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The relation between the free energy of interaction and binding

John A. Schellman
Institute of Molecular Biology, University of Oregon, Eugene, OR 97403 (USA)

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Abstract

For ordinary stoichiometric interactions the extent of binding and the free energy of interaction follow parallel and well understood courses as a function of ligand concentration. This simple picture changes when interactions are very weak and the solutions are concentrated and non-ideal. In these cases, which include most instances of protein denaturation or unfolding, binding must be understood as a competition between the ligand and the principal solvent. Under these circumstances unusual phenomena can arise such as a changes in sign of the thermodynamic binding as concentration is varied. The most striking instances occur when strongly interacting reagents apparently do not bind at all. This paper discusses the paradoxical fact that the free energy of interaction is strongest when thermodynamic binding (selective interaction) goes to zero. Examples are cited where these phenomena have been observed experimentally in agreement with the theory presented here.

Keywords: Thermodynamics of binding; Free energy of binding; Solvation; Preferential

1. Introduction

The concept of binding is intimately associated with the existence of attractive interactions between molecular species, but the relationship can be more subtle than is generally assumed. The purpose of this note is to illustrate the non-intuitive relation which can develop between interaction free energy and binding when the binding is very weak and solutions are concentrated and non-ideal. The discussion will be directed toward the interaction of a number of small ligands with a macromolecule. Small-molecule interactions are then simplified cases of the general results.

Binding, whether of a physical or chemical nature, generally evokes an image of an identifiable, geometric complex. The analysis of the formation of such complexes is the standard theory of associative equilibrium and is part of every chemist's education. This type of binding, which depends on a discrete number of well defined equilibrium relations, will be referred to as stoichiometric binding. Though it is generally recognized that the stability of stoichiometric complexes may depend on the solvent, it is not usually considered necessary to consider explicitly the fact that the formation of the complex involves the displacement of solvent molecules from

Correspondence to: Prof. J.A. Schellman, Institute of Molecular Biology, University of Oregon, Eugene, OR 97403 (USA). Fax: (503) 346-5891.

the region of contact of the two or more associating molecules. This can be seen from the equilibrium equations themselves which do not explicitly make reference to the solvent.

2. Theory

2.1 Stoichiometric binding

Stoichiometric binding can be studied by spectroscopic, titration or dialysis methods. This usually involves a determination of the concentrations of free and bound ligand species from which equilibrium constants can be determined. The results are best analyzed in terms of the binding polynomial [1]

$$\Sigma_b = \sum_{n=0}^{N} K_n A_3^n,\ K_0 = 1., \tag{1}$$

where N is the number of binding sites and A_3 is the activity of the ligand, component 3. For N independent, identical sites the binding polynomial is given by $\Sigma_b = (1 + KA_3)^N$. The free energy of interaction, i.e., the excess free energy of the macromolecule generated by the presence of the ligand, is defined by the thermodynamic relation,

$$\mu_2 = \mu_2^0 + RT \ln m_2 + \Delta\mu_2^{int}$$
$$= \mu_2^0 + RT \ln m_2 + RT\beta_2$$

and is given by [2,3]

$$\Delta\mu_2^{int} \equiv RT\beta_2 = -RT \ln \Sigma_b, \tag{2}$$

$\Delta\mu_2^{int}$ is the excess free energy of component 2 relative to an ideal solution in pure solvent component 1. Alternatively it is the transfer free energy of a mole of component 2 from an ideal solution in solvent 1 into the solvent mixture of 1 and 3. β_2 is the standard symbol for the excess free energy in solution theory and is $\Delta\mu_2^{int}$ in units of RT [4]. $\Delta\mu_2^{int}$ represents interactions between macromolecules (which will be ignored by assuming high dilution) and interactions between component 2 and component 3. The binding isotherm (number of moles of ligand bound as a function of concentration) is then given by

$$\langle \nu \rangle = \left(\frac{\partial \ln \Sigma_b}{\partial \ln A_3} \right) = -\left(\frac{\partial \beta_2}{\partial \ln A_3} \right)$$
$$= -\frac{1}{RT}\left(\frac{\partial \Delta\mu_2^{int}}{\partial \ln A_3} \right) \tag{3}$$

These standard and well known results have been presented for contrast with the discussion which follows. The results can be generalized to more than one ligand species.

