

# Theory of Free Energy and Entropy in Noncovalent Binding

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## 1. Introduction

Noncovalent binding provides an invisible wiring diagram for biomolecular pathways and is the essence of host–guest and supramolecular chemistry. Decades of theoretical and experimental studies provide insight into the determinants of binding affinity and specificity. The many practical applications of targeted molecules have also motivated the development of computational tools for molecular design aimed at drug discovery<sup>1</sup> and, to a lesser extent, the design of low molecular weight receptors.<sup>2–6</sup> Nonetheless, there remain unresolved questions and challenges. The rules of thumb for maximizing binding affinity are unreliable, and there is still a need for accurate methods of predicting binding affinities for a range of systems.

Many researchers are now renewing progress in this area by delving deeper into the physical chemistry and modeling of noncovalent binding.<sup>1,7</sup> Important themes today include the use of more refined models of interatomic energies,<sup>8–12</sup> computational<sup>13–23</sup> and experimental<sup>24–34</sup> characterization of configurational entropy changes on binding, enhanced techniques for sampling molecular conformations and extracting free energies from simulations,<sup>35–43</sup> and the exploitation of advances in computer hardware.<sup>44–53</sup>

Such efforts require both a quantitative understanding of interactions at the atomic level and a means of mapping these interactions to macroscopically observable binding affinities. Statistical thermodynamics provides the required mapping, and the theoretical underpinnings of binding thermodynamics appear to be well-established.<sup>14,54–56</sup> Nonetheless, analyses of binding, both experimental and theoretical, still frequently employ nonrigorous frameworks, which can lead to puzzling or contradictory results. This holds especially in relation to entropy changes on binding, which can be both subtle and controversial. For example, although the change in translational entropy when biomolecules associate has been discussed in the literature for over 50 years, it remains a subject of active discussion.<sup>15,18,56–71</sup>

This review of the theory of free energy and entropy in noncovalent binding aims to support the development of well-founded models of binding and the meaningful interpretation of experimental data. We begin with a rigorous but hopefully accessible discussion of the statistical thermodynamics of binding, taking an approach that differs from and elaborates on prior presentations; background material and detailed derivations are provided in Supporting Information. Then we use this framework to provide new analyses of topics of long-standing interest and current relevance. These include the changes in translational and other entropy components on binding; the implications of correlation for entropy; multivalency (avidity) and the relationship between

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intermolecular and intramolecular binding; and the applicability of additivity in the interpretation of binding free energies.

Several caveats should be noted. First, we focus on general concepts rather than specific systems or computational methods, the latter having been recently reviewed.<sup>1,7</sup> Second, our formulation of binding thermodynamics is based on classical statistical mechanics, although occasional reference is made to quantum mechanics when the connection is of particular interest. Finally, we have not attempted to be exhaustive in citing the literature but hope to have provided enough references to offer a useful entry.

## 2. Free Energy, Partition Function, and Entropy

This section provides the groundwork for our treatment of binding thermodynamics by introducing and analyzing two central concepts, the free energy and its entropy component.

Section 1 of Supporting Information provides background material and further details.

The free energy can be calculated from a microscopic description of the system in question. The Helmholtz free energy,  $F$ , which is particularly useful for the condition of constant volume, is given by

$$F = -k_{\text{B}}T \ln Q \quad (2.1)$$

where  $k_{\text{B}}$  is Boltzmann's constant and  $T$  is absolute temperature. If the coordinates (and perhaps momenta) of the system are collectively denoted as  $\mathbf{x}$  and its energy function is  $E(\mathbf{x})$ , then the partition function,  $Q$ , is given by the configurational integral

$$Q = \mathcal{N} \int d\mathbf{x} e^{-\beta E(\mathbf{x})} \quad (2.2)$$

where  $\beta = (k_{\text{B}}T)^{-1}$  and the constant  $\mathcal{N}$  is inserted to render  $Q$  unitless; its particular value does not have any significance here. The average energy of the system is

$$\langle E \rangle = \int d\mathbf{x} E(\mathbf{x}) \rho(\mathbf{x}) \quad (2.3)$$

in which  $\rho(\mathbf{x})$ , the equilibrium probability density in  $\mathbf{x}$ , is

$$\rho(\mathbf{x}) = \frac{e^{-\beta E(\mathbf{x})}}{\int d\mathbf{x} e^{-\beta E(\mathbf{x})}} \quad (2.4)$$

As shown in Section 1 of Supporting Information, the entropy is

$$S = \frac{-F + \langle E \rangle}{T} \quad (2.5a)$$

$$= -k_{\text{B}} \int d\mathbf{x} \rho(\mathbf{x}) \ln \rho(\mathbf{x}) + k_{\text{B}} \ln \mathcal{N} \quad (2.5b)$$

The entropy given by eq 2.5b will be referred to as the configurational entropy. The constant term,  $k_{\text{B}} \ln \mathcal{N}$ , serves to cancel the units of  $\rho(\mathbf{x})$  inside the logarithm and does not have any consequence when differences in entropy are calculated.

The following subsections introduce ideas that provide a basis for the theory of binding and its analysis in sections 3–8.

### 2.1. Entropy for Single and Multiple Energy Wells

The entropy can be viewed as a measure of uncertainty, and, for an energy function with multiple energy wells, there are two different sources of uncertainty. One arises from motions within a single well, the other arises from transitions between the different energy wells. The configurational entropy may be decomposed into two parts, one corresponding to each source of uncertainty, as now illustrated for a one-dimensional system with coordinate  $x$ .

To start, let us consider the case where the energy has a single minimum, located at  $x = x_0$ . When only a small region around the energy minimum makes a significant contribution to the configurational integral, a harmonic approximation may be made,

$$E(x) \approx E_0 + k(x - x_0)^2/2 \quad (2.6)$$

where  $k$  is the curvature of the energy function at  $x = x_0$ . The partition function is then

$$Q = \mathcal{N} \left( \frac{2\pi}{\beta k} \right)^{1/2} e^{-\beta E_0} \quad (2.7)$$

and the free energy is

$$F = E_0 - k_B T \ln \left[ \mathcal{N} \left( \frac{2\pi}{\beta k} \right)^{1/2} \right] \quad (2.8)$$

The average energy and entropy are given by

$$\langle E \rangle = E_0 + \frac{k_B T}{2} \quad (2.9a)$$

$$S = k_B \ln \left( \frac{2\pi}{\beta k} \right)^{1/2} + \frac{k_B}{2} + k_B \ln \mathcal{N} \quad (2.9b)$$

As the curvature,  $k$ , decreases, the energy well becomes broader, the system explores a broader range of positions, and the entropy increases.

When the energy function  $E(x)$  has multiple local minima, we can break the configurational integral of eq 2.2 into pieces, each covering an energy well,  $i$ <sup>16,72–74</sup>

$$Q = \sum_i Q_i \quad (2.10a)$$

$$Q_i = \mathcal{N} \int_i dx e^{-\beta E(x)} \quad (2.10b)$$

The subscript “ $i$ ” in eq 2.10b signifies that the integration is restricted to energy well  $i$ . Within each energy well, one can normalize the probability density  $\rho(x)$ ,

$$\rho_i(x) \equiv \rho(x)/p_i, \quad x \in \text{well } i \quad (2.11)$$

where  $p_i = Q_i/Q$  is the probability of finding the system in energy well  $i$ . The average energy  $\langle E \rangle$  and the entropy  $S$  can be obtained from  $\rho(x)$  according to eqs 2.3 and 2.5b, respectively. For each well, one can obtain the corresponding quantities  $\langle E \rangle_i$  and  $S_i$  from  $\rho_i(x)$ .

We now establish relations of  $\langle E \rangle$  and  $S$  with  $\langle E \rangle_i$  and  $S_i$ . By breaking the integral of eq 2.3 into individual wells and writing  $\rho(x)$  as  $p_i \rho_i(x)$ , one can easily verify that

$$\langle E \rangle = \sum_i p_i \langle E \rangle_i \quad (2.12)$$

while eq 2.5b becomes<sup>75,76</sup>

$$S = \sum_i p_i S_i - k_B \sum_i p_i \ln p_i \quad (2.13)$$

Note that, whereas the average energy (eq 2.12) is simply a weighted average of the energy associated with each well, the entropy (eq 2.13) includes a second term that is akin to the entropy of mixing. Intuitively, eq 2.13 states that the total configurational entropy is the weighted average of the entropies  $S_i$  of the individual wells plus the entropy associated with the distribution of the system across the energy wells  $i$ . This observation is just a molecular instance of the more general composition law of entropy.<sup>77</sup> In the present context, the two terms of the configurational entropy are often referred to, respectively, as the vibrational and conformational entropies.<sup>75,78</sup> It is worth noting that the same expression would be obtained if we partitioned configuration space in

some other manner; in the mathematical sense at least, there is nothing special about a partitioning into energy wells.

## 2.2. Uncorrelated versus Correlated Coordinates

The degree of correlation among different coordinates significantly affects the magnitude of the entropy. Correlation means that knowing the value of one coordinate reduces the uncertainty regarding the value of another coordinate. Therefore, in general, correlation reduces the entropy.

We denote the components of  $\mathbf{x}$  as  $x_1, x_2, \dots$ . If each coordinate contributes an additive term to the energy, so that  $E(\mathbf{x}) = E_1(x_1) + E_2(x_2) + \dots$ , then motions along the coordinates are uncorrelated with each other, and the partition function of the multidimensional system is the product of the partition functions associated with individual coordinates:

$$Q = \mathcal{N}_1 \int dx_1 e^{-\beta E_1(x_1)} \times \mathcal{N}_2 \int dx_2 e^{-\beta E_2(x_2)} \times \dots \quad (2.14)$$

As a result, the joint probability density is given by the product of the one-dimensional marginals:

$$\rho(x_1, x_2, \dots) = \rho(x_1)\rho(x_2)\dots \quad (2.15)$$

Correspondingly, the free energy, average energy, and entropy can be written as sums of contributions from each coordinate. In particular,

$$S = \sum_i S_i \quad (2.16a)$$

where

$$S_i = -k_B \int dx_i \rho(x_i) \ln \rho(x_i) + k_B \ln \mathcal{N}_i \quad (2.16b)$$

The concept of uncorrelated coordinates can be generalized to a situation in which  $x_1, x_2, \dots$ , each refer to nonoverlapping groups of coordinates rather than to individual coordinates.

