*Journal of Thermal Analysis and Calorimetry, Vol. 72 (2003) 93–103*

# NEW METHODS FOR DATA ANALYSIS OF ISOTHERMAL TITRATION CALORIMETRY

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## Abstract

Heat divided by ligand concentration *vs.* heat, similar to the Scatchard plot, was introduced to obtain the equilibrium constant ($K$) and the enthalpy of binding ($\Delta H$) using isothermal titration calorimetry data. Values of $K$ and $\Delta H$ obtained by this linear pseudo-Scatchard plot for a system with a set of independent binding sites (such as binding fluoride ions on urease and monosaccharide methyl α-*D*-mannopyranoside on concavalin A) were remarkably like that obtained from a normal fitting Wiseman method and other our technical methods. On applying this graphical method to study the binding of copper ion on myelin basic protein (MBP), a concave downward curve obtained was consistent with the positive cooperativity in the binding. A graphical fitting by simple method for determination of thermodynamic parameters was also introduced. This method is general, without any assumption and restriction made in previous method. This general method was applied to the product inhibition study of adenosine deaminase.

**Keywords:** adenosine deaminase, concavalin A, enthalpy of binding, ligand binding, myelin basic protein, Scatchard plot, titration calorimetry, urease

## Introduction

Calorimetry is the principal source of thermodynamic information, and it is indeed both a very versatile and sensitive technique [1]. It is a very general method due to the fact that practically all physical, chemical and biological processes are accompanied by heat exchange. So, calorimetry is one of the most powerful tools for expanding knowledge and understanding in many fields of science and technology [2, 3]. The principal calorimetric techniques that have contributed are differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC) [4]. DSC experiments perturb the system under study by scanning temperature followed by recording the heat capacity of the system against temperature [4–6]. The nature and magnitude of the process that stabilize biomacromolecules and transition states for conversion of native to denatured form are investigated by DSC experiments [6–9]. The energetics of biochemical reactions or molecular interactions at constant temperature are measured by ITC [10]. Experiments are performed by titration of a reactant into a sample solu-

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*1388–6150/2003/ $ 20.00*

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tion containing the other reactant(s) necessary for reaction. After each addition, the heat released or absorbed as a result of the reaction is monitored by the isothermal titration calorimeter. The total concentration of titrant is the independent variable under experimental control. Thermodynamic analysis of the observed heat effects that permits quantitative characterization of the energetic processes associated with the binding reaction [10].

ITC gives invaluable information about biomacromolecule-ligand interaction [11–13], allosteric transition [14], protein denaturation [15–17], enzyme inhibition [18, 19], quality, safety and shelf-life of materials and material stability [20–23]. Different methods have been reported for data analysis of ligand binding study by ITC [19, 24–27]. The principal of these methods is to fit the experimental data in an equation relating equilibrium constant, molar enthalpy of binding and reactants concentration. The Wiseman method [27] for data analysis has extensively used for ligand binding study by ITC and a computer program needs for using this method [28–34].

The most common presentation of ligand-biomacromolecule binding data is the Scatchard plot [9, 11, 35, 36]. For a biomacromolecule which has $g$ binding sites, and in which binding sites are characterized by identical intrinsic association binding constant, $K_a$, and independent of each other without interacting (that is, occupancy of one site does not affect the probability of binding to any other), from mass action equation, Scatchard showed that:

$$\frac{\nu}{[L]} = K_a (g - \nu) \tag{1}$$

where $\nu$ is the number of moles of ligand bound per mole of biomacromolecule, and $[L]$ is the free concentration of ligand. Hence, the Scatchard plot, $\nu/[L]$ *vs.* $\nu$, is linear for system which have one identical and independent set of sites, so-called noncooperative system [9, 35]. The Scatchard plot will be upward-curved for anticooperative system, which by interacting between identical binding sites lead to binding at one site decreasing the affinity of others. The Scatchard plot will be downward-curved for cooperative system, which by interacting between identical binding sites lead to binding at one site increasing the affinity of others [9, 35].