Both $\ln \Sigma_b$ and $\partial \ln \Sigma_b / \partial \ln A_3$ are monotonically increasing functions of the concentration of component 3. $\ln \Sigma_b$ increases monotonically with component 3 because both activities and equilibrium constants are positive quantities and because of a stability criterion (p. 226 of [5]) which states that the activity of a component always increases with its concentration. $\partial \ln \Sigma_b / \partial \ln A_3$ increases monotonically because its slope, $\partial^2 \ln \Sigma_b / \partial \ln A_3^2$, equals the mean square fluctuation of n and is always positive [2]. Thus increasing the concentration of component 3 increases the extent of binding and makes the free energy of interaction more negative. This is the standard situation, so commonplace as to be considered intuitive.

2.2 Selective interaction thermodynamics

Thermodynamics provides a more general definition of binding which includes stoichiometric binding but extends to very small interactions which are too weak or subtle to be considered stoichiometrically. The thermodynamic measure of binding, called selective or preferential interaction, is defined by the thermodynamic formula

$$\Gamma'_{23} = \left(\frac{\partial m_3}{\partial m_2} \right)_{T,P,\mu_3} \tag{4}$$

where component 1 is the principal solvent, component 2 the molecule whose binding properties are under consideration and component 3 is the ligand, which is often a cosolvent. Thus binding, or interaction, is deduced by thermodynamic cor-

relations between species. Equation (4) is intuitive, since it defines the binding as the increase in molality of component 3 which must accompany an increase in molality of component 2 to keep the chemical potential of component 3 constant. [1] This phenomenological approach includes cases where the nature of the association is unknown and solvent participation is inherent in the process. It may also be shown that selective interaction leads to a coupling of the fluctuations of component 2 and component 3 [6], so that it may also be measured with scattering techniques, most effectively with X-rays and neutrons [7]. Experimental evidence for weak, selective interaction as opposed to stoichiometric binding will be discussed at the end of this paper.

A straightforward thermodynamic derivation [8,6] shows that

$$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3} = -\left(\frac{\partial \mu_2}{\partial \mu_3}\right)_{T,P,m_2} \tag{5}$$

The chemical potential of the ligand, or cosolvent, is most conventionally represented in terms of the thermodynamic activity, A_3, on some convenient concentration scale,

$$\mu_3 = \mu_3^0 + RT \ln A_3.$$

On the other hand, following Scatchard, it is advantageous to express the non-ideality of the macromolecule in terms of the excess free energy, $\Delta\mu_2^{int}$ of eq. (2) Substituting the formulas for μ_2 and μ_3 into (4) and (5), we obtain

$$\Gamma'_{23} = -\left(\frac{\partial \beta_2}{\partial \ln A_3}\right)_{T,P,m_2} \tag{6}$$

a formula which was discussed extensively by Casassa and Eisenberg [4]. Compare eq. (6) with eq. (3). Equation (6) demonstrates immediately that a zero value for the selective interaction can arise either from the absence of interaction (μ_2 independent of A_3), *or from a maximum or minimum in the excess free energy produced by component 3*. As discussed above, the latter condition does *not* arise with stoichiometric binding.

Two theoretical forms for the excess free energy, β_2, have been used to describe the interaction of proteins with ligands and cosolvents. The first stems from a binding polynomial approach discussed above and the other represents β_2 as a power series in the concentration or activity of the ligand. The latter approach is more general and has a theoretical basis in the work of MacMillan and Mayer [9] and Kirkwood and Buff [10], but the series converges very badly for stoichiometric binding. The former is useful for strong, stoichiometric interactions between the protein and the ligand, the latter for the weak interactions of cosolvents where the concept of binding is confused by the fact that cosolvents are in such large concentrations that they occupy sites at the surface of the protein even in the absence of a driving force.

