Journal of the Iranian Chemical Society, Vol. 3, No. 1, March 2006, pp. 1-21.

# JOURNAL OF THE Iranian Chemical Society

# A Review on the Ligand Binding Studies by Isothermal Titration Calorimetry

A.A. Saboury

Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran

(Received 20 December 2005, Accepted 1 February 2006)

Thermodynamics of biomacromolecule ligand interaction is very important to understand the structure function relationship in proteins. One of the most powerful techniques useful to obtain additional information about the structure of proteins in biophysical chemistry field is isothermal titration calorimetry (ITC). An ITC experiment is a titration of a biomacromolecule solution by a solution containing a reactant (ligand) at constant temperature to obtain the exchanged heat of the reaction. The total concentration of ligand is the independent variable under experimental control. There are many reports on data analysis for ITC to find the number of binding sites (g), the equilibrium constant (K), the Gibbs free energy of binding process ( $\Delta G$ ), the enthalpy of binding ( $\Delta H$ ) and the entropy of binding ( $\Delta S$ ). Moreover, ITC gives information about the type of reaction, electrostatic and hydrophobic interactions, including determination of cooperativity characterization in binding process by calculating the Hill coefficient (n). A double reciprocal plot and a graphical fitting method are two simple methods used in the enzyme inhibition and metal binding to a protein. Determination of a binding isotherm needs more ITC experiments and more complex data analysis. Protein denaturation by ligand includes two processes of binding and denaturation so that ITC data analysis are more complex. However, the enthalpy of denaturation process obtained by ITC help to understand the fine structure of a protein.

**Keywords:** Isothermal titration calorimetry, Ligand binding, Metal binding, Enzyme inhibition, Protein denaturation, Enthalpy of binding, Equilibrium constant

## 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. Hence, calorimetry is one of the most powerful tools for expanding knowledge and understanding in many fields of science and technology [2-5]. Isothermal titration calorimetry (ITC) is a principal calorimetric technique, which can measure the energetics of biochemical reactions or molecular interactions at constant temperature is measured by ITC [4-5]. Experiments are performed by addition of a reactant to a sample solution containing the other reactant(s) necessary for reaction; see Fig. 1a. After each addition, the heat released or absorbed as a result of the reaction is monitored by the isothermal titration calorimeter as a peak of power against time for each injection; see Fig. 1b. The total concentration of titrant is the independent variable under experimental control; see Fig. 1c. Thermodynamic analysis of the observed heat effects that permits quantitative characterization of the energetic processes is associated with the binding reaction. ITC gives invaluable information about biomacromolecule-ligand interaction [6-23], protein denaturation [24-28], allosteric transition [29-30], enzyme inhibition [31-35], quality, safety and shelf-life of materials and material stability [36-41].

<sup>\*</sup>Corresponding author. E-mail: saboury@ut.ac.ir



**Fig. 1.** (a) ITC experimental set up. (b) After each addition, the heat released or absorbed as a result of the reaction is monitored by the isothermal titration calorimeter as a peak of power against time for each injection. (c) The heat of each injection can be calculated from the surface area under the peak. (d) The heat of interaction is obtained against the total concentration of titrant, as the independent variable under experimental control.

Different methods have been reported for data analysis of ligand binding study by ITC [42-60]. 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 [45] for data analysis has extensively used for ligand binding studies by ITC and a computer program is needed for using this method [61-67]. We have presented an equation, with a useful linear graphical method 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 [31,48-50]. A simple graphical



fitting method for determination of thermodynamic parameters has also been introduced, which has been applied to inhibitor binding on the enzymes [32,46,50]. The Scatchard plot [68-70], as a binding isotherm for ligand-protein interaction in a set of identical and independent binding sites, can be easily obtained by carrying out two different ITC experiments [51]. A calorimetric data analysis has also been introduced to obtain the binding isotherm for a set of independent or interacting binding sites [52,53]. Development of ITC data analysis methods is one of the most important researchs in the field of thermodynamic for protein ligand interaction. Here, we attempt to review a number of recent ITC data analysis

Saboury

methods and their applications.

#### **Materials and Methods**

Jack bean urease (JBU; MW = 545.34 kDa), myelin basic protein (MBP; MW = 18.5 kDa) from bovine central nervous system (CNS), adenosine deaminase from calf intestinal mucosa (ADA; MW = 34.5 kDa), human serum albumin (HSA; MW = 66.5 kDa), concavalin A and Tris-HCl were obtained from Sigma Chemical Co. Highly purified preparations of human growth hormone (hGH; MW = 22kDa) were provided by the National Research Center of Genetic Engineering and Biotechnology (NRCGEB), Tehran. Sodium fluoride, ethylurea (I) and (N,N)dimethylurea (X)were purchased from Alderich Chemical Co. Copper nitrate and calcium nitrate were purchased from Merck Co. Nickel nitrate hexahydrate was purchased from Riedel-dehaen Co. Solutions were made in double-distilled water. Tris-HCl solution (30 mM), pH = 7.00 was used as a buffer for JBU and HSA. 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<sup>-1</sup>, 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 a 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 titration vessel including a stirrer, which contained 2 ml urease (2.5  $\mu$ M). In the second experiment, copper solution (100  $\mu$ M) was injected into the calorimetric stirred titration vessel, which contained

1.8 ml MBP, 13.5  $\mu$ M or 27.0  $\mu$ M. In the third experiment, inosine solution (2 mM) was injected into the calorimetric stirred titration vessel, which contained 1.8 ml ADA, 21.7  $\mu$ M. In the fourth experiment, calcium nitrate solution (2 mM) was injected by into the calorimetric stirred titration vessel, which contained 1.8 ml hGH, 35  $\mu$ M. In the fifth experiment, methyl  $\alpha$ -D-mannopyranoside solution (50 mM) was injected by 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, hGH and concavalin A was 50, 35, 15, 20, and 20  $\mu$ l, respectively.

To study of HSA-Ni<sup>2+</sup> interaction, two experiments were done. In the first experiment, the calorimeter cell contained 2 ml of HSA in relatively high concentration (100  $\mu$ M) and the nickel solution in relatively low concentration (5 µM) was injected into the calorimeter stirred titration cell with a Hamilton syringe in aliquots of 10 µl. The ligand was diluted to 25-240 nM upon ten times of 10 µl injections into the 2 ml calorimeter cell. Thin (0.15 mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. In the second experiment, the calorimeter cell contained 1.5 ml of HSA in relatively low concentration (1  $\mu$ M) and the nickel solution in relatively high concentration (50 µM) was injected into the calorimeter cell. The nickel was diluted upon 20 times of 25 µl injection into the calorimetric titration cell these two experiments provided different thermodynamic parameters.

In JBU inhibition by I and X, a solution containing both of I and X (500 mM, respect to each one) was injected into the calorimetric vessel, which contained 2 ml urease, 0.9  $\mu$ M, including Tris buffer (30 mM), pH 7.0. Injection of I and X solution into the perfusion vessel was repeated 20 times, and each injection included 30  $\mu$ l reagents.

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**

Ligand binding study in a biomacromolecule including one binding site is the simplest study, which is the best one for our calorimetry study in the first. Determination of the number of binding sites in a set of non-cooperative system can be considered as the second in the study. Determination of the binding isotherm for a set of binding sites is the last one in our calorimetry study.

