12.3 Equations used for fitting ITC data

Introduction

This section describes the equations that are the basis for fitting ITC data with MicroCal ITC software.

In this section

This section contains the following subsections:

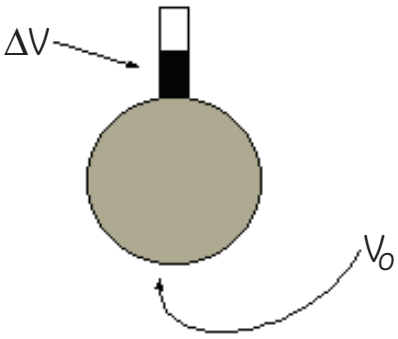
12.3.1 Displaced volume effects

The table describes the parameters used in the following equations.

Parameter	Description
V_0	active cell volume
ΔV_i	i^{th} injection volume
ΔV	total volume injected
M_t^0	initial bulk concentration of cell material
M_t	bulk concentration of cell material in the active volume
X_t	bulk concentration of injectant in the active volume
$[syr]$	concentration of injectant in the syringe

Note: *It will be assumed throughout that the macromolecule M is in the cell at an initial bulk concentration (moles/l) before the first injection, and the ligand X to be injected is initially at zero concentration in the cell.*

The working volume (grey) of the lollipop-shaped cell is V_0, the size of the i^{th} injection is ΔV_i and the total liquid, which has been injected at any point during the experiment, ΔV, is simply the sum of the individual ΔV_i for all injections.



Both the cell and the cell stem are filled with macromolecule solution, but only the active volume is monitored calorimetrically. Each injection drives liquid from the active volume into the cell stem (darkened portion representing ΔV). Consequently, in a typical experiment, M_t decreases slightly (~1%) with each injection. We assume no mixing occurs between the active volume and the cell stem, so the average bulk concentration of macromolecule in ΔV is the computed to be the average of M_t^0 and M_t. Taking this into account, conservation of mass requires that

$$M_t^0 V_0 = M_t V_0 + \frac{1}{2}(M_t + M_t^0)\Delta V$$ Equation (1)

so that

$$M_t = M_t^0 [\frac{1 - \frac{\Delta V}{2V_0}}{1 + \frac{\Delta V}{2V_0}}]$$ Equation (2)

Using similar reasoning, it is shown that X_t can be defined as follows:

$$[syr]\Delta V = X_t V_0 + \frac{1}{2} X_t \Delta V$$ Equation (3)

so that

$$X_t = [syr]\Delta V \frac{1}{V_0 + \frac{1}{2}\Delta V}$$

$$X_t = [syr]\frac{\Delta V}{V_0}[\frac{1}{1 + \frac{\Delta V}{2V_0}}]$$

$$X_t = [syr]\frac{\Delta V}{V_0}[\frac{1 - \frac{\Delta V}{2V_0}}{1 - [\frac{\Delta V}{2V_0}]^2}]$$

$$X_t = [syr]\frac{\Delta V}{V_0}[1 - \frac{\Delta V}{2\Delta V_0}]$$

if we assume that $[\frac{\Delta V}{2V_0}]^2$ is approximately or equal to 0. Equation (4)

The above expressions for M_t and X_t are used by MicroCal ITC software to correct for displaced volume effects, which occur with each injection.

12.3.2 Single set of identical sites

The table describes the parameters used in the following equations.

