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## Model binding experiments with cucurbit[7]uril and p-sulfonatocalix[4]arene support use of explicit solvation term in governing equation for binding equilibria

Daryl Eggers

*San Jose State University*, [daryl.eggers@sjsu.edu](mailto:daryl.eggers@sjsu.edu)

Adam Brewer

*San Jose State University*

Kimberly J. Cacatian

*San Jose State University*

L. Allison Camat

*San Jose State University*

Dominic Castagnoli

*San Jose State University*

*See next page for additional authors*

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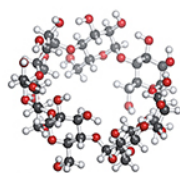
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**Authors**

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# Model binding experiments with cucurbit[7]uril and *p*-sulfonatocalix[4]arene support use of explicit solvation term in governing equation for binding equilibria

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Department of Chemistry, San José State University, San José, CA, USA

## ABSTRACT

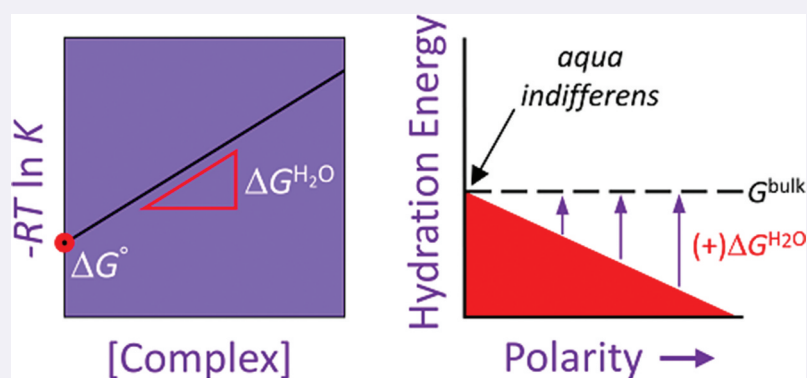
The thermodynamics of model host–guest-binding reactions is examined in depth using isothermal titration calorimetry. In conflict with classical thermodynamics, the results indicate that the equilibrium-binding quotient,  $K$ , is not a constant for all pairings. This outcome is predicted by an equation for binding equilibria that includes an explicit term for the change in solvation free energy that accompanies the formation of a binary complex. Application of this framework to the experimentally observed concentration dependence of  $K$  allows one to obtain the energetic contribution of the solvent, a linked equilibrium denoted here as  $\Delta G^{\text{H}_2\text{O}}$ . The estimated values of  $\Delta G^{\text{H}_2\text{O}}$  are large and unfavourable for the binding of selected guest molecules to two hosts, cucurbit[7]uril and *p*-sulfonatocalix[4]arene. Intriguingly, the estimated values of  $\Delta G^{\text{H}_2\text{O}}$  are near zero for the binding of two hydrophobic guest molecules to  $\beta$ -cyclodextrin, leading to a thought-provoking discussion on the driving force behind the hydrophobic effect.

## ARTICLE HISTORY

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Binding thermodynamics; isothermal titration calorimetry; solvation energy; hydration; hydrophobic effect






## Introduction

Understanding the forces that govern the binding of two molecules is a fundamental goal of supramolecular chemistry. When the medium for interaction is water, the knowledge gained from supramolecular models becomes relevant to understanding the forces that govern biological systems at the molecular level.

The application of thermodynamics to reaction equilibria has a long history, but the classical equations for binding equilibria, as found in many textbooks, may be

impeding progress. Why should the same equation used for gas-phase binding equilibria be applicable to binding equilibria in solution? In the case of water, it is generally accepted that changes in solvent energy underlie the hydrophobic effect, a dominant force in binding and conformational equilibria, yet the classical thermodynamic equations for binding do not account for solvent effects in a discernible way. In a related issue, physical chemists often invoke an (empirically-derived) activity coefficient and label systems ‘nonideal’ when the

**CONTACT** Daryl K. Eggers  [daryl.eggerts@sjsu.edu](mailto:daryl.eggerts@sjsu.edu)  Department of Chemistry, San José State University, One Washington Square, San, CA 95192-0101, USA

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experimental data do not match well with the expectation of classical thermodynamics. The current study dares to ask, is the system truly nonideal, or is it the governing equation that is nonideal?

In 2013, Castellano and Eggers first suggested a modified equation for binding equilibria that includes an explicit term for the change in solvent energy that accompanies the formation of a non-covalent complex [1]. The approach, further elaborated in 2020 [2], acknowledges the fact that the reactants and products of a binding reaction may not be in contact with the same number of solvent molecules. In brief, the new derivation appends the classical thermodynamic framework with a chemical potential for the subset of water molecules that move to a new environment after complex formation. The solvent-balanced reaction captures the change in energy due to release of water molecules from the surfaces of the reactants to the bulk solution, a linked reaction equilibrium that contributes to the observed thermodynamics. The proposed relationship is summarised by Equations 1–3 below, for the binding of two reactants, A and B:

$$\Delta G^{xn} = RT \ln \frac{[AB]}{[A][B]} + [AB]\Delta G^{H2O} + \Delta G^\circ \quad (1)$$

where  $R$  is the gas constant,  $T$  is the temperature,  $[AB]$  is the concentration of the binary complex, and  $\Delta G^{H2O}$  is the change in hydration free energy, or, more generally, the change in solvation energy. The standard-state free energy,  $\Delta G^\circ$ , is a constant obtained from combining the standard-state potentials ( $\mu^{\circ,x}$ ), activity coefficients ( $\gamma_i^x$ ), and reference concentrations ( $[x]^\circ$ ) of each species  $x$  in solution  $i$ , as given by the following:

$$\Delta G^\circ = (\mu^{\circ,AB} - \mu^{\circ,A} - \mu^{\circ,B}) + RT \ln \frac{\gamma_i^{AB}}{\gamma_i^A \gamma_i^B} - RT \ln \frac{[AB]^\circ}{[A]^\circ [B]^\circ} \quad (2)$$

