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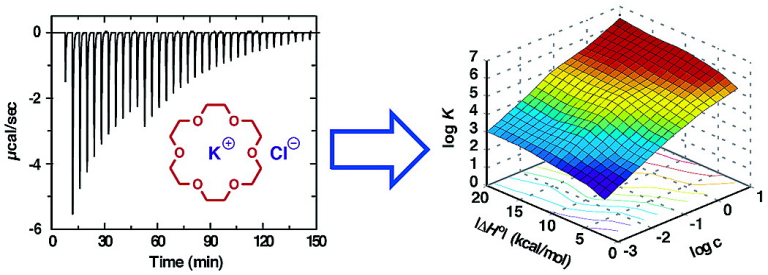

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On the Value of *c*: Can Low Affinity Systems Be Studied by Isothermal Titration Calorimetry?

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Abstract: Isothermal titration calorimetry (ITC) allows the determination of $\Delta G°$, $\Delta H°$, and $\Delta S°$ from a single experiment and is thus widely used for studying binding thermodynamics in both biological and synthetic supramolecular systems. However, it is widely believed that it is not possible to derive accurate thermodynamic information from ITC experiments in which the Wiseman "*c*" parameter (which is the product of the receptor concentration and the binding constant, K_a) is less than ca. 10, constraining its use to high affinity systems. Herein, experimental titrations and simulated data are used to demonstrate that this dogma is false, especially for low affinity systems, assuming that (1) a sufficient portion of the binding isotherm is used for analysis, (2) the binding stoichiometry is known, (3) the concentrations of both ligand and receptor are known with accuracy, and (4) there is an adequate level of signal-to-noise in the data. This study supports the validity of ITC for determining the value of K_a and, hence, $\Delta G°$ from experiments conducted under low *c* conditions but advocates greater caution in the interpretation of values for $\Delta H°$. Therefore, isothermal titration calorimetry is a valid and useful technique for studying biologically and synthetically important low affinity systems.

Introduction

Molecular recognition underpins all biological processes from enzymatic catalysis to signal transduction and also the pharmaceutical intervention in such processes for the treatment of disease.[1] Although the objective of most research in medicinal and supramolecular chemistry is the design of compounds that achieve high affinity recognition, there are many important low affinity systems in biology, most notably carbohydrate−protein[2] and albumin−ligand interactions,[3] and also in chemistry, for example, cyclodextrin−ligand systems[4] as models for catalysis[5] and vehicles for drug delivery.[6] Furthermore, it has long been appreciated[7] that high affinity ligands can be constructed by tethering two complementary low affinity compounds together, a concept that remains important in drug design strategies such as structure−activity relationships by nuclear magnetic resonance (SAR by NMR).[8] A detailed understanding of the enthalpic and entropic contributions to binding can also be of considerable utility to the process of drug design.[9]

Isothermal titration calorimetry (ITC) allows $\Delta G°$, $\Delta H°$, and $T\Delta S°$ for a ligand−receptor interaction to be determined in a single experiment by measuring stepwise changes in the enthalpy of interaction during the course of a titration experiment.[10] ITC has thus found widespread applicability in the study of biological systems[11] involving protein−ligand, protein−nucleic acid, and protein−protein interactions and also in supramolecular chemistry, most notably for complexes of both cyclodextrins (CDs)[4a] and crown ethers.[12] The successful extraction of thermodynamic parameters from calorimetric data relies upon the use of nonlinear least squares curve fitting while employing an appropriate model that describes the interaction under study. Ever since highly sensitive titration microcalorimeters became

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commonly available in the late 1980s,[13] this fitting process has typically been undertaken using the so-called Wiseman isotherm.[14,15] For the most simple case of 1:1 ligand−receptor (X−M) binding,

$$X + M \rightleftarrows MX \quad (1)$$

the Wiseman isotherm (eq 2) relates the stepwise change in heat of the system normalized with respect to moles of ligand added per injection ($dQ/d[X]_t$) to the absolute ratio of ligand to receptor concentration ($X_R = [X]_t/[M]_t$) at any point during the course of the titration:

$$\frac{dQ}{d[X]_t} = \Delta H^\circ V_0 \left[\frac{1}{2} + \frac{1 - X_R - r}{2\sqrt{(1 + X_R + r)^2 - 4X_R}}\right] \quad (2)$$

where

$$\frac{1}{r} = c = K_a[M]_t = \frac{[M]_t}{K_d} \quad (3)$$

and V_0 is the effective volume of the calorimeter cell. (Although small molecules that bind metal ions are usually referred to as ligands, herein we use the calorimetry convention that the ligand is the species in the syringe (i.e., the titrant) and the receptor is the species in the calorimeter cell at the beginning of the experiment.) Wiseman et al. noted[14] that the shape of such a binding isotherm, for a simple noninteracting one site model, changes according to the product of the association constant (K_a) and the (macromolecular) receptor concentration ($[M]_t$, which they referred to as the c value (eq 3). The c value may also be thought of as the ratio of the receptor concentration and the dissociation constant, K_d. More generally, for receptors with several identical noninteracting binding sites,

$$c = nK_a[M]_t \quad (4)$$

wherein n is the number of binding sites per receptor M. For c values higher than ca. 10, the curve is clearly sigmoidal (Figure 1a) with a slope around the end point of the titration that tends toward vertical as c approaches infinity. It is often stated[2b,10,14,15,16a] that curve fitting is optimal in the so-called "experimental window" of c values of 10−500, whereas the fitting process is "erroneous" and that it is "not feasible" to obtain useful information for cases in which $c < 10$ and certainly for $c < 1$.