Because of the large number of molecules involved and the indefiniteness of the interactions it is not possible at present to develop a complete theory of selective interaction. Recently [11,12], however, it has been shown that all of the qualitative features of competitive solvent interaction can be represented by a simple independent binding model. The theory makes use of binding polynomials, but with the essential difference that both the principal solvent (component 1) and the cosolvent (component 3) must be included as ligands. For the interaction at a single site the binding polynomial is $\Sigma_b = A_1 + KA_3$, and the interaction parameter is [2]

$$\beta_2 = -\ln(A_1 + KA_3) \tag{7}$$

[1] There are in fact three definitions of selective interaction associated with three experimental arrangements: dialysis, isopiestic and electrochemical. These differ in what is kept constant in the derivative above. The prime indicates constant T, P, μ_3 in eq. (4). For macromolecules the differences between these definitions is small and calculable when necessary. We have used the definition which behaves most simply. See [8] and [12] for definitions and interrelations.

[2] The primitive binding polynomial is in fact symmetric in components 1 and 3. $\Sigma_b = K_1A_1 + K_3A_3$. The form $A_1 + KA_3$ indicates that solvation by component 1 has been selected as the standard state and K is now the equilibrium constant for the *interchange* of components 1 and 3 on the site [11,12].

where A_1 and A_3 are mole fraction activities of the components and K is the equilibrium constant for the interchange

$$I1 + 3 = I3 + 1.$$

I1 and I3 represent occupancy of the site by molecules of 1 and 3. Mole fraction activities are used to put the two solvent components on an equal footing. On the other hand practical concentrations are always on a molar or molal basis. A complete discussion, with formulas, of the interconversion of concentrations, activities and activity coefficients is given in [13]. We find it convenient to express quantities like A_1 and A_3,

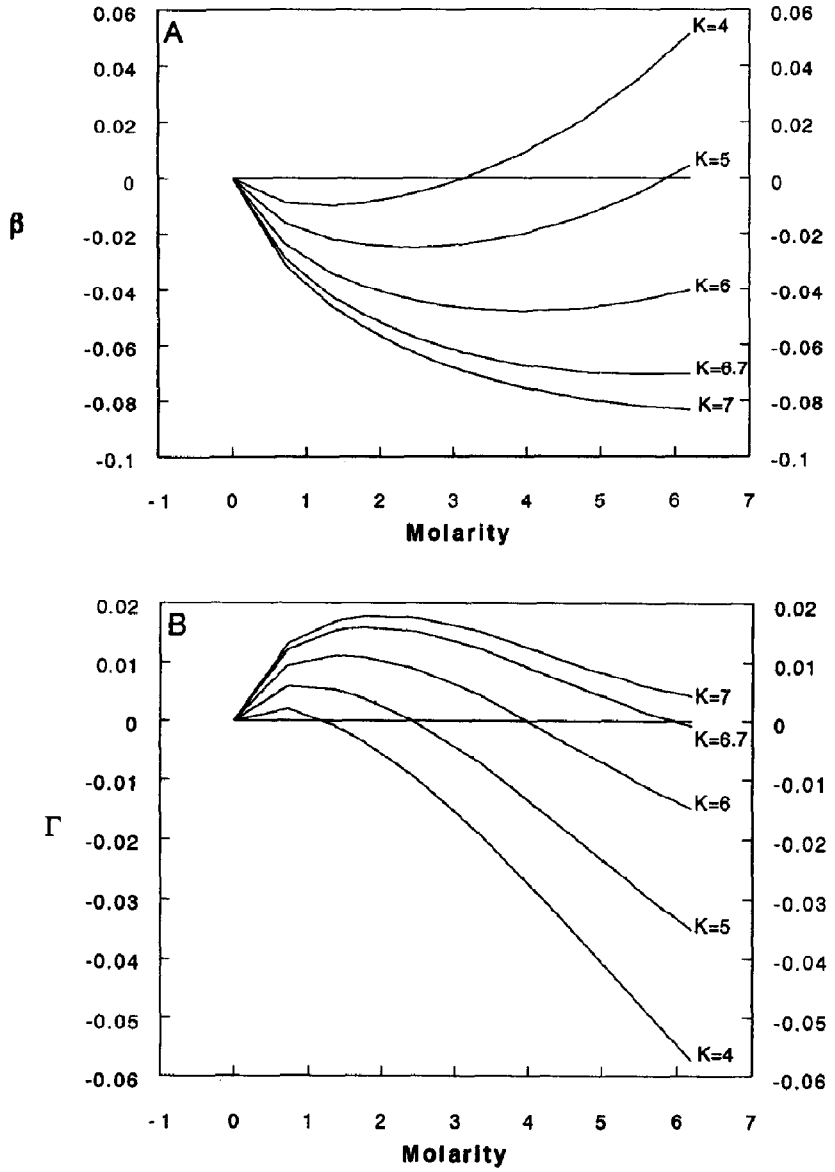

Fig. 1. β_2 and Γ'_{23} as functions of the molarity of guanidinium chloride for various values of the exchange constant K. K is on the mole fraction basis. (A) Excess free energy, and (B) selective interaction.

which are needed in the theory as fitted polynomials in terms of molarity and molality. This permits the direct application of the relations of this paper in terms of the experimental quantities.