At equilibrium,  $\Delta G^{xn} = 0$ , and Equation 1 may be reduced to

$$\Delta G^\circ = -RT \ln K - [AB]^{eq} \Delta G^{H2O} \quad (3)$$

With regard to Equation 3,  $K$  is the concentration ratio of complex to free reactants at equilibrium in the direction of association. Note that the value of  $\Delta G^\circ$  is equal to  $\Delta G^{xn}$  only when the concentration of complex approaches infinite dilution and the ratio of complex to free reactants is unity; it is not necessary to define a standard-state concentration of 1 M (that behaves as if it is infinitely dilute) using this framework [2]. As with the classical equilibrium equation, all concentrations are treated as dimensionless quantities but must be inserted in the same units, typically molarity. One should be aware that the values of  $\Delta G^{H2O}$

and  $\Delta G^\circ$  are dependent on the solution conditions, including the buffer choice and the presence of secondary solutes that do not participate directly in the binding reaction but may alter the time-averaged free energy of all water in the system,  $G^{bulk}$ . The activity coefficients in Equation 2 are assumed to be constant for the experimental conditions employed in the current study. Historically, the activity coefficients have been set to unity for reactions performed in dilute solutions, but the coefficients need only be of constant value in order to be combined with the other parameters that define  $\Delta G^\circ$  in Equation 2. Additional notes on the interpretation of  $\Delta G^{H2O}$  are shared in the Supplementary Material.

Previously, Equation 3 has been applied successfully to the chelation of calcium(II) cation by EDTA [1] and to an inclusion complex formed between rubidium cation and a cryptophane molecule [2], both in aqueous solutions. Equation 3 has also been applied to the formation of lanthanide-container complexes in organic solvents [3–7]. In the current study, Equation 3 is tested against data obtained with three model supramolecular compounds, cucurbit[7]uril (CB7), *p*-sulfonatocalix[4]arene (SC4), and  $\beta$ -cyclodextrin ( $\beta$ CD).

## Materials and methods

### Reagents

Cucurbit[7]uril hydrate was purchased from Strem Chemicals (cat. no. 07–1325), and *p*-sulfonatocalix[4]arene hydrate was obtained from TCI (cat. no. S0469). Stock solutions of CB7 (5 mM) and SC4 (3 mM) were titrated with 1.0 M KOH and brought up to the desired volume in 2.0 mM potassium phosphate buffer, pH 6.7–7.0. The SC4 stock required four equivalents of KOH to neutralise the four sulphonic acid groups, and the CB7 stock required slightly less than 1 equivalent of base to neutralise acids of crystallisation. In the case of CB7 samples, the stock was diluted with a buffer containing 7.0 mM KCl in addition to 2 mM potassium phosphate to maintain the total  $K^+$  concentration at 10 mM. In the case of SC4 samples, the dilution buffer contained 12 mM KCl and 2 mM potassium phosphate to maintain the total  $K^+$  concentration at 15 mM.  $\beta$ -Cyclodextrin was obtained from Sigma Aldrich (cat. no. C4767), and stock solutions of  $\beta$ CD (14 mM) in 10 mM potassium phosphate buffer were made by temporarily warming the solution to 40°C to enhance the speed of dissolution. The guest molecules of tetramethylammonium chloride, putrescine (dichloride salt), and sodium glycochenodeoxycholate were purchased from Sigma Aldrich. Adamantane-1-carboxylic acid was obtained from Maybridge, and phenylalanine derivatives were obtained from Bachem Americas.

### Isothermal titration calorimetry

Isothermal titration calorimetry was performed with a Microcal instrument, model VP-ITC (Malvern Panalytical), using the analysis programmes provided by the manufacturer (Origin software). All solutions were degassed under vacuum (ThermoVac). Prior to each calorimetry run, the sample cell (1.45 mL volume) was cleaned and rinsed with one volume of the corresponding buffer prior to loading. For CB7 and SC4 trials, the injection syringe was filled with the desired guest molecule at a concentration 10-fold higher than the host concentration in the sample cell. In the case of  $\beta$ CD, the ITC locations were reversed with the guest molecule in the cell and  $\beta$ CD in the injection syringe. The analogue input range and reference power settings were adjusted in accordance with the sample concentrations and expected peak output for a given run. In a typical ITC run, 27 injections of 10  $\mu$ L volume were used for starting cell concentrations between 0.10 and 1.00 mM; 54 injections of 5  $\mu$ L volume were employed for starting concentrations above 1.00 mM in the cell. In all trials, the titration was initiated with a 2  $\mu$ L injection that was discarded from the analysis. A control run obtained from injecting the syringe component into buffer only was subtracted from the corresponding binding trials before analysis. Uncertainty in each  $K$  value is reported as the average error following multiple ITC trials at each condition ( $n \geq 3$ ). The errors in free energy values were estimated by re-analysing the slope of the corresponding lines when the maximum and minimum errors of each endpoint were used (at highest and lowest concentration).