It must be emphasized that working with $10 \leq c \leq 500$ is always preferable as receptor saturation can be achieved by adding as little as 2 equiv of ligand. However, for many low affinity systems, for example, protein−carbohydrate[2] and CD−ligand[4] interactions, it is not possible to achieve c values in the preferred range on account of limited solubility of receptors and/or ligands. In some instances, it is possible to use displacement assays,[16] in which a higher affinity ligand that binds competitively with the ligand under study is used to bring the titration curve back into the usual experimental window.

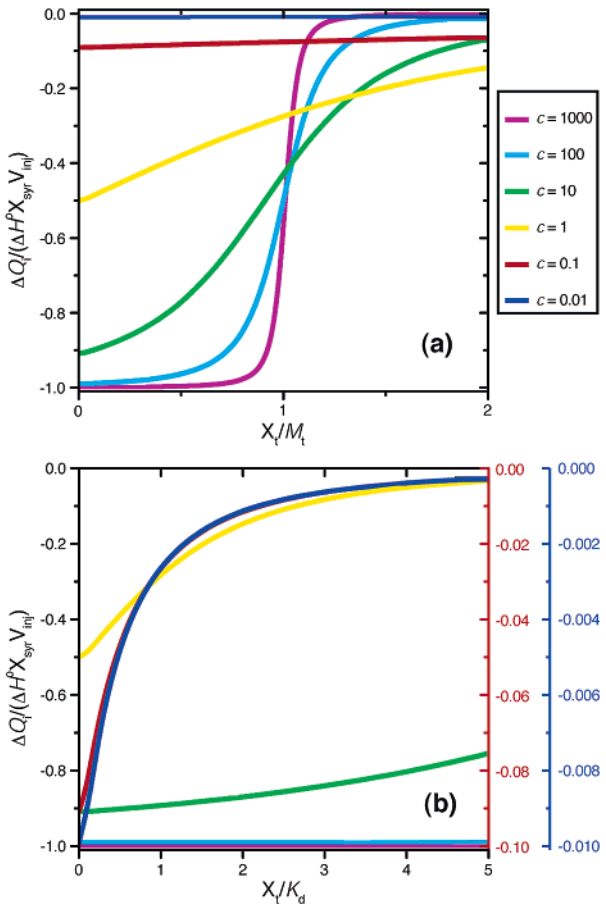

Figure 1. (a) The shape of an ITC titration curve varies with c, that is, the product of the receptor concentration and the association constant. (b) An alternative depiction of the Wiseman isotherm in which the heat released per injection (normalized with respect to moles of added ligand and ΔH°) is plotted vs the ratio of ligand concentration and the dissociation constant. The red and blue curves are magnified by factors of 10 and 100, respectively, to emphasize their similar curved shapes.

However, this approach is usually only applicable to studies that involve dissecting the contributions of fragments of a high affinity ligand. Therefore, researchers working with low affinity systems have nevertheless published,[17] with some confidence, a considerable body of data that conventional thinking would dismiss as erroneous. This apparent contradiction in the literature remains to be addressed adequately, and more detailed guidelines defining the possibilities and limitations for working outside the recommended experimental window are unfortunately lacking. Herein we aim to address the question of curve fitting at low values of c, and guidelines for designing ITC experiments for low affinity systems are proposed.

Experimental Section

All reagents were purchased from Sigma-Aldrich and were of the highest purity available, and solutions were prepared with purified water (Purite). Stock solutions of metal ions were prepared in volumetric

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flasks from anhydrous salts to give a concentration of 1.0 M and further diluted to give the desired concentrations. Concentrations of 1,4,7,-10,13,16-hexaoxacyclooctadecane (18-crown-6) were checked using ^{1}H NMR spectroscopy by comparison with 5 mM ethanol as an internal standard. Experiments involving Concanavalin A (Con A) were conducted in 100 mM sodium acetate buffer at pH 5.2, and the protein subunit concentration was determined by UV absorbance at 280 nm.[18] All solutions were degassed prior to use.

ITC was performed on a MicroCal VP-ITC unit which was calibrated using the built-in electrical calibration check and by titration of $BaCl_2$ into 18-crown-6.[10c] All experiments were conducted at 25 °C with the exception of the Con A titrations which were performed at 27 °C. Potassium chloride titrations involved the addition of 1.3−8 μL aliquots of salt solutions at concentrations of 0.25−1 M at typically 4 min intervals into 18-crown-6 solutions at 0.1−100 mM. Barium chloride experiments used salt concentrations of 6−100 mM and crown ether concentrations between 0.01 and 9 mM. Additions of methyl α-mannopyranoside (10 μL, 3.57−6.95 mM) were added at 4 min intervals to solutions of Con A (8−670 μM). Initial concentrations of ligands and receptors were selected using a spreadsheet implemented in Microsoft Excel (see below), to achieve final receptor saturations of ca. 80% for low c values and 90−99% for high c values, based on $K_a \approx 100$ M^{-1} for KCl/18-crown-6 and $K_a \approx 5000$ M^{-1} for $BaCl_2$/18-crown-6.

Heats of dilution determined in the absence of receptors were subtracted from the titration data prior to curve fitting. Additionally, an initial 2 μL injection was discarded from each dataset in order to remove the effect of titrant diffusion across the syringe tip during the equilibration process. Curve fitting was undertaken in Origin v. 5.0 using the standard noninteracting one site model supplied by MicroCal. Initial values for the fitting parameters were typically at least 2 orders of magnitude different in K_a and 10 kcal mol^{-1} different in $\Delta H°$ from the corresponding values at convergence. For experiments with $c < 5$, the stoichiometry parameter, n, was fixed to 1.0. Titrations of KCl/18-crown-6 were run in triplicate, and errors quoted for these experiments are 95% confidence limits calculated as twice the standard deviation of the mean parameter values. Otherwise, errors from experimental data are those returned by the nonlinear least-squares fitting module of Origin.