An important example of uncorrelated coordinates is presented by the total energy of a classical system, which is the sum of the potential energy and the kinetic energy. The additivity of these two forms of energy means that they contribute multiplicative factors to the full partition function, one depending only on spatial coordinates and the other depending only on momenta, which together completely specify the state of a classical system in phase space. This result has important consequences, which are further discussed in the next subsection. Here we illustrate the separability of potential energy and kinetic energy for the case of a one-dimensional harmonic oscillator. The total energy for this system is

$$E(x, p) = E_0 + k(x - x_0)^2/2 + p^2/2m$$

where  $p$  is momentum and  $m$  is mass. The partition function

$$Q = \mathcal{N} \int dx dp e^{-\beta E(x,p)}$$

can be evaluated by integrating over  $x$  and  $p$  separately. The integral over  $x$  is given by eq 2.7, and the integral over  $p$  gives  $(2\pi m/\beta)^{1/2}$ , so the full partition function is



$$Q = \mathcal{N} \frac{2\pi}{\beta\omega} e^{-\beta E_0} \quad (2.17)$$

where  $\omega = (k/m)^{1/2}$  is the angular frequency. When this classical expression is derived as the high-temperature limit of a quantum treatment, one finds the multiplicative constant  $\mathcal{N}$  to be  $1/h$ , the inverse of Planck's constant. For the more general case of a polyatomic molecule, each atom, by its motion in three dimensions, contributes a factor  $(1/h)^3$  to the multiplicative constant  $\mathcal{N}$ .

Now let us examine the more common situation in which  $E(\mathbf{x})$  is not given by  $E_1(x_1) + E_2(x_2) + \dots$ , so that motions along the different coordinates of the system do correlate. In this case, the entropy of the system is no longer equal to the sum of the contributions from the marginal entropies, as in eq 2.16a for the uncorrelated case. In fact, as proven in Section 1 of Supporting Information, the actual entropy of any system is always less than or equal to the sum of the marginal entropies, and the equality holds only when the coordinates are uncorrelated. In the proof, we derive the following result for the entropy of a system with two coordinates,  $x_1$  and  $x_2$ :

$$S = -k_B \int dx_1 \rho(x_1) \ln \rho(x_1) - k_B \int dx_1 \rho(x_1) \int dx_2 \rho(x_2|x_1) \ln \rho(x_2|x_1) + k_B \ln \mathcal{N} \quad (2.18)$$

The first term gives  $S_1$  (cf. eq 2.16b). However, unless  $x_1$  and  $x_2$  are uncorrelated, the second term cannot be reduced to  $S_2$ . Nonetheless, one can still refer to this term as the entropy associated with  $x_2$  in the following sense. The quantity  $-k_B \int dx_2 \rho(x_2|x_1) \ln \rho(x_2|x_1)$  constitutes the entropy due to motion along  $x_2$  for a given value of  $x_1$ ; the second term is just this entropy averaged over the equilibrium distribution of  $x_1$ . We will return to this interpretation of the second term in subsection 2.4. In eq 2.18,  $x_1$  and  $x_2$  have been set up in different roles:  $x_1$  is kept as an "explicit" coordinate for detailed attention, whereas  $x_2$  is treated as a "bath" or "implicit" coordinate that is readied for suppression through an approximate or mean-field model. Note that, from a mathematical standpoint, either variable can be chosen as explicit; i.e., an equally valid formulation for  $S$  is obtained when  $x_1$  and  $x_2$  are interchanged in eq 2.18.

The difference,  $I_{12} \equiv S_1 + S_2 - S$ , known as the pairwise mutual information, provides a measure of the degree of correlation between two coordinates.<sup>79–81</sup> This concept generalizes to systems with more than two coordinates in two ways. First, the pairwise mutual information also can be applied when  $x_1$  and  $x_2$  are not single coordinates but rather nonoverlapping subsets of coordinates. For example,  $x_1$  might represent the coordinates of a receptor and  $x_2$  might represent those of its ligand. In addition, one can define higher-order mutual information terms.<sup>79–81</sup> For example, the entropy of a system with three coordinates,  $x_1$ ,  $x_2$ , and  $x_3$ , can be written as

$$S = \sum_{i=1}^3 S_i - \sum_{j>i}^3 I_{ij} + I_{123} \quad (2.19)$$

where  $I_{123}$  is the third-order mutual information.

## 2.3. Molecular Partition Function: Rigid versus Flexible Formulations

In order to formulate the statistical thermodynamics of noncovalent binding, we need expressions for the partition functions of the free receptor and ligand, which are in general flexible, polyatomic molecules, and their bound complex. This subsection reviews and analyzes two approaches to writing these molecular partition functions.

### 2.3.1. Rigid Rotor, Harmonic Oscillator Approximation

The first approach is to use the rigid rotor, harmonic oscillator (RRHO) approximation.<sup>82,83</sup> This approximation was first formulated in order to make a quantum mechanical treatment of molecular motions mathematically tractable.<sup>84,85</sup> Treating a molecule as essentially rigid, so that its internal motions comprise only vibrations of small amplitude, allows one to approximate the energy contributions—both kinetic and potential—of its translational, rotational, and vibrational motions as uncoupled from each other. This allows the partition function to be factorized, as discussed in subsection 2.2. Overall translation of the molecule contributes a factor

$$Q_t = V \left( \frac{2\pi m}{\beta h^2} \right)^{3/2} \quad (2.20a)$$

to the full partition function of the molecule, where the molecular mass  $m$  appears because of the contribution of the kinetic energy of overall translation. This expression is the classical approximation to the quantum mechanical sum over states. For future reference, we note that  $Q_t$  leads to an expression for translational entropy known as the Sackur–Tetrode equation (subsection 5.3). The assumption of rigidity in the RRHO treatment allows the moments of inertia to be approximated as constants so that the rotational contribution to the partition function can be written as

$$Q_r = 8\pi^2 \left( \frac{2\pi}{\beta h^2} \right)^{3/2} (I_1 I_2 I_3)^{1/2} \quad (2.20b)$$

where  $I_1$ ,  $I_2$ , and  $I_3$  are the molecule's three principal moments of inertia, and we have again used the classical approximation. Finally, a quantum mechanical treatment of the internal vibrations, each with angular frequency  $\omega_i$ , contributes a factor

$$Q_{\text{vib}} = e^{-\beta E_0} \prod_i \frac{e^{-\beta h\omega_i/4\pi}}{1 - e^{-\beta h\omega_i/2\pi}} \quad (2.20c)$$

The full partition function of the molecule then takes on the familiar form

$$Q = Q_t Q_r Q_{\text{vib}} \quad (2.21)$$

### 2.3.2. Flexible Molecule Approach

The second approach to formulating the molecular partition function is not new, but we are not aware of a term for it in the literature. In order to distinguish it from the RRHO approximation, we call it the flexible molecule (FM) approach. The FM approach relies upon classical statistical thermodynamics, which is routinely and appropriately used for Monte Carlo and molecular dynamics simulations of biomolecular systems. In the classical approximation, the kinetic energy of each atom makes a fixed contribution to

the partition function that is independent of conformation and potential energy. In particular, these kinetic energy contributions to the partition function do not change when two molecules bind to form a noncovalent complex, so they do not affect the binding free energy. This means that classical calculations of the binding constant can completely neglect kinetic energy and, hence, that any correctly formulated classical treatment of binding yields results that are independent of the masses of the atoms. By the same token, each atom contributes a factor  $(1/h)^3$  to the multiplicative constant  $\mathcal{N}$  in the partition function (subsection 2.2), so the binding free energy is also independent of  $\mathcal{N}$  and, hence, of Planck's constant  $h$ . As a corollary, any dependence on mass or Planck's constant of a quantum mechanical expression for the free energy or entropy of binding should disappear when  $\beta \rightarrow 0$ , since in such a limit the quantum result must reduce to classical statistical mechanics. This cancellation is demonstrated below for the classical limit of the RRHO treatment.

When the kinetic energy contribution to the partition function is omitted, as allowed by classical statistical thermodynamics, what is left is the configurational integral over spatial coordinates. There is no longer any need to consider the coupled kinetic energies of overall rotation and conformational change, and the approximation of fixed moments of inertia becomes unnecessary so the unrealistic rigid rotor approximation can be eliminated. It is still convenient to separate the  $n$  spatial coordinates of the molecule into 3 coordinates for overall translation, 3 more for overall rotation, and  $(3n - 6)$  internal coordinates. Overall translation contributes a factor of  $V$ , the total volume, to the configuration integral, and overall rotation contributes a factor of  $8\pi^2$ . The internal contribution can be written as

$$Q_{\text{in}} = \int d\mathbf{x} J(\mathbf{x}) e^{-\beta E(\mathbf{x})} \quad (2.22)$$

where  $\mathbf{x}$  now refers to internal coordinates, and  $J(\mathbf{x})$  is a Jacobian factor whose form depends upon the choice of internal coordinates.<sup>86-90</sup> The multiplicative constant  $\mathcal{N}$  is omitted, since, as noted above, its presence has no consequence on the final result for the binding free energy  $\Delta G_b$ . The partition function of the molecule is now

$$Q = 8\pi^2 V \int d\mathbf{x} J(\mathbf{x}) e^{-\beta E(\mathbf{x})} \quad (2.23)$$

Note that relying on the classical approximation from the outset allows us to consider conformational variations of any amplitude and form, so that the FM approach is not restricted to the small amplitude, vibrational model of internal motion in the RRHO treatment and, hence, is far more suitable for flexible biomolecules. For this reason, and because of its simplicity, the developments in this paper rely on the FM approach except as otherwise noted.

## 2.4. Solvation and a Temperature-Dependent Energy Function

In a fully microscopic description of a system, the potential energy is completely determined by the coordinates. However, it is often useful to consider only a subset of coordinates explicitly and model the rest of them implicitly. In particular, for a molecule in solution phase, one may treat the coordinates of the solute molecule explicitly but the coordinates of the solvent implicitly. This treatment is rigorous

when the energy function of the explicit coordinates is replaced by a potential of mean force that includes one contribution from the internal energy of the solute and another from the net effect of the solvent on the conformational energetics of the solute.<sup>55</sup> If the solute coordinates are denoted by  $\mathbf{x}$ , one may write the potential of mean force,  $W(\mathbf{x})$ , as

$$W(\mathbf{x}) = U(\mathbf{x}) + V_{\text{solv}}(\mathbf{x}) \quad (2.24)$$

where  $U(\mathbf{x})$  is the internal potential energy obtained as if the molecule were in vacuum and  $V_{\text{solv}}(\mathbf{x})$  is the solvation free energy. Because the latter accounts implicitly for the thermal motions of solvent molecules, it depends on temperature.