### Modelling of the governing equation

Equation 3 was modelled using MATLAB by MathWorks. In brief, the total concentrations of host and guest molecules ( $H_t$  and  $G_t$ ) were set as known inputs, and a mass balance expression replaced the concentration of the free (unbound) reactants in the governing equation, where  $X$  is the unknown concentration of bound complex at equilibrium:

$$\begin{aligned} [H] &= H_t - X \\ [G] &= G_t - X \end{aligned}$$

The value of  $G_t$  after each injection was adjusted by a constant increment, set up as an array of 28 injections of 10  $\mu$ L volume at a concentration 10-fold higher than the starting concentration of the host molecule,  $H_t$ . For simplicity, the dilution effect of each injection on the cell concentration was omitted from the model, unlike the true experiment and data analysis programme provided by Microcal. The two free energy parameters,  $\Delta G^\circ$  and

$\Delta G^{\text{H}_2\text{O}}$ , were set as constants that approximate the experimentally obtained values for one of the model systems. The concentration of bound complex ( $X$ ) was solved by MATLAB after each injection from the following expression, in accord with Equation 3:

$$\Delta G^\circ = -RT \cdot \ln\left(\frac{X}{(H_t - X)(G_t - X)}\right) - X \cdot \Delta G^{\text{H}_2\text{O}} \quad (4)$$

Minimum and maximum bounds were placed on the roots for  $X$  using the computational knowledge engine WolframAlpha. Typically, two or three roots were obtained from the analysis, but only one root was a real number in the correct concentration range. After solving for  $X$ , the equilibrium quotient within the natural logarithm term was calculated, as well as the heat released after each injection. The heat was determined by multiplying a constant molar binding enthalpy by the increase in  $X$  relative to the previous injection. The simulated results were plotted in MS Excel against the molar ratio of total guest concentration to total host concentration after each injection, as normally presented in ITC graphs.

### Results

If Equation 3 is a valid relationship for binding equilibria, then the equilibrium ratio for association,  $K$ , should be observed to vary with the concentration of binary complex. Experimentally, this hypothesis can be tested by altering the concentration of the binding reactants and measuring the corresponding equilibrium ratio. As long as the change in solvation energy is nonzero and a reasonably high concentration of complex can be achieved without surpassing the solubility limit (millimolar range), a variable equilibrium ratio should be detected. For this reason, we refer to  $K$  as the equilibrium ratio or equilibrium quotient, but never as the 'equilibrium constant.'

Analysis of Equation 3 is best achieved graphically by plotting the term  $-RT \ln K$  as a function of the complex concentration at equilibrium. In the results that follow, all  $K$  values were obtained by isothermal titration calorimetry using the manufacturer's fitting programme. The concentration of the complex that corresponds to the computer-estimated value of the equilibrium quotient was taken to be the concentration of the limiting reactant in the calorimeter cell, one injection prior to exceeding a 1:1 molar ratio of injectant to binding partner in the cell. When the value of  $K$  is greater than  $10^4$ , as characteristic for all model systems in this study, most of the limiting reactant exists in a binary complex. The 1:1 titration point falls in the steepest region of the titration curve that most strongly reflects the estimated value of  $K$ , as further examined in the Discussion section. For analysing the

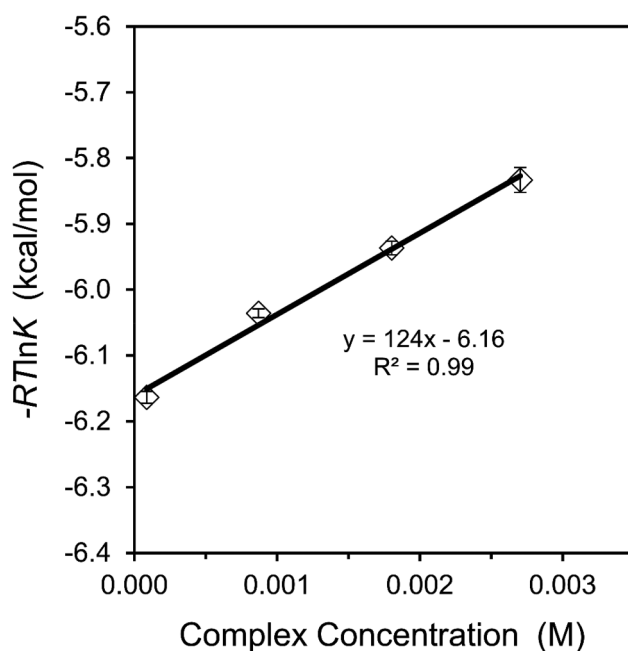
data, the dilution effect due to the accumulated volume of multiple injections was taken into account. For example, if the experiment started with 1.00 mM of host molecule A in the ITC cell, the total concentration of host (and complex) at the 1:1 titration point was found to be 0.87 mM due to the added volume.

A graphical representation of the results plotted as  $-RT\ln K$  versus complex concentration should suggest a linear fit for which the slope is equal to  $\Delta G^{\text{H}_2\text{O}}$  and the y-intercept is  $\Delta G^\circ$ , if Equation 3 correctly captures the thermodynamic role of water in binding equilibria. In the present study, we apply this approach to three popular host–guest binding systems. Quantifying the thermodynamic contribution of the solvent in binding equilibria by this method may be advantageous for many applications of supramolecular chemistry. In general, the lack of experimental hydration energies has been a persistent obstacle for computational chemists who desire experimental values to evaluate their force fields and electrostatic models [8].

### Binding of phenylalanine derivatives to CB7

Cucurbit[7]uril binds to a wide variety of guest molecules, most of which contain a positive charge that interacts favourably with the dipolar ring of oxygen atoms that line the portal on each side of the cavity [9,10]. CB7 is known to bind several amino acid molecules, with a preference for the aromatic side chain of phenylalanine [11]. Unsurprisingly, the binding affinity of free amino acids in water is enhanced as the pH is lowered below the  $\text{pK}_a$  of the  $\alpha$ -carboxylate group, removing a negative charge. We attempted to measure the concentration-dependent binding of CB7 with the carboxy-amidated derivative of phenylalanine (H-Phe-NH<sub>2</sub>), but this guest molecule approached the upper limit in binding affinity that can be measured reliably by ITC without a competitive inhibitor. A value of  $K = 1.89 \times 10^7$  was obtained for H-Phe-NH<sub>2</sub> at a host concentration of 0.10 mM CB7 (Supplementary Material, Figure S1). This value for  $K$  is an order of magnitude larger than that obtained for the unmodified zwitterion at a similar concentration [12]. Thus, we switched to an N-acetylated version of the molecule (Ac-Phe-NH<sub>2</sub>) that has no charged groups.