Parameter	Description
K	binding constant
n	number of sites
$[M]$	free concentration of macromolecule in the active volume
$[X]$	free concentration of ligand in the active volume
Θ	fraction of sites occupied by the ligand X
ΔH	molar heat of ligand binding

$$K = \frac{\Theta}{(1 - \Theta)[X]}$$

Equation (5)

$$X_t = [X] + n\Theta M_t$$

Equation (6)

Combining Equation (5) and Equation (6) gives

$$\Theta^2 - \Theta[1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} + \frac{X_t}{nM_t}] = 0$$

Equation (7)

The total heat content Q of the solution contained in V_0 (determined relative to zero for the unliganded species) at fractional saturation Θ is

$$Q = n\Theta M_t \Delta H V_0$$

Equation (8)

Solving the quadratic Equation (7) for Θ and then substituting this into Equation (8) gives

$$Q = \frac{nM_t\Delta HV_0}{2}[1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} - \sqrt{(1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t})^2 - \frac{4X_t}{nM_t}}]$$

Equation (9)

The value of Q above can be calculated (for any designated values of n, K, and ΔH) at the end of the i^{th} injection and designated $Q(i)$. The parameter of interest for comparison with experiment, however, is the change in heat content from the completion of the i^{-1} injection to completion of the i injection. The expression for Q in Equation (9) only applies to the liquid contained in volume V_0. Therefore, after completing an injection, it is obvious that a correction must be made for displaced volume (i.e., ΔV_i = injection volume) since some of the liquid in V_0 after the i^{-1} injection will no longer be in V_0 after the i^{th} injection, even though it will contribute to the heat effect (assuming the kinetics of reaction and mixing are fast) before it passes out of the working volume V_0. The liquid in the displaced volume contributes about 50% as much heat effect as an equivalent volume remaining in V_0. The correct expression for heat released, $\Delta Q(i)$, from the i^{th} injection is

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_0}\left[\frac{Q(i) + Q(i-1)}{2}\right] - Q(i-1) \qquad \text{Equation (10)}$$

The process of fitting experimental data then involves:

1 initial guesses (which most often can be made accurately enough by MicroCal ITC software) of n, K, and ΔH

2 calculation of $\Delta Q(i)$ for each injection and comparison of these values with the measured heat for the corresponding experimental injection

3 improvement in the initial values of n, K, and ΔH by standard Marquardt methods

4 iteration of the above procedure until no further significant improvement in fit occurs with continued iteration

12.3.3 Two sets of independent sites

Using the same definition of symbols as above for set 1 and set 2, we have

$$K_1 = \frac{\Theta_1}{(1 - \Theta_1)[X]}$$
$$K_2 = \frac{\Theta_2}{(1 - \Theta_2)[X]}$$

Equation (11)

$$X_t = [X] + M_t(n_1\Theta_1 + n_2\Theta_2)$$

Equation (12)

Solving Equation (11) for Θ_1 and Θ_2 and then substituting into Equation (12) gives

$$X_t = [X] + \frac{n_1 M_t[X]K_1}{1 + [X]K_1} + \frac{n_2 M_t[X]K_2}{1 + [X]K_2}$$

Equation (13)

Clearing Equation (13) of fractions and collecting like terms leads to a cubic equation of the form

$$[X^3] + p[X^2] + q[X] + r = 0$$

Equation (14)

where,

$$p = \frac{1}{K_1} + \frac{1}{K_2} + (n_1 + n_2)M_t - X_t$$
$$q = (\frac{n_1}{K_2} + \frac{n_2}{K_1})M_t - (\frac{1}{K_1} + \frac{1}{K_2})X_t + \frac{1}{K_1K_2}$$
$$r = \frac{-X_t}{K_1K_2}$$

Equation (15)

Equation 14 and Equation 15 can be solved for $[X]$ either in closed form or (as done in MicroCal ITC Software) numerically by using Newton's Method if parameters n_1, n_2, K_1, and K_2 are assigned. Both Θ_1 and Θ_2 may then be obtained from Equation 11 above. As discussed in *Section 12.3.2 Single set of identical sites, on page 166*, the heat content after any injection *i* is equal to

$$Q = M_t V_0 (n_1 \Theta_1 \Delta H_1 + n_2 \Theta_2 \Delta H_2)$$

Equation (16)

After a similar correction for the displaced volume, the pertinent calculated heat effect for the i^{th} injection is

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_0}\left[\frac{Q(i) + Q(i-1)}{2}\right] - Q(i-1)$$

Equation (17)

which may be used in the Marquardt algorithm to obtain best values for the six fitting parameters.