## Ligand Binding in one Binding Site (1:1 Stochiometry)

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

$$M + L \longrightarrow ML \qquad K = [M] [L]/[ML] \qquad (1)$$

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,  $q_{\text{max}} \propto [M]_{\text{total}}$ , or  $q_{\text{max}} \propto [M] + [ML]$ . Therefore, it can be concluded [31,50]:

$$\frac{q}{q_{\text{max}}} = \frac{[ML]}{[M] + [ML]}$$
(2)

Because of the equilibrium assumption, [ML] can be expressed in terms of [L], [M], and K, using equation (1). This substituting for [ML] gives:

$$\frac{q}{q_{\max}} = \frac{([M][L]/K)}{[M] + ([M][L]/K)}$$

$$\frac{q}{q_{\text{max}}} = \frac{[L]}{K + [L]}$$
(3)

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  $\Delta H = q_{\text{max}}$ . Hence, the equation (3) can be rearranged to yield [48-50]:

$$\frac{q}{[L]} = K_a \left(\Delta H - q\right) \tag{4}$$

where  $K_a$  is the association binding constant ( $K_a = 1/K$ ). Equation (4) resembles the Scatchard equation. 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. 2 [49-50]. JBU has twelve binding sites for fluoride ions as a competitive inhibitor for urease. Figure 2a shows the heat of each injection, and Fig. 2b shows the cumulative heat related to each total concentration of *L*. The total concentration of fluoride ion is much higher 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. 2c. The values of  $K_a$  and  $\Delta H$  obtained from axis intercepts are [49-50]:

$$K_{\rm a} = 1.07 \text{ mM}^{-1}$$
 (K = 0.93 mM)  $\Delta H = -12.42 \text{ kJ mol}^{-1}$ 

The association equilibrium constant value obtained from this

\_



#### Ligand Binding Studies by Isothermal Titration Calorimetry

**Fig. 2.** (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 300 K. (b) The cumulative heat of ligand binding related to each total concentration of fluoride, calculated from Fig. a. (c) q/[L] vs. q, similar to the Scatchard linear plot, according to the equation (4), using data shown in Fig. b. (d) The double reciprocal linear plot of 1/q vs. 1/[L], according to the equation (5), using data shown in Fig. b.

type of Scatchard plot is approximately equal to the value obtained from assay of enzyme activity in the presence of fluoride ion [31].

A double reciprocal equation form of equation (3) can be written as follow [31,49-50]:

$$\frac{1}{q} = \frac{1}{\Delta H} + \frac{K}{\Delta H} \times \frac{1}{[L]}$$
(5)

By a double reciprocal linear plot of 1/q vs. 1/[L], two important thermodynamic parameters of  $\Delta H$  and K can be

easily obtained from the intercepts axis. 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 fluoride ion to JBU (see Fig. 2d). The dissociation binding constant (*K*) and the molar enthalpy of binding ( $\Delta H$ ) were 0.03 mM and -12.42kJ mol<sup>-1</sup>, respectively. These results are markedly consistent with the results obtained by analysis of the linear plot of q/[L]vs. q. Equations (4) and (5) have been used extensively in the enzyme inhibition [33,35,50], metal [6,71] and sugar binding to proteins studies [48,50].

For the total heat of reaction (q) due to the isothermal



Fig. 3. (a) The heat change of ethylurea (*I*) and (N,N)dimethylurea (*X*) binding to JBU for 20 cumulative, automatic injections, each of 30 ml, of mixed solution *I* and *X*, of 0.5 M each, into the sample cell containing 2 ml urease solution at a concentration of 0.5 mg ml<sup>-1</sup> at pH 7.0 (Tris; 30 mM) and 300 K. (b) The heat of binding for one mole of *I* and one mole of *X* to the binding sites on JBU, assuming (◊) unilateral reaction which is compared by (•) theoretical values according to equation (6) using results obtained by non-linear least-squares fitting. The theoretical curve has been resoluted for (□) *I* and (Δ) *X*, according to the first and second terms of equation (6), respectively.

titration of macromolecule by solution containing the two ligands I and X, equation (3) should be written with two terms as follows [72]:

Least squares fitting [73]. The results are [72]:

$$q = q_{\rm I} + q_{\rm X} = \frac{\Delta H_{\rm I}[I]}{K_{\rm I} + [I]} + \frac{\Delta H_{\rm X}[X]}{K_{\rm X} + [X]}$$
(6)

By measuring the total heat of reaction at any fixed concentration of *I* and *X*, the dissociation equilibrium constants ( $K_{\rm I}$  and  $K_{\rm X}$ ) and the molar enthalpies of binding ( $\Delta H_{\rm I} = q_{\rm I,max}$  and  $\Delta H_{\rm X} = q_{\rm X,max}$ ) for these ligands can be obtained.

The data obtained from isothermal titration calorimetry of JBU with ligands ethylurea (I) and (N,N)dimethylurea (X) is shown in Fig. 3a, which shows the heat change on each injection. Figure 3b shows the heat of binding relative to each total concentration of I and X [72]. The total concentration of I and X is much more than the total concentration of binding sites on macromolecule with one binding site. So, it can be assumed that the total and free concentrations of ligand are approximately equal. The experimental values of heat at any fixed concentrations of I and X have also been fitted to equation (6) using a computer program for non-linear

$$K_{\rm I} = 26.3 \text{ mM}$$
  $K_{\rm X} = 28.4 \text{ mM}$   
 $\Delta H_{\rm I} = -10.2 \text{ kJ mol}^{-1}$   $\Delta H_{\rm X} = -10.8 \text{ kJ mol}^{-1}$ 

By using these values, the calculated total heat change and the contributions from each ligand are also plotted in Fig. 3b. Experimental and theoretical values are in agreement with maximum errors of 0.1 mM and 0.1 kJ mol<sup>-1</sup> for *K* and  $\Delta H$  values, respectively. In addition, the dissociation equilibrium constants measured by this method were consistent with the inhibition constants obtained from assay of enzyme activity in the presence of *I* and *X* [74].

Figure 4a shows the Scatchard plot,  $v/[Cu^{2+}]_f vs. v$ , where  $[Cu^{2+}]_f$  is the free concentration of copper ion and v 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 [68-70]. A concave downward curve, as shown in Fig. 4a, 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 [75],

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

where g,  $K_a$  and n are the number of binding sites, association 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 nonlinear least-square fitting computer program [73]. The results are: g = 2,  $K_a = 0.38 \,\mu\text{M}^{-1}$ and n = 1.6 [13,76]. The best-fit curve of the experimental binding data was then transformed to a Scatchard plot as shown in Fig. 4a. A simple method for calculating intrinsic association equilibrium constants for system with two cooperative sites  $(K_1 \text{ and } K_2)$  has been introduced from the Scatchard plot [77]. It has been shown that, in the limit as vapproaches 0,  $\nu/[Cu^{2+}]_f = 2K_1$  and when  $\nu = 1$ , or at halfsaturation,  $v/[Cu^{2+}]_f = (K_1K_2)^{1/2}$ . Thus,  $K_1$  can be obtained from the ordinate intercept of a Scatchard plot and  $K_2$  is derived from the value of  $v/[Cu^{2+}]_{f}$  at half-saturation. The results obtained from Fig. 4a are  $K_1 = 0.083 \ \mu\text{M}^{-1}$  and  $K_2 = 1.740 \ \mu\text{M}$ <sup>-1</sup>. So, occupation of the first site has produced an appreciable enhancement 21 of the binding affinity of the second site.

The raw data obtained from isothermal titration calorimetry of MBP interaction with copper ion was shown in Fig. 4 [13,50]. Figure 4b is showing the heat of each injection and Fig. 4c 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):

$$[Cu2+]_t = [Cu2+]_f + [Cu2+]_b = [Cu2+]_f + \nu [MBP]_t$$
(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 (v) showed that the molar enthalpies of binding are -13.5 and -14.8 kJ mol<sup>-1</sup> in the first and second binding sites, respectively [13,50].