The Ac-Phe-NH<sub>2</sub> derivative was soluble enough in water to test a CB7 concentration range of 0.10 to 3.0 mM. For this study, the total concentration of potassium ion was maintained at 10 mM for all trials. The equilibrium quotient for this pairing varied from  $3.30 \times 10^4$  at the lowest concentration to  $1.89 \times 10^4$  at the highest concentration (complete dataset in Supplementary Material). This range in binding affinity is reasonable in

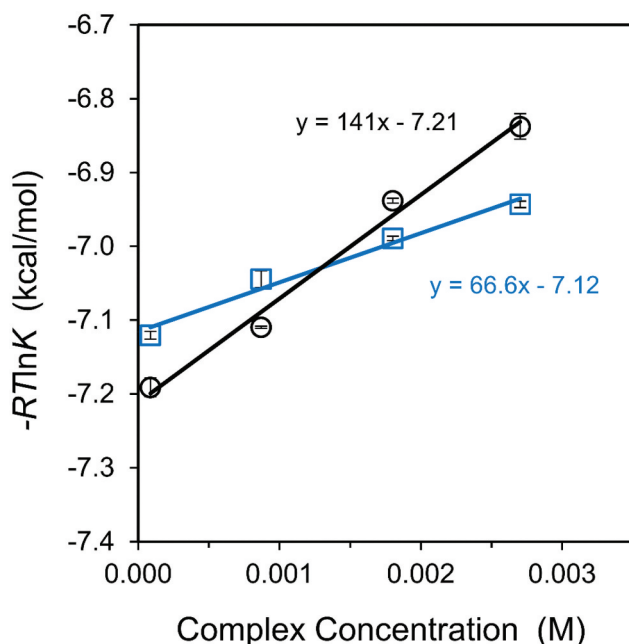


**Figure 1.** Binding of Ac-Phe-NH<sub>2</sub> to CB7 in 2.0 mM phosphate buffer containing 10 mM potassium ion at 25°C. The linear relationship is consistent with Equation 3, for which the y-intercept is  $\Delta G^\circ$  and the slope is  $\Delta G^{\text{H}_2\text{O}}$ . Error bars are shown and are approximately the same size as the symbols. The tabulated values of  $K$  and  $\Delta H^{\text{ITC}}$  as a function of concentration with uncertainties may be found in the Supplementary Material, table S1.

comparison to the value obtained by ITC for the structurally related tripeptide, Gly-Phe-Gly, for which  $K$  was reported to be  $2.2 \times 10^4$  at a concentration between 0.1 and 0.5 mM in 10 mM sodium phosphate buffer [13]. As analysed in Figure 1, this host–guest pairing yields an excellent fit to Equation 3. The thermodynamic parameters obtained for the binding of CB7 with Ac-Phe-NH<sub>2</sub> are  $\Delta G^{\text{H}_2\text{O}} = +120 \pm 10$  kcal/mol, and  $\Delta G^\circ = 6.16 \pm 0.02$  kcal/mol.

### Binding of putrescine and TMA to SC4

To further explore the generality of Equation 3 for interpreting the role of solvent in host–guest-binding equilibria, *p*-sulfonatocalix[4]arene was tested. At pH 7, the four sulphonate groups of SC4 are negatively charged, and therefore this host molecule also has a preference for cationic guest molecules [14]. Putrescine (1,4-diaminobutane) was selected as a guest because it is one of the series of highly soluble diammonium compounds at pH 7 known to bind SC4 [15]. For all SC4 binding studies, the concentration of  $\text{K}^+$  was maintained at 15 mM, and the host concentration was varied from 0.10 to 3.0 mM. For putrescine as the guest, the resulting  $K$  values ranged from  $18.7 \times 10^4$  (lowest concentration) to  $10.3 \times 10^4$



**Figure 2.** Binding of putrescine (black, circles) and TMA (blue, squares) to SC4 in 2.0 mM phosphate buffer containing 15 mM potassium ion at 25°C. The tabulated values of  $K$  and  $\Delta H^{\text{ITC}}$  as a function of concentration may be found in the Supplementary Material, table S2.

(highest concentration). **Figure 2** reveals this host–guest combination also yields a good fit to Equation 3, leading to values of  $\Delta G^{\text{H}_2\text{O}} = +140 \pm 20$  kcal/mol, and  $\Delta G^\circ = 7.21 \pm 0.02$  kcal/mol.

Another study was undertaken with the host–guest system of SC4 and tetramethylammonium ion (TMA). At 25°C, the equilibrium ratio for association ranged from  $16.6 \times 10^4$  (lowest concentration) to  $12.3 \times 10^4$  (highest concentration). These  $K$  values are higher than the reported value of  $7.9 \times 10^4$  obtained by NMR titration, but the NMR study was performed in 100 mM phosphate buffer and, therefore, in the presence of a much higher concentration of a competitive cation [16]. As seen in **Figure 2** (blue line, squares), a linear correspondence with Equation 3 is apparent, leading to free energy estimates of  $\Delta G^{\text{H}_2\text{O}} = +67 \pm 4$  kcal/mol, and  $\Delta G^\circ = 7.12 \pm 0.02$  kcal/mol.