When the system composed of a single solute molecule surrounded by the solvent is described by the potential of mean force,  $W(\mathbf{x})$ , the entropy is given by

$$S = \frac{-F + \langle W \rangle}{T} - \left\langle \left( \frac{\partial W}{\partial T} \right)_V \right\rangle \quad (2.25)$$

as shown in Supporting Information. Here, the second term arises from the temperature dependence of  $W$ , specifically its solvation free energy term in eq 2.24, and the notation  $(\ )_V$  indicates a partial derivative taken at constant volume. The two terms of eq 2.25 are referred to as configurational entropy and solvation entropy, respectively,<sup>4</sup> where the configurational entropy results from motions of the solute molecule and the solvation entropy results from motions of the solvent molecules. It may be unexpected that this separation can be effected, given that solute and solvent motions are correlated with each other, so it is worth noting that the precise meaning of this decomposition is conveyed by eq 2.18, if  $x_1$  and  $x_2$  are taken to represent solute and solvent degrees of freedom, respectively. (See Supporting Information for further details.)

## 3. Binding Free Energy and Binding Constant

Suppose that two molecules, A and B, can bind to form a complex C. Now consider a system consisting of the three species of solute molecules present at concentrations  $C_\alpha$ ,  $\alpha = A, B$ , and C. (The solvent is treated implicitly.) We assume that the only significant interactions are between the binding molecules when complexed. In section 2 of Supporting Information, we derive the change in Gibbs free energy when one A molecule and one B molecule bind to form a single complex under constant pressure, a typical experimental condition. The result is

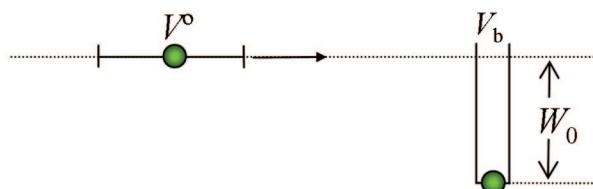
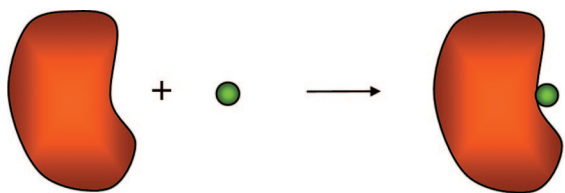
$$\Delta G_b = \mu_C - \mu_A - \mu_B \quad (3.1a)$$

$$= -k_B T \ln \frac{C_A C_B K_a}{C_C} \quad (3.1b)$$

where  $\mu_\alpha$  is the chemical potential of molecule  $\alpha$  and  $K_a$  is the binding constant defined as<sup>82</sup>

$$K_a \equiv \frac{Q_C/V}{(Q_A/V)(Q_B/V)} \quad (3.2)$$

Intuitively, dividing each molecular partition function by  $V$  removes the factor of  $V$  present in each partition function (see eqs 2.20a and 2.23) to yield volume-independent



**Figure 1.** Interpretation of the standard binding free energy. Upon binding, the volume available to the ligand is changed from  $V^\circ$  to  $V_b$ . At the same time, the ligand gains interaction energy  $W_0$ .

quantities. The significance of  $K_a$  is that it determines the concentrations of the three species of molecules when the binding reaction reaches equilibrium, where the binding free energy is 0. The resulting equilibrium concentrations satisfy

$$\frac{(C_C)_{\text{eq}}}{(C_A)_{\text{eq}}(C_B)_{\text{eq}}} = K_a \quad (3.3)$$

Experimentalists often use the dissociation constant, which is simply the inverse of  $K_a$ .

When the three species of molecules are all present at a “standard” concentration  $C^\circ$ , normally 1 M, the standard binding free energy is

$$\Delta G_b^\circ = -k_B T \ln(C^\circ K_a) \quad (3.4)$$

In using this equation to convert a measured  $K_a$  to the standard free energy of binding, if  $K_a$  is given in  $\text{M}^{-1}$ , then  $C^\circ$  is to be assigned a numerical value of 1 M. If  $K_a$  is given in other units, then  $C^\circ$  must be adjusted accordingly; for example, if  $K_a$  is in  $\text{nM}^{-1}$ , then  $C^\circ$  must be assigned a numerical value of  $10^9$  nM. The standard binding free energy and the binding constant provide, on different scales, equivalent measures of binding affinity.

A fact often overlooked in the literature is that the value of  $\Delta G_b^\circ$  depends on the choice of the standard concentration. The inverse of the standard concentration may be thought of as a standard volume  $V^\circ$ , which is the average volume occupied by a single molecule at standard concentration. When  $C^\circ = 1$  M, then  $V^\circ = 1661 \text{ \AA}^3$ . It can be shown that  $\Delta G_b^\circ$  is also the free energy difference between two states illustrated in Figure 1 (for the case of a square-well binding interaction). In the initial state, the ligand is allowed to translate within the volume  $V^\circ$  and freely rotate, but the receptor is fixed in a particular position and orientation; no restriction is placed on internal motions of either molecule, but there is no interaction between them. In the final state, the receptor is still fixed in its position and orientation, and the ligand now moves in the binding site of the receptor and interacts with it. Clearly, the equilibrium between these two states, and hence  $\Delta G_b^\circ$ , is affected by the initial volume,  $V^\circ$ , available to the ligand. The role of  $C^\circ$  may be further clarified by considering a room-temperature simulation of one molecule each of A and B in a cubic box of volume  $V$ . If the equilibrium probability of finding the molecules bound is  $p$ ,

then  $C_C = p/V$ , and  $C_A = C_B = (1 - p)/V$ , so, from eqs 3.3 and 3.4,  $K_a = pV/(1 - p)^2$  and  $\Delta G_b^\circ = -k_B T \ln(C^\circ V) - k_B T \ln[p/(1 - p)^2]$ . If, for example,  $V = 10^6 \text{ \AA}^3$ , the first term in  $\Delta G_b^\circ$  here will be  $-3.8$  kcal/mol at room temperature.

Let us now illustrate the calculation of the binding constant for three examples of increasing complexity.

### 3.1. Binding of Two Atoms to Form a Diatomic Complex

Arguably the simplest binding model is the formation of a diatomic molecule from two atoms. Here we consider the molecule to be held together by a harmonic potential (cf. eq 2.6)

$$E(r) = E_0 + k(r - r_0)^2/2 \quad (3.5)$$

where  $r$  is the interatomic distance.

#### 3.1.1. Classical Rigid Rotor, Harmonic Oscillator Treatment

As discussed in subsection 2.3, the molecular partition function in the RRHO treatment includes both potential and kinetic energy contributions. Before binding, each atom has only translational degrees of freedom, so their partition functions are given by eq 2.20a as

$$Q_\alpha = V \left( \frac{2\pi m_\alpha}{\beta h^2} \right)^{3/2} \quad (3.6a)$$

where  $\alpha = A$  or  $B$  and  $m_\alpha$  is the mass of the subscripted atom. After binding, the diatomic molecule has three translational degrees of freedom, two rotational degrees of freedom, and one vibrational degree of freedom. Here we use the high-temperature, or classical, limit (eq 2.17) of the quantum treatment for vibration (eq 2.20c). The partition function of the complex,  $C$ , is

$$Q_C = \left[ V \left( \frac{2\pi m_C}{\beta h^2} \right)^{3/2} \right] \left[ \frac{8\pi^2 I}{\beta h^2} \right] \left[ \frac{2\pi}{\beta h \omega} e^{-\beta E_0} \right] \quad (3.6b)$$

where  $m_C = m_A + m_B$  is the mass of the diatomic molecule;  $I = mr_0^2$  is the moment of inertia about the center of mass, where  $m \equiv m_A m_B / m_C$  is the reduced mass; and  $\omega = (k/m)^{1/2}$  is the angular frequency of the interatomic vibration. Inserting eqs 3.6a and 3.6b into eq 3.2, we find

$$K_a = 4\pi r_0^2 \left( \frac{2\pi}{\beta k} \right)^{1/2} e^{-\beta E_0} \quad (3.7)$$

Thus, as anticipated in subsection 2.3, the final classical result for  $K_a$  is independent of masses and Planck's constant, even though factors of mass and Planck's constant appear in the individual terms of the RRHO treatment. Note that the pre-exponential factor is the volume effectively accessible to one atom when the other is treated as fixed (see eq II.14a of Supporting Information and cf. eq 3.14b).

#### 3.1.2. Flexible Molecule Treatment

The FM approach to the molecular partition function (subsection 2.3.2) excludes kinetic energy from the outset, because the contributions of kinetic energy to the partition functions will cancel in the final result. In the present case,



the atoms prior to binding have only translational degrees of freedom, so  $Q_A = Q_B = V$ . After binding, the diatomic complex has overall translation and interatomic relative motion. The partition function is

$$Q_C = V \int dr 4\pi r^2 e^{-\beta E(r)} \quad (3.8)$$

where we have used spherical coordinates to represent the relative separation and integrated out the polar and azimuthal angles to yield the factor  $4\pi$ . From these partition functions, we find the binding constant to be

$$K_a = \int dr 4\pi r^2 e^{-\beta E(r)} \quad (3.9)$$

If we now assume that the force constant,  $k$ , is great enough that  $r$  deviates minimally from  $r_0$ , we recover the expression of eq 3.7.

### 3.2. Binding of a Polyatomic Receptor with a Monatomic Ligand

Consider the binding of a monatomic ligand (e.g., a simple ion), B, to a polyatomic receptor, A. The ligand has only translational degrees of freedom, so  $Q_B = V$ . The partition function of the receptor, according to eq 2.23, is given by

$$Q_A = 8\pi^2 V \int d\mathbf{x}_A J_A(\mathbf{x}_A) e^{-\beta E_A(\mathbf{x}_A)} \quad (3.10a)$$

where  $\mathbf{x}_A$  represents the internal coordinates of the receptor and  $E_A(\mathbf{x}_A)$  is its energy, which is understood to represent the potential of mean force with the solvent degrees of freedom averaged out. For the complex, the set of internal coordinates,  $\mathbf{x}_C$ , consists of all the internal coordinates of molecule A, along with the separation,  $\mathbf{r}$ , of the ligand from molecule A:  $\mathbf{x}_C = (\mathbf{x}_A, \mathbf{r})$ . Similar to eq 3.10a, we find the partition function of the complex to be

$$Q_C = 8\pi^2 V \int d\mathbf{x}_C J_A(\mathbf{x}_A) e^{-\beta E_C(\mathbf{x}_C)} \quad (3.10b)$$

However, we have to stipulate that the integration is restricted to the region of configurational space where the complex is deemed formed,<sup>55,91,92</sup> as further discussed in subsection 3.3. For example, the complex cannot be considered as formed in a configuration with  $r = \infty$ . The energy of the complex can be written in the form

$$E_C(\mathbf{x}_C) = E_A(\mathbf{x}_A) + w(\mathbf{x}_A, \mathbf{r}) \quad (3.11)$$

where the second term, which represents the energy arising from receptor–ligand interactions, vanishes at  $r = \infty$ .