Error free simulated datasets for the 1:1 binding model were calculated using a Microsoft Excel spreadsheet based on eq 2. As liquid is displaced from the effective portion of the cell each time that an addition of ligand is made,[14] corrected concentrations of receptor and ligand following the ith injection ($[M]_{t(i)}$ and $[X]_{t(i)}$, respectively) were calculated using equations described in the MicroCal VP-ITC manual:[19]

$$[M]_{t(i)} = [M]_{t(0)}\left(\frac{1 - \frac{\Delta V_{(i)}}{2V_0}}{1 + \frac{\Delta V_{(i)}}{2V_0}}\right) \quad (5)$$

$$[X]_{t(i)} = \frac{\Delta V_{(i)}[X]_{syr}}{V_0}\left(1 - \frac{\Delta V_{(i)}}{2V_0}\right) \quad (6)$$

where $[M]_{t(0)}$ is the initial receptor concentration, $\Delta V_{(i)}$ is the sum total volume of ligand added following the ith injection, and $[X]_{syr}$ is the concentration of ligand in the syringe. All simulations involved 25 additions of 10 μL, and appropriate starting concentrations of ligands and receptors were chosen in order to achieve final receptor occupancies of 90% for $c = 10$ and 80% for $c = 1$, 0.1, 0.01, and 0.001. The values of $\Delta H°$ used were 2, 5, 10, 15, and 20 kcal mol^{-1} and $K_a = 10^1$, 10^2, 10^3, 10^4, and 10^5 M^{-1}. The values of K_a were extended to 10^6 and 10^7 M^{-1} for $c = 1$ and 10, respectively.

Random error was added in Origin to nine replicates of each set of stepwise heat changes simulated in microcalories. The added error had a Gaussian distribution with a mean of zero and a standard deviation of 0.5 μcal or 0.5% of each simulated value, whichever was greater. The simulated data were then normalized with respect to the number of moles of ligand added per injection and plotted versus $[X]_t/[M]_t$. Curve fitting was conducted with the standard noninteracting one site model with $n = 1.0$ for all datasets and with initial K_a and $\Delta H°$ values chosen to differ from the simulated values as described above. Errors in the values of K_a, $\Delta G°$, and $\Delta H°$, thus obtained, were determined by comparison with those values used for the simulations. The mean error plus two standard deviations was plotted versus $\log(K_a)$ in Matlab 6.5 for each set of replicates. The values of $\log(K_a)$ required to give threshold error levels of 0.4 and 1.0 kcal mol^{-1} for $\Delta G°$ and $\Delta H°$, respectively, were determined by linear interpolation and combined to create 3-D surfaces and contour plots.

Results

MCl_x/18-Crown-6 Titrations. To evaluate the applicability of ITC for studying low affinity systems ($K_a < 10\,000$ M^{-1}) under low c value conditions, an experimental system was sought which would allow titrations to be conducted in the range $0.01 < c < 10$. However, all protein−ligand and CD−ligand systems that were investigated were found to be unsuitable on account of poor solubility of either the protein or alternatively the ligand, in the case of the CD systems. Poor solubility of the receptor and the ligand, in particular, is a common problem in ITC but is more often an issue when dealing with low affinity systems, which, by convention, require to be studied at higher concentrations in order to satisfy the c value rule. Alternatively, if titrations are conducted at a low receptor concentration ($[M]_t \ll K_d$), then the final ligand concentration must still be several times higher than K_d (eq 7). Consequently, the classic supramolecular system of 18-crown-6 and potassium chloride ($K_a \approx 100$ M^{-1} in water) was chosen for study.[12] Initial experiments were undertaken to determine what final receptor occupancy should be achieved in order to provide an adequate portion of the binding isotherm for curve fitting. Experiments were conducted at $c = 0.1$ using equal numbers of injections but differing concentrations of KCl in order to achieve 70%, 80%, and 90% saturation of the receptor by the end of the titration (Figure 2). These experiments also have the effect of providing differing proportions of the data points describing the early more variable part of the binding isotherm and the later more constant part. Thus different weightings for the early and later portions of the isotherm are achieved in the curve fitting without changing the weighting given to individual data points. However, very little difference in the fitting parameters was observed across this series of experiments, and consequently a final receptor occupancy of 80% was selected for the low c value experiments.

Data for titrations run in triplicate using 0.1−100 mM 18-crown-6 are summarized in Table 1 and Figure 3. The values of $\Delta G°$ obtained from curve fitting, with the stoichiometry set at $n = 1$ for $c < 5$, showed little variation over the range of c values tested. However, $\Delta H°$ exhibited a clear trend toward larger negative values as the 18-crown-6 concentration, and hence c increased. Without knowing if $\Delta H°$ is constant across such a wide range of receptor concentrations, it is not clear whether this result reflects inaccuracies in curve fitting at low

