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Experimental Support for a Desolvation Energy Term in Governing Equations for Binding Equilibria

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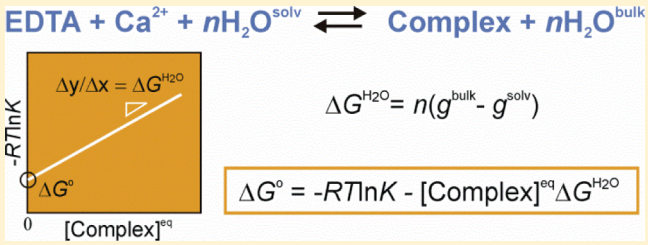
Supporting Information

ABSTRACT: This study introduces a new thermodynamic framework for aqueous reaction equilibria that treats water as a coreactant in the development of a general binding equation. The approach features an explicit consideration for the change in hydration that occurs when two solvated surfaces come into contact. As an outcome of this framework, the standard-state free energy of binding is defined by the summation of two terms: the traditional term ($-RT \ln K_i$) plus a desolvation free-energy term that is weighted by the number of complexes formed at equilibrium. The new formalism suggests that the equilibrium ratio, K_i, is not a constant and that the observed concentration dependence of K_i may be used to obtain the molar desolvation energy and the standard-state free energy at infinite dilution. The governing equation is supported by results from isothermal titration calorimetry using the chelation of calcium(II) by EDTA as a model binding reaction. This work may have far-reaching implications for solution thermodynamics, including an explanation for the oft-noted discrepancy between the enthalpy values obtained by calorimetry and those from the van't Hoff approach.



INTRODUCTION

The binding of two molecules to form a noncovalent complex is a fundamental step in many chemical and biological processes. Historically, binding equilibria in the liquid phase have been characterized in terms of the numbers of bound and unbound molecules at equilibrium with no explicit consideration for solvent effects. The neglect of the solvent, however, may be detrimental to understanding the driving forces behind molecular binding, especially when the solvent is water.

In most physical or biophysical textbooks, the thermodynamic activity of water is treated as a constant; this view is deemed rational on the basis that the solvent concentration is constant. In the case of binding equilibria, the water activity is presumed to be incorporated into the equilibrium constant, K, which, depending on the specific reaction, may also be denoted as the stability constant, the affinity constant, the association constant, or the (reciprocal) dissociation constant.

Disconcertingly, the thermodynamic activity of water depends on other physical properties in addition to its concentration. For a given subset of water molecules, the average free energy is a complicated function of the number and orientation of hydrogen bonds between neighboring molecules (enthalpy) and the randomness of their positions (entropy), and both of these thermodynamic properties are greatly influenced by the presence of a surface or solute; the enthalpy and entropy of water can change considerably without a significant change in number density (concentration). For aqueous reaction equilibria, it becomes appropriate to include water in the balanced reaction because the change in surface chemistry between the reactant and product is accompanied by the rearrangement of a subset of water molecules that interact with each species. Furthermore, the reaction may be characterized by the release of waters of hydration to the bulk phase, in which case it seems imperative to include a term in the governing equation that accounts for the average free energy of bulk water. Although it may be acceptable to ignore the standard-state free energy of water because water appears on both sides of the balanced reaction, the contributions of the perturbed water molecules next to the reactants do not cancel and should not be supplanted by a constant.

The idea that water molecules near a surface may differ in energy from the molecules at a distance away from the surface (bulk water) is not a new concept; a patch of high-energy water next to a nonpolar surface has been hypothesized to underlie the mechanism of the hydrophobic effect for decades,[1,2] and recently developed computational software packages allow one to pinpoint solvation hot spots on the surface of macromolecules as targets for drug interaction.[3] It is clear that the biophysical community does not view water as a passive bystander,[4] yet the governing equations for binding and conformational equilibria fail to account for the process of solvation/desolvation. In the work that follows, a means to resolve this problem is proposed in the form of a new equation that appends the classical relationship with a term for the free energy of desolvation. The equation, derived here from the chemical potentials of all participating species, is supported

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firmly by experimental binding data obtained with an ultrasensitive isothermal titration calorimeter.

This work has broad implications for solution thermodynamics. The theory and results indicate that the classical definition of the Gibbs free energy of a reaction, $-RT \ln K$, is applicable only for the specific case of infinite dilution, which is a stipulation with important consequences for experimentalists who never measure thermodynamic properties at infinite dilution.

THEORETICAL METHODS

Consider the hypothetical binding reaction

$$\mathrm{P} + \mathrm{L} + n\mathrm{H_2O}^{\mathrm{solv}} \leftrightarrow \mathrm{Q} + n\mathrm{H_2O}^{\mathrm{bulk}}$$

where P represents a protein or receptor molecule, L denotes a specific ligand or a binding partner of P, and Q represents a 1:1 complex between the two reactants (P/L). The notations $n\mathrm{H_2O}^{\mathrm{solv}}$ and $n\mathrm{H_2O}^{\mathrm{bulk}}$ refer to the subpopulation of n water molecules in the solvation shells of the reactants that are released to the bulk phase upon the formation of a single molecule of complex Q. For a specific solution condition i, the change in free energy of this reaction may be expressed in terms of the chemical potentials, μ_i, of each product species minus the reactant species

$$\Delta G_i^{\mathrm{rxn}} = \mu_i^{\mathrm{Q}} + N\bar{\mu}_i^{\mathrm{bulk}} - (\mu_i^{\mathrm{P}} + \mu_i^{\mathrm{L}} + N\bar{\mu}_i^{\mathrm{solv}}) \quad (1)$$