On this paper we present an equation with a useful graphical method, very similar to the Scatchard plot in the ligand binding studies, to obtain equilibrium constant and enthalpy of binding by ITC data for noncooperative systems with one set of identical and independent binding sites. This new representation of ITC data shows the positive cooperativity in the binding set. A graphical fitting simple method for determination of thermodynamic parameters was also introduced.

## *Materials and methods*

Jack bean urease (JBU; *MW*=545.340), myelin basic protein (MBP; *MW*=18.500) from bovine central nervous system (CNS), adenosine deaminase from calf intestinal mucosa (ADA; *MW*=34.500), concavalin A and Tris-HCl were obtained from Sigma Chemical Co. Sodium fluoride and methyl $\alpha$-*D*-mannopyranoside were purchased

from Aldrich Chemical Co. Copper nitrate was purchased from Merck Co. Solutions were made in double-distilled water. Tris-HCl solution (30 mM), pH=7.00 was used as a buffer for JBU. Tris-HCl solution (30 mM), pH=7.20 was used as a buffer for MBP. Phosphate solution (50 mM), pH=6.90 for concavalin A was used as a buffer.

Equilibrium dialysis

Experiments were carried out at 300 K using an MBP solution with a concentration of 0.25 mg $mL^{-1}$, of which 2 mL aliquots were placed in dialysis bags and equilibrated with 2 mL of the copper solution, covering the required concentrations range for over 96 h. Corrections for inequalities arising from Donnan effects were negligible at the ionic strength used. The free copper concentrations in equilibrium with complexes of MBP-copper were assayed by the atomic absorption (Perkin Elmer, model 603) method.

Isothermal titration calorimetry

The isothermal titration microcalorimetric experiments were performed with the 4-channel commercial microcalorimetric system, Thermal Activity Monitor 2277, Thermometric, Sweden. Each channel is a twin heat-conduction calorimeter where the heat-flow sensor is a semiconducting thermopile (multi-junction thermocouple plates) positioned between the vessel holders and the surrounding heat sink. The insertion vessel was made from stainless steel. Sodium fluoride (40 mM) was injected by use of a Hamilton syringe into the calorimetric stirred titration vessel, which contained 2 mL urease (2.5 μM). In the second experiment, copper solution (100 μM) was injected into the calorimetric stirred titration vessel, which contained 1.8 mL MBP, 13.5 μM. In the third experiment, inosine solution (2 mM) was injected into the calorimetric stirred titration vessel, which contained 1.8 mL ADA, 21.7 μM. In the fourth experiment, methyl α-*D*-mannopyranoside solution (50 mM) was injected into the calorimetric stirred titration vessel, which contained 2 mL concavalin A, 0.04 mM. The injection volume in each step for JBU, MBP, ADA and concavalin A was 50, 35, 15 and 20 μL, respectively. Thin (0.15 mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. The calorimetric signal was measured by a digital voltmeter that was part of a computerized recording system. The heat of each injection was calculated by the 'Thermometric Digitam 3' software program. The heat of dilution of the ligand solution was measured as described above except that the enzyme was excluded. The enthalpy of dilution was subtracted from the enthalpy of enzyme-ligand interaction. The enthalpy of dilution of enzyme is negligible. The microcalorimeter was frequently calibrated electrically during the course of the study.

## Results and discussion

Consider a solution containing ligand $L$, and a biomacromolecule ($M_g$) that contains $g$ sites capable of binding the ligand. If the multiple binding sites on a biomacromolecule are identical and independent, the ligand binding sites can be reproduced by a model sys-

tem of monovalent molecules ($M_g \rightarrow gM$) with the same set of association equilibrium constant ($K_a$) values. Thus, the reaction under consideration can be written:

$$M+L \leftrightarrow ML \quad K_a=[ML]/[M][L] \tag{2}$$

By titration of a solution containing '*M*' with a solution of ligand *L*, the equilibrium reaction is moved toward increasing concentration of *ML* complex. The heat value of reaction depends on the concentration of *ML* complex ($Q \propto [ML]$). Moreover, the maximal value of heat that would be observed when all the *M* is present as *ML*, that is, $Q_{max} \propto [M]_{total}$, or $Q_{max} \propto [M]+[ML]$. Therefore, it can be concluded:

$$\frac{Q}{Q_{max}} = \frac{[ML]}{[M]+[ML]} \tag{3}$$

Because of the equilibrium assumption, [*ML*] can be expressed in terms of [*L*], [*M*], and $K_a$, using Eq. (2). This substituting for [*ML*] gives:

$$\frac{Q}{Q_{max}} = \frac{K_a[M][L]}{[M]+K_a[M][L]}$$

or

$$\frac{Q}{Q_{max}} = \frac{K_a[L]}{1+K_a[L]} \tag{4}$$

By assuming that all of the single-site macromolecules (*M*) are converted to the *ML* complex, the heat value of the reaction per mole of single-site biomacromolecule is calculated. However, this assumption is only true at a large excess of ligand *L*, because of the equilibrium between *M* and *ML*. The absolute heat values of the reaction *vs.* the ligand concentration will be a rectangular curve. So, the molar enthalpy of binding can be obtained by extrapolation of the heat of reaction to a large excess of ligand *L*; where $\Delta H=Q_{max}$. Hence, Eq. (4) can be rearranged to yield

$$\frac{Q}{[L]} = K_a(\Delta H - Q) \tag{5}$$

which resembles the Scatchard equation (Eq. (1)). In fact, *Q* is proportional to ν. So, we can replace ν in Eq. (1) by *Q* to give Eq. (5). As *g* is the maximum value of ν, $\Delta H$ is the maximum value of *Q*. By measuring the total heat of reaction at any fixed concentration of *L*, the association equilibrium constant and the molar enthalpies of binding ($\Delta H$) for the ligand *L* can be obtained, by using the linear plot of *Q*/[*L*] *vs*. *Q*. This plot is very similar to the normal Scatchard plot.

The data obtained from isothermal titration calorimetry of JBU with ligand fluoride ion is shown in Fig. 1. JBU has twelve binding sites for fluoride ions as a competitive inhibitor for urease. Figure 1a shows the heat of each injection, and Fig. 1b shows the cumulative heat related to each total concentration of *L*. The total concentration of fluoride ion is much more than total concentration of binding sites on biomacromolecule with one binding site. Therefore, it can be assumed that the total

and free concentrations of ligand are approximately equal. The linear plot of $Q/[L]$ *vs*. $Q$ is shown in Fig. 1c. The values of $K_a$ and $\Delta H$ obtained from axis intercepts are:

$$K_a = 1.07\ \text{m}M^{-1} \quad \Delta H = -12.42\ \text{kJ mol}^{-1}$$

The association equilibrium constant value obtained from this type of Scatchard plot is approximately equal to the value obtained from assay of enzyme activity in the presence of fluoride ion [18, 37].

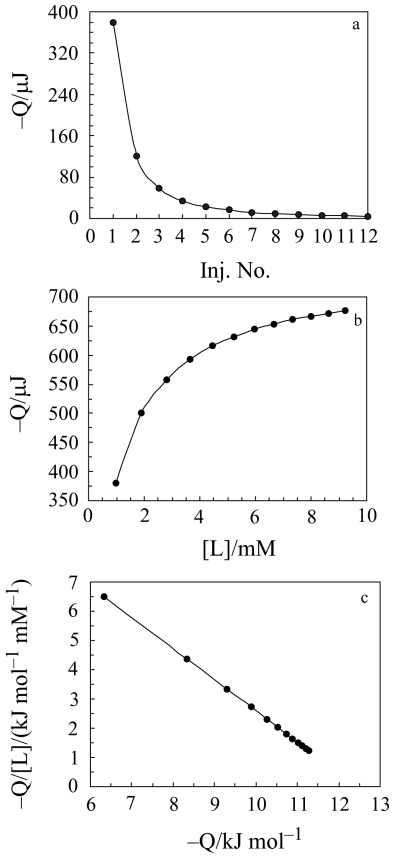


**Fig. 1** a – The heat of fluoride binding on urease for 12 automatic cumulative injections, each of 50 μL, of sodium fluoride solution 40 mM, into the sample cell containing 2 mL protein solution at a concentration of 2.5 μM at pH=7.0 (Tris 30 mM) and 27°C, b – the cumulative heat of ligand binding related to each total concentration of fluoride, calculated from Fig. 1a and c – $Q/[L]$ *vs*. $Q$, similar to the Scatchard linear plot, according to Eq. (5), using data shown in Fig. 1b