The new representation of titration calorimetric data, pseudo-Scatchard plot, has been shown in Fig. 4d. 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 Scatchard like 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 Hill coefficient for a concave downward curve, as shown in Fig. 3d, can be obtained similar to the Scatchard plot, as shown in Fig. 4a, as below [13,69]:

$$n = \frac{1}{1 - (v_m/g)} = \frac{1}{1 - (0.75/2)} = 1.6$$
 (9)

$$n = \frac{1}{1 - (q_m/q')} = \frac{1}{1 - (260 \ \mu J/690 \ \mu J)} = 1.6$$
(10)

where  $v_m$  is the v value at the maximum point of Scatchard plot (Fig. 4a) and  $q_m$  and q' are heat values at the maximum point and horizontal-intercept of Scatchard like plot (Fig. 4d), respectively.

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. Following, 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:

$$[L]_{t} = [L] + [ML] \tag{11}$$

$$[M]_{t} = [M] + [ML] = (K [ML]/[L]) + [ML]$$
(12)

Equation (11) can be solved for [L] and then substituted into the equation (12), and rearranged to give a quadratic equation with the real root [46,50]:

$$[ML] = \{(B + K) - [(B + K)^{2} - C]^{\frac{1}{2}}\}/2$$
(13)

where

$$B = [M]_t + [L]_t \qquad C = 4 [M]_t [L]_t \qquad (14)$$

7





**Fig. 4.** (a) The Scatchard plot of binding copper ion by MBP at 300 K. The best-fit curve of the experimental binding data was transformed to a Scatchard plot using equation (7) with g = 2,  $K_a = 0.38 \,\mu\text{M}^{-1}$  and n = 1.6. (b) The heat of copper binding on MBP for 21 automatic cumulative injections, each of 35  $\mu$ l, of copper, 100  $\mu$ M, into the sample cell containing 1.8 ml MBP solution at a concentration of 13.5  $\mu$ M. The last injection was 350  $\mu$ l. (c) The heat of binding *vs*. total concentration of copper ion, calculated from Fig. b. (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 sum of heat evolutions following the  $i^{th}$  titration step,  $q_i$ , can be expressed as

 $q_{\rm i} = \Delta H \, V_{\rm i} \, [ML]_{\rm i} \tag{15}$ 

where  $V_i$  is the volume of the reaction solution and  $\Delta H$  is the

enthalpy of binding. Combination of equations (13) and (15) will lead to [46,50]

$$\Delta H = 1/A_i \{ (B_i + K) - [(B_i + K)^2 - C_i]^{\nu_2} \}$$
(16)

where

$$A_i = V_i / 2q_i \tag{17}$$

Equation (16) contains two unknowns, *K* and  $\Delta H$ . A series of reasonable value for *K* is inserted into equation (16) 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*.

The data obtained from isothermal titration microcalorimetry of ADA interaction with inosine is shown in Fig. 5. Figure 5a shows the heat of each injection and Fig. 5b shows the heat related to each total concentration of inosine. The plots of  $\Delta H vs. K$ , according to equation (16), for first 10 injections are shown in Fig. 5c. The intersection of curves gives [32]:

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

The conformity of dissociation binding constants (K) obtained from thermodynamic and kinetic studies are observed [46]. The calorimetric data analysis using equation (16) is a graphical fitting model, which extensively has been applied to studies on the enzyme inhibition [32-34,50] and metal binding to proteins [8,58-60,78-79].

These two 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. Design enzyme inhibitors and measurements of the effect of inhibitors on the enzyme are very important in biological science [80-99].

### Ligand Binding in a Set of Binding Sites

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

$$M + L \longrightarrow ML \qquad K = [M][L]/[ML] \qquad (18)$$

If  $\alpha$  is defined as the fraction of free binding sites on the

biomacromolecule,  $M_0$  is the total biomacromolecule concentration and  $L_0$  is the total ligand concentration, then the free concentrations of monovalent molecule, [M], and ligand, [L], as well as the concentration of bound ligand, [ML], can be deduced as follow [56,58]:

$$[ML] = g(1-\alpha) M_0 \tag{19}$$

$$[L] = L_0 - [ML] = L_0 - g (1 - \alpha) M_0$$
(20)

$$[M] = gM_0 - [ML] = gM_0 - g(1 - \alpha) M_0 = \alpha gM_0$$
(21)

Substitution of free concentrations of all these components in equation (18) gives [56,58]:

$$K = (\frac{\alpha}{1-\alpha}) L_0 - \alpha g M_0$$

or

$$\alpha M_0 = \left(\frac{\alpha}{1-\alpha}\right) \frac{1}{g} L_0 - \frac{K}{g}$$
(22)

The value of  $1 - \alpha$  as the fraction of occupied binding sites on the biomacromolecule can be obtained from every desired point on the titration curve of heat intensity *vs.* total concentration of ligand, as shown in Fig. 6b, using the relationship [56,58]

$$1 - \alpha = \frac{q}{q_{\text{max}}} \tag{23}$$

where q represents the heat value at a certain  $L_0$  and  $q_{max}$  represents the heat value upon saturation of all biomacromolecule. If q and  $q_{max}$  are calculated per mole of biomacromolecule then the molar enthalpy of binding for each binding site ( $\Delta H$ ) will be  $\Delta H = q_{max}/g$ . Combination of equations (22) and (23) yields [56,58]:

$$\frac{\Delta q}{q_{\text{max}}} M_0 = \left(\frac{\Delta q}{q}\right) L_0 \frac{1}{g} - \frac{K}{g}$$
(24)

where  $\Delta q = q_{\text{max}} - q$ . Therefore, the plot of  $(\Delta q/q_{\text{max}}) M_0 vs$ .  $(\Delta q/q) L_0$  should be a linear plot by a slope of "1/g" and the



vertical-intercept of K/g, which g and K can be obtained.

The raw data obtained from isothermal titration calorimetry of hGH interaction with calcium ion was shown in Fig. 6 [56]. Figure 6a shows the heat of each injection and Fig. 6b shows the total cumulative heat of related to each total concentration of calcium ion,  $[Ca^{2+}]_t$ . The heat values in these figures have been expressed in terms of total amount of protein (63 nano-mole) in the calorimetric sample cell. These raw calorimetric data can be used to show the heat of binding of calcium ions per mole of hGH *vs*. total concentration of  $Ca^{2+}$ , Fig. 6c, or *vs*. total concentration of the protein, Fig. 6d.