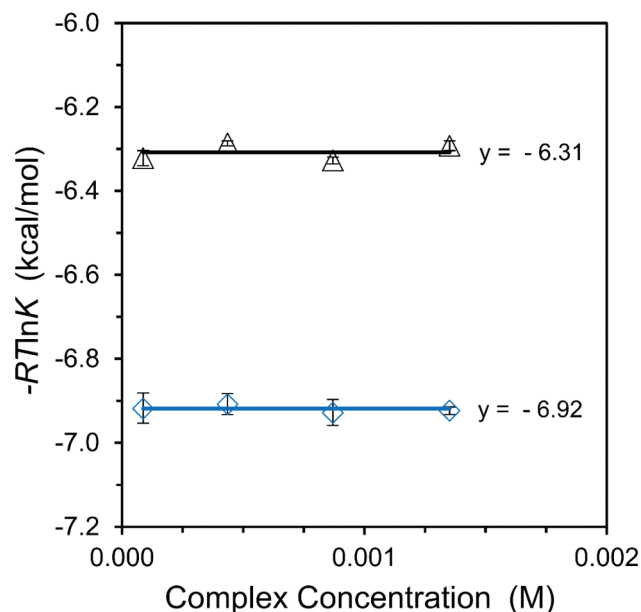
### Binding of $\beta$ CD with non-polar guests

Binding measurements were also pursued with  $\beta$ -cyclodextrin and two guest molecules. Most of the reported guest molecules that bind well to  $\beta$ CD are hydrophobic compounds of low water solubility [17], presenting a technical challenge to this study because measurements across multiple concentrations in the millimolar range are desired. ITC trials were completed

with adamantane-1-carboxylate ( $\text{AC}^-$ ) and with the bile acid, sodium glycochenodeoxycholate (GCDC). For these investigations, the normal ITC setup was reversed such that the guest molecule resides in the cell and the host molecule is loaded into the syringe. In this manner, the binding affinity for each guest was measured in the concentration range of 0.10–1.5 mM in 10 mM potassium phosphate buffer.

Interestingly, the binding of  $\text{AC}^-$  to  $\beta$ CD displayed no significant change in  $K$ , with an average value of  $4.21 (\pm 0.16) \times 10^4$  across all concentrations tested (**Figure 3**). Our measured value of  $K$  is slightly higher than the reported value of  $3.90 \times 10^4$  in 100 mM sodium phosphate [18] and another reported value of  $3.84 \times 10^4$  in 50 mM phosphate buffer [19]. With respect to Equation 3, one concludes that  $\Delta G^{\text{H}_2\text{O}} \approx 0$  kcal/mol, and  $\Delta G^\circ = 6.31 \pm 0.03$  kcal/mol for the binding of  $\text{AC}^-$  with  $\beta$ CD.

Similarly, the equilibrium quotient was found to be constant for the binding of GCDC with  $\beta$ CD in **Figure 3** (blue line, diamonds). For this pairing, the average binding quotient is  $11.8 (\pm 0.6) \times 10^4$  across all concentrations tested, a value positioned between the two reports of  $10.8 \times 10^4$  [20] and  $14.0 \times 10^4$  [21] for the same pairing in 50 mM phosphate buffer. The corresponding free energies for binding of GCDC with  $\beta$ CD are  $\Delta G^{\text{H}_2\text{O}} \approx 0$  kcal/mol, and  $\Delta G^\circ = 6.92 \pm 0.04$  kcal/mol.



**Figure 3.** Binding of  $\text{AC}^-$  (black, triangles) and GCDC (blue, diamonds) to  $\Delta$ CD in 10 mM potassium phosphate at 25°C. The horizontal lines indicate  $\Delta G^{\text{H}_2\text{O}}$  is near zero for both guest molecules. The tabulated values of  $K$  and  $\Delta H^{\text{ITC}}$  as a function of concentration may be found in the Supplementary Material, table S3.



