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Notes & Tips

Isothermal titration calorimetry at very low c

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Abstract

In the study of 1:1 binding, $M + X \rightleftarrows MX$, isothermal titration calorimetry (ITC) can be used successfully at values of $c = K[M]_0$ well below the value 1.0 that is often considered its lower limit. However, analysis of low-c ITC data may require freezing the stoichiometry parameter n, and that is thought to be prohibitive for biological systems, where n can be poorly known. Here it is noted that the least-squares estimates of the binding constant K are virtually independent of errors in n at low c, permitting reliable determination of K and, from its temperature dependence, ΔH° and n, down to $c = 10^{-4}$ or lower, ligand solubility permitting.

In the study of 1:1 binding processes, isothermal titration calorimetry (ITC)[1] is generally thought to be at its best when the product of the concentration of the reagent in the cell (titrate M) and the binding constant K, designated historically as c [1], is in the range 1–1000. The standard analysis of ITC data yields estimates of K, ΔH°, and a third parameter usually designated n and called the site number or stoichiometry number, where the latter recognizes its effect of adjusting the starting titrate concentration $[M]_0$ to be compatible with the stated concentration of titrant (X) in the syringe. Recently there has been increased interest in binding processes that fall below $c = 1$ [2], from inherent low affinity, low solubility, or combinations thereof that limit $c = K[M]_0$. Biophysically relevant processes in this category include cyclodextrin–ligand binding, carbohydrate–protein interactions, some protein–ligand and protein–protein processes, and fragment-based drug design [3–7]. Indeed, results have been reported down to $c < 0.1$ in such studies [2,3]. However, a severe limitation arises from the very strong correlation between ΔH° and n in the analysis of low-c data [8], which makes it impossible to determine both of these simultaneously. The usual solution is to freeze n in the analysis because it is often considered known from prior knowledge of the binding process and confidence in the prepared solution concentrations. Indeed, such knowledge is required in many methods for studying binding [9]. However, n can be ill-defined in biological systems, and this has appeared to prohibit the direct use of ITC on such systems at low c, forcing workers to resort to competitive displacement approaches [5]. The purpose of the present note is to point out that as c decreases below 0.1, the LS-estimated value of K becomes *practically immune to errors in n*, meaning that K can be reliably estimated by direct ITC down to $c < 10^{-3}$, even when n is very poorly known. Then repetition of the experiments over a range of temperature can yield ΔH° from a van't Hoff analysis [10] and thence n by comparison with the original ΔH° estimates.[2]

The insensitivity of K to n can be appreciated by considering the equilibrium relation for the process,

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[1] *Abbreviations used:* ITC, isothermal titration calorimetry; LS, least-squares; SE, standard error.

[2] A global LS analysis of all T-dependent experiments represents a more elegant way to accomplish this task. Alternatively, if the determined value of n differs significantly from the originally assumed value, the ordinary analysis can be redone in a refinement step.

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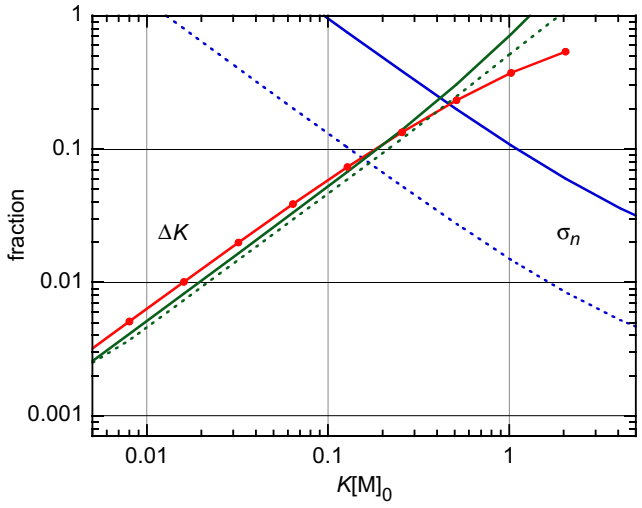

Fig. 1. Relative statistical and systematic errors in n and K as functions of c. Results for n from optimized five-injection scheme of [11], for $h = 0.1$ cal/L (solid) and $h = 1$ cal/L (dashed). Illustrated systematic errors in K result from freezing $n = 1$ when the true value is 2 (curve with points, negative error) or 0.2 (positive error), including results for the latter at $h = 1$ cal/L (dashed).