With the partition functions for the receptor, ligand, and complex, the binding constant is found as

$$K_a = \frac{\int d\mathbf{x}_C J_A(\mathbf{x}_A) e^{-\beta E_C(\mathbf{x}_C)}}{\int d\mathbf{x}_A J_A(\mathbf{x}_A) e^{-\beta E_A(\mathbf{x}_A)}} = \frac{\int d\mathbf{x}_A d\mathbf{r} J_A(\mathbf{x}_A) e^{-\beta E_C(\mathbf{x}_A, \mathbf{r})}}{\int d\mathbf{x}_A J_A(\mathbf{x}_A) e^{-\beta E_A(\mathbf{x}_A)}} \quad (3.12)$$

We now define a potential of mean force in  $\mathbf{r}$ :

$$e^{-\beta W(\mathbf{r})} \equiv \frac{\int d\mathbf{x}_A J_A(\mathbf{x}_A) e^{-\beta E_C(\mathbf{x}_A, \mathbf{r})}}{\int d\mathbf{x}_A J_A(\mathbf{x}_A) e^{-\beta E_A(\mathbf{x}_A)}} \quad (3.13a)$$

$$= \langle e^{-\beta(E_C(\mathbf{x}_A, \mathbf{r}) - E_A(\mathbf{x}_A))} \rangle_A = \langle e^{-\beta w(\mathbf{x}_A, \mathbf{r})} \rangle_A \quad (3.13b)$$

where  $\langle \dots \rangle_A$  represents an average over the equilibrium distribution of the internal coordinates of molecule A, computed as if the ligand was absent. Equation 3.13a expresses  $W(\mathbf{r})$  in terms of the ratio of two configurational integrals, one for the bound state in which the ligand is at a relative separation  $\mathbf{r}$  from the receptor and the other for the unbound state in which the ligand is at infinite separation. The potential of mean force  $W(\mathbf{r})$  can thus be seen as the change in free energy when the ligand is brought from infinite separation to a separation  $\mathbf{r}$  from the receptor. In terms of  $W(\mathbf{r})$ , the binding constant can now be written as

$$K_a = \int_b d\mathbf{r} e^{-\beta W(\mathbf{r})} \quad (3.14a)$$

where the subscript “b” signifies that the integration is restricted to the region where the ligand is deemed bound. This result can be compared with eq 3.9. Here  $W(\mathbf{r})$  plays the role of the potential energy arising from the interaction between the receptor and the ligand.

Usually the ligand is found in one distinct binding site. A simple illustration is provided by a square-well form of the potential of mean force  $W(\mathbf{r})$ , which takes a value  $W_0$  when the ligand is in a binding region of volume  $V_b$  and value 0 elsewhere (see Figure 1). Then

$$K_a = V_b e^{-\beta W_0} \quad (3.14b)$$

### 3.3. General Expression for $K_a$

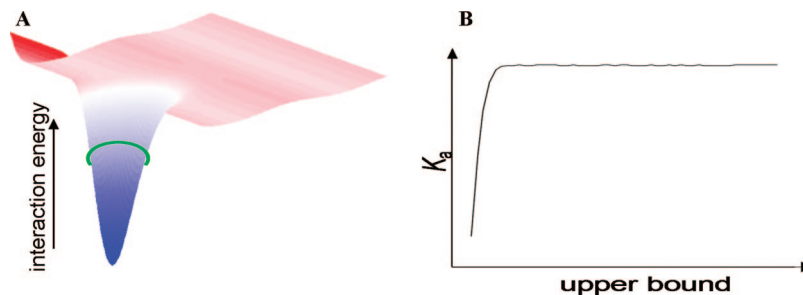
More generally, the ligand also has internal coordinates, so a complete specification of the complex requires not only the relative separation  $\mathbf{r}$  between the ligand and the receptor but also their relative orientation  $\boldsymbol{\omega}$ , along with coordinates specifying the conformation of the ligand. As in eq 3.13a, one can again define a potential of mean force  $W(\mathbf{r}, \boldsymbol{\omega})$ , whereby the binding constant can be expressed as

$$K_a = (8\pi^2)^{-1} \int_b d\mathbf{r} d\boldsymbol{\omega} J(\boldsymbol{\omega}) e^{-\beta W(\mathbf{r}, \boldsymbol{\omega})} \quad (3.15)$$

Details of the derivation can be found in section 3 of Supporting Information. A practical issue in the calculation of  $K_a$  via eq 3.15 is the specification of the region of integration (as indicated by the subscript “b”) for the complex.<sup>55,93</sup> In order to be considered as a separate species in a thermodynamic sense, the complex must be dominated by well localized configurations with very low values of  $W(\mathbf{r}, \boldsymbol{\omega})$  (Figure 2A), which make the overwhelming contributions to  $K_a$ . As long as the specified region of integration covers these configurations, an unequivocal value for  $K_a$  will be obtained (Figure 2B).<sup>55,91,92</sup>

## 4. Enthalpy–Entropy Decomposition of Binding Free Energy

The “standard” binding entropy under constant pressure is



**Figure 2.** (A) Diagram of a receptor–ligand potential of mean force  $W(\mathbf{r}, \omega)$ , shown as an energy surface in two dimensions with the bound state defined by the deep energy well. The boundary of the bound state can be specified by an upper bound, indicated by a green ring, of  $W(\mathbf{r}, \omega)$  so long as the well is only big enough to hold a single ligand at a time.<sup>91</sup> (B) Sketch of the binding constant associated with the energy well in (A) as a function of the upper bound of  $W(\mathbf{r}, \omega)$ . The binding constant is seen to be virtually independent of the upper bound so long as the lowest energy configurations are included.

$$\Delta S_b^\circ = k_B \ln(C^\circ K_a) + k_B T \left( \frac{\partial \ln K_a}{\partial T} \right)_P \quad (4.1)$$

in which we have neglected a small term arising from the fact that, under constant pressure, the volume and, hence, the concentration vary with temperature (section 2 of Supporting Information). Note that the binding entropy inherits the dependence of the binding free energy on the standard concentration.

The binding enthalpy is

$$\Delta H_b = k_B T^2 \left( \frac{\partial \ln K_a}{\partial T} \right)_P \quad (4.2)$$

which has no dependence on concentration. Thus, changing the arbitrarily selected value of  $C^\circ$  shifts both the standard free energy and the standard entropy of binding, but does not affect the enthalpy change.

Binding is sometimes referred to as enthalpy-driven or entropy-driven, depending on the signs and magnitudes of  $\Delta H_b$  and  $\Delta S_b^\circ$ . However, this distinction is not especially meaningful because it is based on the incorrect assumption that the zero value of  $\Delta S_b^\circ$  has physical significance. In reality, as just noted, the value of  $\Delta S_b^\circ$  depends on the choice of the standard concentration, whose conventional value of 1 M is arbitrary and historical in nature. In fact, there is no special physical significance to a zero value of either  $\Delta S_b^\circ$  or  $\Delta G_b^\circ$ . This point does not bear on the phenomenon of entropy–enthalpy compensation, however, because changing  $C^\circ$  shifts all values of  $\Delta S_b^\circ$  by a constant and, therefore, does not influence any correlation that may exist between  $\Delta S_b^\circ$  and  $\Delta H_b$ .

The example of a monatomic ligand binding a polyatomic receptor provides insight into the entropy component of the binding free energy. First let us specialize to the square-well potential of mean force, with the further assumption that the well depth,  $W_0$ , and the volume,  $V_b$ , of the binding region are both temperature-independent. Inserting eq 3.14b in eq 4.1, we find

$$\Delta S_b^\circ = k_B \ln(C^\circ V_b e^{-\beta W_0}) + \frac{1}{T} W_0 \quad (4.3a)$$

$$= k_B \ln(C^\circ V_b) = k_B \ln \left( \frac{V_b}{V^\circ} \right) \quad (4.3b)$$

which can be viewed as the entropy change when the volume available to the ligand changes on binding from the standard volume  $V^\circ$  to the volume accessible to the ligand after

binding,  $V_b$  (Figure 1). The result in this case can be identified as the change in translational entropy.

Now consider a general form of  $W(\mathbf{r})$ . Using eq 3.14a, we have

$$\Delta S_b^\circ = k_B \ln(C^\circ K_a) + k_B T \frac{\int_b d\mathbf{r} \left( \frac{\partial e^{-\beta W(\mathbf{r})}}{\partial T} \right)_P}{\int_b d\mathbf{r} e^{-\beta W(\mathbf{r})}} \quad (4.4a)$$

$$= k_B \ln(C^\circ K_a) + \frac{1}{T} \langle W(\mathbf{r}) \rangle_b - \left\langle \left( \frac{\partial W(\mathbf{r})}{\partial T} \right)_P \right\rangle_b \quad (4.4b)$$

The last result is formally similar to eq 2.25. By comparing against eq 4.3a, we can still identify the first two terms of eq 4.4b as the change in translational entropy:

$$\Delta S_t^\circ \equiv k_B \ln(C^\circ K_a) + \frac{1}{T} \langle W(\mathbf{r}) \rangle_b \quad (4.5)$$

The binding entropy can now be written as

$$\Delta S_b^\circ = \Delta S_t^\circ - \left\langle \left( \frac{\partial W(\mathbf{r})}{\partial T} \right)_P \right\rangle_b \quad (4.4c)$$

By further equating  $\Delta S_t^\circ$  to  $k_B \ln(V_b/V^\circ)$ , one can interpret the  $V_b$  thus obtained as the effective volume accessible to the ligand in the bound state. The change in translational entropy is further developed in subsection 5.3. (A calculation of  $V_b$  is illustrated in subsection 2.2 of Supporting Information.) For the present case of a monatomic ligand, the change in translational entropy apparently corresponds to what has been termed the “association entropy”.<sup>94</sup> The second term in eq 4.4c contains entropy contributions from internal motions and solvation.

Equation 4.5 for the change in translational entropy can also be applied to a flexible, polyatomic ligand, when averaging is taken not only over receptor and solvent degrees of freedom but also over the internal and rotational coordinates of the ligand to arrive at a potential of mean force,  $\bar{W}(\mathbf{r})$ , which still depends only on the relative separation  $\mathbf{r}$  (ref 55; see eq III.6 of Supporting Information).