## Discussion

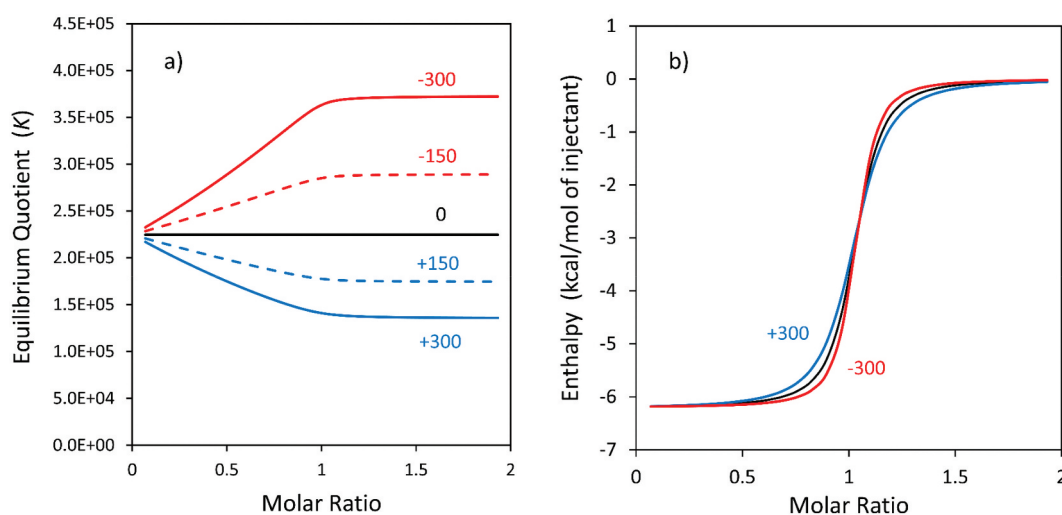
### Importance of buffer composition

Because this study is interested in noncovalent interactions in the context of biology, all of the model-binding systems in this report were investigated near pH 7. In the cases of CB7 and SC4, the common biological buffer Tris (tris[hydroxymethyl]aminomethane) was avoided because the protonated form of the buffer is a cation that competes with guest molecules for binding to the host cavity. One should note that the counterion used in phosphate buffers can also compete for binding. For example, sodium ion has been reported to bind CB7 with an equilibrium quotient of  $130 \text{ M}^{-1}$  [22], and potassium ion has been reported to bind SC4 with an equilibrium quotient of  $217 \text{ M}^{-1}$  [23]. For this reason, a low concentration of 2.0 mM potassium phosphate was employed as the buffer for CB7 and SC4 reactions in the current study. Furthermore, it was found to be critical to account for potassium from the initial neutralisation of the acidic stock solutions of CB7 and SC4 by supplementing the dilution buffers with an equivalent amount of KCl (7.0 mM KCl added for CB7 dilutions and 12 mM KCl for SC4 dilutions). In this way, a constant concentration of the weak competitor,  $\text{K}^+$ , was maintained across all target concentrations of the same host and guest molecule. In preliminary experiments completed without KCl addition, the measured binding quotients were larger in magnitude at the lower end of the tested concentration range, leading to  $\Delta G^{\text{H}_2\text{O}}$  values about two-fold higher than the values reported here.

### Fitting concentration-dependent titration curves with algorithm based on classical binding equation

Modern calorimeters typically include an analysis software package that treats the equilibrium quotient as a constant, presenting a dilemma for this study. If the equilibrium for a binding reaction is dependent on the concentration of the formed complex, then the equilibrium quotient should change slightly after each injection during the progress of a titration experiment, as more and more complex is formed. This expectation is realised in a modelling study, as depicted in Figure 4(a). If the change in solvation free energy is positive, the equilibrium quotient will decline for the first half of the titration, and if the change in solvation free energy is negative, the equilibrium quotient will increase. Note that  $K$  approaches a constant value near the midpoint of the titration which corresponds to a 1:1 molar ratio of total guest to total host. The last half of the titration is characterised by a nearly constant value for  $K$  because the reactant that started in the calorimeter cell has become the limiting reactant and because nearly all of the limiting reactant has been converted to complex (when  $K$  is sufficiently large).

The simulated ITC titration curves in Figure 4(b) were generated from the three datasets in Figure 4(a) that correspond to  $\Delta G^{\text{H}_2\text{O}}$  values of +300, 0, and  $-300 \text{ kcal/mol}$ . Note that the change in curvature is subtle for these relatively large values of  $\Delta G^{\text{H}_2\text{O}}$ , and that the three curves nearly coincide at the beginning and end of the titration. The main deviation occurs between the molar ratio of



**Figure 4.** (A) Predicted changes in the equilibrium quotient during the progress of an ITC experiment, and (b) corresponding titration curves, as obtained from the modelling of Equation 3. Model assumptions: total host concentration in ITC cell = 1.0 mM,  $\Delta G^\circ = 7.3 \text{ kcal/mol}$ , and  $\Delta H^{\text{ITC}} = -6.2 \text{ kcal/mol}$ . The number next to each curve indicates the input value for  $\Delta G^{\text{H}_2\text{O}}$  in units of kcal/mol. The solid black line in each panel corresponds to the classical equation, for which  $K = 2.25 \times 10^5$  (constant) and  $\Delta G^{\text{H}_2\text{O}} = 0$ . All lines were constructed from 28 datapoints that mimic 28 injections of an ITC trial. See Methods section for modelling details.

0.5 and 1.5, with the lines intersecting near a ratio of 1:1. For this reason, it is important that ITC protocols employ an adequate number of injections to characterise the middle portion of the titration curve. For the experimental results reported here, the calorimeter software's estimation of  $K$  is paired with the concentration of complex at the 1:1 point. The perfect match might occur at a slightly smaller concentration than the 1:1 point, but it cannot be greater than this value. If the best match is a smaller concentration, then the  $\Delta x$  value between any two points on the characteristic  $x$ - $y$  plots (Figures 1 and 2) will be slightly smaller, and the slope will be slightly larger. Thus, our estimated values for  $\Delta G^{\text{H2O}}$  may be viewed as conservative in magnitude.

### Nonideality in solution thermodynamics

The three results for host-guest interactions involving CB7 and SC4 follow Equation 3, as demonstrated by the linear relationships indicated in Figures 1 and 2. This approach to obtaining the change in hydration free energy was questioned following our first publication on the topic, but an alternative explanation for the observed concentration dependence of  $K$  was not provided other than stating that the high charge densities of the  $\text{Ca}^{2+}$  and EDTA reactants lead to a system of 'strong nonideality' [24]. It is clear that reactants of high charge density will bind water strongly and, therefore, should deviate from the classical equation for binding equilibria if the classical equation does not account properly for the release of water molecules upon formation of a complex. We disagree with the notion that water requires no explicit consideration because 'no water is added or consumed in the course of the reaction' [24]. Our approach recognises that (1) a subset of water is *added* to the bulk phase when the complex interacts with fewer water molecules than the free reactants, and (2) there is no parameter in the classical equation that reflects the chemical potential of a bulk water molecule, a side-product of the reaction.

With regard to solutions of nonideal behaviour, as one should expect upon addition of a secondary solute at high concentration, nonideality is taken into account by the change in activity coefficients in the expression for  $\Delta G^\circ$  (Equation 2). When comparing a reaction in a concentrated (nonideal) solution to the same reaction in a dilute solution, the standard-state free energy in the concentrated system is given by Equation 5 for which the activity coefficients are no longer unity.

$$\Delta G^\circ(\text{obs}) = \Delta G^\circ(\text{dilute}) + RT \ln \frac{Y_i^{\text{AB}}}{Y_i^{\text{A}} Y_i^{\text{B}}} \quad (5)$$

Thus, the observed value of  $\Delta G^\circ$  is expected to change by a constant due to changes in the activity coefficients of the reactants, but this does not preclude the simultaneous existence of a concentration-dependent equilibrium, as reflected in  $\Delta G^{\text{H2O}}$ . Furthermore, if the secondary solute alters the surface hydration of the reactants to a different extent than it influences the bulk water free energy, then the value of  $\Delta G^{\text{H2O}}$  also will be altered. In graphical terms, both the slope and  $y$ -intercept may change in the characteristic plot when a secondary solute is present.

