii) The denaturant binding model (Aune & Tanford, 1969), in which $\delta\Delta G$ is set equal to the free energy of binding of denaturant to groups newly exposed on protein unfolding; (iv) Tanford's model (Tanford, 1964, 1970), which states that the denaturation free energy increment, $\delta\Delta G$, is equal to the sum of the transition free energies from water to denaturant solution, $\delta g_{tr,i}$, of newly exposed groups of type i on unfolding:

$$\delta\Delta G = \sum \alpha_i n_i \delta g_{tr,i} \qquad (1)$$

where n_i is the total number of groups i and α_i is the fractional change in their degree of exposure.

Pace (1975, 1986) has examined critically these various analyses of urea and Gdn·HCl induced denaturation transitions. There is reasonable agreement in the values of $\delta\Delta G$. The parameter Δn, however, varies greatly. Procedures (i) and (ii) lead to values of 10–20 (Pace, 1975), and procedure (iii) is insensitive and results in Δn values that can vary by a factor of 5 depending on the value of the assigned binding constant (Pace, 1975). The Δn values resulting from procedure (iv) are equal to 20–35% of the total number of peptide groups plus nonionizable amino acid side chains (Tanford, 1970; Pace, 1986). The Δn values given by the first two procedures lead to values of the ratio $\delta\Delta G/\Delta n$ of ~−1.0 kcal/mol, which, at times, have been interpreted as the contribution of each newly bound denaturant molecule to the protein on unfolding. If this is accepted as a binding free energy, then one would expect a binding constant of ~1.0 M^{-1}, which leads to the expectation that effects would take place at concentrations much lower than 3–8 M. Furthermore, recent calorimetric titration measurements (Pfeil et al., 1991; Makhatadze & Privalov, 1992) of the binding of Gdn·HCl and urea to unfolded proteins are consistent with interaction free energy contributions of ~−1.0 and ~−0.2 kcal/mol, respectively, for each denaturant molecule bound to the protein.

This apparent puzzle can be resolved if the process of protein–denaturant interaction, whether in the denaturation or in the binding process, is examined in terms of the thermodynamics of weak binding systems, i.e., in terms of preferential interactions (Kirkwood & Goldberg, 1950; Stockmayer, 1950; Timasheff et al., 1957; Timasheff & Kronman, 1959; Casassa & Eisenberg, 1964) with the recognition that, in such thermodynamic processes, the interactions of a protein with solvent components must be accounted for over the *entire* protein–solvent interface. In order to do this, let us (1) define rigorously the thermodynamic control by cosolvent (e.g., urea) of the protein unfolding equilibrium, (2) define the thermodynamic meaning of binding, and (3) establish the relation between occupancy of sites on

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[1] Abbreviations: Gdn·HCl, guanidine hydrochloride; RNase, ribonuclease A.

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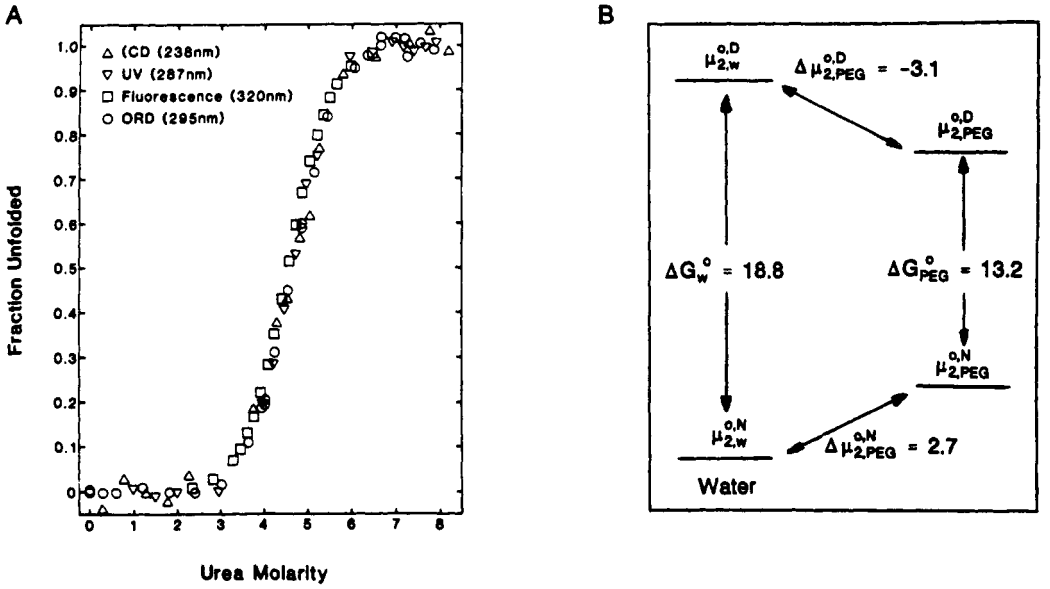

FIGURE 1: Effect of denaturants on protein unfolding. (A) Transition curve as measured experimentally by a number of techniques for the urea denaturation of ribonuclease T1 [reproduced from Thomson et al. (1989) with permission]. (B) Thermodynamic analysis of the effect of 20% poly(ethylene glycol) 1000 on the unfolding of chymotrypsinogen at 20 °C: it is shown that the denaturation free energy increment due to PEG addition, $\Delta G^{\circ}_{PEG} - \Delta G^{\circ}_{w} = -5.6$ kcal/mol, is equal to the change in transfer free energy from water to the cosolvent system as a result of protein unfolding, $\Delta\mu^{\circ,D}_{2,PEG} - \Delta\mu^{\circ,N}_{2,PEG} = -5.8$ kcal/mol. [Calculated from the data of Lee and Lee (1987).]

a protein by a denaturant and its global thermodynamic interaction with the entire protein–solvent interface.

