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Modeling complex equilibria in isothermal titration calorimetry experiments: Thermodynamic parameters estimation for a three-binding-site model

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ABSTRACT

Isothermal titration calorimetry (ITC) is a powerful technique that can be used to estimate a complete set of thermodynamic parameters (e.g., K_{eq} (or ΔG), ΔH, ΔS, and n) for a ligand-binding interaction described by a thermodynamic model. Thermodynamic models are constructed by combining equilibrium constant, mass balance, and charge balance equations for the system under study. Commercial ITC instruments are supplied with software that includes a number of simple interaction models, for example, one binding site, two binding sites, sequential sites, and n-independent binding sites. More complex models, for example, three or more binding sites, one site with multiple binding mechanisms, linked equilibria, or equilibria involving macromolecular conformational selection through ligand binding, need to be developed on a case-by-case basis by the ITC user. In this paper we provide an algorithm (and a link to our MATLAB program) for the nonlinear regression analysis of a multiple-binding-site model with up to four overlapping binding equilibria. Error analysis demonstrates that fitting ITC data for multiple parameters (e.g., up to nine parameters in the three-binding-site model) yields thermodynamic parameters with acceptable accuracy.

Titration calorimetry has been used for the simultaneous determination of K and ΔH for more than 40 years [1–4]. Isothermal titration calorimetry is now routinely used to directly characterize the thermodynamics of biopolymer binding interactions [5–13]. Knowledge of the thermodynamic profiles for drug–receptor binding interactions greatly enhances drug design and development [14–17]. Isothermal titration calorimetry (ITC) instruments (available from GE Healthcare (Microcal) and TA Instruments (Calorimetry Sciences)) have adequate sensitivity to measure heat effects as small as 0.1 μcal, making it possible to directly determine binding constants as large as 10^8 to 10^9 M^{-1}. Even larger values for K may be estimated from competitive binding experiments [18,19].

To take full advantage of the powerful ITC technique, the user must be able to design the optimum experiment, understand the nonlinear fitting process, and appreciate the uncertainties in the fitting parameters K, ΔH, and n. ITC experiment design and data analysis have been the subject of numerous publications [5,6,14–23]. Recent reviews of isothermal titration calorimetry describe the ease of use of modern microcalorimeters [18,19,24]. Several papers have described modern uses of isothermal titration calorimetry to study a broad range of chemical equilibria in numerous ways [25–27]. For example, ITC studies are now being used to identify possible binding mechanisms for ligand–DNA complexes based on their thermodynamic signatures [28]. ITC experiments exploring iron binding to *Escherichia coli* ferritin were accompanied by a model describing the equations for three independent binding sites [29], while ITC studies of histone nucleoplasm interactions were best fit with a site-specific cooperative model including four equilibrium constants and four enthalpy changes [30]. Examples of the use of ITC experiments to unravel the complicated binding equilibria often occurring in biology are limited since the analysis tools provided by the ITC industry cover only the simplest cases.

The improved sensitivity of the current ITC instruments has resulted in the ability to accurately estimate thermodynamic parameters for multiple overlapping binding equilibria. Fig. 1 shows three unique thermograms that might result from the titration of a system exhibiting two overlapping binding processes. Fig. 1B and C were simulated from a model for a system having two binding processes, wherein K_1 is much greater than K_2 and ΔH_1 is not equal to ΔH_2. These two thermograms show a clear distinction between the first and the second binding process, which can be differentiated when fitting data based on the differences in enthalpy change. However, Fig. 1A also demonstrates a simulation for a system exhibiting two binding sites in which the binding affinities have the same relative magnitude as the previous cases, $K_1 \gg K_2$, but with ΔH_1 equal to ΔH_2. In this case, it is not possible to distinguish the two overlapping equilibria and only the weaker

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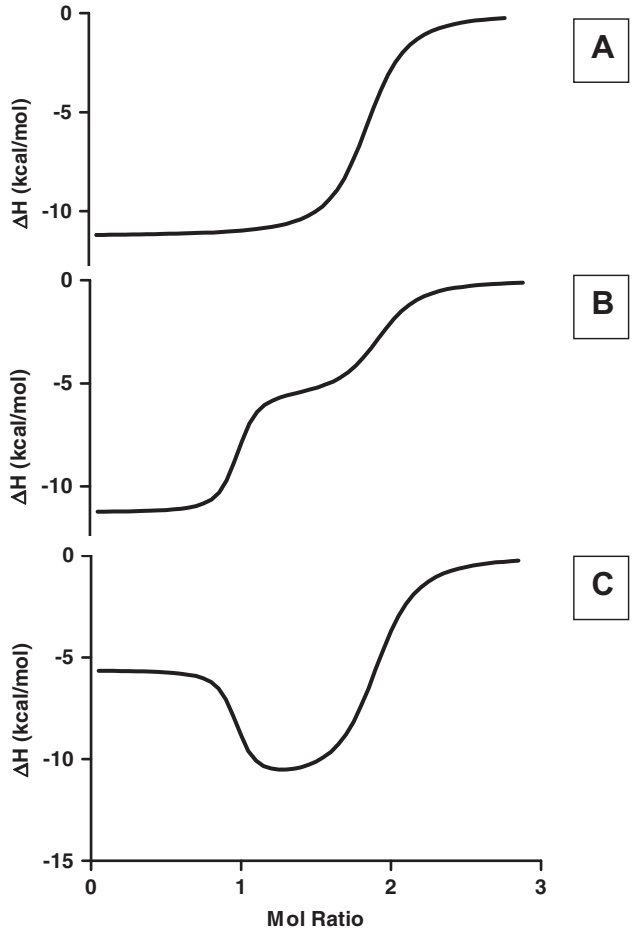