A double reciprocal equation form of Eq. (5) can be written as below:

$$\frac{1}{Q} = \frac{K}{\Delta H}\frac{1}{[L]} + \frac{1}{\Delta H} \tag{6}$$

Hence, the linear plot of $1/Q$ *vs.* $1/[L]$ gives similar results to the linear plot of $Q/[L]$ *vs.* $Q$. This method was applied to the binding of monosaccharide methyl α-*D*-mannopyranoside to concavalin A at pH 6.9 and temperature of 25°C (Fig. 2). The dissociation binding constant ($K$) and the molar enthalpy of binding ($\Delta H$) were 0.147 mM and −28.61 kJ mol$^{-1}$, respectively. These results were markedly consistent with the results obtained from previous method [34, 38].

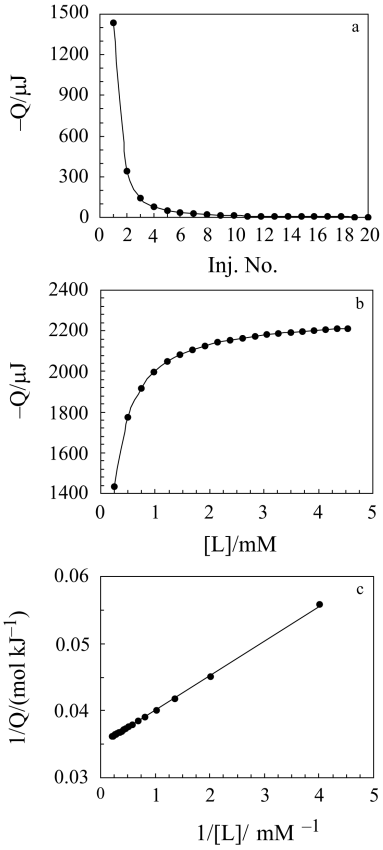


**Fig. 2** a – The heat of methyl α-*D*-mannopyranoside binding on concavalin A for 20 automatic cumulative injections, each of 20 μL, of sodium fluoride solution 40 mM, into the sample cell containing 2 mL concavalin A solution at a concentration of 40 μM at pH=6.9 (phosphate 50 mM) and 25°C, b – the cumulative heat of ligand binding related to each total concentration of methyl α-*D*-mannopyranoside, calculated from Fig. 2a and c – $1/Q$ *vs.* $1/[L]$, according to Eq. (6), using data shown in Fig. 2b

Figure 3a shows the Scatchard plot, $\nu/[Cu^{2+}]_f$ *vs.* $\nu$, where $[Cu^{2+}]_f$ is the free concentration of copper ion and $\nu$ defined to be moles of bound copper ions per mole of total MBP. The shapes of the Scatchard plots are clearly characteristic of different types of cooperativity. A concave downward curve, as shown in Fig. 3a, describes a

system with positive cooperativity. For obtaining approximated values of binding parameters, it might be possible to fit the binding data to Hill equation [39],

$$\nu = \frac{g(K_a([Cu^{2+}]_f)^n}{1+(K_a([Cu^{2+}]_f)^n} \quad (7)$$

where $g$, $K_a$ and $n$ are the number of binding sites, binding constant, and Hill coefficient, respectively. The binding data for the binding of copper ions to MBP have been fitted to the Hill equation using a computer program for nonlinear least-square fitting [40]. The results are: $g$=2, $K_a$=0.38 μM$^{-1}$ and $n$=1.6. The best-fit curve of the experimental binding data was then transformed to a Scatchard plot as shown in Fig. 3a. A simple method for calculating intrinsic association equilibrium constants for system with two cooperative sites ($K_1$ and $K_2$) has been introduced from the Scatchard plot [41]. It has been shown that, in the limit as ν approaches 0, $\nu/[Cu^{2+}]_f=2K_1$ and when ν=1, or at half-saturation, $\nu/[Cu^{2+}]_f=(K_1K_2)^{1/2}$. Thus, $K_1$ can be obtained from the ordinate intercept of a Scatchard plot and $K_2$ is derived from the value of $\nu/[Cu^{2+}]_f$ at half-saturation. The results obtained from Fig. 3a are $K_1$=0.083 μM$^{-1}$ and $K_2$=1.740 μM$^{-1}$. So, occupation of the first site has produced an appreciable enhancement 21 of the binding affinity of the second site.