Thermodynamic Definition of the Effect of Denaturants on the Protein Unfolding Equilibrium; Preferential Interactions. The effect of a denaturant on the stability of a protein is usually expressed thermodynamically with respect to one of two reference states: (i) the denaturant solution of the given composition; (ii) pure water. For the analysis, let us adopt the notation of Scatchard (1946) that components 1, 2, and 3 are water, protein, and denaturant.

In the first reference state, the effect of a denaturant on protein stability is expressed thermodynamically in terms of the Wyman linkage relation (Wyman, 1948, 1964; Wyman & Gill, 1990). According to the Wyman theory, if two reactions, such as protein denaturation and interaction of a ligand with the protein occur together, then the effect of ligand on the chemical equilibrium ($K = [D]/[N]$ for the denaturation equilibrium, $N \rightleftharpoons D$), at the given ligand concentration, m_3, is given by

$$\left(\frac{\partial \ln K}{\mathrm{d} \ln a_3}\right)_{T,P,m_2} = \frac{(\partial\mu_3/\partial m_2)^{N}_{T,P,m_3} - (\partial\mu_3/\partial m_2)^{D}_{T,P,m_3}}{(\partial\mu_3/\partial m_3)_{T,P,m_2}} = \left(\frac{\partial\mu_2}{\partial\mu_3}\right)^{N}_{T,P,m_2} - \left(\frac{\partial\mu_2}{\partial\mu_3}\right)^{D}_{T,P,m_2} \quad (2)$$

where μ_i is the chemical potential of component i, m_i is its molal concentration, and a_i is its activity: $\mu_i = \mu_i^{\circ} + RT \ln a_i$. The denominator on the right-hand side of eq 2 is the self-interaction of the ligand (γ_i is the activity coefficient of component i):

$$\left(\frac{\partial\mu_3}{\partial m_3}\right)_{T,P,m_2} = RT\left(\frac{1}{m_3} + \frac{\partial \ln \gamma_3}{\partial m_3}\right)_{T,P,m_2} \quad (3)$$

The quantity $(\partial\mu_3/\partial m_2)_{m_3} = (\partial\mu_2/\partial m_3)_{m_2}$, which expresses the mutual perturbations of the chemical potentials of the protein and ligand by each other, is known as the *preferential interaction parameter* (Kirkwood & Goldberg, 1950; Stockmayer, 1950; Casassa & Eisenberg, 1964; Timasheff & Kronman, 1959). The perturbation of the chemical potentials leads to a redistribution of solvent components in the domain of the protein, since

$$\frac{(\partial\mu_3/\partial m_2)_{T,P,m_3}}{(\partial\mu_3/\partial m_3)_{T,P,m_2}} = -\left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3} \quad (4)$$

With the minor approximation that $(\partial m_3/\partial m_2)_{T,P,\mu_3} \approx (\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (Stigter, 1960), this parameter is equal to the binding measured experimentally at dialysis equilibrium, $\bar{\nu}_3$ in Scatchard (1949) notation. It is known as the *preferential binding.* In the case of a reaction, such as the denaturation equilibrium, combination of eqs 2 and 4 gives

$$\left(\frac{\partial \ln K}{\partial \ln a_3}\right)_{T,P,m_2} = \left(\frac{\partial m_3}{\partial m_2}\right)^{D}_{T,P,\mu_3} - \left(\frac{\partial m_3}{\partial m_2}\right)^{N}_{T,P,\mu_3} = \Delta\nu_3^{N-D} \quad (5)$$

The Wyman plot, therefore, tells the change in preferential binding (Tanford, 1969, 1970; Aune & Tanford, 1969; Schellman, 1978) *at the given cosolvent concentration* at which the slope is being measured and the direction in which a ligand (denaturant) will displace an equilibrium at that particular cosolvent concentration, as $\Delta\nu_3$ may be positive (denaturation), negative (stabilization), or zero (no effect).

The preferential binding parameter at either end state of the equilibrium may itself be positive, negative, or zero, depending on whether the interaction of a protein with the cosolvent containing solution is thermodynamically favorable, unfavorable, or indifferent, i.e., $(\partial\mu_3/\partial m_2)_{T,P,m_3}$ is negative, positive, or zero (Timasheff & Kronman, 1959). This means that, at any given solvent composition, m_3, the binding value measured by dialysis equilibrium may be positive, negative, or zero. A measured *negative* binding stoichiometry (preferential exclusion of cosolvent) means that the solvent vicinal to the protein contains an excess of water over the bulk solvent. Application of the Gibbs–Duhem equation to eq 4 gives for

this preferential hydration (Timasheff, 1963)

$$\left(\frac{\partial m_1}{\partial m_2}\right)_{T,\mu_1,\mu_3} = -\frac{m_1}{m_3}\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3} \quad (6)$$

With pure water as the reference state, the change in standard free energy of denaturation when the system is transferred from water to the denaturant solution, $\delta\Delta G^{\circ,N-D}$, is given by the difference between the transfer free energies from water to the denaturant solution of the protein in the denatured and native states, $\delta\Delta\mu_{2,tr}^{N-D}$ (Tanford, 1964). This is equivalent to $\sum\alpha_i n_i \delta g_{i,tr}$ in Tanford's (1970) model. Specifically

$$\delta\Delta G^{\circ,N-D} = \Delta G_{m_3}^{\circ,N-D} - \Delta G_w^{\circ,N-D} = \Delta\mu_{2,tr}^{D} - \Delta\mu_{2,tr}^{N} \quad (7)$$

where the subscripts w and m_3 mean pure water and an aqueous solution of denaturant of concentration m_3, and $\Delta\mu_{2,tr}$ is the transfer free energy of the protein from water to a denaturant solution at concentration m_3; the superscripts N and D mean protein in the native and denatured states. An example is given in Figure 1B, which is the effect of poly(ethylene glycol) 1000 on the stability of chymotrypsinogen (Lee & Lee, 1987). It is clear that the difference between the free energies of denaturation in water and PEG 1000 is defined exactly by the change in transfer free energy between the native and denatured states of the protein.