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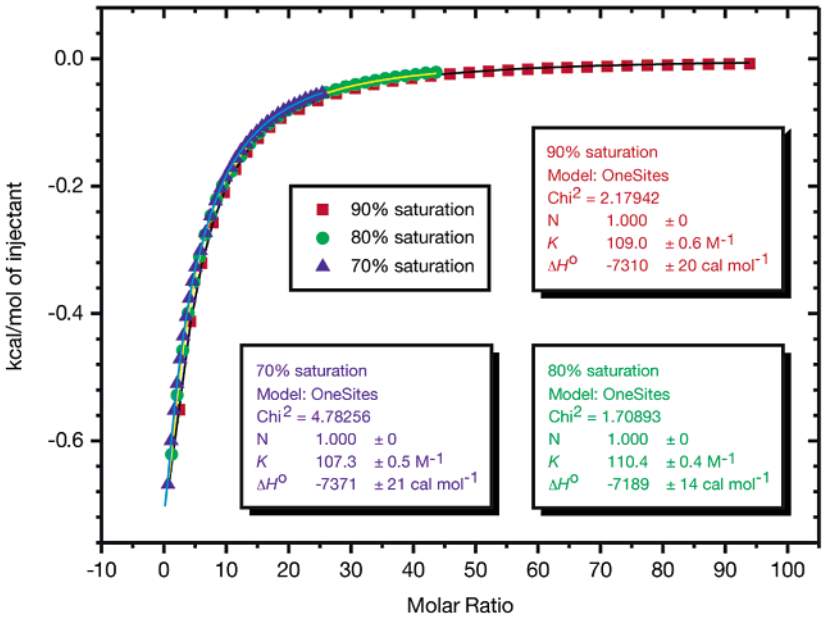

Figure 2. Titration of 18-crown-6 (1 mM) with KCl to achieve 70%, 80%, and 90% final receptor occupancy in 35 equal additions of ligand solution (best fitting lines are shown in cyan, yellow, and black, respectively). Variation of the proportion of data points describing the early part of the curve and final receptor occupancy have little effect on the fitting parameters. Residuals of the fitting are similar to those shown in Figure 3f.

Table 1. Selected Thermodynamic Parameters for 18-crown-6 Binding KCl at 25 °C

c^a	K_a/M^{-1}	$\Delta G°$/kcal mol^{-1}	$\Delta H°$/kcal mol^{-1}	n
10	117 ± 6^b	-2.82 ± 0.03	-7.86 ± 0.10	0.99 ± 0.01
5	104 ± 2	-2.75 ± 0.01	-7.82 ± 0.06	1.00 ± 0.02
1	119 ± 4	-2.83 ± 0.02	-7.59 ± 0.06	1
0.1	110 ± 2	-2.78 ± 0.01	-7.19 ± 0.14	1
0.05	111 ± 2	-2.78 ± 0.01	-7.28 ± 0.08	1
0.01	117 ± 8	-2.82 ± 0.04	-6.88 ± 0.28	1

[a] Approximate values based on $c = 100[\text{18-crown-6}]_t$. [b] Errors are twice the standard deviation calculated from three replicates of each titration.

values of c. At higher concentrations, one would expect that chemical activity coefficients will deviate from unity, and under nonideal conditions, it is possible that the free energy or enthalpy changes for the higher c value experiments, in particular, could be affected. However, as K_a represents a ratio of activities, it is also possible that any changes in the chemical activity coefficients may effectively cancel one another to give comparable values of K_a and hence $\Delta G°$.[20] The necessity of using high ligand concentrations when working with low affinity systems means that deviation of chemical activity coefficients from unity is unavoidable. However, we note that there are special cases, for example, the comparative study of complexation of enantiomers,[20] where activity coefficients for related ligands will nevertheless be identical, thus allowing fair comparison of their interactions at any concentration.

It can be seen in Figure 3d that the control experiment involving dilution of potassium chloride into water provides a nonlinear curve, indicating that some alternative equilibrium process could be coupled to complexation with 18-crown-6. Considering that this experiment involves only the addition of a 250 mM solution of KCl to water, the additional equilibrium process occurring is presumably dissociation of KCl ion pairs.[21] At 250 mM, 6.8% of potassium ions will be present as ion pairs,[21] whereas, following the first injection, less than 0.1% of ions in the cell will exist as ion pairs and this rises to only 1.5% by the end of the titration. Consequently, there is a significant contribution to the observed heat changes in the dilution experiment resulting from ion pair dissociation. Whereas these dilution processes can be removed explicitly from the experimental titration data by subtraction on a point-by-point basis, any effects of further ion pair dissociation that may occur as a consequence of removing potassium ions from the ion pair equilibrium through complexation by 18-crown-6 will remain. However, the ion pair equilibrium has a small enthalpy change and a very large dissociation constant (ca. 3 M),[21] reflecting the very low concentrations of ion pairs present during the experiments. Therefore any enthalpy changes from further ion pair dissociation would be expected to be very small. Analysis of the 18-crown-6 titration data using Sigurskjold's displacement model,[16b] modified to accommodate having the low affinity complex (i.e., KCl ion pairs) in the syringe, returned values for K_a and $\Delta H°$ that were essentially identical to those from the simple one site model and within the experimental errors listed in Table 1. Therefore we conclude that the effects of the ion pair equilibrium are negligible and that the system is described well by the one site model. Furthermore it would thus appear that the variable values for $\Delta H°$ do not arise from the effects of coupled equilibria.

An alternative source of error which could have an impact on the value of $\Delta H°$ would be errors in the concentrations of either the ligand or receptor. Although great care was taken in preparing all of the solutions used, in the absence of an independent determination of binding stoichiometry during the fitting process (as is the case for low c values where the stoichiometry must be imposed), the possibility that small errors in concentration could be the source of the change in enthalpy values seen across the series of titrations can not be completely