Fig.1. Representative two-site ITC titration experiment thermograms. In all three cases, the stoichiometry demonstrates that two ligand molecules are binding to the receptor molecule. (A) A system wherein the enthalpy changes for binding of both ligands are experimentally indistinguishable. (B) A system wherein the binding site with higher affinity is accompanied by a more exothermic enthalpy change. (C) A system wherein the higher binding affinity site demonstrates a smaller exothermic enthalpy change than the lower binding affinity site.

binding process (K_2) can be modeled. The equivalence of the binding enthalpy changes for the two overlapping processes causes this system to be modeled as a single binding process with $n = 2$.

The three plots shown in Fig. 1 demonstrate the increased complexity that is often seen in ITC thermograms as the number of binding process is increased. ITC thermograms must be understood prior to modeling the data. Understanding the binding profiles ensures that the models being applied to the data give an accurate representation of the chemical equilibria being observed. Furthermore, error analysis of algorithms being applied to analyze ITC thermograms ensures best-fit solutions are accurate. Several papers have been published on methods for evaluating the error introduced into ITC results from either experimental or data fitting considerations [32–39]. In general, the potential for using the ITC method to estimate the number of parameters needed to describe more complex thermodynamic binding models is underappreciated.

In this work, we describe the construction of algorithms that can be used to model ITC data obtained on systems exhibiting three or more binding sites described with overlapping equilibrium constants. We have also used simulated data and the Monte Carlo method [31,36,39] to evaluate the uncertainties and cross-correlation in the binding parameters (K, ΔH, and n). One- and two-site systems are used to compare our nonlinear fitting routines to those in the program commercially available from GE Healthcare (Microcal, Northampton, MA, USA; Origin 7.0). Simulated data are used to evaluate the three-binding-site algorithm implemented in MATLAB (version R2012a, 2012; The MathWorks, Natick, MA, USA) for the accuracy of multiple parameters (K_{1-3}, ΔH_{1-3}, and n_{1-3}) determined in an ITC experiment. The analysis techniques described in this paper can be extended to ITC data obtained on other complex systems if the user can construct the appropriate thermodynamic model for the binding interactions and/or linked equilibria. One novel aspect of this work is that our n-sites program for the analysis of systems having up to four binding sites is described in detail in the Supplementary material and the fully functional analysis program can be downloaded from our web site (http://lewis.chemistry.msstate.edu/download.html).

Materials and methods

Multiple-binding-site model

Algorithms for modeling ITC thermograms demonstrating up to four overlapping binding processes were developed using MATLAB software. The algorithms model the binding affinity, K_j; the molar enthalpy change, ΔH_j; and the total stoichiometric ratio, n_j; for each of the $j = 1$ to 4 binding process using nonlinear regression techniques. The mass balance and equilibrium equations were manipulated to produce an $(n + 1)$th degree polynomial for the n-binding sites, with free ligand as the indeterminate.

General-binding-site model

The thermodynamic model algorithms were developed from a combination of the appropriate mass balance and equilibrium constant expressions. Eqs. (1) and (2) are the generalized mass balance and binding equilibrium equations, respectively. Each equation is written in a simplified form that can be expanded to include n-binding site:

$$L_t = [L] + M_t \sum_{1}^{j=n} (n_j \Theta_j), \tag{1}$$

$$K_j = \frac{\Theta_j}{(1 - \Theta_j)[L]} \Rightarrow \Theta_j = \frac{[L]K_j}{1 + [L]K_j}. \tag{2}$$

Eq. (1) establishes that at any point in the titration, the total ligand present in the reaction vessel (L_t) must be either bound to one of the n-binding sites, $M_t \sum_1^{j=n} (n_j \Theta_j)$ or free in the reaction vessel ($[L]$). The equilibrium constants in Eq. (2) have been rewritten to express the fraction of process j bound (Θ_j) as a function of the binding affinity, K_j, and the presence of free ligand. Substitution of Θ_j into Eq. (1) and expanding yields a $(n + 1)$th degree polynomial where $[L]$ is the indeterminate:

$$[L]^{k+1} + \alpha_k [L]^k + \alpha_{k-1} [L]^{k-1} + \ldots + \alpha_0 = 0. \tag{3}$$

Eq. (3) represents a simplified form of this polynomial, where α_i represents the ith coefficient. The coefficients, α_i, are derived from ligand concentration, macromolecule concentration, stoichiometric ratios, and binding affinities. Calculating the roots of the polynomial shown in Eq. (3) determines the concentration of free ligand present in the reaction vessel after the ith injection.

Once the concentration of free ligand is determined, substitution of $[L]$ into Eq. (2) yields the fraction of site n bound after the ith injection. The total heat produced from the start of the titration through the ith injection, Q_i, can be calculated using Eq. (4),

$$Q_i = M_t V_o \sum_{1}^{i=n} n_i \theta_i \Delta H_i, \tag{4}$$