What Is the Relation between the Thermodynamic Interaction Parameters in the Two Reference States? The transfer free energy of a protein from water to an aqueous cosolvent system (e.g., 8 M urea), being the total free energy change due to the addition of the cosolvent to the aqueous protein solution, is related to the variation of the preferential interaction of the protein with the ligand by

$$\Delta\mu_{2,tr} = \int_0^{m_3} (\partial\mu_2/\partial m_3)_{T,P,m_2}\, dm_3 \quad (8)$$

For the denaturation equilibrium, the change in $\Delta\mu_{2,tr}$ is, therefore, the integral of the Wyman equation:

$$\delta\Delta\mu_{2,tr}^{N-D} = \int_0^{m_3} [(\partial\mu_2/\partial m_3)_{T,P,m_2}^{D} - (\partial\mu_2/\partial m_3)_{T,P,m_2}^{N}]\, dm_3 \quad (9)$$

From eq 8, it is clear that knowledge of the preferential interaction at a single solvent composition cannot reveal the effect of an added solvent component on a protein relative to water. In fact, *the transfer free energy and preferential interaction parameter may have opposite signs*. This is vividly illustrated by the system native β-lactoglobulin in aqueous $MgCl_2$ at pH 3.0 (Arakawa et al., 1990). The variations of $(\partial m_3/\partial m_2)_{T,P,\mu_3}$, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$, and $\Delta\mu_{2,tr}$ as a function of ligand concentration for this system are compared in Figure 2. The three manifestations of the same interactions follow totally different dependencies on the concentration of the ligand. The binding, measured by dialysis equilibrium, expressed as $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$, is at first strongly negative (preferential exclusion); it attains a minimum at 1 M salt; above this it becomes less negative, crosses zero at 2.5 M salt, and becomes positive above that salt concentration. These experimental results reflect a thermodynamic preferential interaction parameter, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$, that starts at a high positive value at low salt concentration (unfavorable) and decreases linearly until it becomes negative (favorable) after crossing zero at 2.5 M salt. The transfer free energy, on the other hand, is unfavorable (positive) at all salt concentrations. It increases until 2.5 M salt, at which point it reaches a maximum of 42.8 kcal/mol and continues unfavorable but decreasingly so at higher salt concentrations. The striking

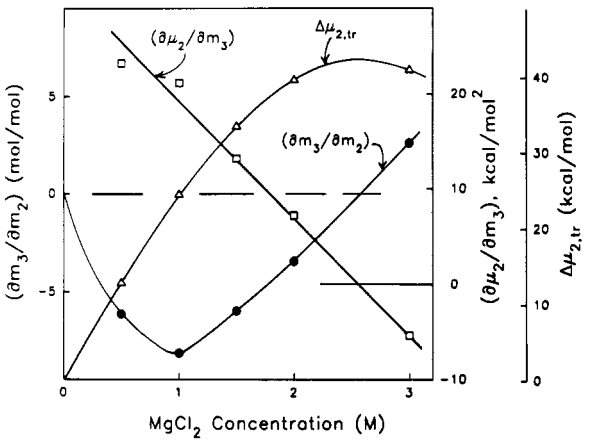

FIGURE 2: Variation with component 3 concentration of preferential binding, $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (●), the preferential interaction parameter, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ (□), and the transfer free energy, $\Delta\mu_{2,tr}$ (△), for the system β-lactoglobulin in aqueous $MgCl_2$ solution at pH 3.0. [Plotted from the data of Arakawa et al. (1990).] It is to be noted that $\Delta\mu_{2,tr}$ is maximal at the point where the binding measured by dialysis equilibrium is zero.

fact is that, at 3 M salt, the thermodynamic interactions with respect to the two reference states are opposite in sign. They are unfavorable with respect to water, but favorable with respect to the solvent of the given salt concentration.

A very important and interesting solvent composition point is the one at which preferential interaction is zero and the dialysis equilibrium experiment gives a result of *no binding*. In the system of Figure 2, this is found at 2.5 M salt. This means that, at that solvent composition, the thermodynamic interactions of the protein with water or salt are perfectly balanced and its surface occupancy by the two is in the same proportion as their presence in the solvent. This may be naively interpreted as total thermodynamic indifference. In fact, the truth is just the opposite. At that point the transfer free energy of the protein from water to the mixed solvent is at an apex. For β-lactoglobulin in 2.5 M $MgCl_2$, it has a positive value of 42.8 kcal/mol, i.e., the interaction with this solvent system is highly unfavorable relative to water. *Thermodynamic indifference requires that there be no interactions between protein and ligand at all solvent compositions*, i.e., that the solvent compositions in the bulk and in the immediate domain of the protein be identical at all cosolvent concentrations: $\Delta\mu_{2,tr}$ must have a zero value at all solvent compositions. This automatically leads to zero values of $(\partial m_3/\partial m_2)_{T,P,\mu_3}$ and $(\partial\mu_2/\partial m_3)_{T,P,m_2}$.