or, by rearranging,

$$\Delta G_i^{\mathrm{rxn}} = \mu_i^{\mathrm{Q}} - \mu_i^{\mathrm{P}} - \mu_i^{\mathrm{L}} + N(\bar{\mu}_i^{\mathrm{bulk}} - \bar{\mu}_i^{\mathrm{solv}}) \quad (2)$$

where N represents the total number of water molecules involved in the equilibrium and $\bar{\mu}_i^{\mathrm{bulk}}$ and $\bar{\mu}_i^{\mathrm{solv}}$ refer to the average chemical potentials (per water molecule) for the two subpopulations that define the bulk phase and the solvation shell, respectively. The bar above each water potential is a reminder that this term refers to a subset of water molecules and that this term is independent of the overall concentration of water in the solution. For reactants P, L, and Q, it is customary to substitute the following expression for their chemical potentials

$$\mu_i^{\mathrm{x}} = \mu^{\mathrm{o,x}} + RT \ln a_i^{\mathrm{x}} \quad (3)$$

where $\mu^{\mathrm{o,x}}$ is the standard-state potential of component x and a_i^{x} is the relative activity of x in solution i.[5] The activity term in eq 3 is typically replaced by the concentration of the species multiplied by a corresponding activity coefficient such that $a_i^x = \gamma_i^x[\mathrm{x}]_i$, where γ_i^x is the activity coefficient and $[\mathrm{x}]_i$ denotes the molar concentration of species x. In the framework presented here, the activity coefficients are viewed as unity ($\gamma_i^x = 1$) because under most conditions the explicit treatment of water in eq 2 will account for deviations from the classical equation.[6]

After substitution of eq 3 into eq 2 for components P, L, and Q, the three standard-state potentials may be combined to obtain the standard-state free energy constant, ΔG°. These substitutions lead to the following expression:

$$\Delta G_i^{\mathrm{rxn}} = RT \ln \frac{[\mathrm{Q}]_i}{[\mathrm{P}]_i[\mathrm{L}]_i} + \Delta G^\circ + N(\bar{\mu}_i^{\mathrm{bulk}} - \bar{\mu}_i^{\mathrm{solv}}) \quad (4)$$

The water potentials that appear in eqs 2 and 4 deserve special consideration. Because the chemical potential of water is a complicated function of hydrogen-bond strength and molecular orientation and not a strong function of its own number density that is relatively constant, eq 3 is not an appropriate substitution for the solvent potential. As an alternative, the chemical potentials of water may be expressed as free-energy values using the following relationship

$$\bar{\mu}_i^{\mathrm{H_2O}} = \bar{g}^{\mathrm{o,H_2O}} + \bar{g}_i^{\mathrm{H_2O}} \quad (5)$$

where $\bar{g}^{\mathrm{o,H_2O}}$ is the standard-state free energy of water per mole of water and $\bar{g}_i^{\mathrm{H_2O}}$ denotes the free energy per mole of a specific subset of water molecules (bulk phase or solvation shell) in solution i. Note that the standard-state free energy of water will cancel out when eq 5 is substituted into eq 4 because water is both a reactant and product of the balanced reaction. The total number of participating water molecules per reaction volume, N, is related by stoichiometry to the product concentration as follows

$$N = n[\mathrm{Q}]_i \quad (6)$$

where n is the number of water molecules released to the bulk phase per molecule of complex Q that is formed. Substituting eqs 5 and 6 into eq 4 leads to a general equation for binding equilibria in aqueous solution:

$$\Delta G_i^{\mathrm{rxn}} = RT \ln \frac{[\mathrm{Q}]_i}{[\mathrm{P}]_i[\mathrm{L}]_i} + n[\mathrm{Q}]_i(\bar{g}_i^{\mathrm{bulk}} - \bar{g}_i^{\mathrm{solv}}) + \Delta G^\circ \quad (7)$$

Equation 7 may be simplified further by defining the desolvation energy, $\Delta G_i^{\mathrm{H_2O}}$

$$\Delta G_i^{\mathrm{H_2O}} = n(\bar{g}_i^{\mathrm{bulk}} - \bar{g}_i^{\mathrm{solv}}) \quad (8)$$

leading to a final expression for binding reactions in aqueous solution:

$$\Delta G_i^{\mathrm{rxn}} = RT \ln \frac{[\mathrm{Q}]_i}{[\mathrm{P}]_i[\mathrm{L}]_i} + [\mathrm{Q}]_i \Delta G_i^{\mathrm{H_2O}} + \Delta G^\circ \quad (9)$$

At equilibrium, $\Delta G_i^{\mathrm{rxn}} = 0$, and the rearrangement of eq 9 leads to the following

$$\Delta G^\circ = -RT \ln \frac{[\mathrm{Q}]_i^{\mathrm{eq}}}{[\mathrm{P}]_i^{\mathrm{eq}}[\mathrm{L}]_i^{\mathrm{eq}}} - [\mathrm{Q}]_i^{\mathrm{eq}} \Delta G_i^{\mathrm{H_2O}} \quad (10)$$

where $[\mathrm{x}]_i^{\mathrm{eq}}$ denotes the concentration of components P, L, and Q at equilibrium in solution i. Following tradition, the equilibrium concentrations within the natural logarithm term are replaced by the equilibrium ratio, K_i, yielding the final equilibrium expression:

$$\Delta G^\circ = -RT \ln K_i - [\mathrm{Q}]_i^{\mathrm{eq}} \Delta G_i^{\mathrm{H_2O}} \quad (11)$$