The related plot, according to the equation (24), for the binding of calcium ions by hGH is shown in Fig. 7a. The linearly of the plot has been examined by different estimated values for  $q_{\text{max}}$  to find the best value for the correlation coefficient (near to one). The best linear plot with the correlation coefficient value of 0.99 was obtained using a



Fig. 5. (a) The heat of inosine binding on ADA for 20 automatic cumulative injections, each of 15µl, of inosine solution, 2 mM, into the sample cell containing 1.8 ml ADA solution at a concentration of 0.75 mg ml<sup>-1</sup> (21.7 µM). (b) The heat of binding *vs.* total concentration of inosine, calculated from Fig. a. (c)  $\Delta H vs. K$  for first 10 injections in the reasonable *vs.* of *K*, according to equation (16). The coordinates of intersection point of curves give true value for  $\Delta H$  and *K*.

value of  $-3290 \ \mu\text{J}$  (equal to  $-52.2 \ \text{kJ} \ \text{mol}^{-1}$ ) for  $q_{\text{max}}$ . The amounts of g and K, obtained from the slope and verticalintercept plot, are 3 and 52  $\mu$ M, respectively. Dividing the  $q_{\text{max}}$ value of  $-52.2 \ \text{kJ} \ \text{mol}^{-1}$  by g = 3, therefore, gives  $\Delta H = -17.4 \ \text{kJ} \ \text{mol}^{-1}$  [56]. According to data shown in Fig. 6, the total cumulative heats respect to kJ mol<sup>-1</sup> are known in any different values of  $M_0$  and  $L_0$ ; therefore, A<sub>i</sub>, B<sub>i</sub>, and C<sub>i</sub> are known in all titration steps. A series of reasonable value for K is inserted into equation (16) 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. The plots of  $\Delta H vs$ . K, according to equation (16), for all injections are shown in Fig. 7b. The intersection of curves gives [56]:  $K = 52 \ \mu\text{M}$  and  $\Delta H = -17.4 \ \text{kJ} \ \text{mol}^{-1}$ .

The conformity of K and  $\Delta H$  values obtained from two methods are observed.



Fig. 6. (a) The heat of calcium binding on hGH for 20 automatic cumulative injections, each of 20 μl, of calcium, 2 mM, into the sample cell containing 1.8 ml hGH solution at initial concentration of 35 μM at 300 K. (b) The total cumulative heat of binding vs. total concentration of calcium ion, calculated from Fig. a. (c) The heat of binding calcium ions per mole of hGH vs. total concentration of calcium ions, calculated from Fig. b. (d) The heat of binding calcium ions per mole of hGH vs. total concentration of the protein. The initial concentration of hGH was 35 μM.

The calorimetric method described recently allows obtaining the number of binding sites (g), the molar enthalpy of binding site ( $\Delta H$ ) and the dissociation equilibrium constant (*K*) for a set of biomacromolecule binding sites. The lack of a suitable value for  $q_{\text{max}}$  to obtain a linear plot of ( $\Delta q/q_{\text{max}}$ )  $M_0$  vs. ( $\Delta q/q$ )  $L_0$  may be related to the existence of non-identical

binding sites or the interaction between them. This method has also been applied to study on the binding of chromium ions by  $\beta$ -lactoglobulin-A [100]. There is a set of six identical and independent binding sites for Cr<sup>3+</sup> by a dissociation binding constant of 124  $\mu$ M and the molar enthalpy of binding -17.8



**Fig. 7.** (a) The best linear plot of  $(\Delta q/q_{\text{max}}) M_0 vs. (\Delta q/q) L_0$ , according to the equation (21), using a value of  $-3290 \,\mu\text{J}$  (equal to  $-52.2 \,\text{kJ mol}^{-1}$ ) for  $q_{\text{max}}$  to obtain the best correlation coefficient value (R<sup>2</sup> = 0.99). Values of g and K can be obtained from the slope and the vertical-intercepts, respectively. (b)  $\Delta H vs. K$  for all 20 injections in the reasonable values of K, according to equation (16), using data in Fig. 6. The coordinates of intersection point of curves give true value for  $\Delta H$  and K.

kJ mol<sup>-1</sup> [100]. Using this method also showed that there is a set of three identical and non-interacting binding sites for magnesium ions. The intrinsic dissociation equilibrium constant and the molar enthalpy of binding were 46  $\mu$ M and -17.7 kJ mol<sup>-1</sup>, respectively [58].

## Calorimetric Data Analysis for Determination of Binding Isotherm

A calorimetric data analysis allows the measurement of the complete set of thermodynamic parameters. The binding isotherm for ligand-protein interaction is easily obtained by carrying out two different ITC experiments. In the first experiment, the enthalpy of binding for one mole of ligand to one mole of binding site on a protein is obtained, which is used in the second experiment to obtain the binding isotherm and finding number of binding sites (g) and the equilibrium constant (*K*). This method was used in the first time to study the interaction between nickel ions (Ni<sup>2+</sup>) with human serum albumin (HSA).

In the first experiment, the calorimeter cell contained

protein (HSA) in relatively high concentration and the ligand(nickel) solution in relatively low concentration was Injected into the calorimeter vessel. The results are shown in Fig. 8a [51]. At this step, the enthalpy of binding one mole of ligand to one mole of binding site  $(\Delta H)$  can be obtained. Under these conditions, the protein is much in excess over the ligand during the whole titration experiment and the injected ligand is completely bound to the surface of protein, provided the association binding constant is sufficiently large. Each 10 µl injection of ligand thus produces the same heat of binding. Dividing this heat of binding  $(-1.826 \mu J)$  by the molar amount of added nickel  $(10^{-11} \text{ mol })$  finally leads to the molar enthalpy of binding ( $\Delta H$ ) equal to -36.520 kJ mol<sup>-1</sup>, assuming complete ligand binding [51]. The injection of the ligand into the pure buffer without protein gives a small heat of dilution, which nevertheless was subtracted as a small correction term in the final evaluation of  $\Delta H$ .

Measurement of the binding isotherm is done in the second experiment. At this step, a protein less concentrated solution is contained in the calorimeter cell and a ligand more



concentrated solution is injected *via* the titration syringe. The data obtained from this step is shown in Figs. 8b and 8c. Figure 8b shows the heat of each injection and Fig. 8c shows the heat related to each total concentration of nickel. After each addition of the ligand solution (25  $\mu$ l) into the calorimeter cell (initially 1.5 ml), ligand may be bound to the protein and some binding site removed from the binding process. Hence, with increasing ligand concentration in the reaction vessel less and less binding site on the protein is available for binding. The heat of reaction is therefore no longer constant, but decreases with each ligand injection. After k injections of nickel ion solution into the calorimetric titration vessel, the fraction of nickel ion bound to the protein is given

by [51]



**Fig. 8.** (a) The heat of nickel ions binding on HSA for 10 automatic cumulative injections, each of 10  $\mu$ l, of nickel nitrate solution 5  $\mu$ M, into the sample cell containing 2 ml protein solution at a concentration of 100  $\mu$ M at pH 7.0 (Tris 30 mM) and temperature of 300 K. (b) The heat of nickel binding on HSA for 20 automatic cumulative injections, each of 25  $\mu$ l, of Ni<sup>2+</sup> solution 50  $\mu$ M, into the sample cell containing 1.5 ml HSA solution at a concentration of 1.0  $\mu$ M at pH 7.0 and temperature of 300 K. (c) Thecumulative heat related to each total concentration of nickel ion divided by total concentration of HAS in the calorimetric titration vessel.

$$X^{(k)} = \frac{n_b^{(k)}}{n_t}$$
(25)

where  $n_b^{(k)}$  and  $n_t$  are the molar amount of bound nickel after k injection and the total amount of nickel in the vessel, respectively. They can be calculated by [51]

$$n_{b}^{(k)} = \frac{\sum^{k} q_{i}}{\Delta H}$$
(26)

$$n_{t} = k \times V_{inj} \times L_{o}$$
(27)

where  $\Sigma^k q_i$  is the cumulative heat of the first k injections,  $V_{inj}$  is the injected volume of ligand in each titration step, and  $L_0$  is

### Ligand Binding Studies by Isothermal Titration Calorimetry

the ligand concentration of the stock solution.