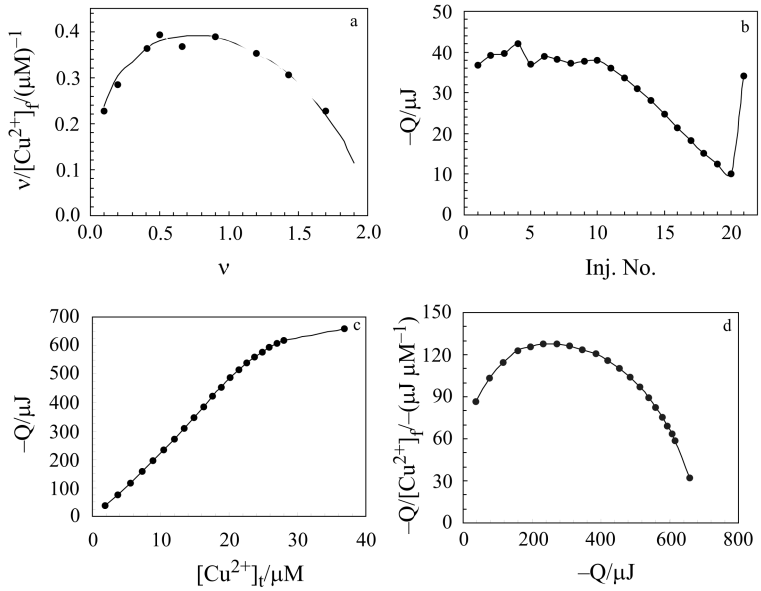


**Fig. 3** a – The Scatchard plot of binding copper ion by MBP at 27°C. The best-fit curve of the experimental binding data was transformed to a Scatchard plot using Eq. (7) with $g$=2, $K_a$=0.38 μM$^{-1}$ and $n$=1.6, b – the heat of copper binding on MBP for 21 automatic cumulative injections, each of 35 μL, of copper, 100 μM, into the sample cell containing 1.8 mL MBP solution at a concentration of 13.5 μM. The last injection was 350 μL, c – the heat of binding *vs*. total concentration of copper ion, calculated from Fig. 3b and d – a new representation of titration calorimetric data, very similar to the Scatchard plot, shows positive cooperativity in two binding sites for copper ions

The raw data obtained from isothermal titration calorimetry of MBP interaction with copper ion was shown in Fig. 3. Figure 3b is showing the heat of each injection and Fig. 3c is showing the heat of related to each total concentration of copper ion, $[Cu^{2+}]_t$. These raw calorimetric data can be used to show the heat of binding copper ions per mole of MBP ($\Delta H$) *vs*. total concentration of copper ions or *vs*. moles of bound copper ions per mole of total MBP ($\nu$) using Eq. (8):

$$[Cu^{2+}]_t=[Cu^{2+}]_f+[Cu^{2+}]_b=[Cu^{2+}]_f+\nu[MBP]_t \quad (8)$$

where $[Cu^{2+}]_b$ is the bound concentration of copper ion and [MBP] is the total concentration of MBP. The plot of $\Delta H$ *vs*. moles of bound copper ions per mole of total MBP ($\nu$) showed that the molar enthalpies of binding are –13.5 and –14.8 kJ $mol^{-1}$ in the first and second binding sites, respectively [42].

The new representation of titration calorimetric data, pseudo-Scatchard plot, has been shown in Fig. 3d. This concave downward curve describes a system with positive cooperativity in copper ions binding to MBP. It is concluded that a new representation of isothermal titration calorimetric data as a pseudo-Scatchard plot, which is used in these systems, can be developed for other systems to find the type of cooperativity in the binding. It is predicted that the pseudo-Scatchard is a concave upward curve for negative cooperativity in the binding (anticooperative system).