What Is the Molecular Meaning of Preferential Binding? In pure water, by definition, all surface sites (loci, areas) on a protein molecule must be in contact with water molecules. The binding of one molecule of ligand to a site on a protein molecule, or even a thermodynamically neutral temporal occupancy of that site by the ligand, must, therefore, displace water molecules. This is illustrated on Figure 3. Therefore, the free energy of binding at a site, ΔG^b, must be the difference between the intrinsic free energies of interaction of the protein with the ligand, ΔG^L, and with water, ΔG^W (Timasheff & Kronman, 1959; Timasheff, 1963):

$$\Delta G^b = \Delta G^L - \Delta G^W \quad (10)$$

with corresponding intrinsic water and ligand binding constants, K_W and K_L. The binding constant measured in aqueous medium, then, is the exchange constant, K_{ex} (Schellman,

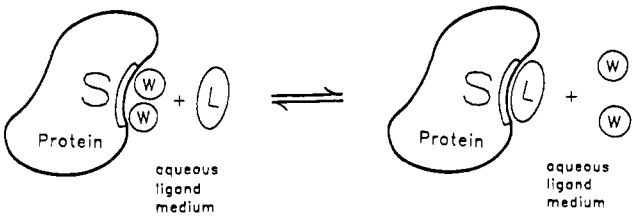

FIGURE 3: Schematic representation of the competition between a ligand molecule, L, and water, W, at site, S, on a protein, as defined by eqs 10 and 11.

1987a, 1990), for the reaction

$$P{\cdot}H_2O_m + L \overset{K_{ex}}{\rightleftharpoons} PL + mH_2O$$

$$K_{ex} = \frac{K_L}{K_W} = \frac{[PL][H_2O]^m}{[P{\cdot}H_2O_m][L]} \tag{11}$$

and $\Delta G^b = -RT \ln K_{ex}$.

Equations 10 and 11 define preferential interaction in terms of a competition equilibrium: they state that the observed binding is the consequence of the difference in affinity of a locus on a protein for ligand and for water (Timasheff & Kronman, 1959). Thus, when $\Delta G^L < \Delta G^W$ and $K_L > K_W$, the binding measured in a thermodynamic equilibrium experiment (e.g., dialysis equilibrium) is positive (there is preference for ligand at the site, i.e., preferential binding). In the opposite case, $\Delta G^L > \Delta G^W$ and $K_L < K_W$, the measured binding is negative (there is preference for water at the site, i.e., preferential hydration). When the binding of ligand is strong (most biochemical effectors, enzyme–substrate, etc.), $\Delta G^L \ll \Delta G^W$ and $K_L \gg K_W$, then $\Delta G^b \simeq \Delta G^L$ and $K_{ex} \simeq K_L$, and the contribution from the displacement of water may be neglected. When the interactions are weak, however, as in the case of denaturants (urea, Gdn·HCl), the ligand and water intrinsic binding constants are of similar magnitude and water must be taken into account specifically.

Let us define the relation between the equilibrium eqs 10 and 11 and the thermodynamic interaction parameters $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ and $(\partial m_3/\partial m_2)_{T,P,\mu_3}$. For a single site

$$\left(\frac{\partial\mu_2}{\partial m_3}\right)^{\text{per site}}_{T,P,m_2} = \left(\frac{\partial\Delta G^b}{\partial m_3}\right)_{T,P,m_2} \tag{12}$$

Then for all sites on a protein molecule, by eq 8, $\Delta\mu_{2,tr} = \sum_{i=1}^{n}\Delta G^b$, i.e., the transfer free energy of the protein from water to the cosolvent system is given by the sum of the local interactions, each of which may be favorable if, for a given site, $\Delta G_i^L < \Delta G_i^W$, or unfavorable if $\Delta G_i^L > \Delta G_i^W$. Combination of eqs 12 and 10 permits to decompose formally the preferential interaction parameter measured in a global binding experiment (i.e., dialysis equilibrium) into contributions from the ligand and water at any given ligand concentration (Timasheff & Kronman, 1959):

$$\left(\frac{\partial\mu_2}{\partial m_3}\right)_{T,P,m_2} = \left(\frac{\partial\mu_2}{\partial m_3}\right)^{(3)}_{T,P,m_2} - \left(\frac{\partial\mu_2}{\partial m_3}\right)^{(1)}_{T,P,m_2} \tag{13}$$

where the terms on the right-hand side represent contributions to the perturbation of the chemical potential of the protein by the occupancy of sites by ligand (3) and water (1) in equilibrium with each other. Consideration of eq 13 in conjunction with eqs 4 and 6 leads heuristically to the relation between the preferential binding measured by dialysis equilibrium and the effective total numbers of ligand and water molecules actually found at loci on the protein molecule, B_3 and B_1, respectively (Timasheff & Inoue, 1968; Inoue & Timasheff, 1972; Kupke, 1973; Reisler et al., 1977):

$$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3} = B_3 - \frac{m_3}{m_1}B_1 \tag{14}$$

It should be noted that, while $(\partial m_3/\partial m_2)_{T,P,\mu_3}$ is a true thermodynamic quantity, B_1 and B_3 are not. Let us examine in greater detail the implications of this relation for binding measurements.