Rearranging eq 4.5, we find that the binding free energy can be written as



$$\begin{aligned}\Delta G_b^\circ &= -k_B T \ln(C^\circ K_a) = \langle W(\mathbf{r}) \rangle_b - T\Delta S_t^\circ \\ &= \langle W(\mathbf{r}) \rangle_b - k_B T \ln\left(\frac{V_b}{V^\circ}\right)\end{aligned}\quad (4.6)$$

There is an intuitively helpful analogy here between the first term and the enthalpy change on binding, as well as between the second term and the entropy change on binding, and this analogy would become an identity if  $W(\mathbf{r})$  were temperature-independent. It is sometimes hypothesized that the binding free energy can be modeled as the result of additive contributions from the chemical groups making up a compound. There may indeed be cases where the average receptor–ligand potential of mean force,  $\langle W(\mathbf{r}) \rangle_b$ , can be approximated in the group-additive form,

$$\langle W(\mathbf{r}) \rangle_b = \sum_i \langle W_i(\mathbf{r}) \rangle_b \quad (4.7)$$

where  $i$  refers to different groups of the receptor and  $\langle W_i(\mathbf{r}) \rangle_b$  is the contribution of group  $i$  to  $\langle W(\mathbf{r}) \rangle_b$ . On the other hand, it is not clear that the translational entropy term of the standard binding free energy in eq 4.6 can be accurately modeled as additive in nature. Section 8 returns to this issue of group additivity.

## 5. Changes in Configurational Entropy upon Binding

As the discussion in sections 2 and 4 indicates, the change in entropy upon binding can be decomposed into a part associated with changes in solute motions, the configurational entropy, and a part associated with changes in solvent motions, the solvent entropy.<sup>76</sup> This decomposition can be understood as an application of eq 2.18 in which  $x_1$  and  $x_2$  stand for the solute and solvent degrees of freedom, respectively. The solvent part of the entropy change relates to the temperature-dependence of the difference in solvation free energy between the bound and unbound states (subsection 2.4), and has contributions from hydrophobic and electrostatic interactions between the receptor and the ligand. The configurational entropy relates to the changes in mobility of the receptor and ligand upon binding and can be estimated through evaluation of the first term in eq 2.18 by various means, including the quasi-harmonic approximation in Cartesian<sup>95–98</sup> or torsional<sup>37</sup> coordinates, scanning methods,<sup>21</sup> the M2 method,<sup>16,76</sup> the mutual information expansion,<sup>81</sup> the nearest-neighbor method,<sup>99</sup> or a combined mutual information/nearest neighbor approach.<sup>100</sup>

We now address the experimental evaluation of configurational entropy and the further decomposition of configurational entropy into translational, rotational, and internal parts, with particular attention to the long-standing issue of changes in translational entropy on binding.

### 5.1. Experimental Determination of Configurational Entropy Changes

The total change in entropy on binding can be determined experimentally by measuring  $K_a$  at different temperatures and then computing the enthalpy and entropy components of the binding free energy by taking temperature derivatives. Alternatively, isothermal titration calorimetry can be used to measure the binding free energy via the equilibrium constant and the binding enthalpy via heat release, and from

these the binding entropy can be computed. However, neither approach can separate the configurational and solvation parts of the binding entropy.

Elegant neutron-scattering methods have been used to probe differences in the vibrational density of states before and after enzyme–inhibitor binding, yielding an estimated 4 kcal/mol configurational entropy change that, somewhat surprisingly, favors binding.<sup>33</sup> It is worth noting that density of states measurements were done only for the free and complexed protein, so the configurational entropy associated with the free ligand was not subtracted from the final result. As a consequence, the experiment may not report on the full change in configurational entropy upon binding, and accounting for the free ligand might reverse the sign of the final result. Interestingly, a follow-up computational study<sup>34</sup> suggests that the measured entropy change is an average over proteins trapped in different local energy wells due to the low temperature at which the experiment was done. The reported penalty thus appears to represent a change in vibrational entropy without a contribution from the conformational entropy, in the sense of eq 2.13.

NMR relaxation data are uniquely able to provide structurally correlated information about the configurational entropy change upon binding.<sup>25–32</sup> The NMR order parameter  $\mathcal{J}^2$  of a bond vector  $\mathbf{n}$ , often that of a backbone amide N–H bond, is a measure of the extent of orientational fluctuation of  $\mathbf{n}$ . The order parameter is defined as<sup>101</sup>

$$\mathcal{J}^2 = \frac{4\pi}{5} \sum_{m=-2}^2 \left| \int d\mathbf{n} \rho(\mathbf{n}) Y_{2m}(\mathbf{n}) \right|^2 \quad (5.1a)$$

where  $\rho(\mathbf{n})$  is the probability density of  $\mathbf{n}$ . The entropy associated with the orientational motion of  $\mathbf{n}$  can be written as (cf. eq 2.5b)

$$S_{\text{om}} = -k_B \int d\mathbf{n} \rho(\mathbf{n}) \ln \rho(\mathbf{n}) \quad (5.1b)$$

It is clear that  $\mathcal{J}^2$  and  $S_{\text{om}}$  are related. For the diffusion-in-a-cone model of orientational motion, in which the polar angle of  $\mathbf{n}$  is restricted to between 0 and an upper limit  $\theta_0$ , both  $\mathcal{J}^2$  and  $S_{\text{om}}$  can be easily obtained. Using  $\cos \theta_0$  as an intermediate variable to relate  $S_{\text{om}}$  directly to  $\mathcal{J}^2$ , one finds<sup>102,103</sup>

$$S_{\text{om}} = k_B \ln \{ \pi [3 - (1 + 8\mathcal{J}^2)^{1/2}] \} \quad (5.1c)$$

The order parameters of backbone amides and side-chain methyls of a protein can be determined before and after ligand binding. These results can be converted into entropic contributions by a relation like eq 5.1c and summed over individual residues, to yield information on the change in configurational entropy of the protein upon binding. Such studies yield unique and valuable insight into the contribution of conformational fluctuations to the binding affinity, but the numerical results should be interpreted with caution.<sup>27</sup> First, a model for bond orientational motion other than diffusion-in-a-cone will yield a different relation between  $S_{\text{om}}$  and  $\mathcal{J}^2$ . Second, summing the contributions of individual bond-vectors effectively makes the assumption that their motions are independent; in reality, motions of neighboring bonds can be highly correlated, and the numerical consequences of such correlations for the entropy (see subsection 2.2.2) are still uncertain. At least one study has argued that the entropic consequences of pairwise correlations are minor,

although correlations expected to be large due to short-ranged chain connectivity were not assessed.<sup>104</sup> Finally, order parameters are sensitive only to a subset of motions, both in time scale (picoseconds to nanoseconds) and in type (motions that affect bond orientations), so some important coordinates, such as translation of a bound ligand relative to the receptor, may not be captured. It is worth mentioning recent work<sup>105</sup> directed at clarifying the connections between bond-vector fluctuations and conformational distributions, as such connections can help interpret order parameters in terms of well-defined coordinate fluctuations.

## 5.2. Decomposition of Configurational Entropy

As discussed in subsection 2.3, the  $3n$  coordinates of a molecule or complex with  $n$  atoms can be separated into 3 overall translational coordinates; 3 overall rotational coordinates; and  $3n - 6$  internal coordinates. Accordingly, following eq 2.19, the complete configurational entropy can be formally decomposed as

$$S = S_t + S_r + S_{in} - I_{t,r} - I_{t,in} - I_{r,in} + I_{t,r,in} \quad (5.2a)$$

where  $S_t$ , for example, is the entropy associated with the marginal probability density of the 3 overall translational coordinates,  $I_{t,r}$  is the mutual information measuring correlation between overall translational and overall rotational motion, and  $I_{t,r,in}$  measures the third-order correlation among all three sets of coordinates. However, both the second- and third-order mutual information terms in eq 5.2a are identically zero, rigorously so in the FM approach (subsection 2.3.2) and as a basic assumption in the RRHO approach (subsection 2.3.1). When using the FM approach for a bimolecular complex, one may furthermore separate the two molecules' 6 relative translational and rotational coordinates (indicated by  $t'$  and  $r'$ ) from the remaining  $3n - 12$  internal coordinates (indicated by  $in'$ ) and write the following entropy decomposition

$$S = S_t + S_r + S_{t'} + S_{r'} + S_{in'} - I_{t,r'} - I_{t',in'} - I_{r',in'} + I_{t',r',in'} \quad (5.2b)$$

where the identically zero mutual information terms involving overall translation and overall rotation are omitted. However, this separation of the relative translational and rotational coordinates of the complex from its other internal coordinates is not customary in the RRHO approach. Each subset of coordinates in eq 5.2b could be still further decomposed, and such decomposition would reveal additional correlations among, for example, the 3 relative translational coordinates.

The decomposition of entropy changes on binding has been the subject of considerable discussion in the literature (e.g., refs 14, 18, 58–61, 67, 70, and 106–108). However, little attention has been given to the mutual information terms, i.e., to the entropic consequences of correlation, even though the mutual information terms can be similar in magnitude to the individual entropy terms, as, indeed, suggested by recent studies.<sup>76,109</sup>

## 5.3. Changes in Translational Entropy on Binding

Changes in translational entropy on binding have been addressed with particular vigor in the theoretical literature, as noted in the Introduction. This is, perhaps, appropriate,

given that the essence of binding is that two molecules that initially move about independently become attached to one another and then translate together. The present section uses the theoretical framework presented in the preceding sections to analyze various views of translational entropy from a consistent standpoint.

### 5.3.1. Change in Translational Entropy in the FM Approach

For a monatomic ligand, the change in translational entropy in the FM approach is formally defined by eq 4.5. In the context of eq 5.2, this result can be interpreted as the difference between the sum of the overall and relative translational entropy terms of the complex, C, and the sum of the overall translational entropy terms of the receptor, A, and ligand, B:

$$\Delta S_t^\circ = S_t^\circ(C) + S_{t'}(C) - S_t^\circ(A) - S_t^\circ(B) \quad (5.2c)$$

Because the same standard volume,  $V^\circ$ , applies to all three species, one has  $S_t^\circ = k_B \ln V^\circ$ , for A, B, and C. If the effective volume accessible to the ligand after binding is  $V_b$ , then  $S_{t'}(C) = k_B \ln V_b$ . Thus, we obtain  $\Delta S_t^\circ = k_B \ln(V_b/V^\circ)$ , as already found in section 4.