The method introduced for ligand binding study in fluoride interaction with JBU includes an assumption that the concentration of bound ligand is negligible in comparison with the total concentration of ligand. Here, it is introduced another method without this assumption. When our assumption is not true, the general method developed here will be useful. It is defined:

$$[L]_{total}=[L]+[ML] \quad (9)$$

$$[M]_{total}=[M]+[ML]=(K[ML]/[L])+[ML] \quad (10)$$

Equation (9) can be solved for $[L]$ and this then substituted into the Eq. (10) which can then be rearranged to give the quadratic equation its only real root is:

$$[ML]=\{(B+K)-[(B+K)^2-C]^{1/2}\}/2 \quad (11)$$

where

$$B=[M]_{total}+[L]_{total}\,C=4[M]_{total}[L]_{total} \quad (12)$$

The sum of heat evolutions following the $i^{th}$ titration step , $Q_i$, can be expressed as

$$Q_i=\Delta H V_i[ML]_i \quad (13)$$

where $V_i$ is the volume of the reaction solution and $\Delta H$ is the enthalpy of binding. Combination of Eqs (11) and (13) will lead to

$$\Delta H=1/A_i\{(B_i+K)-[(B_i+K)^2-C_i]^{1/2}\} \quad (14)$$

where

$$A_i=V_i/2Q_i \quad (15)$$

Equation (14) contains two unknowns, $K$ and $\Delta H$. A series of reasonable value for $K$ is inserted into Eq. (14) and corresponding values for $\Delta H$ are calculated and the graph $\Delta H$ *vs*. $K$ is constructed. Curves of all titration steps will intersect in one point, which represents the true value for $\Delta H$ and $K$.

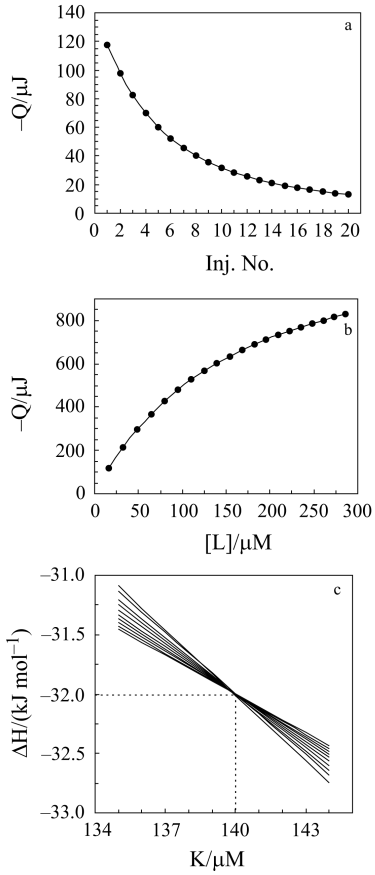


**Fig. 4** a – The heat of inosine binding on ADA for 20 automatic cumulative injections, each of 15 μL, of inosine solution, 2 mM, into the sample cell containing 1.8 mL ADA solution at a concentration of 0.75 mg $mL^{-1}$ (21.7 μM), b – the heat of binding *vs.* total concentration of inosine, calculated from Fig. 4a and c – $\Delta H$ *vs*. $K$ for first 10 injections in the reasonable values of $K$, according to Eq. (14) and data from Fig. 4b. The coordinates of intersection point of curves give true value for $\Delta H$ and $K$

The data obtained from isothermal titration microcalorimetry of ADA interaction with inosine is shown in Fig. 4. Figure 4a shows the heat of each injection and Fig. 4b shows the heat related to each total concentration of inosine. The plots of $\Delta H$ *vs*. $K$, according to Eq. (14), for first 10 injections are shown in Fig. 4c. The intersection of curves gives:

$$K=140\ \mu M\quad \Delta H=-32\ kJ\ mol^{-1}$$

The conformity of dissociation binding constants ($K$) obtained from thermodynamic and kinetic studies is observed [43].

Our proposed data analysis for isothermal titration calorimetry is expected useful for a set of binding sites, especially for enzyme inhibition study and measurement of inhibition constant.

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The financial support of the Research Council of University of Tehran is gratefully acknowledged.

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