As shown in the cited papers, for a number of independent sites,

$$\Delta\mu_2^{\text{int}} = -RT\sum_j \left\{ \ln f_1 + \ln\left[1 + \left(\frac{K_j f_3}{f_1} - 1\right)\chi_3\right]\right\} \tag{8}$$

where f and χ stand for activity coefficients and concentrations on the mole fraction scale and K_j is the exchange constant for the jth site on the mole fraction scale. The new features, which are characteristic of selective interaction, are the appearances of the activity of the principal solvent and the quantity $K_{\text{eff}} = (K_j(f_3/f_1) - 1)$, which acts like an effective equilibrium constant. When K_j is large, the quantity -1 can be ignored and $K_{\text{eff}} \equiv K_j f_3/f_1$. Also, when K_j is large the titration of a site occurs over a small range of low concentrations so that activity coefficients do not vary to an appreciable extent. This is the justification for the common practice of defining the equilibrium constant in terms of concentrations rather than activities.

The situation is very different when K is small. The factor -1 cannot be ignored and titrations of sites go over large ranges of concentration, so that K_{eff} can vary considerably with concentration and can even change sign, though it will never be less than -1. When K is less than one it means that component 1 is preferentially bound at the site. In this case the K_{eff} is usually negative.

The selective interaction can be obtained from eqs. (6) and (8)

$$\Gamma'_{23} = \sum_{\text{all sites}} \left(\frac{K_{\text{eff}}\chi_3}{1 + K_{\text{eff}}\chi_3}\right) \tag{9}$$

This looks like a normal Langmuir type surface binding but K_{eff} is not an equilibrium constant and will usually vary with concentration. A somewhat different formula is required for electrolytes [12].

3. Results and discussion

It should be realized that the selective interaction formalism is completely symmetric with respect to components 1 and 3; the apparent asymmetry of the above equations comes from the fact that we have chosen component 1 as the principal solvent component and are therefore studying the binding of component 3 in this medium. It is sometimes useful to look at selective interaction in the reverse way [8,12].

Though eqs. (8) and (9) are based on an independent site model, they reproduce all the "unusual" behavior observed for selective interaction and macromolecular stabilization or destabilization in concentrated reagents. Figure 1 shows the behavior predicted by eqs. (8) and (9) for several values of K when component 3 is guanidinium chloride. Only as K becomes large ($K > 7$ on the mole-fraction scale, $K > 0.14$ on the molarity or molality scale) do these curves resemble the monotonic behavior of stoichiometric site binding. At low values for K the free energy and the thermodynamic binding can have maxima, minima and changes in sign. If the solutions are ideal, $K_{\text{eff}} = K - 1$ is a constant and the system behaves like stoichiometric binding, though with an altered value of K [11]. In solutions like guanidinium chloride, or urea, which show positive deviations from Raoult's law, the ratio of activity coefficients f_3/f_1 becomes smaller and smaller as the solution becomes concentrated until eventually Kf_3/f_1 becomes smaller than unity and negative binding starts to occur. As long as the effective K is positive, the free energy is diminished by the binding, but as soon as K_{eff} becomes negative the free energy starts to increase. This produces a minimum in the free energy curve where $\Gamma = 0$, in accordance with eq. (6). T. Lilley has, in fact, used eq. (6) in the form

$$\beta_2 = -\int_0^{A_3} \frac{\Gamma'_{23}}{A_3} \mathrm{d}A_3 \tag{10}$$

to evaluate the free energy of interaction of denaturants with proteins [14].

Though there has been a continued tendency to use the original stoichiometric binding formalism to interpret solvent denaturation [15,16], there

are many experimental papers where it is clear that only selective interaction with weak binding can describe the experimental results. We close with a review of some examples.