Thermodynamic Binding and Protein–Ligand Contacts. Binding can be measured in two ways. The first encompasses true thermodynamic techniques, such as dialysis equilibrium. It measures the total preferential interaction of the protein with solvent components (both ligand and water) at all sites on the surface of the protein molecule but gives no information on site occupancy by water and ligand, respectively. The second type of measurement involves nonthermodynamic techniques which detect protein–ligand contacts manifested by, say, the perturbation of a spectral property or the evolution or uptake of heat in a calorimetric titration experiment. The binding reported by these techniques is the number of ligand molecules in contact with the protein, B_3, but not the equilibrium quantity $(\partial m_3/\partial m_2)_{T,P,\mu_3}$. When binding is strong, the second term on the right-hand side of eq 14 vanishes as $m_3 \ll m_1$ and $B_3 \approx (\partial m_3/\partial m_2)_{T,P,\mu_3}$. In the case of weak binding, such as with urea or Gdn·HCl, the two terms on the right-hand side of eq 14 are of similar magnitude and B_3 must always be greater than $(\partial m_3/\partial m_2)_{T,P,\mu_3}$. Hence, protein–ligand contact detecting techniques, measuring only B_3, are not capable of giving a true global thermodynamic description of the interactions of proteins with weakly binding ligands, such as denaturants and stabilizers.

Let us examine now how the two types of information can be combined to give a molecular description of the binding of weak ligands, such as denaturants. The surface of a protein molecule can be described as a mosaic of sites that can interact with water and with ligand, each with its own affinity.[2] *There can be no vacant sites*; all sites must be occupied, be it simply by van der Waals contacts. This is illustrated in Figure 4, where the protein chain is drawn as a linear model: as a time average, each site carries either an excess of water or ligand, or it is neutral (Timasheff & Inoue, 1968). The thermodynamic interaction at each site i is expressed by $(\partial m_3/\partial m_2)^{(i)}_{T,P,\mu_3}$. The global preferential interaction parameter measured in a thermodynamic binding experiment is

$$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3} = \sum_{i=1}^{n}\left(\frac{\partial m_3}{\partial m_2}\right)^{(i)}_{T,P,\mu_3} \tag{15}$$

The i sites on the protein surface can be classified into three general categories, depicted schematically in Figure 5: (1) those that are indifferent to being in contact with water or cosolvent ($B_3/B_1 = m_3/m_1$); (2) those that have little affinity for the ligand relative to water ($B_3/B_1 < m_3/m_1$; preferential exclusion); (3) those that have a significant affinity for ligand and, therefore, exhibit water–ligand exchange ($B_3/B_1 > m_3/$

[2] Sites in the present context should be regarded as statistical entities on the protein surface with which solvent makes contacts and not as specific loci at which a solvent molecules complexes in definite geometry (such as a substrate-binding site on an enzyme).

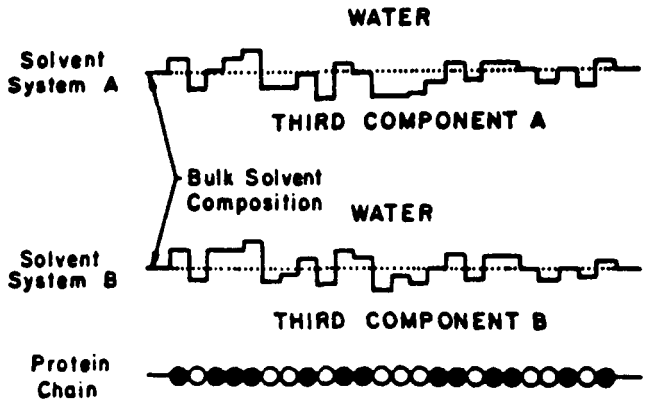

FIGURE 4: Schematic representation of the preferential interactions of solvent components at all individual sites on a protein represented as an extended unfolded chain. The sites have been classified into those with preferential affinity for cosolvent (●) and for water (○). The solvent composition along the protein chain is represented by the solid lines for two cosolvents, A and B, that interact with the protein by similar mechanisms, e.g., urea and Gdn·HCl. The bulk solvent composition is shown by the dotted lines. As a time average, sites at which the solid line is above the dotted line are occupied by ligand; those at which the solid line is below the dotted line are occupied by water. The difference in deviation from the dotted line represents differences in affinity. [Reprinted with permission from Timasheff and Inoue (1968).]

m_1; preferential binding).[3] What are their contributions to the total preferential interaction? The indifferent sites make no contribution to the thermodynamic interaction, and so they can be neglected. The sites from which ligand is excluded (Kupke, 1973; Reisler et al., 1977) make no contribution to B_3. They must be occupied, however, by a number of water molecules, which we will call nonexchangeable, B_1^{Nex}. Those that can interact with the ligand will be occupied by either ligand or water depending on the magnitudes of the exchange constants and the ligand concentration; for them the occupancies are expressed by B_3 and B_1^{exch}. The measured global preferential binding is the sum of all three occupancies:

$$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3} = \sum_1^{\ell} B_3 - \frac{m_3}{m_1}\left(\sum_1^{\ell} B_1^{exch} + B_1^{Nex}\right) \quad (16)$$

The transfer free energy, $\Delta\mu_{2,tr}$, being a global property of the protein, is then the summation of contributions from interactions at all sites, both exchangeable and nonexchangeable:

$$\Delta\mu_{2,tr} = \Delta\mu_{2,tr}^{exch} + \Delta\mu_{2,tr}^{Nex} = \int_0^{m_3}\left(\frac{\partial\mu_2}{\partial m_3}\right)_{T,P,m_2}^{exch} dm_3 + \int_0^{m_3}\left(\frac{\partial\mu_2}{\partial m_3}\right)_{T,P,m_2}^{Nex} dm_3 \quad (17)$$