In practical application of eqs 4–11, all concentrations are treated as dimensionless quantities but must be expressed in the same units (typically molarity). Thus, the magnitudes of the free-energy values are dependent on the definition of the standard state and on the choice of the concentration units. In this work, we refer to K_i as the equilibrium ratio as opposed to the equilibrium constant because, as will be shown in the experimental results, K_i may vary with reactant concentration. Using the thermodynamic framework introduced here, this concentration dependence is understood to originate from the presence of the desolvation energy term on the right side of eq 11. If the initial reactant concentrations are tested over an appropriate range and if the desolvation energy is nonzero (i.e., $\bar{g}_i^{\mathrm{bulk}} \neq \bar{g}_i^{\mathrm{solv}}$), then the concentration dependence of K_i should

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be detectable by biophysical methods. The subscript i in the notation for K_i and $\Delta G_i^{H_2O}$ is retained in the general equations to denote that the value of each parameter is dependent on the specific solution conditions in which the measurements were performed; the free energy of bulk water depends on all species in the solution, including buffers and secondary solutes that do not participate directly in the reaction.[6]

EXPERIMENTAL METHODS

All chemical reagents were obtained from Fisher Scientific. Stock solutions of calcium chloride and sodium ethylenediaminetetraacetate (EDTA) were made at room temperature using ultrapure water (Millipore, Milli-Q system). Solutions were supplemented with 0.150 M 2-(4-morpholino)-ethane sulfonic acid buffer (MES) and adjusted to pH 6.2 at room temperature with a potassium hydroxide solution. An increase of 0.06 pH units was noted upon decreasing the temperature from 25 to 5 °C, and a decrease of 0.15 pH unit was observed upon increasing the temperature from 25 to 50 °C; the minor pH deviation is not expected to alter the results beyond the reported error of precision (±5% for K values).

The isothermal titration calorimetry was performed with a Microcal instrument, model VP-ITC, using the analysis programs provided by the manufacturer (Origin software). All solutions were degassed under vacuum (ThermoVac). Prior to each calorimetry run, the sample cell was incubated with 25 mM EDTA for a minimum of 1 h to saturate the nonspecific binding sites on the wall of the sample cell. After the incubation solution was removed, the sample cell was rinsed once and loaded with the EDTA solution of a desired concentration. The injection syringe was filled with $CaCl_2$ at a concentration 10-fold higher than the EDTA concentration in the sample cell. The analog-input range and reference power settings were adjusted in accordance with the sample concentrations and the expected peak output for a given run. (See Figure S1 in the Supporting Information for the sample output and instrument settings at each concentration.) Most binding experiments consisted of 54 injections; the first injection was 2.0 μL, and the subsequent injections were all 5.0 μL in volume. For the calorimetry runs starting with the highest concentration of $CaCl_2$ in the syringe (125 mM), the first injection was 2.0 μL, followed by 106 injections of 2.5 μL. In all trials, the first injection peak was discarded from the analysis because of the unavoidable error in the actual injection volume after filling and transferring the syringe. The initial error in the volume may be minimized by repositioning the plunger downward by a short distance prior to placing the injection syringe into the sample cell.[7] For each calcium concentration, reference data were recorded by injecting $CaCl_2$ into a control buffer solution containing no EDTA, and the recorded enthalpies were subtracted from the corresponding binding experiments prior to regression analysis. A sample ITC run is shown in Figure 1.

RESULTS

The thermodynamic framework introduced here leads to the following testable prediction: if eq 11 is correct for a general binding reaction at equilibrium and if $\Delta G°$ and ΔG^{H_2O} are constants for a given reaction pair in a given solution i, then an increase in complex formation, $[Q]^{eq}$, should increase the contribution of the desolvation energy term and lead to a corresponding change in the value of K_i. The concentration-dependent behavior of K_i is investigated in the current study by

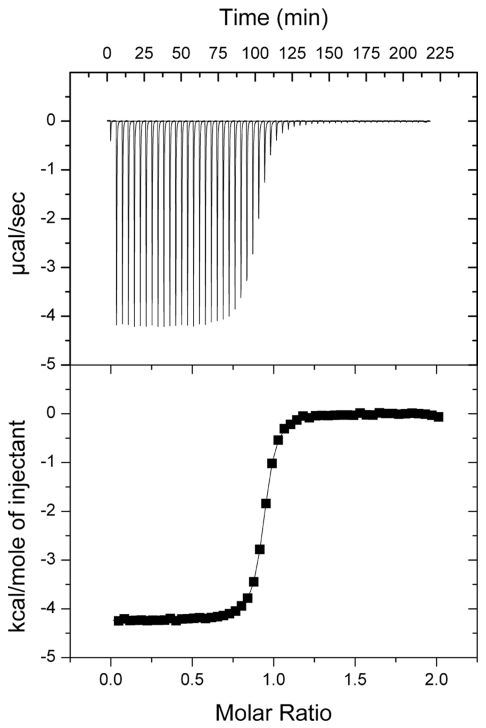

Figure 1. Sample calorimetry experiment for the binding of Ca^{2+} to EDTA in 150 mM MES buffer, pH 6.2. The top panel is a thermogram showing the heat released per injection versus time, and the bottom panel is the binding isotherm showing the integrated peak results plotted against the corresponding molar ratio of Ca^{2+}/EDTA. Experimental conditions are 5.00 mM $CaCl_2$ in the syringe, 0.500 mM EDTA in the sample cell, and 25 °C. Additional ITC runs are given in Figure S1.

using the interaction between calcium(II) and EDTA as a model binding reaction.