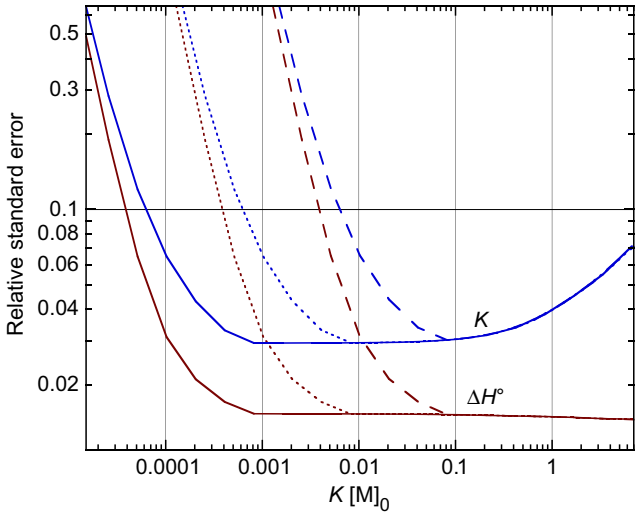

Fig. 2. Relative standard errors in K and ΔH° when n is frozen, as a function of c for $h = 0.1$ cal/L. Solid curves represent results when $[X]_0$ in the syringe is limited to 10^4 times $[M]_0$; the fine and broad dashed curves are for this ratio dropped to 10^3 and 10^2, respectively. Cell volume is taken as 1.4 ml and total titrant volume 0.25 ml.

$M + X \rightleftarrows MX$. For low c, one must titrate to a great excess of X to achieve significant conversion of M to MX [2,8], meaning $[X]_0 \gg [M]_0$. Then $[X] \approx [X]_0$ and we have

$$K[X]_0 \approx \frac{[MX]}{[M]}. \tag{1}$$

Changes in n are tantamount to changes in $[M]_0$, which produce concomitant changes in [M] and [MX], leaving the ratio on the right hand side of Eq. (1) unchanged. For example, if $K[X]_0 = 1$, we have 50% conversion to complex; doubling n from 1.0 to 2.0 doubles both [MX] and [M] but leaves the ratio unchanged, as long as [X] remains close to $[X]_0$. On the other hand, the observed heat for the ith injection of titrant is proportional to $\Delta[MX] \times \Delta H^\circ$, so doubling n means halving ΔH°.

These points are illustrated quantitatively in Fig. 1, which shows the increase in σ_n with decreasing c and the corresponding decreasing sensitivity of K to errors in n.[3] The results for σ_n bracket the range h ($=[M]_0 \Delta H^\circ$) = 0.1–1 cal/l for the key parameter that governs overall experimental sensitivity [8]. They were computed for a five-injection scheme optimized with respect to the injection volume to deal specifically with low-signal or heat-starved reactions [11]. Hence they are specific to these considerations and to the statistical error in the data [12]. On the other hand, the systematic error in K produced by specified systematic errors in n is much less sensitive to such assumptions and should hold generally.

[3] Monte Carlo calculations have verified the computed statistical SE in the parameters in the low-c range of interest here and have shown that K, n, $(n \times \Delta H^\circ)$, and $1/\Delta H^\circ$ are approximately normal, even to relative errors comparable to the parameters in magnitude [8]. On the other hand ΔH° is strongly nonnormal when n is fitted. More recent MC computations show that n remains roughly normal even when $\sigma_n/n > 10$.

Fig. 1 shows that n can be fitted successfully (<10% SE) down to $c = 1$ for $h = 0.1$ cal/L, and down to $c = 0.1$ for $h = 1$ cal/L. Thus there is no need to freeze n above these c values. Accordingly, the effects on K of systematic errors in n are greater in this region. However, where the systematic errors in K exceed 10%, the corresponding assumed systematic errors in n greatly exceed σ_n and so would appear unreasonable when compared with the fitted values of n.