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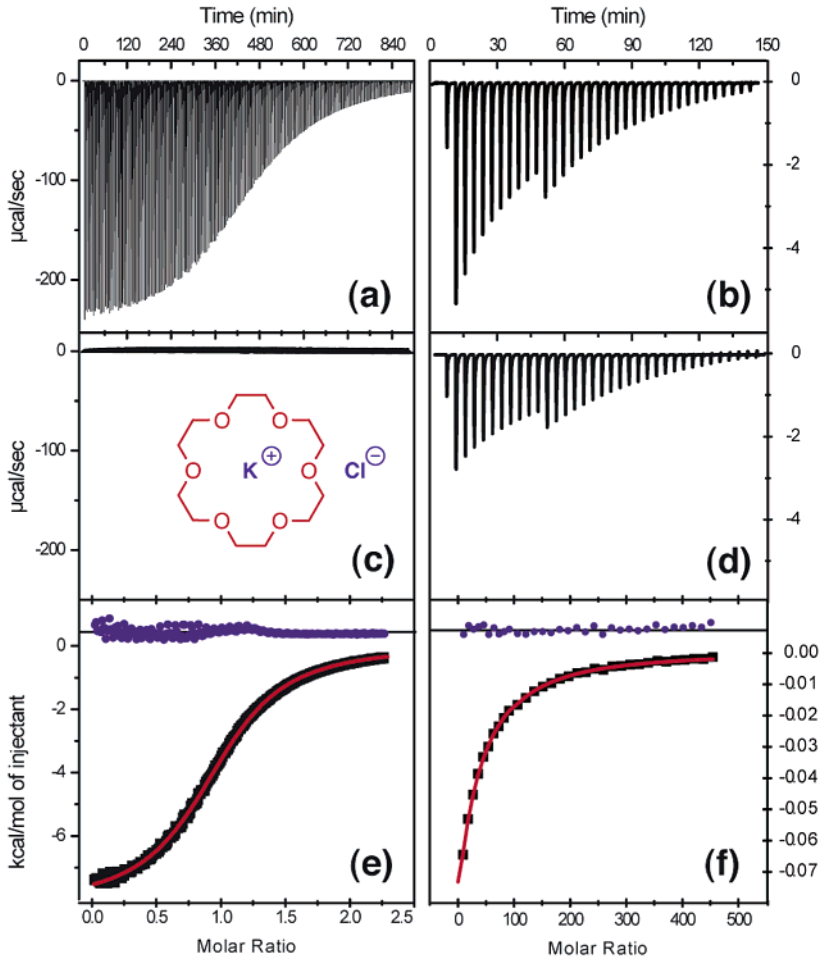

Figure 3. Representative ITC data for the interaction of KCl and 18-crown-6 showing (a) $c = 10$ with 225 consecutive injections of 1.3 μL and (**b**) $c = 0.01$ with an initial injection of 2 μL followed by 10 × 5 μL and 24 × 8 μL. The corresponding heats of dilution are shown in parts c and d, and the integrated data, with best fitting lines in red, are displayed in parts e and f along with residuals of the fitting in blue (scaled-up by a factor of five). The structure of the 18-crown-6/KCl complex is inset in part c.

discounted. There is, however, very good repeatability in $\Delta H°$ for a given value of c, and although this is often taken as a measure of accuracy, we note that if the concentrations contain consistent errors, then so will the determined enthalpy changes.

The effects of inaccuracies in the ligand and receptor concentrations on determining the values of $\Delta H°$ and K_a were assessed using 18-crown-6/Ba^{2+} titration data at $c \approx 50$, 0.5, and 0.05. The results displayed in Figure 4a show that varying the ligand concentration by ±15% has little effect on the calculated values for $\Delta G°$ but gives rise to variations in $\Delta H°$ by as much as ±1.5 kcal mol^{-1} (±20%) for the high c value experiment, whereas, at $c = 0.5$, $\Delta H°$ is largely unaffected by error in the ligand concentration. Error in the receptor concentration similarly has little effect on $\Delta G°$ (Figure 4b), but here it is the low c experiment that returns significant inaccuracies in $\Delta H°$. The situation at $c = 0.05$ was similar to that for $c = 0.5$, except $\Delta H°$ showed even less variation with error in ligand concentration; however, all values of $\Delta H°$ were shifted by +1 kcal mol^{-1} as would be predicted from Figure 5d.

Simulated Titrations. Simulated data containing a predetermined random error were used to extend the investigation to a wider range of K_a and $\Delta H°$ values. The strategy employed was analogous to that adopted by Christensen et al.,[22] in their evaluation of the "entropy titration method" as a means for determining pK_a's for weak acids. Here, data was synthesized using Monte Carlo simulations, the standard error for which was estimated from root-mean-square deviations of residuals following the curve fitting of barium chloride/18-crown-6 titrations conducted at 25 °C with c values in the range 10−100 (data not shown). The standard error thus selected was ±0.5% or 0.5 μcal/injection, whichever was greater for a given addition of ligand.

Mean errors (and their standard deviations) determined from nine replicates of each starting combination of $\Delta H°$, K_a, and c, all containing different random errors, were combined to construct the surface and contour plots shown in Figure 5. The surfaces describe a maximum "acceptable" error in the parameters extracted from the curve fitting process of 0.4 and 1.0 kcal mol^{-1} for the values $\Delta G°$ and $\Delta H°$, respectively. The contour plots indicate which combinations of c and $\Delta H°$ will give errors smaller than the selected threshold values for a system with a given association constant. Thus, acceptable values of c and $\Delta H°$ lie to the right-hand side of a given log(K_a) contour line. In the case of the error in $\Delta H°$, these contour lines are parallel and equally spaced, indicating that, in the range