12.3.4 Sequential binding sites

For sequential binding, the binding constants K_1, K_2, ...K_n must be defined relative to the progress of saturation, so that

$$K_1 = \frac{[MX]}{[M][X]}$$
$$K_2 = \frac{[MX_2]}{[MX][X]}$$
$$K_3 = \frac{[MX_3]}{[MX_2][X]}$$

Equation (18)

In the sequential model, there is no distinction as to which sites are saturated, but only as to the total number of sites that are saturated. If the sites are identical, then there is a statistical degeneracy associated with the sequential saturation since the first ligand to bind has more empty sites of the same kind to choose from than does the second ligand, etc. For identical interacting sites then, we can distinguish between the phenomenological binding constants K_i (defined by Equation (18)) and the intrinsic binding constants K_i^0 where the effect of degeneracies has been removed. The relationship between the two binding constants is given by:

$$K_i = \frac{n - i + 1}{i} K_i^o$$

Equation (19)

All calculations given below, as well as parameters reported from curve-fitting, are in terms of K_i values but the operator may convert to K_i^0 values, if desired, using Equation (19). Since concentrations of all liganded species $[ML_i]$ can be easily expressed in terms of the concentration of the non-liganded species, $[M]$, then the fraction of total macromolecule having i bound ligands, F_i, is simply

$$F_0 = \frac{1}{P}$$
$$F_1 = \frac{K_1[X]}{P}$$
$$F_2 = \frac{K_1 K_2 [X]^2}{P}$$
$$F_n = \frac{K_1 K_2 \ldots K_n [X]^n}{P}$$

Equation (20)

where

$$P = 1 + K_1[X] + K_1K_2[X]^2 + \ldots + K_1K_2\ldots K_n[X]^n$$

$$X_t = [X] + M_t \sum_{i=1}^{n} iF_i \qquad \text{Equation (21)}$$

Once n and values of fitting parameters K_1 through K_n are assigned, then Equation (20) and Equation (21) may be solved for $[X]$ by numerical methods (the Bisection method is used). After $[X]$ is known, all F_i may be calculated from Equation (20) and the heat content after the i^{th} injection is determined from

$$Q = M_tV_0(F_1\Delta H_1 + F_2[\Delta H_1 + \Delta H_2] + \ldots + F_n[\Delta H_1 + \Delta H_2 + \Delta H_3 + \ldots + \Delta H_n]) \qquad \text{Equation (22)}$$

and, as before,

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_0}\left[\frac{Q(i) + Q(i-1)}{2}\right] - Q(i-1) \qquad \text{Equation (23)}$$

which then leads into the Marquardt minimization routine.

12.3.5 Enzyme, substrate, inhibitor assay

Assaying enzymes, inhibitors or substrates by calorimetric activity has the major advantage that it works well for any enzyme/substrate/inhibitor system with no prior chemical modification of any participants in the reaction. The rate R_t of the substrate conversion reaction is directly proportional to the power output in the calorimeter cell, i.e.,

$$R_t = \frac{P}{\Delta H V_0}$$ Equation (24)

where,

Parameter	Description
P	power generated by the reaction
ΔH	heat of conversion of the substrate

The units of R_t will be moles/l/sec if P is expressed in µcal/sec, ΔH in µcal per mole of substrate, and V_0 in liters, for example.

If Michaelis-Menten kinetics are assumed then the experimental values for the rate R_t can be expressed as

$$R_t = \frac{k_{cat}[E]_{cat}[S]_t}{[S]_t + K_M(1 + \frac{[I]}{K_I})}$$ Equation (25)

where,

Parameter	Description
k_{cat}	catalytic rate constant for substrate conversion
K_M	Michaelis constant
$[E]_{tot}$	total enzyme concentration
$[S]_t$	instantaneous concentration of substrate
$[I]$	concentration of competitive inhibitor
K_I	inhibition constant

The equation as written is valid both in the absence or presence of a $[I]$ and K_I.