It is a subtle but important conceptual point that the change in translational entropy defined in this way depends on how the relative translational coordinates,  $\mathbf{r}$ , are defined for the complex.<sup>55</sup> There are multiple different and yet correct ways to define this coordinate system. For example,  $\mathbf{r}$  might be a vector from the center of mass of the receptor to the monatomic ligand,<sup>15</sup> or it could equally well be a vector from any atom of the receptor to the ligand (e.g., ref 81). Any such definition of  $\mathbf{r}$  can be used as the translational part of a fully consistent coordinate system for the ligand relative to the receptor, yet each will, in general, lead to a different change in translational entropy upon binding. For example, if  $\mathbf{r}$  is the vector from an atom in the receptor's binding site to the ligand, then the ligand will be found to have low translational entropy after binding. In contrast, if  $\mathbf{r}$  is chosen to be the vector from a receptor atom that is very mobile relative to the binding site, then the ligand may be found to have relatively high translational entropy after binding. Similar considerations apply if one turns the system around and regards the receptor as a polyatomic ligand and the ligand as a small receptor.

Furthermore, different coordinate systems will correspond to different degrees of correlation and, hence, different mutual information terms between relative translational and other coordinates (eq 5.2b). For example, defining the ligand's position with respect to a mobile atom far from the binding site is likely to generate large mutual information between relative translation and the internal degrees of freedom of the receptor. A lower mutual information is likely if the ligand is defined relative to an atom in the binding site. The latter definition is also more intuitively appealing, and one's intuitive choice of a reasonable coordinate system tends to be one that minimizes mutual information.

Of course, the choice of coordinate system cannot affect the *total* change in configurational entropy on binding; it can only affect the *partitioning* of the change in configurational entropy among the translational, rotational, internal, and mutual information parts (eq 5.2a). (It is worth remarking that a correct and complete treatment of these coordinate

system issues can become quite complicated, requiring careful attention to Jacobian contributions, as previously emphasized.<sup>89,90</sup> One may legitimately conclude, therefore, that the change in translational entropy upon binding in general is not in itself a well-defined physical quantity. It is best understood as one part of the larger picture of entropy changes on binding.

### 5.3.2. RRHO Approach to Translational Entropy

In the RRHO approach, all internal motions of the receptor, ligand, and the complex are treated as vibrations. Unlike in the FM approach, the relative translational motions of the bound ligand and receptor are not distinguished from the other internal motions. As a consequence, when the RRHO approach is used, the change in translational entropy upon binding is considered to involve only overall translation of the three species.

The RRHO approach yields eq 2.21 for the partition function of a receptor, ligand or complex. When each species is present at a given concentration, the system is treated as an ideal gas, as justified by the McMillan–Mayer solution theory.<sup>110</sup> Inserting eq 2.21 into eq I.35 of Supporting Information yields the entropy per reactant or product molecule at, for convenience, the standard concentration,  $C^\circ$ :

$$S_{\text{RRHO}}^\circ = S_{\text{RRHO,t}}^\circ + S_{\text{RRHO,r}} + S_{\text{RRHO,vib}} \quad (5.3a)$$

where

$$S_{\text{RRHO,t}}^\circ = k_B \ln \left[ \left( \frac{2\pi m}{\beta h^2} \right)^{3/2} \frac{e^{5/2}}{C^\circ} \right] \quad (5.3b)$$

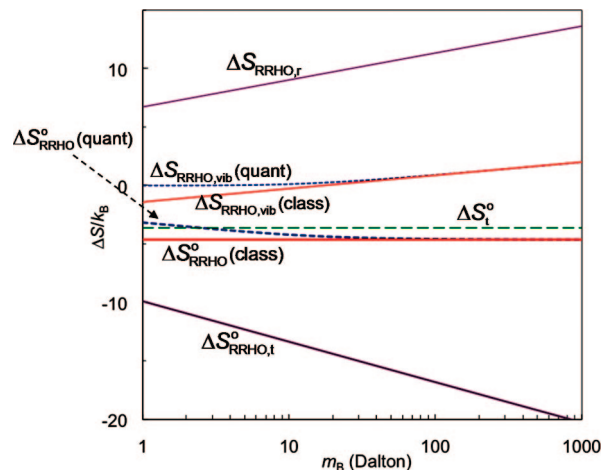
$$S_{\text{RRHO,r}} = k_B \ln \left[ 8\pi^2 \left( \frac{2\pi e}{\beta h^2} \right)^{3/2} (I_1 I_2 I_3)^{1/2} \right] \quad (5.3c)$$

$$S_{\text{RRHO,vib}} = k_B \sum_i \left[ \frac{\beta h \omega_i}{2\pi} (e^{\beta h \omega_i / 2\pi} - 1)^{-1} - \ln(1 - e^{-\beta h \omega_i / 2\pi}) \right] \quad (5.3d)$$

Equation 5.3b for the translational part is called the Sackur–Tetrode equation. The standard binding entropy is finally calculated as

$$\Delta S_{\text{RRHO}}^\circ = \Delta S_{\text{RRHO}}^\circ(\text{C}) - \Delta S_{\text{RRHO}}^\circ(\text{A}) - \Delta S_{\text{RRHO}}^\circ(\text{B}) \quad (5.4)$$

The translational part,  $\Delta S_{\text{RRHO,t}}^\circ$ , of this calculation is sometimes referred to as the change in translational entropy on binding. However,  $\Delta S_{\text{RRHO,t}}^\circ$  is different from  $\Delta S_t^\circ$  introduced by eq 4.5 and equivalently given by eq 5.2c. It is straightforwardly shown that  $\Delta S_{\text{RRHO,t}}^\circ$  is given by  $-S_{\text{RRHO,t}}^\circ$  with  $m$  set to the reduced mass  $m_A m_B / m_C$ . Therefore  $\Delta S_{\text{RRHO,t}}^\circ$  depends on the molecular masses, whereas  $\Delta S_t^\circ$  has no mass dependence. In this connection we note that  $\Delta S_{\text{RRHO,r}}$  and  $\Delta S_{\text{RRHO,vib}}$ , the rotational and vibrational parts of  $\Delta S_{\text{RRHO}}^\circ$ , are also mass-dependent. It turns out that the mass-dependence of  $\Delta S_{\text{RRHO,r}}$  and  $\Delta S_{\text{RRHO,vib}}$  leads to near-complete cancellation of the mass-dependence of  $\Delta S_{\text{RRHO}}^\circ$  as a whole when the quantum expression for the vibrational entropy is used, and to apparently complete cancellation when the classical expression is used.

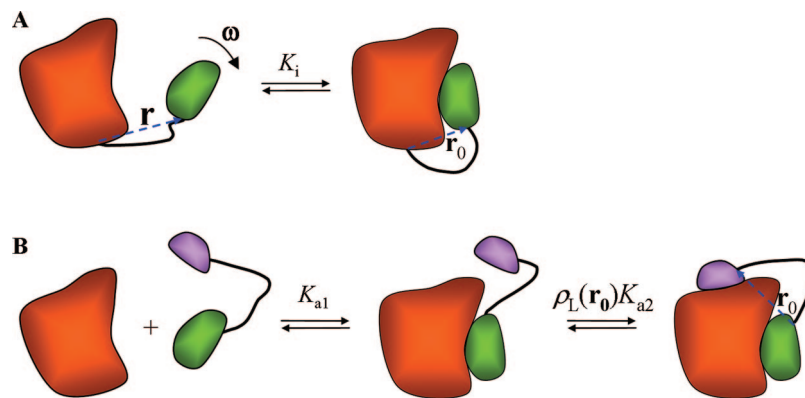


**Figure 3.** Entropy of binding and its parts for a diatomic binding model according to the RRHO approximation. The entropy,  $\Delta S_{\text{RRHO}}^\circ$  of binding in the FM approach is also shown for comparison. As also labeled in the figure, from top to bottom on the left axis, the graphs are as follows:  $\Delta S_{\text{RRHO,t}}^\circ$ ;  $\Delta S_{\text{RRHO,vib}}^\circ$  in the quantum and classical formulations;  $\Delta S_{\text{RRHO}}^\circ$  in the quantum formulation;  $\Delta S_t^\circ$ ;  $\Delta S_{\text{RRHO}}^\circ$  in the classical formulation; and  $\Delta S_{\text{RRHO,t}}^\circ$ . The results are derived in subsection 2.2 of Supporting Information. Parameters used are:  $m_A = 68$  kD,  $k = 500$  kcal/mol/Å<sup>2</sup>,  $r_0 = 5$  Å, and  $T = 300$  K.

The last observation is illustrated in Figure 3, which graphs  $\Delta S_{\text{RRHO}}^\circ$  and its components  $\Delta S_{\text{RRHO,t}}^\circ$ ,  $\Delta S_{\text{RRHO,r}}$ , and  $\Delta S_{\text{RRHO,vib}}^\circ$  for the binding of two atoms to form a diatomic complex held together by a harmonic bond (eq 3.5), as a function of atomic mass. (See subsection 2.2 of Supporting Information.) For  $\Delta S_{\text{RRHO,vib}}^\circ$ , both the quantum mechanical result, from eq 5.3d, and the classical approximation, obtained by taking the  $\beta \rightarrow 0$  limit, are shown. It is clear that, although the separate entropy terms from the RRHO treatment—translational, rotational, and vibrational—all depend upon mass, the total entropy change,  $\Delta S_{\text{RRHO}}^\circ$ , is independent of mass for the classical approximation, and, in the quantum mechanical case, becomes dependent on mass only when the atoms are very light. The mass independence of the classical approximation for  $\Delta S_{\text{RRHO}}^\circ$  is expected, since the binding constant in this case, given by eq 3.7, is independent of mass.

For the diatomic binding model, we show in subsection 2.2 of Supporting Information that  $\Delta S_t^\circ$  is the total binding entropy calculated by the FM approach and that it differs from the RRHO counterpart, i.e., the classical approximation for  $\Delta S_{\text{RRHO}}^\circ$ , by a small amount,  $-k_B$ . For comparison, we also graph  $\Delta S_t^\circ$  in Figure 3. It is clear that  $\Delta S_t^\circ$  differs substantially from  $\Delta S_{\text{RRHO,t}}^\circ$ . Thus, while the two approaches basically agree on the change in total entropy, they differ sharply on the change in translational entropy. As made clear by the preceding discussion, these are really two very different definitions of the change in translational entropy. In the RRHO approach, the relative motions of the receptor and ligand molecules within the complex are not considered to include any translational component; instead, all the entropy associated with its motions is ascribed to the vibrational entropy of the complex, as previously emphasized,<sup>59</sup> and thus does not appear as a component of the translational entropy after binding. In contrast, in the FM approach, the relative translational coordinates of the two bound molecules are treated separately from the other internal coordinates of the complex (eq 5.2b), and the corresponding relative translational entropy balances some of the overall translational entropy lost on binding. It should be emphasized





**Figure 4.** (A) Receptor (orange) and ligand (green) connected by a linker (black), showing the position vector,  $\mathbf{r}$ , relating the ligand to the receptor, and the orientation of the ligand in space  $\omega$ . In the bound state,  $\mathbf{r}$  takes the value  $\mathbf{r}_0$ . (B) Binding of a bivalent ligand can be viewed as the sequential binding of the first fragment followed by intramolecular binding of the second.

that the difference between  $\Delta S_{\text{RRHO},i}^{\circ}$  and  $\Delta S_i^{\circ}$  does not reflect a deficiency in either expression. Instead, these are two different and equally valid definitions of the change in translational entropy on binding. That said, it is worth noting that the assumption of rigidity in treating overall rotation means that the RRHO treatment is not well-suited for studying highly flexible molecules.