The EDTA/Ca^{2+} system is especially appropriate for this work because both reagents are highly soluble in water, allowing one to vary the starting concentrations by over 1 order of magnitude. The EDTA/Ca^{2+} system has been studied for many years[8] and is often recommended as a tutorial exercise for the operation of modern titration calorimeters. One caveat of this experimental system is the fact that Ca^{2+} binding typically involves the release of a proton from EDTA, making the observed equilibrium strongly dependent on pH.[9] To alleviate pH concerns and enhance the precision of the measurements, a relatively high buffer concentration of 150 mM MES, pH 6.2, was employed in all solution studies reported here. Under this buffer condition, changes in the initial and final pH values were found to be negligible for the contents of the sample cell in trials using the most concentrated EDTA solution (12.5 mM). At pH 6.2, EDTA exists in approximately equal proportions of the mono- and diprotonated species,[10] and the binding affinity should fall below the upper limit for practical measurements by ITC.[11]

Another issue for the current investigation is the possibility that the desolvation effects become apparent only at reactant concentrations that far exceed the concentrations normally employed in ITC experiments. This is a concern because calorimeters have an upper limit for the accurate measurement of enthalpy. Also, the curve-fitting algorithms employed for estimating K_i become less reliable as the binding isotherm becomes steeper in the region approaching saturation. As a

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general rule, the c value, defined as the product of K_i and the starting concentration of the reactant in the sample cell in units of molarity, should be less than 1000, although other factors may influence the magnitude of the statistical error.[12]

The Equilibrium Ratio Is Not a Constant for Formation of the EDTA/Ca^{2+} Complex. The values of K_i obtained from calorimetry experiments are reported in Table 1

Table 1. Equilibrium Ratio Values from ITC for EDTA/Ca^{2+} Binding[a]

	reactant concentrations (mM)			
$[EDTA]_0$	0.500	2.50	7.50	12.5
$[CaCl_2]$[b]	0.428	2.14	6.42	10.7
T (°C)	equilibrium ratio, $K_i/10^5$ (M^{-1} ± error of fit)			
5.0	22.3 ± 0.6	16.6 ± 0.8	7.88 ± 1.01	4.86 ± 0.61
25.0	13.4 ± 0.3	10.6 ± 0.3	6.58 ± 0.29	4.48 ± 0.14
37.0	10.6 ± 0.4	9.46 ± 0.32	6.43 ± 0.45	4.43 ± 0.35
50.0	8.53 ± 0.47	8.01 ± 0.47	5.96 ± 0.76	4.24 ± 0.43

[a]All ITC solutions contain 150 mM MES, pH 6.2. The percent error of precision is ≤5% for all K_i values, and is based on three or more trials at each condition. [b]The total $[CaCl_2]$ in the ITC cell one injection prior to surpassing a 1:1 molar ratio of Ca^{2+}/EDTA.

for four initial concentrations of EDTA, $[EDTA]_0$, measured at four different temperatures (5, 25, 37, and 50 °C). The effective calcium concentration in the working volume of the sample cell just prior to surpassing a 1:1 molar ratio of both reactants is also given in Table 1; the relevance of this concentration is discussed later.

As seen in Table 1, the equilibrium ratio for the formation of the EDTA/Ca^{2+} complex is not a constant at any given temperature. At 25 °C, K_i varies 3-fold, from a high-K_i value of 13.4×10^5 M^{-1} in the lowest starting concentration of EDTA to a low-K_i value of 4.48×10^5 M^{-1} at the highest EDTA concentration. Thus, the selected range of reactant concentrations is adequate to observe a significant change in the equilibrium ratio by isothermal titration calorimetry. Table 1 also indicates that the concentration dependence of K_i becomes stronger with decreasing temperature; the largest range of K_i values occurs at 5 °C. Under the given experimental conditions, the concentration dependence of K_i is not attributed to nonideal solution behavior because the reactant concentrations are in the millimolar range, which is far below their solubility limits and far below the molar concentrations that are typically required to influence binding equilibria by the addition of secondary solutes.

The buffer concentration was also observed to have an influence on the observed equilibrium; at 25 °C and at the lowest initial concentration of 0.50 mM EDTA, the equilibrium ratio was found to increase nearly 2-fold from 13.4×10^5 in 150 mM MES to 24.1×10^5 in 10 mM MES. The latter K_i value is comparable to the value of 20.1×10^5 M^{-1} reported by Christensen et al. under similar conditions.[13] The dependence on buffer concentration is expected if the buffering reagent alters the average free energy of bulk water, which is a defining parameter for the desolvation energy term. In this case, one would infer that MES buffer increases the free energy of the bulk water phase, thereby increasing ΔG^{H_2O} and reducing the formation of the EDTA/Ca^{2+} complex in accord with eqs 8 and 11. The buffer effect may also be attributed, in part, to a low but measurable binding affinity between Ca^{2+} and MES; the competition between metal−chelator interactions and metal−buffer interactions should reduce the magnitude of K_i with increasing buffer concentration.[13]

With the exception of the lowest EDTA concentration in Table 1, all of the calorimetry results correspond to c values that exceed the recommended limit of 1000. Consequently, several trials were repeated to see if reducing the injection volume from 5.0 to 2.5 μL would have any effect on the reported value of K_i. If high c values are an issue, then reducing the injection volume should yield a more reliable fit and a more accurate value of K_i by (a) reducing the magnitude of the total enthalpy per injection, leading to a less-steep transition region for the binding isotherm and (b) providing more data points within the transition region that dictates the best fitting curve. The outcome of the injection volume test indicated that a smaller volume is beneficial only for the highest EDTA concentration of 12.5 mM; at the next lower concentration of 7.5 mM EDTA, the same K_i value was obtained for injection volumes of 2.5 and 5.0 μL (data not shown). For the data set at 12.5 mM EDTA, reducing the injection volume led to slightly larger values of K_i, as reported in the last column of Table 1. It should be noted that the standard injection volume of 5.0 μL, as used in this work, is less than the 10 μL volume reported most commonly in the literature for similar experiments and that the reduced injection volumes translate into much longer titration runs. For example, a single ITC run employing the 2.5 μL injection protocol takes about 6 h to complete.