Ligand–Water Exchange and Total Thermodynamic Interaction. The relation between preferential interactions and the exchange reaction has been addressed by Schellman (1978, 1987a,b, 1990) in a series of important theoretical papers. Treating explicitly the model of one independent site at which one molecule of ligand replaces one molecule of water (similar to that depicted in Figure 3), Schellman (1990) has shown that the preferential interaction *per exchangeable site* is related to the exchange equilibrium constant, K_{ex}, expressed on the molal scale, by

$$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3}^{per\ site} = \frac{\left(K_{ex} - \frac{1}{m_1}\right)m_3}{1 + K_{ex}m_3} \quad (18)$$

This differs from the usual strong binding isotherm by having the binding constant, K, in the numerator replaced by $(K_{ex} - 1/m_1)$, which takes into account the binding of water at the exchangeable sites. This reconciles the requirement that an equilibrium constant must be positive, while binding measured at thermodynamic (dialysis) equilibrium can assume negative values, as it frequently does (Timasheff, 1963; von Hippel et al., 1973; Pittz & Timasheff, 1978; Timasheff & Arakawa, 1988). The extent of site occupancy, θ, and the net free energy of the interaction of the protein with the ligand at a site, ΔG_b, at any solvent composition are given by (Schellman, 1990)

$$\theta = \frac{K_{ex}m_3}{1 + K_{ex}m_3} \quad (19)$$

$$\Delta G_b = -RT \ln (a_W + K'_{ex}a_L) \quad (20)$$

where the activities, a_i, and K'_{ex} are expressed in mole fraction units. It must be noted that the activity of water appears explicitly in this equation.[4]

These relations describe the thermodynamics of the interactions at one exchangeable site. The effect of the solvent on the thermodynamic state of the protein, however, is given by the global preferential interactions over the entire protein molecule. These must include the contributions from nonexchangeable sites as well as from exchangeable ones, as depicted in Figure 5. The complete relation between binding at the n exchangeable sites [as expressed within the Schellman (1987a, 1990) model] and global preferential interactions must, therefore, also contain a term for the nonexchangeable water, B_1^{Nex}:

$$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3} = n\frac{\left(K_{ex} - \frac{1}{m_1}\right)m_3}{1 + K_{ex}m_3} - \frac{m_3}{m_1}B_1^{Nex} \quad (21)$$

Relation between the Thermodynamic and Molecular Descriptions of Binding: Urea and Gdn·HCl. Let us apply now this complete theory of the binding of weak ligands to the only four systems for which the necessary data are available. These are fully unfolded lysozyme and ribonuclease A (RNase)[1] in 8 M urea and 6 M Gdn·HCl. Table I gives all the experimentally determined parameters. The preferential binding values, $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$, listed in column 1, were measured by dialysis equilibrium (Lee & Timasheff, 1974; Prakash et al., 1981). The total number of sites available to urea or Gdn·HCl binding, n (column 2), and the number of sites occupied by the ligands at the given denaturant concentrations, B_3 (column 3), were obtained from careful microcalorimetric titration measurements followed by a Scatchard-type analysis (Makhatadze & Privalov, 1992). The number of exchangeable sites occupied by water in equilibrium with the denaturant is then equal to $n - B_3$. The total effective

[3] Since the reference state is protein in water, there is no class of sites listed at which there is preferential exclusion of water; since these would correspond to strong binding of ligand, they are contained in the third class, with a high value of the exchange constant.

[4] The frequently used equation, $\Delta G_b = RT \ln (1 + K_B a_L)$, where K_B is a binding constant, is not valid for weakly interacting systems (Schellman, 1987a,b), and values of ΔG_b calculated with it do not lead to the transfer free energy.

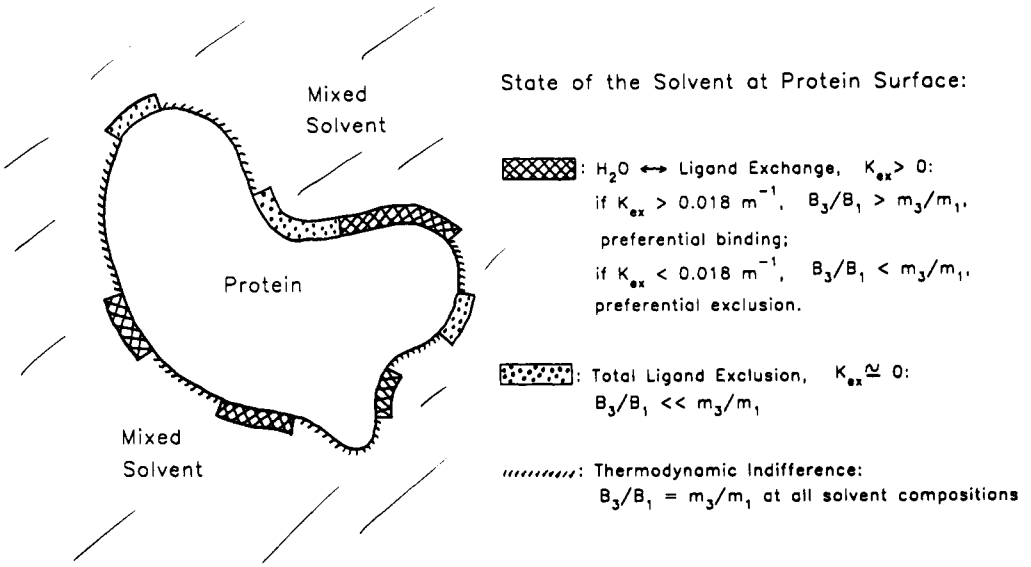

FIGURE 5: Schematic representation of the thermodynamic state of the surface of a protein dissolved in a mixed solvent. The total protein surface must make contact with water or cosolvent (ligand) molecules. Depending on the free energy of interaction with cosolvent, there will be areas on the surface at which (a) cosolvent exchanges with water (these will be occupied by water or cosolvent depending on their relative affinities); (b) cosolvent is excluded; (c) the ratio of cosolvent to water molecules will be the same as in the bulk solvent medium (thermodynamic indifference).