After k injections of nickel ion solution into the calorimetric titration vessel, the fraction of nickel ion bound to the protein multiplied by the total concentration of ligand in the cell,  $[L]_t$ , gives the bound ligand concentration,  $[L]_b$ ; that is,  $[L]_b^{(k)} = X^{(k)}[L]_t^{(k)}$ . Now, the average number of moles of ligand bound per mole of protein (v) can be calculated by the bound ligand concentration,  $[L]_b$ , divided by the total concentration of protein in the calorimetric vessel,  $[P]_t$ . Hence, it is possible to calculate v after k injection according to [51]

$$v^{(\kappa)} = \frac{X^{(k)}[L]_{t}^{(k)}}{[P]_{t}^{(k)}} = \frac{\Sigma^{k} q_{i}}{(k \times V_{inj} \times L_{o}) \Delta H} \times \frac{[L]_{t}^{(k)}}{[P]_{t}^{(k)}}$$
(28)

The most common presentation of ligand-biomacromolecule binding data is the Scatchard plot [68-70]. 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 [68-70]:

$$\frac{v}{[L]} = K_a \left( g - v \right) \tag{29}$$

where [*L*] is the free concentration of ligand ([*L*] = [*L*]<sub>t</sub> – [*L*]<sub>b</sub>). Hence, the Scatchard plot,  $\nu/[L] \nu s$ .  $\nu$ , is linear for system which have one identical and independent set of sites, so-called noncooperative system [68-70]. 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 [68-70]. Figure 9 shows the Scatchard plot for the interaction between HSA and Ni<sup>2+</sup>, which obtained from data shown in Fig. 8c using equation (28). Here, the Scatchard plot is linear showing that a set of 8 identical and independent binding sites for nickel ions with intrinsic association binding constant of 0.57  $\mu$ M<sup>-1</sup> [51].

The calorimetric method described above allows the measurement of the complete set of thermodynamic



Fig. 9. The Scatchard plot of binding nickel ions by HSA at pH 7.0 and temperature of 300 K. Here, the Scatchard plot is linear resulting a set of eight identical and independent binding sites with association binding constant of  $0.57 \ \mu M^{-1}$ .

parameters for a set of identical and independent binding sites. The Scatchard plot as a binding isotherm for ligand-protein interaction can be easily obtained by carrying out two different experiments. In the first experiment, the enthalpy of binding for one mole of ligand to one mole of binding site ( $\Delta H$ ) is obtained, which is used in the second experiment to obtained the binding isotherm and finding of equilibrium constant ( $K_a$ ). Observation of a linear plot, as the Scatchard plot, shows a setof identical and independent binding sites on the surface of a protein. Any deviation from the linearly of the Scatchard plot may be related to the non-identically or interacting between binding sites.

A simple general method for determination of binding isotherm in the protein-ligand interaction has been introduced using isothermal titration calorimetric data [52-53]. It is a general method to ligand binding study on a biomacromolecule having a set of interacting or non-interacting binding sites. In the first time, this method was applied to the study of the interaction of myelin basic protein (MBP) from bovine central nervous system with divalent copper ion at 300 K in Tris buffer solution at pH = 7.2. The binding isotherm for copper-MBP interaction was easily obtained by carrying out titration calorimetric experiment in two different



**Fig. 10.** (a) The heat of copper binding on MBP for 28 automatic cumulative injections, each of 35  $\mu$ l, of copper, 100  $\mu$ M, into the sample cell containing 1.8 ml MBP solution at two initial concentrations of 13.5  $\mu$ M (•) and 27.0  $\mu$ M (O). (b) The heat of binding *vs*. total concentration of copper ion, calculated from Fig. a. (c) The heat of binding copper ions per mole of MBP (*q*) *vs*. total concentration of Copper ions, calculated from Fig. b. (d) The heat of binding copper ions per mole of MBP (*q*) *vs*. total concentration of MBP. The initial concentration of MBP was 13.5  $\mu$ M (•) and 27.0  $\mu$ M (O).

concentrations of MBP. The raw data obtained from isothermal titration calorimetry of MBP interaction with copper ion in two different concentrations of the protein is shown in Fig. 10 [52]. Figure 10a shows the heat of each injection and Fig. 10b shows 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, Fig. 10c, or *vs*. total concentration of the protein, Fig. 10d.

In general, there will be "g" sites for binding of ligand molecules per protein macromolecule and v is defined as the average moles of bound ligand per mole of total protein. At any constant heat value due to the binding of ligand molecules per mole of protein (q), the free concentration of ligand ( $L^{\text{free}}$ )



**Fig. 11.** At any constant heat value due to the binding of ligand molecules per mole of protein (q), the free concentration of ligand  $(L^{\text{free}})$  and v are also constant at equilibrium on both two curves of (q) against total concentration of either ligand or.

and v are also constant at equilibrium on both two curves of (q) against total concentration of either ligand or protein obtained in two titration experiments at two different concentrations of a protein (such as Fig. 11). Ligand molecules exist in two forms of free and bound. Hence,  $L^{\text{free}} = L - L^{\text{bound}}$ , where L and  $L^{\text{bound}}$  are the total and bound concentration of ligand, respectively. The titration calorimetric experiment has been carried out in two concentrations of the protein, shown by 1 and 2. Equality of  $L^{\text{free}}$  at any constant value of q on both titration curves (such as Fig. 11) results the equation [52-53]:

$$L_1 - L_1^{\text{bound}} = L_2 - L_2^{\text{bound}}$$
(30)

By applying  $v = L^{\text{bound}}/M$ , where *M* is the total concentration of the protein, and equality of v at any constant value of *q* on both titration curves, it can be deduced equation (31) from equation (30) [52-53].

$$L_1 - \nu M_1 = L_2 - \nu M_2 \tag{31}$$

This equation can be rearranged to give equation (32).

$$v = \frac{L_2 - L_1}{M_2 - M_1}$$
(32)

Then  $L^{\text{free}}$  can be calculated by equation (33), which obtains

from substitution of v from equation (32) into the equation  $L^{\text{free}} = L_1 - vM_1$  [52-53].

$$L^{\text{free}} = \frac{L_1 M_2 - L_2 M_1}{M_2 - M_1}$$
(33)

One can then calculate v and  $L^{\text{free}}$  from equation (32) and (33), respectively. This procedure is repeated over the range of q values that span the titration curves, thus yielding a full range of values of v and  $L^{\text{free}}$ . In this way, one can obtain a binding isotherm for protein ligand interaction by two calorimetric titration curves at two different concentration of a protein.

The binding isotherm, as shown in Fig. 12a, or the Scatchard plot  $(v/[Cu^{2+}]_f vs. v)$ , as shown in Fig. 12b, for MBP interaction with copper ions has been obtained. Results obtained by this calorimetric method are in agreement with our previous results; see Fig. 4a [13,50]. The above described calorimetric method allows obtaining the binding isotherm for measurement of the complete set of thermodynamic parameters in protein ligand binding studies. The binding isotherm for ligand-protein interaction can be easily obtained by carrying out titration calorimetric experiment in two different concentrations of a protein. This method has been applied to the binding of theophiline by ADA [53] and also copper ion by hGH [54].