Linear Relationship between $RT \ln K_i$ and Reactant Concentration. If eq 11 is a valid expression for binding equilibria, then an xy plot of the quantity $-RT \ln K_i$ versus the concentration of the complex at equilibrium should yield a linear fit for which the slope and y intercept correspond to the desolvation energy and standard-state free energy, respectively. Because the binding ratio is expected to exceed 10^4 M^{-1}, the concentration of the complex at equilibrium is nearly equal to the concentration of the limiting reactant to 3 significant figures. For the ITC experiments, the limiting reactant is the component that occupies the syringe for the early stages of the titration (i.e., until a 1:1 molar ratio is achieved), and the component that was loaded into the sample cell becomes the limiting reagent after the 1:1 molar ratio is surpassed. Unfortunately, the available software packages for analyzing ITC data treat the equilibrium ratio as a constant, whereas the equation to be tested predicts that the equilibrium ratio will change slightly after each injection in accord with the change in the limiting reactant concentration and the (unknown) values of $\Delta G°$ and ΔG^{H_2O}. Thus, a direct test of eq 11 using K_i values obtained from the current ITC methodologies is not straightforward; it is not possible to obtain unique values for K_i, $\Delta G°$, and ΔG^{H_2O} from a single titration run. As an approximation, one may assume that the ITC-generated value of K_i corresponds most closely to the limiting reactant concentration near the middle of the titration curve just prior to reaching a 1:1 molar ratio of Ca^{2+}/EDTA. This is a reasonable assumption because the nonlinear regression analysis of the data is highly dependent on the points in the transition zone that precede the attainment of the 1:1 reactant ratio. The concentration of Ca^{2+} corresponding to the penultimate injection required to reach a 1:1 ratio is denoted here as the limiting concentration and is given in Table 1 below the corresponding EDTA concentration at the beginning of each titration.

As seen in Figure 2, the predicted linearity of $-RT \ln K_i$ versus the limiting reactant concentration is upheld for the

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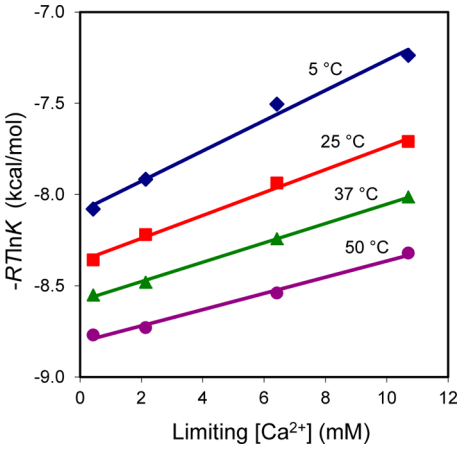

Figure 2. Calorimetry data support the inclusion of the desolvation energy term in the governing equation for binding equilibria. Each K_i value in Table 1 was utilized to calculate $-RT \ln K$ and plotted against the corresponding concentration of calcium, also given in Table 1. The linear relationship at each temperature is consistent with eq 11, for which the y intercept is $\Delta G°$ and the slope is ΔG^{H_2O}.

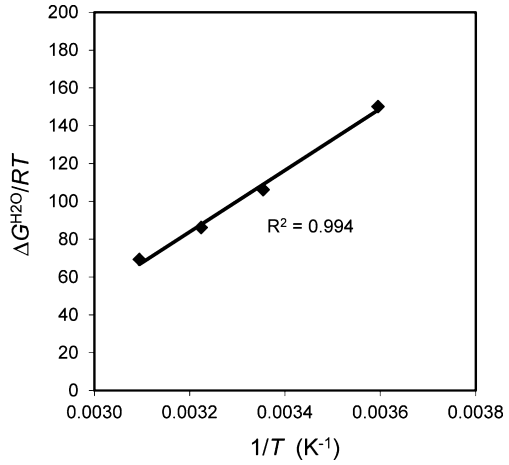

Figure 3. Temperature-dependent analysis of the desolvation energy for the binding of Ca^{2+} to EDTA. Values of ΔH^{H_2O} = +324 kcal/mol and ΔS^{H_2O} = +0.87 kcal/mol·K are obtained from the slope and y intercept of the line, respectively. These quantities represent the energy contributions of water per mole of complex formed. Table 2 shows the values of ΔG^{H_2O} versus temperature.

EDTA/Ca^{2+} system, providing strong support for the thermodynamic framework; the exceptional correlation is unlikely to be a coincidence of the experimental approach.

The values of $\Delta G°$ and ΔG^{H_2O}, as obtained from the plots in Figure 2, are listed in Table 2. The linear fit is commendable,

Table 2. Standard-State Free Energy, Desolvation Free Energy, and Goodness of Linear Fit for EDTA/Ca^{2+} Binding[a]

T (K)	$\Delta G°$ (kcal/mol)	ΔG^{H_2O} (kcal/mol)	R^2
278.15	−8.09	+83.0	0.990
298.15	−8.37	+62.9	0.994
310.15	−8.58	+53.1	0.999
323.15	−8.81	+44.5	0.992

[a]Free energies obtained from Figure 2 by application of eq 11.

with $R^2 \geq 0.99$ for each data set. Relative to the standard-state free energy, the desolvation energy is larger in magnitude and opposite in sign at each temperature for this binding reaction (Table 2). A positive value for the desolvation free energy is in agreement with the negative free energy of hydration values reported for calcium and acetate ions.[14] The unfavorable desolvation energy term for the formation of the EDTA/Ca^{2+} complex reflects the kosmotropic character of calcium and carboxylate ions, species known to bind water strongly.[15] Thus, the sign of the resulting desolvation energy provides further support for the thermodynamic approach employed here.