The calculations in Fig. 1 employed the key result that sets the range of the titration [8],

$$R_m = \frac{6.4}{c^{0.2}} + \frac{13}{c}, \tag{2}$$

where R_m is the ratio $[X]_0/[M]_0$ in the cell after the last (mth) injection. At low c the second term dominates, and we see by comparing with Eq. (1) that this gives ~93% conversion to complex. As long as this condition can be satisfied, the relative standard errors in both K and ΔH° (with n frozen) remain approximately constant as c is decreased [8,11], as shown in Fig. 2. Thus the statement that ITC is limited to the range $c = 1$–1000 (or sometimes 10–500) is a misperception based on the default adoption of $R_m = 2$, which fails to achieve significant complexation when $c < 1$.

On the other hand there are practical limitations to working at low c. As Fig. 2 shows, finite solubility of X must eventually force deviation from Eq. (2) and high $[X]_0$ can mean large blank corrections [11]. One might expect only minor loss of precision in titrating just to 50% conversion, because the total reaction heat is more than half that obtained by using Eq. (2) (93% conversion); and, from Eq. (1), the required $[X]_0$ is smaller by more than an order of magnitude. However, the calculations show

that 50% conversion yields a fourfold increase in σ_K as compared with 93% complexation. With 75% conversion, the precision loss is less than a factor of 2. These dependences are responsible for the sharp rises in the curves in Fig. 2 at small c. Still, even the least favorable situation gives K with <20% uncertainty down to $c = 0.003$. This, for example, might be the case with $\Delta H^\circ = 10$ kcal/mol, $[M]_0 = 0.01$ mM, and 1.0 mM maximum solubility for X.[4]

These same solubility limitations also affect competitive displacement approaches for weak binding [5] because one must start the competitive titration with M mostly converted to MX, which requires large $[X]_0/[M]_0$ in the cell. However, this approach does have a solubility advantage, because one starts with X in the cell instead of in the syringe. The problem of determining n is effectively solved by the titration with the strong binder, together with the assumption that the weak binding process involves the same sites.

To make this comparison more instructive, the weakest binding case in [5] yielded $K = 48 \pm 7\ M^{-1}$ and $\Delta H^\circ = -2.1 \pm 0.2$ kcal/mol. The experiments employed $[M]_0 = 0.08$ mM, giving $c = 0.0038$ and $h = 0.17$ cal/L. With $[X]_0 = 50$ mM in the cell at the outset, Eq. (1) yields 70% complexation. Direct titration to that level would require a syringe concentration about five times higher than that and should yield about 4% relative standard error in K and 2% in ΔH° for 5 injections, rising to 6 and 4% for 20 injections (as used in [5]). However, if 50 mM is taken as the limiting titrant solubility (and hence the syringe concentration), the direct titration range is reduced by a factor of five and the uncertainties rise to 19 and 15%. At the other extreme, titration to 93% conversion, with volume optimization of 5 injections, yields 2 and 1% uncertainties. Of course, n cannot be determined without data as a function of T, and then the true uncertainties for both n and ΔH° would be reduced by the inherent lower precision of the van't Hoff analysis [10].

In summary, direct ITC can yield reliable estimates of the binding constant K at very low c, even when n is poorly known, because there the required freezing of n in the analysis has no effect on the determined value of K. Experiments run over a range of temperatures can then yield ΔH° and n via a van't Hoff analysis, as is required for estimating ΔH° in most methods of studying binding. The precision with which K can be estimated depends on a number of experimental parameters, but the relative SE is roughly independent of c as long as the titration is extended to ~93% complexation, as dictated by Eq. (2). Titrant solubility may limit the range of titration, and in such cases the competitive displacement approach may win out, because in that method the titrant is prepared in the cell instead of by dilution from the syringe. However, direct titration should be valuable for systems not readily treated by the competitive displacement method and as a consistency check on the results for those that are.

[4] The volume optimization study [11] showed that, at low c and low h, the most precise parameter estimates come from the minimal number of injections—three when all parameters are fitted and two when n is frozen. The precision gains from volume optimization were typically a factor of 3–5 compared with a 10-injection, constant v_i procedure. However, more recent calculations have shown that volume optimization yields little improvement unless the titration is extended to the great excess of titrant dictated by Eq. (2). For example, titration to 70% complexation takes five times less titrant and shows only ~10% precision improvement with volume optimization for a given number of injections.

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