## Protein Denaturation and Determination of the Enthalpy of Denaturation

Denaturation studies are capable of yielding information about the native state in terms of its cooperativity, intrinsic stability and the nature of the forces required to maintain its tertiary structure. Denaturation for providing additional information on the structure, properties and function of a protein can be brought about in many ways. These include thermal denaturation, by raising the temperature, [101-102] chemical denaturation by urea or guanidinum chloride [103], denaturation by surfactant [24-25,27,104-107] and denaturation by other materials [28]. The interactions of proteins with ionic surfactants differ from their interactions with other ligands in three important respect [27]: (1) Ionic surfactants having hydrocarbon-chain lengths of eight or more carbon atoms are the most potent protein denaturants known. (2) Unlike all other classes of ligand, except hydrogen ion,

Ligand Binding Studies by Isothermal Titration Calorimetry



Fig. 12. The binding isotherm (a) and the Scatchard plot (b) of binding copper ion by MBP at 300 K. The bestfit curve of the experimental binding data was transformed to both binding isotherm and the Scatchard plot using equation (6) with g = 2,  $K = 0.38 \mu M^{-1}$  and n = 1.6.

they combine with most native proteins in multiple equilibria, i.e. many equivalents per mole of protein. Surfactants combine in still larger quantities with proteins that have been unfolded. (3) With respect to the amphipathic nature of surfactants and proteins, it is well established that there are two kinds of interactions in the binding of ionic surfactants to proteins. The electrostatic interaction, which is accompanying by preliminary hydrophobic interaction is occurred initially, followed by a more extensive pure hydrophobic interaction [24]. The predominant denaturing of a protein is related to the first interaction, in which neutralization of charges at the surface of the protein perturb the balance of forces in the protein structure [24-25].

An interesting research has been carried out to study on the homologous interactions of а series of n-alkyl trimethylammonium bromides for a better understanding of the energetics of the interactions between JBU and cationic surfactants. The enthalpy of unfolding for JBU from this interaction could be reported. The interaction of JBU with dodecyl trimethylammonium bromide (DTAB), tetradecyl trimethylammonium bromide (TTAB), and hexadecyl trimethylammonium bromide (HTAB) has been studied by isothermal titration calorimetry at 300 K and equilibrium dialysis at temperatures of 300 K and 310 K in alkaline

solution at pH 10. The molar enthalpy of binding has been calculated from binding data, which have been obtained from equilibrium dialysis in terms of the Wyman binding potential theory [108] related to the van't Hoff relation [109]. The interaction of protein with surfactant includes two processes of the ligand binding and the protein unfolding. Hence, the molar enthalpy of urease unfolding ( $\Delta H_u$ ) by this cationic surfactant could be determined by subtraction of the observed molar calorimetric enthalpy ( $\Delta H_{obs}$ ) and the molar enthalpy of binding ( $\Delta H_b$ ) according to the equation (34) [25-26]:

$$\Delta H_{\rm obs} = \Delta H_{\rm b} + \Delta H_{\rm u} \tag{34}$$

Figure 13 shows the various enthalpies ( $\Delta H_{obs}$ ,  $\Delta H_b$  and  $\Delta H_u$ ) against total concentrations of DTAB, TTAB and HTAB [25]. Figure 14 plots the variation of  $\Delta H_u$  with respect to the total concentration of surfactants [25]. All curves are step-like and it seems that there is a semi-stable transition state in the process of urease unfolding. By extrapolation of these curves at their plateau regions, the molar enthalpy of unfolding for each plateau step can be estimated. In terms of this analysis, the molar enthalpy of unfolding for the first plateau is equal to 6550 kJ mol<sup>-1</sup> and for the second, 650 kJ mol<sup>-1</sup>. The extrapolation points are remarkably alike for both plateau





**Fig. 13.** Molar enthalpies of interaction: ( $\Box$ )  $\Delta H_{obs}$ , ( $\Delta$ )  $\Delta H_b$ , ( $\nabla$ )  $\Delta H_u$  between JBU and *n*-alkyl trimethylammonium Bromides: (a) DTAB, (b) TTAB, (c) HTAB as a function of total concentration of surfactant at pH 10 and



**Fig. 14.** Changes of the unfolding enthalpy  $(\Delta H_u)$  for JBU in interaction with cationic surfactants with different tails, as a function of total concentration (*c*) of surfactant: ( $\Box$ ) DTAB, ( $\Delta$ ) TTAB, ( $\nabla$ ) HTAB at pH 10 and 300 K.

regions. The total molar unfolding enthalpy of JBU is approximately equal to 7200 kJ mol<sup>-1</sup> (13.2 J g<sup>-1</sup>). This method for determination of molar enthalpy of unfolding due to the binding of a surfactant has been applied for horse radish peroxides [26] and DNA [7]. Also, denaturation of HSA by 2,2'-bipyridineglycinato palladium(II) chloride, as a anticancer drug has been investigated using equation (24) [28].

### ACKNOWLEDGEMENTS

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

### REFERENCES

- J.T. Edsall, H. Gutfreund, Biothermodynamics, John Wiley & Sons, New York, 1984, Chapter 6.
- [2] I. Jelesarov, H.R. Bosshard, J. Mol. Recognit. 12 (1999)3.
- [3] I. Wadso, Thermochim. Acta 394 (2002) 305.
- [4] E. Freire, O.L. Mayorga, M. Straume, Anal. Chem. 62 (1990) 950A.
- [5] S. Leavitt, E. Freire, Current Opinion in Structural Biology 11 (2001) 560.
- [6] A.A. Saboury, M.U. Dahut, S. Ghobadi, J. Chamani, A.A. Moosavi-Movahedi, J. Chin. Chem. Soc. 45 (1998) 667.
- [7] S.Z. Bathaie, A.A. Moosavi-Movahedi, A.A. Saboury, Nuc. Acid Res. 27 (1999) 1001.
- [8] A.A. Saboury, F. Karbassi, Thermochimica Acta 362 (2000) 121.
- [9] G. Ataie, A.A. Moosavi-Movahedi, A.A. Saboury, G.H. Hakimelahi, J. Ru. Hwu, S.C. Tsay, Int. J. Biol. Macromol. 27 (2000) 29.
- [10] M.R. Housaindokht, J. Chamani, A.A. Saboury, A.A. Moosavi-Movahedi, Bull. Kor. Chem. Soc. 22 (2001) 145.

- [11] A.A. Saboury, S. Saeidian, H. Sanati, A.A. Moosavi-Movahedi, F. Alasti, J. Chin. Chem. Soc. 48 (2001) 827.
- [12] A. Arabzadeh, S.Z. Bathaie, H. Farsam, M. Amanlou, A.A. Saboury, A.A. Moosavi-Movahedi, Inter. J. Pharmac. 237 (2002) 47.
- [13] A. Mahmoudi, K. Nazari, A.A. Moosavi-Movahedi, A.A. Saboury, Thermochim. Acta 385 (2002) 33.
- [14] D. Ajloo, A.A. Moosavi-Movahedi, G.H. Hakimelahi, A.A. Saboury, H. Gharibi, Coll. Surf. B: Biointerfaces 26 (2002) 185.
- [15] A.A. Saboury, Biologia 57 (2002) 221.
- [16] S.Z. Bathaie, A.A. Moosavi-Movahedi, B. Ranjbar, A.A. Saboury, Coll. Surf. B: Biointerfaces 28 (2003) 17.
- [17] J. Chamani, A.A. Moosavi-Movahedi, A.A. Saboury, M. Gharanfoli, G.H. Hakimelahi, J. Chem. Thermodyn. 35 (2003) 199.
- [18] A.K. Bordbar, A.R. Ghaderi, E. Safaei, S. Tangestaninejad, A.A. Saboury, A.A. Moosavi-Movahedi, Bull. Kor. Chem. Soc. 24 (2003) 547.
- [19] H. Mansoori Torshizi, M. Eslami Moghaddam, A.A. Saboury, Acta Biochim. Biophys. Sin. 35 (2003) 886.
- [20] A. Gharanfoli, A.A. Moosavi-Movahedi, S. Safarian, J. Chamani, A.A. Saboury Thermochim. Acta 411 (2004) 37.
- [21] A.A. Moosavi-Movahedi, A.R. Golchin, K. Nazari, J. Chamani, A.A. Saboury, S.Z. Bathie, S. Tangestani-Nejad, Thermochim. Acta 414 (2004) 233.
- [22] K. Nazari, A.R. Golchin, A.A. Moosavi-Movahedi, A.A. Saboury, G.H. Hakimelahi, A. Shokravi, S. Tangestani-Nejad, Thermochim. Acta 428 (2005) 157.
- [23] N.S. Sarraf, S. Mamaghani-Rad, F. Karbassi, A.A. Saboury, Bull. Kor. Chem. Soc. 26 (2005) 1051.
- [24] A.A. Saboury, A.K. Bordbar, A.A. Moosavi-Movahedi, Bull. Chem. Soc. Jpn. 69 (1996) 3031.
- [25] A.A. Saboury, A.K. Bordbar, A.A. Moosavi-Movahedi, J. Chem. Thermodyn. 28 (1996) 1077.
- [26] K. Nazari, A.A. Saboury, A.A. Moosavi-Movahedi, Thermochim. Acta 302 (1997) 131.
- [27] A.A. Moosavi-Movahedi, A.A. Saboury, Jour. Chem. Soc. Pak. 21 (1999) 248.
- [28] A.A. Saboury, A.A. Shamsaei, A.A. Moosavi-