Additional information on aqueous solution thermodynamics may be gleaned from analyzing the desolvation energies as a function of temperature. The evaluation of the ΔG^{H_2O} values in Table 2 by a van't Hoff plot yields a straight line from which the enthalpy and entropy of desolvation may be obtained (Figure 3). The desolvation of EDTA and calcium(II) is characterized by a large and unfavorable change in the enthalpy of water, ΔH^{H_2O} = +324 kcal/mol of complex formed, which is balanced somewhat by a favorable change in the entropy of water, ΔS^{H_2O} = +0.87 kcal/mol·K. It is important to note that ΔH^{H_2O} and ΔS^{H_2O} refer to the water molecules released from the reacting ions upon complex formation and do not represent the overall changes in enthalpy and entropy of the system; ΔH^{H_2O} and ΔS^{H_2O} do not include the contributions of the metal−chelator interaction.

When the standard-state free energies in Table 2 are analyzed using a van't Hoff plot, the slope and intercept yield values of $\Delta H°$ = −3.65 kcal/mol and $\Delta S°$ = +0.016 kcal/mol·K (R^2 = 0.99, Figure S2). Thus, all standard-state values ($\Delta G°$, $\Delta H°$, and $\Delta S°$) are smaller than the corresponding desolvation terms by 1 to 2 orders of magnitude. This outcome is consistent with the idea that aqueous binding equilibria are often governed by relatively small differences in large opposing forces, even for the reaction between two small molecules like calcium(II) and EDTA.

In the traditional analysis, van't Hoff plots are employed with standard-state free energies calculated by the relationship $\Delta G° = -RT \ln K$. When this approach is applied to each concentration-specific data set in Table 1, widely differing values for the standard-state enthalpy (ΔH^{vH}) are obtained, as summarized in Figure 4. For example, at the lowest concentration of EDTA the slope of the van't Hoff plot yields ΔH^{vH} = −3.8 kcal/mol, whereas the slope yields a much smaller enthalpy of −0.52 kcal/mol at the highest concentration of 12.5 mM EDTA. Interestingly, the binding enthalpy, as measured directly from the calorimeter (ΔH^{ITC}), is about −4.3 kcal/mol at 25 °C and is independent of concentration.

DISCUSSION

Physical chemists who favor the classical approach may argue that solvation is considered implicitly by the activity coefficients of each reactant, but the use of activity coefficients does not address two important issues: (a) for a bimolecular binding reaction, the individual species interact with more water molecules in total than the complex formed upon binding and (b) the release of waters of hydration from the surface of the reactants mandates an accounting term for the free energy of the bulk phase. When water is viewed as a coreactant, it becomes apparent that the traditional equation for the standard-state Gibbs free energy of binding represents an unbalanced reaction.

An understandable response to this study is to ask why the concentration dependence of binding has not been noted

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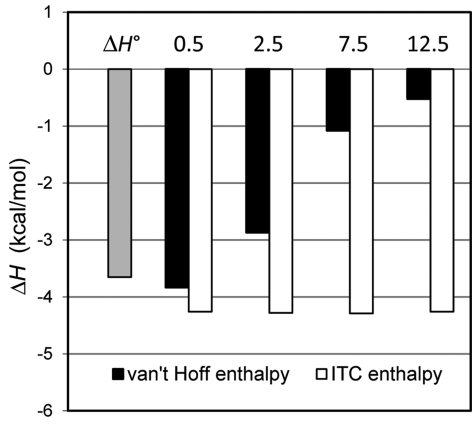

Figure 4. Comparison of the binding enthalpies obtained by three different methods. The $\Delta H°$ value (gray bar) was calculated from a van't Hoff plot using eq 11 and the $\Delta G°$ values in Table 2. The ΔH^{vH} values (black bars) were calculated from a van't Hoff plot using the traditional free-energy relationship $\Delta G° = -RT \ln K$, and the K_i values were obtained at a single starting concentration. The ΔH^{ITC} values (white bars) were obtained from the calorimeter output at 25 °C after subtracting the corresponding control run at each concentration; the ΔH^{ITC} values were weakly dependent on temperature and nearly constant with concentration (Table S1). The numbers on the graph above each pair of bars denote the starting concentrations of EDTA in millimolar.

previously. One possible reason is the fact that most binding experiments are performed at the lowest reactant concentration required to gain a reproducible result. Why would any scientist deplete their precious binding reagents to test a 10-fold-higher concentration if they believed the outcome (equilibrium ratio) would be the same? Also, the operating limits of the instrumentation used to monitor a binding reaction will often dictate the experimentally feasible range of concentrations. For example, most spectroscopic techniques have a maximum detection limit that may prohibit binding experiments at the higher concentrations required to observe a desolvation effect. One speculates that if other concentration-dependent equilibria were noted in the past then there would have been a strong inclination to attribute the results to nonideal solution effects at the higher reactant concentrations and not to pursue the observation any further.