For the concentration range of reactants and buffer conditions employed in the current investigation, the activity coefficients should be constant. A point in support of this argument is the fact that the ratio of water molecules to reactant molecules is very large. For example, at a relatively high reactant concentration of 10 mM, the molar ratio of water to reactant is  $\sim 55 \text{ M}/0.010 \text{ M} = 5500:1$ . Thus, reactant-water interactions should far outnumber reactant-reactant interactions, and a significant change in the activity coefficient of a reactant due to its own presence seems unlikely. Many past binding studies in the literature may have failed to notice a concentration-dependent change in binding affinity simply because the experiments were performed at a single concentration under the presumption that  $K$  is a constant.

### Comparison of model binding pairs

The key results from the current study are summarised in Table 1, along with results from two aqueous binding systems that were published previously. The overall range in binding affinity in Table 1 covers two orders of magnitude in  $K$ , from  $10^4$  to  $10^6$ . It should be recognised that the selected model systems vary considerably in the degree of electrostatic interactions that contribute to binding. At one extreme is the chelation of calcium by EDTA (multivalent charge interactions), and at the other extreme is the interaction between an uncharged host with an uncharged guest, CB7 with Ac-Phe-NH<sub>2</sub>. If one scans down each column of Table 1, no obvious correlations exist between  $\Delta G^\circ$ ,  $\Delta G^{\text{H2O}}$ , and  $\Delta H^{\text{TC}}$  when viewing the pairings from highest to lowest binding affinity at infinite dilution ( $\Delta G^\circ$ ).

Regarding the tabulated values for  $\Delta G^{\text{H2O}}$ , all of the solvation energies are positive, with the notable exception of the two  $\beta\text{CD}$  pairings. The magnitude of these hydration energies supports the concept that molecular recognition, in general, is a fine balance between two large opposing forces [25]; the favourable contribution from the direct interaction of host and guest is opposed by an unfavourable contribution from the solvent for SC4 and CB7.

**Table 1.** Comparison of different model systems in order of descending binding affinity.

Host:Guest	Solution Conditions	$\Delta G^\circ$	$\Delta G^{H2O}$	$\Delta H^{TC}$	<sup>a</sup> Max Conc (mM)
<sup>b</sup> EDTA:Ca <sup>2+</sup>	150 mM MES	-8.37	+63	-4.3	12.5
<sup>c</sup> Crypt:Rb <sup>+</sup>	0.20 M NaOH	-7.93	+77	-5.5	3.0
SC4:Putr <sup>2+</sup>	2 mM KPhos + KCl <sup>d</sup>	-7.21	+140	-4.3	3.0
SC4:TMA <sup>+</sup>	2 mM KPhos + KCl <sup>d</sup>	-7.12	+67	-6.9	3.0
$\beta$ CD:GCDC	10 mM KPhos	-6.92	~0	-6.2	1.5
$\beta$ CD:AC <sup>-</sup>	10 mM KPhos	-6.31	~0	-6.0	1.5
CB7:Ac-Phe-NH <sub>2</sub>	2 mM KPhos + KCl <sup>e</sup>	-6.16	+120	-9.9	3.0

All energy values in units of kcal/mol, obtained at 25°C.

<sup>a</sup>Maximum concentration tested in cell at start of titration.

<sup>b</sup>Results from Castellano and Eggers [1].

<sup>c</sup>Results from Eggers, et al [2].

<sup>d</sup>Total concentration of K<sup>+</sup> adjusted to 15 mM, including buffer counterion.

<sup>e</sup>Total concentration of K<sup>+</sup> adjusted to 10 mM, including buffer counterion.

### Rethinking the driving force of the hydrophobic effect

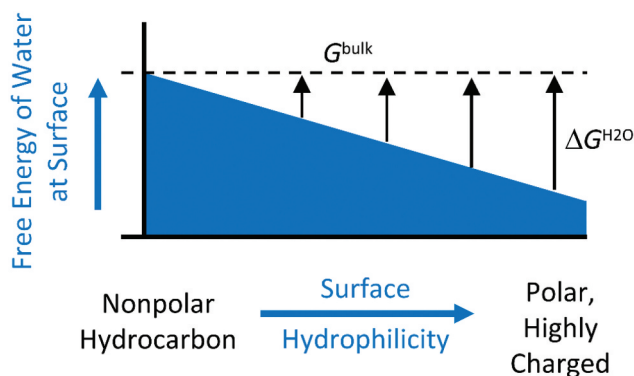
The two results obtained with  $\beta$ CD as host deserve further consideration. The observation that the binding quotients for both AC<sup>-</sup> and GCDC are independent of concentration was initially viewed as an undesirable finding because it does not provide positive support for the proposed relationship, Equation 3. However, it has been previously hypothesised that the change in hydration free energy for a given binding model may be positive, negative, or near zero [1]. Thus, the  $\beta$ CD results do not diminish the relevance of Equation 3. A concentration-independent change in affinity has also been reported for  $\beta$ CD with two derivatives of adamantane, amantadine, and rimantadine, as tested at two concentrations near 0.25 mM and 1.0 mM [24].