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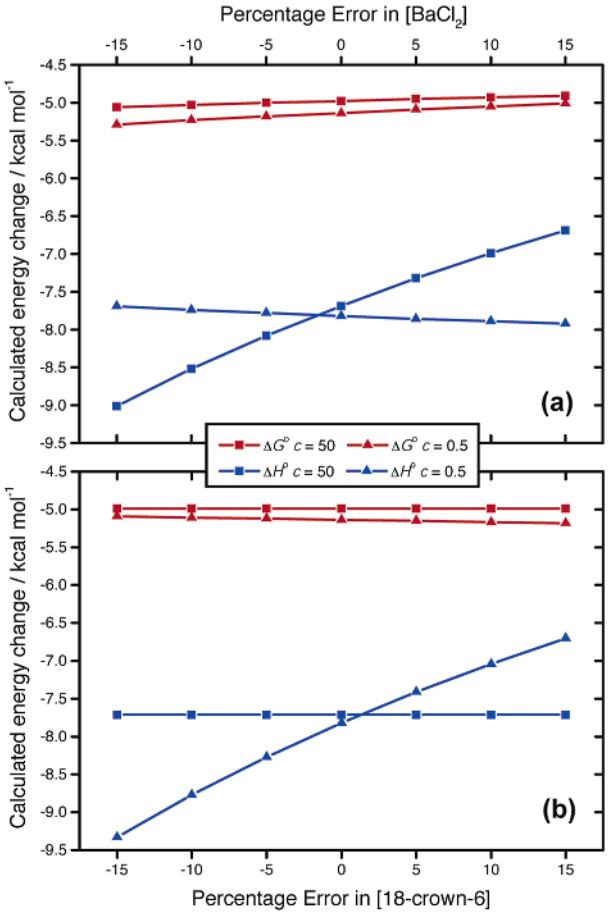

Figure 4. Curve fitting $BaCl_2$/18-crown-6 titration data with error in (a) the $BaCl_2$ and (b) the 18-crown-6 concentrations ranging from 0 to ±15% demonstrates that derivation of $\Delta G°$ is not affected by inaccuracy in the ligand or receptor concentrations, whereas error in $\Delta H°$ is dependent on the value of c. The stoichiometry parameter, n, was fixed at 1.0 for $c = 0.5$ but allowed to float freely for $c = 50$. Error bars lie within the bounds of the data point symbols.

Table 2. Selected Thermodynamic Parameters for Con A Binding Methyl α-Mannopyranoside at 27 °C

c[a]	K_a/M^{-1}	$\Delta G°$/kcal mol^{-1}	$\Delta H°$/kcal mol^{-1}	n
3.5	5280 ± 60[b]	−5.07 ± 0.01	−6.14 ± 0.02	1.01 ± 0.01
0.2	6830 ± 60	−5.23 ± 0.01	−6.32 ± 0.03	1
0.04	6890 ± 320	−5.23 ± 0.03	−4.90 ± 0.13	1

[a] Values based on $c = 5280[\text{ConA}]_t$. [b] Errors quoted are those returned by Origin on the curve fitting.

of c values listed, a receptor concentration of 10 μM will always give rise to an error of ca. ±1 kcal mol^{-1}.

Lectin Binding Studies. The Con A/methyl α-mannopyranoside system was chosen as a suitable protein receptor system to evaluate the results of the data simulations. Experiments conducted with Con A concentrations ranging from close to its solubility limit (ca. 0.67 mM) to 8 μM are summarized in Table 2. In our hands, the highest c value data returned $K_a = 5300$ M^{-1} and $\Delta H° = -6.1$ kcal mol^{-1}, which corresponds to the experiments having c values of 3.5 to 0.04. Figure 5c predicts that, for a system with $\log(K_a) = 3.7$, all three experiments should return $\Delta G°$ values within the chosen error limits of ±0.4 kcal mol^{-1}, whereas Figure 5d indicates that the titration with the lowest c value will fail to give $\Delta H°$ to within 1 kcal mol^{-1} of its correct value. If the highest c value experiment is considered to be the most accurate, then these predictions are true in this case.

Discussion

Low *c* Value Binding Isotherms. A graph displaying the relationship between curve shape and c value (Figure 1a), similar to that described by Wiseman et al.,[14] is often presented as evidence for the failure of curve fitting at low c values. The graph would indicate that, below $c = 1$, the curve becomes a featureless flat line. However, this is only true for the interval $0 \le [X]_t/[M]_t \le 2$. Although this interval is adequate to describe >99% of the binding isotherm for $c = 100$, for systems in which $c \ll 1$, it can be easily shown (Supporting Information) that, for simple 1:1 binding, the bound ligand concentration ([MX]) and, hence, the degree of saturation become dependent principally on the value of K_d rather than on the receptor concentration:

$$[MX] = [X]_t - \frac{[X]_t - [M]_t - K_d + \sqrt{([M]_t - [X]_t + K_d)^2 + 4[X]_t K_d}}{2} \quad (7)$$

Hence, the principal reason for failure in curve fitting for low c systems described by only the region $0 \le [X]_t/[M]_t \le 2$ is that an inadequate fraction of the binding isotherm is used for the calculation. Of course, another source of inaccurate curve fitting can originate from noisy data in systems for which very small amounts of heat are released on each addition of ligand. *This issue is less relevant for low affinity systems which will, by definition, have a higher receptor concentration at a given c value than for a high affinity system*. Thus, more heat will be released per injection for the low affinity system assuming that (1) the systems under comparison have equal values of $\Delta H°$ and (2) an equal number of ligand additions are used to achieve a given level of receptor saturation. Conversely, signal-to-noise will be a more significant problem for high affinity systems studied at low c values, just as signal-to-noise limits the upper bound of K_a that can be studied within the traditional experimental window.[14] Of course, it should be noted that improvements in calorimeter design over the past 15 years have provided greater sensitivity than was available to Wiseman et al.[14]