Discrepancies in the Binding Enthalpy Obtained by Calorimetry versus the van't Hoff Approach: Kudos to Sturtevant. The deviation in the enthalpy values obtained by calorimetry and by the van't Hoff approach at each starting concentration is expected if eq 11 is a valid relationship. The ΔH^{vH} values were obtained from the classical equation for $\Delta G°$ without extrapolating it to infinitely dilute reactant concentrations. If the desolvation energy is positive, then the classical equation yields $\Delta G°$ values that are too positive (too unfavorable), and if the desolvation energy is negative, then the classical equation yields $\Delta G°$ values that are too negative (too favorable). Isothermal titration calorimetry, however, is a direct measure of the experimental heat of binding normalized to the number of complexes formed. Thus, ΔH^{ITC} includes the intrinsic contribution of desolvation and possibly other linked equilibria. In the specific case of calcium(II) binding to EDTA at pH 6.2, a proton is released from EDTA and bound by the buffer molecule, MES. If the enthalpies of the two weak acid reactions do not cancel, then this enthalpy difference is embedded in the measured value of ΔH^{ITC}. The contribution of the proton-linked equilibria may account for the modest difference in the magnitudes of $\Delta H°$ and ΔH^{ITC} reported in Figure 4 for the EDTA/Ca^{2+} system. Linked reaction equilibria, however, should not alter the values of K_i as calculated from ITC measurements because the enthalpy contributions of the linked reactions are directly proportional to the number of binding events; the presence or absence of linked equilibria is inconsequential to the thermodynamic values reported in Tables 1 and 2.

In the mid-1990s, Julian Sturtevant and co-workers reported a discrepancy in the enthalpy values obtained by calorimetry and the van't Hoff approach for three different binding reactions.[16−18] In this series of papers, Sturtevant suggested that the true equilibrium constant, designated as K_T, is a product of the apparent equilibrium, K_{app}, and another term, K', which includes additional parameters to account for variable water activity and for possible interactions between the reactants and the buffering agent.[18] In essence, Sturtevant associated the calorimetry results with the true enthalpy value and associated the van't Hoff analysis with the apparent enthalpy value because the Gibbs free energies, from which the ΔH^{vH} values were calculated, lacked one or more factors that contribute to the thermodynamics. To his credit, Sturtevant also mentioned the possible role of desolvation in all three papers, but he often followed this statement with a resignation to the inherent difficulty in measuring changes in hydration.[17,18]

The disagreement between ΔH^{vH} and ΔH^{ITC} was discussed further in a binding paper by Færgeman et al.,[19] but these authors chose to refer to ΔH^{vH} as the intrinsic (true) enthalpy, whereas Sturtevant would view ΔH^{ITC} as the true enthalpy. In 1997, Chaires suggested that the discrepancy in enthalpy values was due to the statistical error that arises when small changes in heat capacity are neglected.[20] Horn et al. repeated the ITC experiments with two of Sturtevant's original binding systems and concluded that there is no statistical difference between ΔH^{vH} and ΔH^{ITC}, "when experimental setup and analysis are correctly performed".[21] However, using the barium(II) and 18-crown-6 ether-binding system, Mizoue and Tellinghuisen came to a different conclusion; these authors found the discrepancy between ΔH^{vH} and ΔH^{ITC} to be statistically significant, and they attributed the phenomenon to a potential flaw in the standard ITC procedure of subtracting a blank titration run that may not sufficiently account for all background phenomena.[22]

Of particular relevance to the current study, none of the former investigations analyzed the effects of reactant concentration on the observed equilibrium ratio. ITC experiments are typically performed at one concentration, and it is difficult to find literature data for the same binding system that varies in the reactant concentrations by more than 2-fold. In many calorimetry reports, the reactant concentrations are omitted from the methods section, presumably because the authors view concentration as an insignificant factor. In one exceptional paper by Wiseman et al., the reactant concentrations were varied 40-fold for the enzyme/inhibitor pairing of ribonuclease A (RNaseA) and the nucleotide 2′-CMP.[23] The resulting binding ratios for this study ranged from 13.5×10^4 M^{-1} at the lowest concentration to 8.0×10^4 M^{-1} at the highest concentration, and a strong dependence on the potassium acetate buffer concentration was also noted. The authors attributed the concentration-dependent K_i values to the dimerization or aggregation of the protein,[23] but no further biophysical evidence was given to support their hypothesis. The Wiseman results could be interpreted as further evidence of the

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validity of eq 11; the equilibrium ratio varied with concentration because the desolvation energy for the RNaseA binding system is large enough to observe its influence in the range of selected concentrations. We suggest that the concentration dependence of K_i for the RNaseA system is not due to protein aggregation or nonideal solution conditions but rather is due to a natural law of thermodynamics as expressed by eq 11.

In general, the current work most closely aligns with the Sturtevant viewpoint; the classical equation for the Gibbs free energy of binding is missing an important thermodynamic component, and calorimetry methods expose this fundamental oversight. The latent factor is desolvation. One may view the desolvation energy term in eq 11 as the mathematical outcome of moving Sturtevant's K' equilibrium factor outside of the natural logarithm; although, it should be noted that this manipulation will not capture the concentration-dependent character of K_i.

As demonstrated here, eq 11 provides a means to quantify the desolvation energy, which is a driving force that Sturtevant relinquished as experimentally inaccessible. The estimations of $\Delta H°$ and $\Delta S°$ also should be calculated from free energies obtained by eq 11. As highlighted by the dark-shaded bars in Figure 4, the enthalpies obtained by the van't Hoff approach can be highly misleading if the standard-state free energy is calculated by the traditional equation. Contrary to other discussions on linked equilibria,[24] the EDTA/Ca^{2+} binding study demonstrates that desolvation can have a significant influence on the observed equilibrium ratio, depending on the reactant concentrations and the magnitude of the molar desolvation energy, ΔG^{H_2O}.