An important related issue is whether or not the binding of non-polar guests to  $\beta$ CD should be viewed as a good model for the hydrophobic effect. Although  $\beta$ CD is comprised of seven hydrophilic glucose rings joined by  $\alpha$ -1,4-glycosidic bonds, the three hydroxyl groups on each sugar residue are pointing away from the exterior surface, allowing the interior face to define a hydrophobic cavity [26]. Thus, the binding of the adamantane group of AC<sup>-</sup> (a hydrophobic sphere) and the binding of the fused ring system of GCDC (a hydrophobic spike) to  $\beta$ CD do indeed appear to represent reasonable models of non-polar–non-polar interactions and the hydrophobic effect. The hydrophobic nature of the  $\beta$ CD:AC<sup>-</sup> interaction is also consistent with compressibility studies that predict the release of 20–25 water molecules upon complex formation [27]. Our results suggest that the change in hydration free energy for the displaced water molecules is near zero.

Based on this study, it would be misleading to refer to the water confined to the cavity of  $\beta$ CD and other host molecules as ‘high energy water’, as often

described [28,29]. The water of hydrophobic hydration may be higher in energy than water at other surfaces, but water molecules next to a non-polar surface are not necessarily higher in free energy than the average water molecule in the bulk liquid phase. Although the hydrogen-bonding pattern of water molecules at a non-polar surface is expected to be different than the average pattern in the pure liquid phase, the change in enthalpy and entropy of the water upon release from the non-polar surface may cancel out such that the free energy change is negligible. If true, the driving force for the hydrophobic effect in a dilute solution must be attributed to the van der Waals interaction between non-polar groups (dispersion forces), as opposed to the release of high energy water. Our results suggest that non-polar interactions in water are the same energetically as would be found in the gas phase, if it were possible to measure the gas-phase interaction at the same concentration. With these thoughts in mind, it would be fitting to replace the term ‘hydrophobic effect’ with the phrase ‘*aqua indifferens* effect.’

Admittedly, this conclusion regarding the hydrophobic effect is difficult to assimilate because it conflicts with many past theories and computational studies, but the approach shared here is one of the few experimental options described in the literature for quantifying the thermodynamic contribution of water in molecular recognition. Molecular torsion balances represent another experimental technique for probing solvent effects on the weak interactions that define a binding event [30]. However, molecular balances measure an intramolecular equilibrium between two conformers of the same molecule, whereas Equation 3 can be applied to any two molecules of interest and yields a change in hydration free energy directly without by-passing the entropy change due to association (unlike the molecular balance approach). The main limitation to



**Figure 5.** Calibrating the hydrophobic effect relative to the free energy of the bulk aqueous phase,  $G^{\text{bulk}}$ . Hydration free energy is plotted against a generic hydrophilicity scale for the reactant surface. When water molecules are released from a surface upon binding to another surface, the free energy change in hydration is defined as  $\Delta G^{\text{H}_2\text{O}} = G^{\text{bulk}} - G^{\text{surface}}$ . The value of  $\Delta G^{\text{H}_2\text{O}}$  is positive and unfavourable for polar molecules but approaches zero for a non-polar hydrocarbon.

implementing the new governing relationship is the fact that the last term of Equation 3,  $[AB]^{\text{eq}} \cdot \Delta G^{\text{H}_2\text{O}}$ , must be large enough to detect a change in the equilibrium. Experimentally, this means that the complex concentration must surpass 1.0 millimolar or  $\Delta G^{\text{H}_2\text{O}}$  must be significantly larger than the values reported in this study.

The concepts discussed above are reproduced schematically in Figure 5 which emphasises how the value of  $G^{\text{bulk}}$  determines the magnitude and sign of  $\Delta G^{\text{H}_2\text{O}}$  when a subset of water molecules is released from one surface upon binding to another surface. In general, there is a free energy penalty for removing water from a polar surface, but there is no penalty (or benefit) for removing water from a non-polar surface; the estimated value of  $\Delta G^{\text{H}_2\text{O}}$  is zero for the interaction of two non-polar molecules in dilute solution, in accord with our experimental results with the  $\beta\text{CD}$  system.

We caution that  $\Delta G^{\text{H}_2\text{O}}$  is not solely a function of polarity, as presented in Figure 5. There may be some small polar compounds that induce water of equal or higher energy than that of a hydrocarbon. For example, addition of a chemical denaturant, such as urea or guanidinium chloride, may increase the free energy of the bulk aqueous phase relative to dilute solution. Alternatively, the addition of secondary solutes, such as salts of phosphate, sulphate, and acetate from the kosmotropic end of the Hofmeister anion series [31,32], may stabilise binding interactions by decreasing the average free energy of the bulk aqueous phase [33].

## Conclusion

This investigation expands the number of model systems tested against a governing equation for binding equilibria that includes an explicit term for the contribution of water. Two host molecules, cucurbit[7]uril and *p*-sulfonatocalix[4]arene, were found to have concentration-dependent binding equilibria that fit to the proposed equation. These results, obtained in the low millimolar concentration range, are not viewed as arising from solution nonideality. Another host molecule,  $\beta$ -cyclodextrin, was found to be characterised by concentration-independent binding equilibria, indicating that the change in free energy of water is zero for its non-polar guests. If binding to  $\beta\text{CD}$  is an appropriate model for the interaction of non-polar surfaces, and if the thermodynamic framework employed here is valid, then it follows that the hydrophobic effect is not driven by the release of water.

This work suggests a fundamental change in the application of solution thermodynamics to reaction equilibria. The scientific community is encouraged to examine other binding models as a function of concentration to explore further the utility and validity of the experimental approach employed here.

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## ORCID

Daryl K. Eggers  <http://orcid.org/0000-0003-1915-5945>

## Data availability statement

Sample ITC data files and modelling details related to Figure 4 are available on the SJSU ScholarWorks page of the corresponding author at [https://works.bepress.com/daryl\\_eggers/](https://works.bepress.com/daryl_eggers/).

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