Table I: Preferential Binding and Occupancy of Sites[a]

	(1) $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$[b]	(2) n[c]	(3) B_3[c]	(4) B_1^{total}[d]	(5) $B_1^{total}/(n-B_3)$	(6) K_{ex}[e] (m^{-1})	(7) $(\partial m_3/\partial m_2)_{calc}^{per\ exch\ site}$[f]	(8) $(\partial m_3/\partial m_2)_{T,P,\mu_3}^{ex,calc}$	(9) B_1^{Nex}[g]
lysozyme, 8 M urea	12.0	229	72	266	1.69	0.036	0.155	35.5	109
RNase A, 8 M urea	2.0	240	75	332	2.01	0.036	0.155	37.2	167
lysozyme, 6 M Gdn·HCl	14.0	82	64	264	14.7	0.327	0.732	60.0	246
RNase A, 6 M Gdn·HCl	0	74	57	300	17.6	0.327	0.732	54.0	283

[a] $m_1 = 55.56m$; m_3 (for 8 M urea) = 12.53; m_3 (for 6 M Gdn·HCl) = 10.54. [b] Data of Lee and Timasheff (1974) and Prakash et al. (1981). [c] Data of Makhatadze and Privalov (1992). [d] $B_1^{total} = (m_1/m_3)[B - (\partial m_3/\partial m_2)_{T,P,\mu_3}]$. [e] Calculated with eq 19 from the data of Makhatadze and Privalov (1992). [f] Calculated with eq 18 which assumes the exchange of one ligand molecule for one water. [g] Calculated with eq 21.

number of water molecules that occupy nonindifferent sites, both exchangeable and nonexchangeable, B_1^{total}, calculated with eq 14, is listed in column 4. If it is assumed that there are no nonexchangeable sites, then the number of water molecules that exchange with each molecule of the denaturant is equal to $B_1^{total}/(n - B_3)$. The values listed in column 5 give ca. 1.8 water molecules replaced by each molecule of urea and 14–17 water molecules replaced by each guanidinium ion. While the first number appears reasonable, the second one is obviously high. This indicates that, in 6 M Gdn·HCl, there must be nonexchangeable water-occupied sites from which the denaturant is excluded, as expressed by eq 16 and Figure 5.

To establish the contribution of the nonexchangeable sites to the total thermodynamic interaction, it is necessary to establish first the interactions at the exchangeable sites. This requires a model (an exchange stoichiometry) of the binding at these sites. The site occupancy data (B_3, n) were examined, therefore, in terms of the model treated by Schellman (1990) that each exchangeable site can be occupied either by one water molecule or one denaturant molecule. The obtained numbers, therefore, are valid only within this model. Other stoichiometries of water diplacement would give numerically different results, but the general arguments would remain unchanged. The Schellman model sets $B_1^{ex} = n - B_3$. The binding exchange constants, K_{ex}, listed in column 6, were calculated from the experimental values of n and B_3 with eq 19, since $\theta = B_3/n$. These exchange constants lead to the values of $(\partial m_3/\partial m_2)_{T,P,\mu_3}$ per exchangeable site, listed in column 7. For the n exchangeable sites, this results in the expected global preferential interaction values, $(\partial m_3/\partial m_2)_{T,P,\mu_3}^{ex,calc}$, listed in column 8. These are seen to be very much larger than the experimentally measured preferential interactions (column 1), meaning that there must be compensation from other sites that are hydrated and are not available to water–urea or water–Gdn·HCl exchange. Their numbers, calculated with eq 21, are listed in column 9 of Table I. It is evident that both proteins contain a large number of hydrated sites from which the denaturants are excluded. This is consistent with the recent demonstration that the interactions of apolar residues are thermodynamically more unfavorable with urea than with water (Cheek & Lilley, 1988). Taking ribonuclease, we find that, while it contains 240 sites available to occupancy by urea and 74 sites available to Gdn·HCl, it also has, within the exchange model adopted here, close to 170 water molecules that cannot be displaced by urea and some 280 water molecules that cannot be displaced by Gdn·HCl.

What are the thermodynamic contributions of the interactions at the exchangeable and nonexchangeable sites and what are their consequences for the overall thermodynamic interactions? These were calculated from the binding parameters listed in Table I and the pertinent numbers are summarized in Table II. Columns 1 and 2 list (i) the values of the preferential interaction parameter per exchangeable site calculated from the $(\partial m_3/\partial m_2)_{T,P,\mu_3}^{per\ exch\ site}$ values of Table I, column 7, and (ii) the corresponding transfer free energy per exchangeable site. The resulting quantity, $n\Delta\mu_{2,tr}^{per\ exch\ site}$, is the free energy made available by the water–urea or water–Gdn·HCl exchange to the thermodynamic state of the protein at the given denaturant concentration. Its values

Table II: Thermodynamics of the Preferential Interactions[a]