The use of Equation (25) assumes no effects from product inhibition. This assumption has been discussed by Todd and Gomez (Todd, M. J. & Gomez, J. (2001) Analytical Biochemistry 296, 179-187.) and found to be quantitative in many cases. In those cases where product inhibition is significant, then Equation (25) can only be used to express initial rates of reaction prior to accumulation of product.

Todd and Gomez discussed in some detail the two methods by which assays can be carried out in a titration calorimeter, and these are summarized in the following topics.

Method 1: Single injection

Using this approach, the reaction is initiated by the injecting enzyme solution from the syringe into the sample cell containing substrate solution, or vice versa. If desired, a competitive inhibitor may also be included in one solution or the other. The reaction is allowed to go to completion in the calorimeter cell, and the power P is recorded as a function of time t.

Integration of the excess power P associated with the reaction enables ΔH to be determined, i.e.,

$$\Delta H = \frac{\int_0^{\infty} P\,dt}{[S]_{t=0} V_0}$$ Equation (26)

where $[S]_{t=0}$ is the starting substrate concentration. Knowing ΔH, the substrate concentration can be determined as a function of time from the equation:

$$[S]_t = [S]_{t=0} - \frac{\int_0^{t} P\,dt}{\Delta H V_0}$$ Equation (27)

After obtaining the time-dependent rate from Equation (24), these data can be equated to the Michaelis expression in Equation (25) so that the final equation can be fit by nonlinear least squares. In the absence of inhibitor, k_{cat} and K_M are used as variable parameters during iterative fitting. In the presence of inhibitor I, it is best to enter previously determined values of k_{cat} and K_M and use K_I as the only variable fitting parameter.

Method 2: Multiple injections

In this method, multiple injections of substrate solution from the syringe are made into the reaction cell containing enzyme solution (with or without inhibitor). After each injection, a sufficient time is allowed for the instrument to equilibrate at the new power level resulting from the increased substrate concentration. Measurements are carried out quickly enough, however, so that little hydrolysis of substrate takes place relative to the total substrate contained in the cell. That is, $[S]_t$ is calculated directly from the total added substrate assuming no significant hydrolysis.

Equation (24) and Equation (25) are still valid for Method 2, except that R_t and $[S]_t$ now correspond to discrete values of the rate and substrate concentration after each injection, rather than time-dependent values. To determine ΔH from Equation (26), it is necessary to carry out another single-injection experiment where hydrolysis is allowed to go to completion. Having done this, then discrete values of R_t at different $[S]_t$ are calculated, so that Equation (25) can then be fit to obtain best values of k_{cat} and K_M (in the absence of inhibitor). In the presence of a competitive inhibitor, data are also fit to Equation (25) but using k_{cat} and K_M as fixed (results obtained from previous experiment with no inhibitor present) and treating K_I as the only fitting parameter.

12.3.6 Dimer dissociation mode

A protein molecule P, may associate at high concentrations to form a dimer. The dilution of this concentrated protein solution by injection into the calorimeter cell containing buffer can then result in some heat effects from dissociation

$$P_2 \overset{\Delta H_{disc}}{\Leftrightarrow} 2P$$

$$K_D = \frac{(P)^2}{(P_2)}$$

where,

Parameter	Description
(P)	concentration of monomer
(P_2)	concentration of dimer
ΔH_{disc}	heat of dissociation of the dimer
K_D	dissociation constant

It is assumed in this model that the stoichiometry is well-defined, i.e., no aggregates with stoichiometry higher than 2 are present. By measuring heats for a series of injections it is then possible, using curve-fitting, to determine K_D and ΔH_{disc}.

The equivalent monomer concentration C is is defined as

$$C = [P] + 2[P_2]$$

Equation (28)

For solving $[P]$ we have

$$[P] = \frac{\sqrt{1 + \frac{8}{K_D}C} - 1}{\frac{4}{K_D}}$$

Equation (29)

from which $[P_2]$ follows.