Sometimes  $\Delta S_{\text{RRHO},i}^{\circ}$  and  $\Delta S_{\text{RRHO},r}$  are combined with other contributions to empirically construct an estimate of the binding free energy. We note that, if these other contributions do not depend on mass and Planck's constant in a way that makes the binding free energy as a whole independent of mass and Planck's constant as  $\beta \rightarrow 0$ , then such constructions must be internally inconsistent.

### 5.3.3. Free Volume Theories of Translational Entropy

It has been argued that the loss of translational entropy on binding can be markedly overestimated by approaches like those just discussed because, even in their unbound state, molecules A and B are caged by solvent molecules so the “free volume” accessible to them before binding,  $V_f$ , is much smaller than the system volume,  $V$ , or the standard volume,  $V^{\circ}$ , which appear in the expressions above.<sup>64</sup> Accordingly, the effective concentration,  $C = 1/V_f$ , has been inserted into the Sackur–Tetrode equation to compute the translational entropy.<sup>64</sup> (It is not clear why similar arguments have not been applied to the rotational entropy, too, since rotational motion is subject to similar solvent-caging.) This reasoning appears problematic, however, because molecules A and B are restricted to their solvent cages only on a very short time scale. Over the time scale of typical binding measurements, molecules A and B roam the entire system volume. After binding, they still roam the entire volume, but the translational range of molecule B relative to A (or vice versa) is greatly reduced, as discussed above, so there is a substantial loss of translational freedom and entropy. Put differently, A and B still roam the entire volume after binding, but now their motions are highly correlated, and the translational correlation lowers the entropy due to a large mutual information term (subsection 2.2.2). The free volume approach appears to ignore this part of the translational entropy loss.

Another part of the reasoning in support of the free volume viewpoint has been that the drop in entropy when a monatomic gas dissolves in water results from its becoming translationally caged by water molecules. Even if one adopted this view, there would still be an additional translational

entropy loss if the gas atom were then to bind another molecule in solution, due again to an increase in correlation. It is this binding entropy, not the solvation entropy, that is the topic of the present discussion.

An alternative development of the free volume theory has been used to argue that the change in translational entropy on binding is given by

$$\Delta S_{i,\text{free vol}}^{\circ} = k_B \ln(C^{\circ} V_{f,\text{bound}}) - k_B \ln(C_{\text{solvent}} V_{f,\text{solvent}}) - k_B \quad (5.5)$$

(eq 6 in ref 64). Here  $C_{\text{solvent}}$  is the molarity of the solvent (55 M for water), and  $V_{f,\text{solvent}}$  and  $V_{f,\text{bound}}$  represent the free volume accessible to the ligand in solvent versus the binding site. This expression has been rearranged from the original to highlight its similarity to eq 4.3b. In fact, if  $V_{f,\text{bound}}$  is considered equivalent to  $V_b$ , then the differences between eq 5.5 and eq 4.3b cancel for the relative change in translational entropy on binding to two different receptors by the same ligand. Thus, the two equations appear to reflect similar theoretical approaches. On the other hand, it is difficult to determine whether or when the second term in eq 5.5 is correct, especially because, as discussed above, a formula for the change in translational entropy should be considered in the context of a theory for the total change in entropy, and we are not aware of such a context for eq 5.5. It is also unclear whether problems surrounding the so-called communal entropy in this free volume theory, which were highlighted by Kirkwood,<sup>111</sup> have been fully resolved.

## 6. Relation between Intermolecular and Intramolecular Binding

When the ligand is tethered to the receptor, binding becomes intramolecular (Figure 4A). For example, SH3 domains sometimes are found tethered to their proline-rich ligands. The intramolecular binding constant is given by

$$K_i \equiv \frac{(C_C)_{\text{eq}}}{(C_A)_{\text{eq}}} \quad (6.1)$$

where  $C_C$  and  $C_A$  now signify, respectively, the concentrations of the receptor with the tethered ligand in and out of the binding site. Their ratio is determined by the relative probabilities of the single receptor–tether–ligand molecule in two conformations and is unaffected by its total concentration. This marks an important departure from intermo-



lecular binding, where the concentration ratio of bound and free receptors depend on the concentration of the ligand (see eq 3.3). The concentration independence of intramolecular binding may present an advantage which partly accounts for the gene fusion of proteins that function in the bound form.

Intuitively, one may assume that the role of a flexible tether is to fix the ligand at an effective concentration,  $C_{B,eff}$ . Comparison between eqs 6.1 and 3.3 then leads to

$$K_i = K_a C_{B,eff} \quad (6.2a)$$

A formal derivation shows that the effective concentration is given by the probability density,  $\rho_L(\mathbf{r})$ , of the end-to-end vector  $\mathbf{r}$  of the linker:<sup>112</sup>

$$K_i = K_a \rho_L(\mathbf{r}_0) \quad (6.2b)$$

where  $\mathbf{r}_0$  is the end-to-end vector when the ligand is bound to the receptor (Figure 4A) (see section 4 of Supporting Information). Values of the effective concentration as given by  $\rho_L(\mathbf{r}_0)$  for flexible peptide linkers are typically found in the mM range.<sup>112,113</sup> Therefore, tethering the ligand to its receptor achieves the same effect as having the ligand present at a fixed concentration of  $\sim 1$  mM. Intramolecular ligand binding plays prominent roles in regulation of enzyme activities,<sup>113</sup> and tethering by disulfide bond formation has been used as a technique for identifying weak binding ligands.<sup>114</sup> Inasmuch as ligand binding can shift the folding–unfolding equilibrium of a protein toward the folded state, intramolecular ligand binding can also increase the stability of a folded protein.<sup>113</sup>

Tamura and Privalov<sup>65</sup> measured the difference in binding entropies,  $\Delta S_b^\circ - \Delta S_i$ , between a dimeric protein and a cross-linked variant and, like Page and Jencks,<sup>115</sup> attributed the binding-entropy difference to the loss of translational/rotational entropy. Karplus and Janin<sup>68</sup> commented that such an attribution is not valid.

We now use eq 6.2b to analyze  $\Delta S_b^\circ - \Delta S_i$  in more detail. Neglecting the temperature dependence of the probability density  $\rho_L(\mathbf{r}_0)$ , we find that

$$\Delta S_i = \Delta S_b^\circ + k_B \ln[V^\circ \rho_L(\mathbf{r}_0)] \quad (6.3a)$$

or, equivalently,

$$\Delta S_b^\circ - \Delta S_i = -k_B \ln[V^\circ \rho_L(\mathbf{r}_0)] \quad (6.3b)$$

The right-hand side of eq 6.3b can be recognized as the entropy change of the ligand when its concentration is changed from the standard concentration,  $1/V^\circ$ , to the effective concentration,  $\rho_L(\mathbf{r}_0)$ . In essence, there is a difference in ligand concentration in the unbound state between intermolecular and intramolecular binding: it is the standard concentration in the former case but the effective concentration in the latter case. The difference between the intermolecular and intramolecular binding entropies is a reflection of the difference in ligand concentration before binding. When  $\rho_L(\mathbf{r}_0)$  was predicted from a polymer model and used in eq 6.3a, experimental results for  $\Delta S_b^\circ - \Delta S_i$  were reasonably reproduced.<sup>116</sup>

## 7. Multivalent Binding

High affinities can be achieved by multivalent binding, where the receptor has multiple binding sites and the ligand consists of multiple linked fragments, each with affinity to

one site. When two fragments that target neighboring nonoverlapping sites on a receptor are tethered to each other, the binding constant ( $K_{1-2}$ ) of the resulting bivalent ligand can be related to the binding constants ( $K_{a1}$  and  $K_{a2}$ ) of the fragments. The binding can be envisioned to occur stepwise: the first fragment followed by the second fragment (Figure 4B). Upon binding, the first fragment effectively becomes part of the receptor; the binding of the second fragment can then be considered intramolecular. Combining the two steps and using eq 6.2b, we have

$$K_{1-2} = K_{a1} K_{a2} \rho_L(\mathbf{r}_0) \quad (7.1)$$

When  $K_{a1} \rho_L(\mathbf{r}_0)$  and  $K_{a2} \rho_L(\mathbf{r}_0)$  are greater than 1, the affinity of the bivalent ligand will be higher than that of either of the two fragments by itself. (Note that  $K_{1-2}$  is the binding constant for simultaneous association of both fragments with their respective sites. If it happened that  $\rho_L(\mathbf{r}_0)$  and hence  $K_{1-2}$  were extremely small, then the dominant bound species would consist of the two singly bound fragments.) As noted in the preceding section, typical values of  $\rho_L(\mathbf{r}_0)$  are around 1 mM, so that the affinity of the bivalent ligand is expected to be greater than that of one individual fragment when the fragmental binding constants are greater than  $\sim 10^3 \text{ M}^{-1}$ . Fragments with such a modest affinity are relatively easy to find, so covalent linkage between fragments can provide a simple strategy for achieving high affinity. This strategy has found significant successes in practice.<sup>117,118</sup> Moreover, the scheme can be easily extended to treat more than two linked fragments, with each additional fragment providing extra affinity enhancement. For example, a designed trivalent ligand was able to increase the affinity from  $10^6 \text{ M}^{-1}$  to  $3 \times 10^{16} \text{ M}^{-1}$ .<sup>119</sup> Multivalent ligands provide a trivial example of the fact that  $K_a$  does not have an upper limit so long as one places no upper limit on the sizes of the ligand and receptor.

Despite an early warning by Jencks,<sup>120</sup> it is still a common mistake to expect that  $K_{1-2}$  equals the product  $K_{a1} K_{a2}$ , or equivalently, that the binding free energy of a bivalent ligand equals the sum of the binding free energies of the two fragments. A simple indication of the fallacy of this expectation is the mismatch in units,  $\text{M}^{-1}$  for  $K_{1-2}$  vs  $\text{M}^{-2}$  for  $K_{a1} K_{a2}$ . Equation 7.1 lays the foundation for the relation between the binding free energy of the bivalent ligand and those of its fragments. In terms of standard binding free energy, this equation becomes

$$\Delta G_{1-2}^\circ = \Delta G_1^\circ + \Delta G_2^\circ - k_B T \ln[V^\circ \rho_L(\mathbf{r}_0)] \quad (7.2)$$

The first two terms are the additive contributions of the two fragments. The third term, which has already appeared as entropy in eq 6.3b, accounts for the fact that, after the first fragment has bound, the unbound second fragment is present at the effective concentration, not the standard concentration.