	(1) $(\partial\mu_2/\partial m_3)_{T,P,m_2}^{\text{calc per exch site}}$ [b]	(2) $\Delta\mu_{2,tr}^{\text{per exch site}}$ [c] (cal/mol)	(3) $\Delta\mu_{2,tr}^{\text{exch}}$ [d] (kcal/mol)	(4) $\Delta\mu_{2,tr}$ [e] (kcal/mol)	(5) ΔG^{excl} (kcal/mol)	(6) $\Delta G^{\text{excl}}/B_1^{\text{Nex}}$ (cal/mol)
lysozyme, 8 M urea	−5.4	−68	−15.6	−7.0	8.6	+79
RNase, 8 M urea	−5.4	−68	−16.4	−1.2	15.2	+91
lysozyme, 6 M Gdn·HCl	−34	−358	−29.4	−8.2	21.2	+86
RNase, 6 M Gdn·HCl	−34	−358	−26.5	0	26.5	+94

[a] All exchangeable site interactions were calculated from values deduced from the experimental data by application of the one water–one ligand exchange model. [b] Calculated with eq 4 from column 7 of Table I. [c] Calculated with eq 8 with the assumption that $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ is independent of denaturant concentration. [d] $\Delta\mu_{2,tr}^{\text{exch}} = n\Delta\mu_{2,tr}^{\text{per exch site}}$. [e] Calculated with eqs 4 and 8 from the data of Lee and Timasheff (1974) and Prakash et al. (1981), with the assumption that $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ is independent of denaturant concentration.

listed in column 3 show that, for both proteins, interactions with the denaturants are favorable: more than 15 kcal/mol of free energy are made available by the water–urea exchange and close to 30 kcal/mol by the water–Gdn·HCl exchange. The values of the *measured* global transfer free energies, $\Delta\mu_{2,tr}$, listed in column 4, are less negative.[5]

What is the source of the difference between $\Delta\mu_{2,tr}^{\text{exch}}$ and $\Delta\mu_{2,tr}$? It is the compensation by the unfavorable interactions of the two denaturants with those sites at which they cannot exchange with water. These unfavorable interactions amount to the free energy contributions listed in column 5 of Table II, $\Delta G^{\text{excl}} = \Delta\mu_{2,tr} - \Delta\mu_{2,tr}^{\text{exch}}$. While they attain values of >25 kcal/mol of unfavorable free energy, they, in fact, reflect not very large individual contributions from the large number of nonexchangeable hydrated sites, listed in column 6 of Table II. At these sites, contact with water is favored over urea and Gdn·HCl by −70 to −100 cal/mol per site. This corresponds, however, to exchange constants at these sites (using eq 20) that are close to zero, meaning that urea and Gdn·HCl have essentially no affinity for these loci on the protein molecules.

Conclusions. In conclusion, it is evident that, in the denaturant systems, the weak overall thermodynamic interactions with a protein, $\Delta\mu_{2,tr}$, are a consequence of the compensation of strong favorable interactions of the proteins with the denaturants (up to −30 kcal/mol for Gdn·HCl within the used binding model) at the exchangeable sites by almost as strong unfavorable interactions at loci on the protein surface at which the denaturants cannot displace water. Returning to the two approaches to the measurement of protein–denaturant interactions, thermodynamic techniques, such as dialysis equilibrium, give a complete description of the effects of denaturants on the thermodynamic state of the *whole* protein molecule. Techniques that depend on the recognition of protein–ligand contacts, such as calorimetric or spectroscopic titrations, by measuring B_3 as a function of denaturant concentration, can give K_{ex}, n, and B_1^{exch}. They are insufficient, however, to describe fully protein–denaturant interactions. Together, the two types of measurements give both a thermodynamic (preferential interactions, transfer free energy) and a molecular site occupancy accounting (B_3, B_1^{exch}, B_1^{Nex}) description of the interactions.

The same criteria apply to the analysis of the denaturation equilibrium, for which all the parameters must be established in both end states of the reaction. The contribution of the ligand to the free energy of unfolding, $\delta\Delta G^{\circ,\text{N-D}}$ of eq 7, is given by the summation of $\delta\Delta\mu_{2,tr}^{\text{exch}}$ and $\delta\Delta\mu_{2,tr}^{\text{Nex}}$ (see eq 17), which frequently are opposite in sign. The slope of the Wyman plot or similar relations, Δn, is defined by

$$\Delta\left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3}^{\text{N-D}} = \Delta B_3^{\text{N-D}} - \frac{m_3}{m_1}(\Delta B_1^{\text{exch}} + \Delta B_1^{\text{Nex}})^{\text{N-D}} = \Delta n \tag{22}$$

Measurements of $\Delta B_3^{\text{N-D}}$ can lead to the number of exchangeable sites newly exposed to solvent interactions. They cannot give, however, a thermodynamic description of the denaturation equilibrium, and they do not enter explicitly into such an analysis. In fact, it is the identification of Δn measured from denaturation transition curves with the number of newly exposed binding sites that had led to the apparent puzzle stated at the outset. This erroneously sets $\Delta(\partial m_3/\partial m_2)_{T,P,\mu_3} = \Delta B_3^{\text{N-D}}$ and neglects totally the contribution of water. Even identifying Δn with the preferential interactions at newly exposed exchangeable sites, i.e., $\Delta B_3^{\text{N-D}} - (m_3/m_1)\Delta B_1^{\text{exch,N-D}}$, is insufficient. The proper thermodynamic and molecular identifications of Δn are given by eq 22, i.e., Δn is the expression of the contribution of newly exposed groups to the total thermodynamic interaction of the protein with solvent components both at exchangeable and nonexchangeable sites. Since, at present, the necessary data are not available for the interactions of denaturants with proteins in the native state, a complete thermodynamic interaction and site occupancy analysis of the denaturation reaction is not possible.

[5] These carry a certain uncertainty, since they were calculated by eq 7 from the only available values of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$, with the usual assumption that this quantity is independent of denaturant concentration.

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