The heat released q_i when the i^{th} injection of volume dV_i is made into a fixed-volume (V_0) cell will be

$$q_i = \Delta H_{disc}(P_2)_{syr} dV_i - \Delta H_{disc}[(P_2)_i - (P_2)_{i-1}][V_0 + \frac{dV_i}{2}] \quad \text{Equation (30)}$$

The first term in Equation (30) is the heat content of the aggregate contained in the injection volume prior to injection while the second term is the net heat content due to the difference in aggregate present in the cell before and after the injection. The $[V_0 + \frac{dV_i}{2}]$ factor in the final term is an effective volume which takes into account the displacement which occurs in a total-fill cell (see *Section 12.3.1 Displaced volume effects, on page 164*).

Assuming experimental parameters V_0, dV_i, and C_{syr} are known, Equation (28), Equation (29), and Equation (30) are simultaneous equations, which can be solved for q_i whenever values are assigned to K and ΔH_{disc}.

12.3.7 Competitive binding mode

Using conventional ITC methods, binding constants from 10^3 M^{-1} to 10^8 M^{-1} can be measured most accurately. When binding constants significantly exceed 10^8 M^{-1}, instrument sensitivity becomes challenged as concentrations are lowered to the point where quantitative measurements of the binding constant would be possible. On the other hand, binding constants substantially in excess of 10^8 M^{-1} can be measured quantitatively if such strong-binding ligands are studied in competition with a second ligand, which binds competitively but more weakly to the same binding site.

Competitive binding studies are carried out using the strong-binding ligand A as the injectant, with the solution in the cell containing the second competitive ligand B as well as the binding protein P (or other target molecule). This system has two equilibria, which are displaced with each injection, i.e.,

$$A + P \overset{\Delta H_A}{\Leftrightarrow} PA \qquad K_A = \frac{[PA]}{[P][A]}$$

$$B + P \overset{\Delta H_B}{\Leftrightarrow} PA \qquad K_B = \frac{[PB]}{[P][B]}$$

The value of K_B and ΔH_B for the competing ligand are first measured in a conventional ITC experiment, and these parameter values are entered as known parameters when determining K_A from results of the competition experiment. For the competition experiment, the total concentration of competing ligand, $[B]_{tot}$, should be selected such that

$$\frac{"K_A"}{K_B[B]_{tot}} \approx 10^5 - 10^8 M^{-1}$$

where "K_A" is the estimated value of K_A.

The detailed equations used in the fitting model for competitive binding are found in a paper by Sigurskjold (Sigurskjold, B. W. (2000) Analytical Biochemistry 277, 260-266).

12.3.8 Chi-square (Chi^2) minimization

The aim of the fitting procedure is to find those values of the parameters that best describe the data. The standard way of defining the best fit is to choose the parameters so that the sum of the squares of the deviations of the theoretical curve(s) from the experimental points for a range of independent variables is at a minimum.

For ITC models where there is no weighting, the theoretical models can be represented by:

$$y = f(x; p_1, p_2, p_3, \ldots)$$

where:

p_i = the fitting parameters

Hence, the expression for χ^2 simplifies to:

$$\chi^2 = \frac{1}{n^{eff} - p} \sum [y_i - f(x_i; p_1, p_2, \ldots)]^2$$

where:

Parameter	Description
n^{eff}	the total number of experimental points used in the fitting
p	total number of adjustable parameters
y_i	experimental data points
$f(x; p_1, p_2, p_3, \ldots)$	fitting function

Note: *The difference $d = n^{eff} - p$ is usually referred to as the number of degrees of freedom.*

The previous equation states that the Chi-squared value of the fit is equal to the sum of the squares of the deviations of the theoretical curve(s) from the experimental points divided by the number of degrees of freedom (DoF). Since there is no weighting, it can be seen that the calculated values are dependent on the magnitude of the scale and the number of data points. After fitting, this value is reported as Chi^2/DoF.