A first example is the work of von Hippel and his coworkers [17,18] who observed apparent negative binding while using sensitive column techniques to measure the interaction of salts with amide groups. These instances were interpreted as the preferential interaction of amides with water and reported as negative binding constants which are essentially the K_{eff} defined above.

The very extensive work of Timasheff and coworkers See (for example [19–21]) on the effect of solvent components in stabilizing proteins also falls in this category. Selective interaction was measured directly by densimetric methods, and it was shown that stabilizing agents in general are those which display negative selective interaction with proteins, i.e., those agents which induce preferential hydration. Where thermodynamic data is available on the cosolvents, the form of the results agrees with the qualitative predictions of eqs. (7) and (9). One cannot go further than this at present except by considering averages because of the heterogeneous nature of protein–solvent interactions.

Finally there are the denaturing agents like urea or guanidinium chloride, which require high concentrations. According to Tanford, 6*M* guanidinium chloride solutions generate the conditions for maximum unfolding of proteins. On the other hand there is the apparently paradoxical result of Hade and Tanford [22] and Lee and Timasheff [23], that the selective interaction of ribonuclease is zero in 6 *M* guanidinium chloride. [3] Different experimental methods were used in the two investigations (isopiestic distillation and densimetry) so there seems no possibility of a methodological error. These results are in accord, however, with expectations for the selective interaction of guanidinium chloride. The curve for $K = 6.7$ in Fig. 1 was presented as a simulation for the behavior of ribonuclease. It is seen that if one assumes identical, independent binding sites with $K = 6.7$ ($K = 0.12$ on the molality scale), the selective interaction goes to zero at 6 *M* denaturant while the free energy goes to a minimum, i.e., to a maximum favorable interaction with the denaturant. Thus the zero thermodynamic binding of guanidinium chloride at 6 *M* is perfectly consistent with Tanford's conclusion that this constitues the condition of maximum unfolding.

The above, however, is not a proper discussion of the unfolding reaction of ribonuclease in this solvent. Hade and Tanford and Lee and Timasheff measured the interaction of guanidnium chloride with the unfolded form of ribonuclease. Denaturation, on the other hand occurs as a result of interaction with the surfaces of the protein which are exposed as a result of the unfolding. One would need the difference in free energy between the folded and unfolded forms to discuss denaturation. We can conclude only that 6 *M* is the condition of maximum stabilization of the unfolded form with this value for *K*. A second factor is that the surface of a protein is highly heterogeneous. The total free energy and selective interaction result from a sum over all sites and these can have widely varying values of K_{eff}. Transfer free energy experiments indicate a considerable variability of the interaction of guanidine with sidechains, some of which will be repulsive. Positive ions for example will repel guanidinium ions and will contribute negatively to the selective interaction and positively to the free energy of interaction.

Just as this paper was being completed an important paper on this topic appeared by Poklar and Lapanje [24], who studied the selective interaction of β-lactoglobulin at two pH's with urea and three alkyl-ureas. They also calculated excess free energies of the protein using eq. (10). They found minima and changes in sign of the free energy and maxima and changes in sign of the selective interaction. Curves of their data take the form predicted by eqs. (9) and (7) (or 8) using

[3] In their paper Lee and Timasheff also calculated a quantity A_3 as the sum of the number of molecules bound via selective interaction plus the number which is normally present in an assumed hydration layer with the composition of the bulk solution. This is a way of enumerating the number of solute molecules in the neighborhood of a protein but it is not a thermodynamic quantity.

the thermodynamic properties of urea solutions and assuming low values for K_{eff}.

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Glossary of symbols

Subscripts 1, 2, 3	Indicate principal solvent, macromolecule and cosolvent respectively
A_i	Activity of component i on the mole fraction scale
β_{23}	$= \Delta\mu_2^{int}$ in units of RT
f, γ, y	Activity coefficients in the mole fraction, molality and molarity scales, respectively
Γ'_{23}	Selective interaction parameter of component 3 on a site of component 2, defined by eq. (4)
$\Delta\mu_2^{int}$	Change in free energy (chemical potential) of component 2 caused by the addition of component 3 to the solution
Σ_b	Binding polynomial, either stoichiometric or thermodynamic