In view of the thermodynamic issues presented here, it is not surprising that the literature is full of disputes and discussions on the existence and meaning of enthalpy−entropy compensation in binding interactions.[25−29] Manufacturers claim that ITC equipment can generate $\Delta G°$, $\Delta H°$, and $\Delta S°$ from a single titration run, but the validity of this practice needs to be reconsidered. The equations and techniques employed in the current work are expected to lead to a better understanding of enthalpy−entropy relationships by yielding the correct standard-state values and by providing a means to quantify the role of desolvation.

Other Ramifications for Aqueous Solution Thermodynamics. The experimental results given in this study firmly support eq 11 as a valid approach for analyzing binding data. An issue arises, however, when the formalism is applied to the calculation of the nonequilibrium free energy at any discrete set of reactant concentrations (eq 9). If the traditional standard-state condition of 1 M reactant concentrations is inserted into eq 9, the following relationship is obtained:

$$\Delta G_i^{rxn} = \Delta G_i^{H_2O} + \Delta G° \tag{12}$$

Thus, in conflict with current practices in solution thermodynamics, $\Delta G°$ and ΔG^{rxn} are not synonymous at 1 M reactant concentration when water is treated as an explicit reactant. Furthermore, when any pairing of ΔG^{H_2O} and $\Delta G°$ from Table 2 is substituted into eq 12, one obtains a positive value for ΔG^{rxn} because the molar desolvation energy for the binding of Ca^{2+} to EDTA is positive and larger in magnitude than the corresponding value of $\Delta G°$. Equation 12 predicts that (further) binding of Ca^{2+} to EDTA does not occur when all species are present at 1 M concentration; instead, the dissociation of the existing complex molecules is favored.

Because $\Delta G°$ and ΔG^{rxn} are opposite in sign for the EDTA/Ca^{2+} system, the concentration at which the binding affinity switches from complex formation to complex dissociation may be estimated by substituting $K_i = 1$ into eq 11, leading to the following expression:

$$\Delta G° = -[Q]_i^{eq} \Delta G_i^{H_2O} \tag{13}$$

Solving eq 13 for the concentration of the EDTA/Ca^{2+} complex at equilibrium yields $[Q]^{eq} = 0.133$ M, as calculated from the molar free-energy values in Table 2 at 25 °C. A value of 133 mM is more than 10-fold higher than the highest concentration tested in the current ITC study and may be difficult to approach experimentally while maintaining a constant pH. This affinity crossing point should be viewed as a rough estimate because the properties of bulk water are expected to change at the high total reactant and buffer concentrations necessary to achieve this condition and because the average free energy of bulk water is a component of the desolvation energy (eq 8).

In this investigation, the desolvation free energy was found to be a positive value at all temperatures, indicating that the desolvation of Ca^{2+} and EDTA ions disfavors complex formation. Is this result exemplary of all binding interactions? As mentioned previously, it is generally recognized that the hydrophobic effect is driven by the desolvation of the two nonpolar surfaces that interact, and, consequently, ΔG^{H_2O} should be negative for the binding of two nonpolar solutes. Interestingly, eq 11 predicts that the equilibrium ratio will increase with increasing concentrations of hydrophobic reactants, as summarized in Table 3.

Table 3. Sign of the Desolvation Free Energy Determines the Concentration Effect on the Equilibrium

sign of ΔG^{H_2O}	consequence of $[Q]_i^{eq} \uparrow$	binding-pair example
(+)	$K_i \downarrow$	EDTA/Ca^{2+}
~0	K_i constant	many?
(−)	$K_i \uparrow$	two nonpolar solutes

Because hydrophobic molecules have intrinsically low solubilities in water, the prediction that the hydrophobic effect is enhanced with increasing concentration of the nonpolar reactants may be difficult to test; it may be challenging to identify a model hydrophobic-binding reaction that is amenable to ITC experiments over a concentration range similar to that employed in the current work.

CONCLUSIONS

This study combines theory and experiment to test the general idea that water should be treated as a coreactant in the application of thermodynamics to aqueous solutions. More specifically, an equation was derived for aqueous binding equilibria that includes a term for the desolvation of the two binding molecules (or surfaces) that come into contact. One outcome of the proposed thermodynamic framework is that the equilibrium constant is not generally a constant with changing reactant concentrations; this expectation was upheld for the EDTA/Ca^{2+} binding model at pH 6.2 and was also reported for an enzyme/inhibitor-binding model.[23] Thus, it may be appropriate to refer to K_i as the equilibrium ratio and not as the equilibrium constant. In general, the concentration dependence of K_i should not be ascribed to solution nonideality

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unless, of course, one views the innate presence of water as nonideal. For the thermodynamic framework introduced here, nonideality is indicated by a reaction that does not conform to eq 11, as is expected to occur at exceedingly high reactant concentrations or in the presence of certain secondary solutes that bind directly to the reactants.

The concentration dependence of K_i allows one to quantify the role of water in reaction equilibria. For the EDTA/Ca^{2+} binding pair, the desolvation free energy was found to be unfavorable; therefore, this system represents a case where the metal−chelator interaction energy dominates over the desolvation energy. The desolvation free energy is expected to be favorable and negative in sign for other systems, especially those driven by the hydrophobic effect.

Desolvation is likely to be an important driving force in conformational equilibria,[6] including the folding of biomolecules such as proteins and tRNAs. Because these macromolecules are characterized by large surface areas in contact with water, the magnitude of the desolvation energy per mole of folded product may be orders of magnitude larger than the values reported in this work for the formation of the EDTA/Ca^{2+} complex. In the future, many more binding systems should be examined to verify the general applicability of the equations proposed here and to quantify the role of water in each reaction.

■ ASSOCIATED CONTENT

Supporting Information

Sample ITC data for the entire concentration range, van't Hoff analysis of standard-state parameters, and enthalpy values from ITC measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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