In the same paper, Jencks also proposed that  $\Delta G_{1-2}^\circ - \Delta G_1^\circ$  provides an estimate for the interaction energy of the second fragment with the receptor. We can assess Jencks's proposition for the case where the second fragment is a monatomic ligand and the linker is fully flexible. For this case, eqs 7.1 and 3.14b yield  $\Delta G_{1-2}^\circ - \Delta G_1^\circ = \langle W_2(\mathbf{r}) \rangle_{b2} - k_B T \ln[\rho_L(\mathbf{r}_0) V_{b2}]$ . Thus, there are actually two contributions to  $\Delta G_{1-2}^\circ - \Delta G_1^\circ$ . One, consistent with Jencks's view, is the interaction energy,  $\langle W_2(\mathbf{r}) \rangle_{b2}$ . The second,  $-k_B T \ln[\rho_L(\mathbf{r}_0) V_{b2}]$ , arises from the fact that the linker end-to-end vector  $\mathbf{r}$  can

explore all the allowed space in the unbound state but is restricted to the volume  $V_{b2}$  available to the monatomic ligand while bound. This second term is essentially the loss in translational entropy of the monatomic ligand on binding, conditional upon the fact that it is attached to the already bound first fragment via a flexible linker. However, Jencks's proposition does appear to be valid for the special case in which the monatomic ligand is attached to the first fragment via a linker whose conformation and orientation relative to the first fragment are rigidly fixed, because in this case,  $\Delta G_{1-2}^{\circ} - \Delta G_1^{\circ} = W_2(\mathbf{r}_0)$  (eq IV.5d of Supporting Information). This quantity measures the free energy arising from the interaction of the ligand with the receptor while rigidly held at  $\mathbf{r}_0$  by the linker. In short, there is an entropy cost associated with restricting the second fragment to its binding site; this is minimized when a suitable rigid linker is used.

## 8. Ligand Efficiency and Additivity Analysis of Binding Free Energy

There is general expectation that the binding affinity can increase as more functional groups are added to a ligand, and this expectation is met, at least to some extent, by experimental studies.<sup>121,122</sup> On the other hand, there is at least a rough upper limit on the molecular weight for a ligand that is to become a drug, so it can be important to gain as much affinity as possible with a small ligand. Accordingly, the ratio of  $-\Delta G_b^{\circ}$  to  $n_{ha}$ , the number of heavy atoms in the ligand, has been proposed as a figure of merit termed the ligand efficiency.<sup>123</sup> It is worth noting that this quantity depends upon the choice of standard concentration in a nontrivial fashion, as now discussed.

According to eq 4.6,  $\Delta G_b^{\circ}$  consists of two terms, one arising from receptor–ligand interactions and the other from the change in available volume from  $V^{\circ}$  to  $V_b$ . A typical binding constant of  $10^9 \text{ M}^{-1}$  corresponds to a binding free energy  $\Delta G_b^{\circ} = -12.3 \text{ kcal/mol}$  at room temperature. There are no rules for the magnitude of  $V_b$ , but for the sake of argument let us assume a value  $\sim 1 \text{ \AA}^3$ . The corresponding translational entropy term has a value of  $4.4 \text{ kcal/mol}$ , so the interaction energy term must have a value of  $-16.7 \text{ kcal/mol}$ . This simple calculation shows that the interaction energy term dominates the translational entropy term. This dominance helps account for the near-zero  $y$ -intercepts in graphs of  $\Delta G_b^{\circ}$  vs  $n_{ha}$  for different ligands.<sup>121,122</sup> For example, a correlation analysis by Wells and McClendon<sup>122</sup> showed that the binding free energies of 13 ligands targeting different receptors closely followed the relation  $-\Delta G_b^{\circ} = 0.24n_{ha}$ . According to this relation, ligands with 34, 51, and 68 heavy atoms would have binding constants of  $10^6$ ,  $10^9$ , and  $10^{12} \text{ M}^{-1}$ , respectively, and these ligands all have a ligand efficiency of 0.24. Now, consider what would happen if the standard concentration were 1 nM. First, the value of  $\Delta G_b^{\circ}$  corresponding to  $K_a = 10^9 \text{ M}^{-1}$  would be exactly 0, and the translational entropy term would have the same magnitude as the interaction energy term. Second, for the ligands listed above with 34, 51, and 68 heavy atoms and corresponding binding constants of  $10^6$ ,  $10^9$ , and  $10^{12} \text{ M}^{-1}$ , the values of  $\Delta G_b^{\circ}$  would be  $-4.1$ ,  $0$ , and  $4.1 \text{ kcal/mol}$ , respectively. The ligand efficiency,  $-\Delta G_b^{\circ}/n_{ha}$ , would therefore have widely disparate values,  $-0.12$ ,  $0$ , and  $0.06$ , and might no longer be perceived as a useful metric.

A fundamental issue with the definition of ligand efficiency is that the change in translational entropy on binding, and consequently the binding free energy as a whole, are not

group additive. This problem is significantly lessened when we focus on differences in binding free energy among similar ligands for the same receptor, since then we will benefit from the cancellation of the dependence on the standard concentration and at least partial cancellation of the relative translational entropy in the bound state. Consider the case where an additional atom, forming significant favorable interactions with the receptor, is rigidly attached to an existing ligand. The motion of the original ligand within its binding site is assumed to be unperturbed by the added atom. Then, as concluded in the last paragraph of the preceding section, the added atom only contributes to the interaction energy term. Calculations of mutation effects in toy models of protein–protein binding support the dominance of the interaction energy term in the contribution of an additional group bonded to a ligand which already has significant affinity.<sup>92</sup> One may thus conclude that an additive model is much more likely to be successful when applied to relative binding free energies than total binding free energies.<sup>124,125</sup>

Finally, we comment on the practice of discussing the energetic contributions of individual chemical groups in the context of the total binding free energy, as sometimes done in studies of mutational effects on protein–protein binding affinity. To illustrate, suppose that two proteins form a complex with a binding constant of  $10^9 \text{ M}^{-1}$ , which, as mentioned above, corresponds to  $\Delta G_b^{\circ} = -12.3 \text{ kcal/mol}$  at room temperature. Now a point mutation from arginine to alanine is made on one of the proteins. As a result, the binding constant is reduced to  $10^7 \text{ M}^{-1}$ , or equivalently, the binding free energy is raised by  $\Delta\Delta G_b = 2.7 \text{ kcal/mol}$ . It may then be claimed that the arginine residue contributes  $\Delta\Delta G_b/(-\Delta G_b^{\circ}) = 2.7/12.3 = 22\%$  of the binding free energy. This view is problematic in two respects. First, as the preceding discussion makes clear, the bulk of the  $2.7 \text{ kcal/mol}$  contribution of arginine is to the interaction energy term; the mutant likely pays a translational entropy cost similar to that of the wild-type protein. Thus, the presence of the essentially constant translational entropy term in the denominator,  $-\Delta G_b^{\circ}$ , masks any simple interpretation of the ratio  $\Delta\Delta G_b/(-\Delta G_b^{\circ})$ . Second, because the value of  $-\Delta G_b^{\circ}$  depends on the choice of the standard concentration, the value of  $\Delta\Delta G_b/(-\Delta G_b^{\circ})$  also depends on this completely arbitrary quantity. For example, if the standard concentration were 1 nM,  $\Delta G_b^{\circ}$  would be 0, and it would then certainly not be sensible to assign any meaning to the ratio  $\Delta\Delta G_b/(-\Delta G_b^{\circ})$ .

## 9. Concluding Remarks

The theory of noncovalent binding has been formulated and interpreted in a number of different ways in the literature, some more faithful than others to the underlying statistical thermodynamics. We have sought here to provide a unified, rigorous review of this material that addresses not only simple, intermolecular binding but also intramolecular and multivalent binding; to generate insight through simple examples; and to bring out important physical implications of the mathematical formulas. Central observations include the following:

(1) Because the RRHO approximation treats molecules as rigid, it is not well-suited to the partition functions of flexible biomolecules and offers no advantage relative to the FM approach except when a quantum mechanical treatment of vibrational motion is needed.

(2) Although the individual terms of the RRHO approximation have contributions from mass and Planck's constant, in the classical limit, these contributions cancel in the free energy and entropy of binding.

(3) There is no physical significance to a zero value of binding free energy or its entropy component, because these quantities depend upon the standard concentration,  $C^\circ$ , whose value is established by culture rather than physics.

(4) There are two distinct definitions of the change in translational entropy upon binding. In the FM approach, the ligand retains some translational freedom after binding, and the change in translational entropy is related to the volume effectively accessible to it in the bound state, relative to the standard volume  $1/C^\circ$ . In the RRHO, or Sackur–Tetrode, approach, the ligand has no translational motion of its own after binding; only the complex as a whole is considered to translate. Both definitions are legitimate, so long as they are used consistently within the theoretical frameworks from which they derive.

(5) Although entropy is a property of the whole system, it may be partitioned in useful and rigorous ways. For example, partitioning entropy into contributions from different degrees of freedom via conditional probability distributions enables one to define the configurational and solvation entropies, while partitioning into contributions from different regions of configurational space via the composition law of entropy enables one to define vibrational and conformational entropies.

(6) Mutual information terms usefully quantify the entropic consequences of correlation.

(7) When intermolecular binding is made intramolecular through the introduction of a flexible linker between receptor and ligand, the change in the entropy of binding is determined by the statistical properties of the linker. When a new group is linked to an existing ligand to create a bivalent ligand, there is an entropy cost associated with restricting the new group to its binding site, which can be minimized by a suitably chosen rigid linker.

(8) While the partitioning of the binding enthalpy into contributions of chemical groups seems justified, a similar partitioning of the binding entropy is problematic, because of the latter's dependences on the standard concentration and on correlated motions (e.g., overall translation). Consequently, analyses of ligand affinities based on group additivity of the total (as opposed to relative) binding entropy or binding free energy can be misleading.

We hope the present work will be found useful, especially to theorists formulating new methods of computing binding thermodynamics, and to experimentalists deriving insight from thermodynamic and structural data.

## 10. Acknowledgments

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## 11. Supporting Information Available

Background material and detailed derivations for various results presented here. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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