It is therefore more informative to redraw the c value graph as in Figure 1b with $[X]_t/K_d$ along the x-axis. By normalizing the axis with respect to K_d, it is now the high c value lines that appear featureless, whereas the low c value lines adopt curves that tend toward a constant hyperbolic shape (for $c < 0.1$) that does not vary further with decreasing c, except for the scaling along its x- and y-axes. Indeed, scaling along the x-axis is principally dependent on K_d, while the magnitude of the y-axis depends on $\Delta H°$, $[M]_t$, and K_d. It should be noted that this more simple shape is the primary reason why deriving the value of binding stoichiometry (n) fails when working below $c = 1$; the curve fitting becomes over parametrized and often will not converge. Thus it is necessary to fix the value of n, leaving only two variable parameters for fitting. *As little information is now to be gained from having many data points around the equivalence point, it becomes more relevant to add several equivalents of ligand per injection, thus allowing significant stepwise increases in the degree of saturation, which provide improved levels of signal-to-noise in the dataset*. The observa-

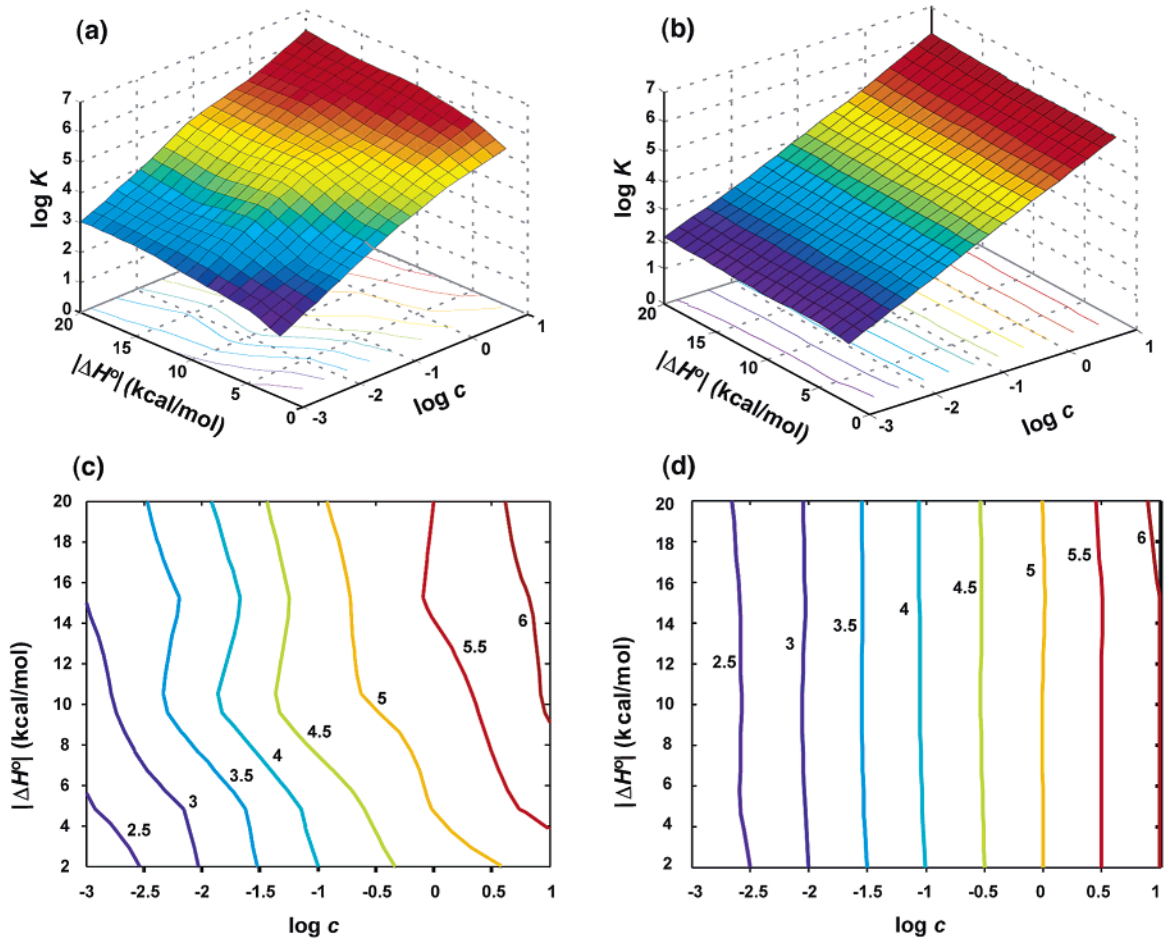

Figure 5. Surface (a and b) and contour (c and d) depictions of the combinations of log(c), log(K_a), and $\Delta H°$ that will give rise to a maximum error of 0.4 kcal mol^{-1} in $\Delta G°$ (a and c) and 1.0 kcal mol^{-1} in $\Delta H°$ (b and d), calculated by curve fitting. To be below this error limit, combinations of log(c) and $\Delta H°$ must lie to the right-hand side of a given log(K_a) contour line.

tions that, for lower values of c, it is preferable to add more than 2 equiv of ligand and that n must be fixed did not escape Wiseman et al. in their seminal paper.[14]

Indeed, the results of titrations of potassium chloride into solutions of 18-crown-6 suggest that determination of K_a is possible well beyond the lower limit of the normal experimental window, assuming that an adequate percentage of the binding isotherm is used. However, the experimental studies are inconclusive concerning whether $\Delta H°$ can also be determined accurately at low c values, as the varying values of $\Delta H°$ for KCl/18-crown-6 could be an intrinsic property of the system or alternatively the consequence of errors in concentrations. Simulated data containing defined random error provide the opportunity to extend the study over a much wider range of theoretical systems while knowing the concentrations and correct values of K_a and $\Delta H°$ explicitly and that there is no possibility of complications from coupled equilibria. Furthermore, variation of $\Delta H°$ for a given combination of K_a and c provides a means of assessing the effects of levels of signal-to-noise in the data. Curve fitting of simulated data concurs with the experimental findings, and quantitative analysis of the resulting errors is summarized in the contour plots shown in Figure 5. The straight vertical lines in Figure 5d indicate that error in the calculated value of $\Delta H°$ does not depend on the absolute enthalpy value, whereas the accuracy of $\Delta G°$ (Figure 5c) and, hence, K_a are much more sensitive to low values of $\Delta H°$ for the system. The fact that contour lines for log(K_a) = 2.5 only just appear in the graphs shown implies that errors in $\Delta G°$ and $\Delta H°$ will always be below the selected threshold values for systems in which $K_a < 10^2$, assuming that (1) the system achieves at least ca. 80% saturation by the end of the titration, (2) there is a measurable change in enthalpy on interaction, and (3) $c > 0.001$.