Movahedi, H. Mansori-Torshizi, J. Chin. Chem. Soc. 46 (1999) 917.

- [29] O.A. Amire, J. Masoudy, A.A. Saboury, A.A. Moosavi-Movahedi, Thermochim. Acta 303 (1997) 219.
- [30] J. Masoudi, A.K. Bordbar, O.A. Amire, A.A. Saboury, A.A. Moosavi-Movahedi, Biophys. J. 72 (1997) 82.
- [31] A.A. Saboury, A.A. Moosavi-Movahedi, J. Enz. Inhib. 12 (1997) 273.
- [32] A.A. Saboury, A. Divsalar, G. Ataie, A.A. Moosavi-Movahedi, M.R. Housaindokht, G.H. Hakimelahi, J. Biochem. Mol. Biol. 35 (2002) 302.
- [33] N. Sarri-Sarraf, A.A. Saboury, A.A. Moosavi-Movahedi, J. Enz. Inhib. Med. Chem. 17 (2002) 203.
- [34] A.A. Saboury, A. Divsalar, G. Ataie, M. Amanlou, A.A. Moosavi-Movahedi, G.H. Hakimelahi, Acta Biochim. Pol. 50 (2003) 849.
- [35] M.U. Dahot, A.A. Saboury, A.A. Moosavi-Movahedi, J. Enz. Inh. Med. Chem. 19 (2004) 157.
- [36] A.K. Bordbar, A.A. Moosavi-Movahedi, A.A. Saboury, Thermochim. Acta 287 (1996) 343.
- [37] A.A. Saboury, M. Miroliae, M. Nemat-Gorgani, A.A. Moosavi-Movahedi, Thermochim. Acta 326 (1999) 127.
- [38] M. Angberg, C. Nystrom, S. Castensson, Acta Pharm. Suec. 25 (1988) 307.
- [39] L.D. Hansen, E.A. Lewis, D.J. Eatough, R.G. Bergstrom, D. DeGraft-Johnson, Pharm. Res. 6 (1989) 20.
- [40] M.J. Koenigbaure, S.H. Brooks, G. Rullo, R.A. Couch, Pharm. Res. 9 (1992) 939.
- [41] A.A. Saboury, N. Sarri-Sarraf, M.U. Dahot, Jour. Chem. Soc. Pak. 26 (2004) 69.
- [42] T.F. Bolles, R.S. Drago, J. Am. Chem. Soc. 87 (1965) 5015.
- [43] M.A. Laandav, M.N. Markovich, L.A. Pyruzyan, Biochim. Biophys. Acta 493 (1977) 1.
- [44] A. Chen, I. Wadso, Biochem. Biophys. Meth. 6 (1982) 307.
- [45] T. Wiseman, S. Williston, J.F. Brandts, L. Lin, Anal. Biochem. 179 (1989) 131.
- [46] M. Ghadermarzi, A.A. Saboury, A.A. Moosavi-Movahedi, Polish J. Chem. 72 (1998) 2024.
- [47] A.A. Saboury, A.A. Moosavi-Movahedi, Biophys. J. 72

(1997) 217.

- [48] A.A. Saboury, Jour. Chem. Soc. Pak. 22 (2000) 204.
- [49] A.A. Saboury, Ind. J. Biochem. Biophys. 37 (2000) 347.
- [50] A.A. Saboury, J. Therm. Ana. Cal. 72 (2003) 93.
- [51] A.A. Saboury, J. Chem. Thermodyn. 17 (2003) 1975.
- [52] A.A. Saboury, J. Therm. Ana. Cal. 77 (2004) 997.
- [53] A.A. Saboury, S. Bagheri, G. Ataie, M. Amanlou, A.A. Moosavi-Movahedi, G.H. Hakimelahi, G. Cristalli, S. Namaki, Chem. Pharm. Bull. 52 (2004) 1179.
- [54] A.A. Saboury, M. Kordbacheh, M.H. Sanati, F. Mizani, M. Shamsipur, M.B. Yakhchali, A.A. Moosavi-Movahedi, Asian J. Chem. 17 (2005) 2773.
- [55] A.A. Saboury, S. Bagheri, G. Ataie, A.A. Moosavi-Movahedi, G.H. Hakimelahi, G. Cristalli, Asian J. Chem. 17 (2005) 233.
- [56] A.A. Saboury, M.S. Atri, M.H. Sanati, M. Sadeghi, J. Therm. Ana. Cal. (2006) in press.
- [57] A.A. Saboury, S. Ghasemi, M.U. Dahot, Ind. J. Biochem. Biophys. 42 (2005) 326.
- [58] A.A. Saboury, M.S. Atri, M.H. Sanati, A.A. Moosavi-Movahedi, G.H. Hakimelahi, M. Sadeghi, Biopolymers 81 (2006) 120.
- [59] M.S. Atri, A.A. Saboury, M. Rezaei-Tavirani, M.H. Sanati, A.A. Moosavi-Movahedi, M. Sadeghi, H. Mansuri-Torshizi, N. Khodabandeh, Thermochim. Acta 438 (2005) 178.
- [60] A.A. Saboury, M.S. Atri, M.H. Sanati, A.A. Moosavi-Movahedi, K. Haghbeen, Int. J. Biol. Macromol. 36 (2005) 305.
- [61] P.R. Connelly, R. Varadarajan, J.M. Sturtevant, F.M. Richards, Biochemistry 29 (1990) 6108.
- [62] J.E. Wilson, A. Chin, Anal. Biochem. 193 (1991) 16.
- [63] F. Zhang, E.S. Rowe, Biochemistry 31 (1992) 2005.
- [64] D. Hamada, S. Kidokoro, H. Fukada, K. Takahashi, Y. Goto, Proc. Natl. Acad. Sci. USA 91 (1994) 10325.
- [65] R. Ramkumar, A. Surolia, S.K. Podder, Biochem. J. 308 (1995) 237.
- [66] C.S. Raman, M.J. Allan, B. Nall, Biochemistry 34 (1995) 5831.
- [67] M.C. Chervenak, E.J. Toone, Biochemistry 34 (1995) 5685.
- [68] G. Scatchard, Ann New York Acad. Sci. 50 (1949) 660.