Errors in Concentrations. As fitting is based on the enthalpy change normalized with respect to added ligand, one would expect that $\Delta H°$ should be affected more significantly by inaccuracy in the ligand concentration than should be the value of K_a. However, data for the interaction of Ba^{2+} with 18-crown-6 indicate that this is only true for higher values of c (Figure 4a). Indeed, whereas the derived value of $\Delta G°$ is largely independent of error in ligand or receptor concentrations at all c values tested, the accuracy of $\Delta H°$ as a function of error in $[X]_t$ or $[M]_t$ depends strongly on c: at high c values, $\Delta H°$ is affected only by accuracy in ligand concentration; conversely, at low c values, it would appear that only accuracy in receptor concentration is of paramount importance. *Therefore, even when a low c experiment is conducted under appropriate conditions of final receptor saturation and with an adequate level of signal-to-noise, the accuracy in the $\Delta H°$ value may be lower than that for K_a and $\Delta G°$, unless particular care is taken in measuring the receptor concentration used.*

In the high c value experimental window, where the stoichiometry of the interaction may be determined independently,

accurate knowledge of only one of the binding partners is sufficient to allow the determination of the other.[23] However, if fixing the value of n is necessary for curve fitting (i.e., in the low c value regime), then inaccuracies in the receptor concentration and, hence, in $\Delta H°$ may go unnoticed. It should be noted that such inaccuracies are not represented in the contour plots, as errors in ligand and receptor concentrations were not considered explicitly during the simulations.

That n must be fixed and known with some accuracy is not in itself a disadvantage relative to other methods for determining binding constants for low affinity systems. Indeed, methods of extracting thermodynamic parameters from solution titration data, whether followed by UV, fluorescence, NMR, electrospray mass spectrometry (ESMS), or radiometric methods, all require the prior assumption of a binding model and knowledge of total ligand and receptor concentrations throughout the titration. With the exception of data acquired by equilibrium dialysis, ESMS, or NMR spectroscopy (for systems in slow exchange), a direct measurement of free ligand concentration is usually not possible, forcing the adoption of the approximation that $[X]_{total} = [X]_{free}$, which is valid for systems in which $c <$ ca. 0.1. In ITC, it is more common to calculate $[X]_{free}$ explicitly during the curve fitting process,[14,15] and this approach is equally valid for all values of c. Furthermore, other key advantages of ITC that are retained regardless of the value of c include (1) the almost universal phenomenon of enthalpy change on complexation, thus negating the requirement for the presence or introduction of an optical or radiometric probe in the system or the necessity of using a coupled process to report binding in the system under study; (2) the applicability of the technique for studying binding between molecules in solution, as opposed to at a surface, for example, in surface plasmon resonance which preferentially requires that the ligand be attached to a surface, a problem for ligands with no scope for modification such as metal ions or those that are fully encapsulated by the receptor on binding or for multivalent systems wherein an inappropriate presentation of ligand groups can lead to contradictory data;[24] (3) the determination of $\Delta H°$ in a single experiment in contrast to the van't Hoff method which requires titrations to be performed at several temperatures;[25] and (4) the ready automation of the titration which provides high precision in the data as a consequence of reducing operator error.

Conclusions

In summary, both the theoretical and experimental data presented herein support extending the accepted experimental window for ITC to much lower values of c, in particular for the low affinity systems. Although the simulated data reveal clear trends in the errors in curve fitting for experiments conducted under various conditions and these observations are in accord with the results of Con A/methyl α-mannopyranoside (Table 2), we would advocate caution in interpreting the contour plots (Figure 5) too literally. First, it should be stated that this work relates to simple systems involving only identical, noninteracting binding sites. The evaluation of uncertainties for systems displaying cooperativity or involving higher order aggregates[26] is beyond the scope of the present study. However, we note that where a system exhibits more complicated mixtures of species in solution, for example, 1:1, 1:2, and 2:2 complexes, dilution of the system, with a concomitant reduction in the value of c, can often allow the data to be analyzed using a simple 1:1 binding model.[26] It must also be emphasized that these results are based on estimated standard errors for the MicroCal VP-ITC system and may not readily transfer to other calorimeters. Furthermore, no attempt has been made to incorporate errors relating to ligand or receptor concentrations, and it should also be noted that, for situations in which the heat of dilution becomes comparable to the heat of interaction, then further errors may be introduced on subtracting background processes from the data. Nevertheless, the simulations do demonstrate that, in principle, there is no reason curve fitting should not give accurate values for K_a and $\Delta H°$, provided that the experiment has been well designed with consideration of the effects of final receptor occupancy and levels of signal-to-noise and that the concentrations of the ligand and receptor are known accurately. It therefore remains the responsibility and the discretion of the individual to assess the validity of ITC results obtained at low values of c. However, the results of this study would support the validity of all values of K_a and, hence, $\Delta G°$ determined under low c conditions but would advocate caution in the interpretation of values for $\Delta H°$. Isothermal titration calorimetry is nevertheless a powerful technique for studying biologically and synthetically important low affinity systems.

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Supporting Information Available: Derivation of eq 7 and examples of simulated binding isotherms and their error analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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