- [69] A.A. Saboury, A.A. Moosavi-Movahedi, Biochem. Educ. 22 (1994) 48.
- [70] A.K. Bordbar, A.A. Saboury, A.A. Moosavi-Movahedi, Biochem. Educ. 24 (1996) 172.
- [71] A.A. Saboury, M.U. Dahut, A.A. Moosavi-Movahedi, Biophys. J. 74 (1998) 369.
- [72] A.A. Saboury, Thermochim. Acta 320 (1998) 97.
- [73] M.L. James, G.M. Smith, J.C. Wolford, Applied Numerical Methods for Digital Computer, 3<sup>rd</sup> ed., Harper and Row Publisher, New York, 1985.
- [74] A.A. Saboury, A.A. Moosavi-Movahedi, J. Enz. Inh. 11 (1997) 217.
- [75] A.V. Hill, J. Physiol. 40 (1910) iv.
- [76] A.A. Saboury, N. Sarri-Sarraf, S. Saeidian, A.A. Moosavi-Movahedi, Biophys. J. 80 (2001) 310.
- [77] W.C. Galley, M. Bouvier, S.-D. Clas, G.R. Brown, L.E. ST-Pierre, Biopolymers 27 (1988) 79.
- [78] A.A. Saboury, M. Sadat-Atri, M. Kordbacheh, H. Ghourchaei, M.H. Sanati, FEBS J. 272 (2005) 390.
- [79] M.S. Atri, M. Rezaei-Tavirani, M.H. Sanati, A.A. Saboury, A.A. Moosavi-Movahed, Biophys. J. 88 (2005) 212.
- [80] G.H. Hakimelahi, M. Pasdar, S. Hakimelahi, A. Khalafi-Nejad, M.N. Soltani, N.-W. Mei, H.-C. Mei, A.A. Saboury, M. Rezaei-Tavirani, A.A. Moosavi-Movahedi, Bioorg. Med. Chem. 10 (2002) 2927.
- [81] G.H. Hakimelahi, K.-S. Shia, M. Pasdar, S. Hakimelahi,
  A.A. Moosavi-Movahedi, A.A. Saboury, A. Khalafi-Nejad, M.N. Soltani-Rad, V. Osyetrov, K.-P. Wang, J.-H. Liao, F.-T. Luo, Bioorg. Med. Chem. 10 (2002) 3489.
- [82] A.A. Moosavi-Movahedi, S. Safarian, G.H. Hakimelahi, G. Ataei, D. Ajloo, S. Panjehpour, S. Riahi, M.F. Moosavi, S. Mardanian, N. Soltani, A. Khalafi-Nezhad, H. Sharghi, H. Moghadamnia, A.A. Saboury, Nucleos. Nucleot. Nuc. Acids 23 (2004) 613.
- [83] G.H. Hakimelahi, A.A. Moosavi-Movahedi, A.A. Saboury, V. Osetrov, G.A. Khodarahmi, K.-S. Shia, Eur. J. Med. Chem. 40 (2005) 339.
- [84] G.H. Hakimelahi, A.A. Moosavi-Movahedi, A.A. Saboury, V. Osetrov, G.A. Khodarahmi, K.-S. Shia, Int. J. Sci. Res. 14 (2005) 59.
- [85] S. Shareefi Borojerdi, K. Haghbeen, A.A. Karkhane, M.

Fazli, A.A. Saboury, Biochem. Biophys. Res. Commu. 314 (2004) 925.

- [86] G. Ataie, S. Safarian, A. Divsalar, A.A. Saboury, A.A. Moosavi-Movahedi, B. Ranjbar, G. Cristalli, S. Mardanian, J. Enz. Inh. Med. Chem. 19 (2004) 71.
- [87] F. Karbassi, K. Haghbeen, A.A. Saboury, B. Ranjbar, A.A. Moosavi-Movahedi, Coll. Surf. B: Biointerfaces 32 (2003) 137.
- [88] K. Haghbeen, B. Zamani, A.A. Saboury, Biophys. J. 84 (2003) 348.
- [89] F. Karbassi, K. Haghbeen, A.A. Saboury, M. Rezaei-Tavirani, B. Ranjbar, Biologia 59 (2004) 319.
- [90] F. Karbassi, A.A. Saboury, M.T. Hassan Khan, M. Iqbal Choudhary, Z.S. Saifi, J. Enz. Inh. Med. Chem. 19 (2004) 349.
- [91] A.A. Saboury, F. Karbassi, K. Haghbeen, B. Ranjbar, A.A. Moosavi-Movahedi, B. Farzami, Int. J. Biol. Macromol. 34 (2004) 257.
- [92] N.S. Sarraf, A.A. Saboury, B. Ranjbar, A.A. Moosavi-Movahedi, Acta Biochim. Pol. 51 (2004) 665.
- [93] K. Haghbeen, A.A. Saboury, F. Karbassi, Biochim. Biophys. Acta (BBA) Gen. 1 Sub. 1675 (2004) 139.
- [94] R. Yousefi, S.K. Ardestani, A.A. Saboury, A. Kariminia, M. Zeinali, M. Amani, J. Biochem. Mol. Biol. 38 (2004) 407.
- [95] M. Amani, A.A. Moosavi-Movahedi, G. Floris, S. Longu, A. Mura, S.Z. Moosavi-Nejad, A.A. Saboury, F. Ahmad, Protein J. 24 (2005) 183.
- [96] N.S. Sarraf, A.A. Saboury, B. Ranjbar, M. Nemat-

Gorgani, Asian J. Chem. 17 (2005) 2385.

- [97] N. Gheibi, A.A. Saboury, H. Mansuri-Torshizi, K. Haghbeen, A.A. Moosavi-Movahedi, J. Enz. Inh. Med. Chem. 20 (2005) 393.
- [98] N. Gheibi, A.A. Saboury, K. Haghbeen, A.A. Moosavi-Movahedi, Coll. Surf. B: Biointerfaces 45 (2005) 104.
- [99] F. Karbassi, N. Gheibi, A.A. Saboury, K. Haghbeen, Biophys. J. 88 (2005) 389.
- [100] A. Divsalar, A.A. Saboury, A.A. Moosavi-Movahedi, Protein J. (2005) in press.
- [101] A.A. Saboury, A.A. Moosavi-Movahedi, Biochem. Educ. 22 (1994) 210.
- [102] A.A. Saboury, A.A. Moosavi-Movahedi, Biochem. Educ. 23 (1995) 164.
- [103] M.H. Moghaddamnia, A.A. Saboury, G.H. Hakimelahi, A.A. Moosavi-Movahedi, J. Chin. Chem. Soc. 44 (1997) 417.
- [104] A.K. Bordbar, A.A. Saboury, M.R. Housaindokht, A.A. Moosavi-Movahedi, J. Coll. Interf. Sci. 192 (1997) 415.
- [105] A.A. Saboury, A.A. Moosavi-Movahedi, K. Nazari, FASEB J. 11 (1997) 257.
- [106] A.A. Moosavi-Movahedi, K. Nazari, A.A. Saboury, Coll. Surf. B: Biointerfaces 9 (1997) 123.
- [107] J. Chamani, M.R. Housaindokht, A.A. Moosavi-Movahedi, A.A. Saboury, Biophys. J. 76 (1999) 103.
- [108] J. Wyman, J. Mol. Biol. 11 (1965) 631.
- [109] G.M. Barrow, Physical Chemistry, 4<sup>th</sup> ed., Chap. McGraw-Hill, New York, 1988.