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APPLICATION OF A SIMPLE CALORIMETRIC DATA ANALYSIS ON THE BINDING STUDY OF CALCIUM IONS BY HUMAN GROWTH HORMONE

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A simple graphical linear method was introduced for isothermal titration calorimetric data analysis in the protein-ligand interaction. The number of binding sites, the dissociation binding constant and the molar enthalpy of binding site can be obtained by using this new isothermal titration calorimetric data analysis method. The method was applied to the study of the interaction of human growth hormone (hGH) with divalent calcium ion at 27°C in NaCl solution, 50 mM. hGH has a set of three identical and independent binding sites for Ca^{2+}. The intrinsic dissociation equilibrium constant and the molar enthalpy of binding are 52 μM and –17.4, respectively. Results obtained by this new calorimetric data analysis are in good agreement with results obtained using our previous method.

Keywords: *calcium, calorimetric method, human growth hormone, isothermal titration calorimetry*

Introduction

Isothermal titration calorimetry (ITC) is one of the most powerful tools for understanding the quantification of biomolecular interactions at constant temperature [1–3]. The correlation of structural and calorimetric measurements is one of the fundamental areas of advance incorporating ITC data. The number of publications on ITC has grown exponentially over the last 10 years, reflecting the general utility of the method [4–5]. ITC gives invaluable information about thermodynamical parameters of ligand interaction [6–10], protein denaturation [11–15], kinetic parameters [16], enzyme inhibition [17–21] and material stability [22–24].

ITC experiments are performed by titration of a reactant into a sample solution containing the other reactant(s) necessary for reaction and the exchanged heat as a result of the reaction is monitored. The total concentration of titrant is the independent variable under experimental control. Thermodynamic analysis of the observed heat effects permits quantitative characterization of the energetic processes associated with the binding reaction. Different methods have been reported for data analysis of ligand binding study by ITC [25–29]. The principal of these methods is based on using nonlinear least square fitting experimental data in an equation relating equilibrium constant, molar enthalpy of binding and reactants concentration [29]. We have presented a number of useful graphical methods in the ligand binding studies, to obtain equilibrium constant and enthalpy of binding by ITC data [30–34]. Determination of the binding isotherm for a set of identical and independent binding sites [35] as well as for a set of identical and dependent binding sites [36–37] have been reported recently. Here, a simple calorimetric data analysis is applied to obtain the number of binding sites, the equilibrium binding constant and the molar enthalpy of binding on the interaction between hGH and calcium ions, and results are compared with results based on our previous data analysis method.

Experimental

Materials and methods

Materials

Highly purified preparations of hGH were provided by the National Research Center of Genetic Engineering and Biotechnology (NRCGEB), Tehran. Protein concentrations were determined from absorbance measurements at 277 nm in 1cm quartz cuvettes. An $E^{1\%}$(277 nm)=9.3 was used as reported by Bewley *et al.* [38]. Calcium nitrate was purchased from Merck Co. All other materials and reagents were of analytical grades, and solutions were made in NaCl 50 mM using double-distilled water.

Methods

The isothermal titration microcalorimetric experiments were performed with the 4-channel commercial microcalorimetric system, Thermal Activity Monitor 2277, Thermometric, Sweden. The titration vessel was made

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from stainless steel. Calcium solution (2 mM) was injected by use of a Hamilton syringe into the calorimetric stirred titration vessel, which contained 1.8 mL hGH (35 μM). Thin (0.15 mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. Injection of calcium solution into the perfusion vessel was repeated 20 times, and each injection included 20 μL reagent. 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 calcium solution was measured as described above except hGH was excluded. Also, the heat of dilution of the protein solution was measured as described above except the aqueous solution, without calcium ion, was injected to the protein solution in the sample cell. The enthalpies of calcium and protein solutions dilution were subtracted from the enthalpy of hGH-calcium interaction. The microcalorimeter was frequently calibrated electrically during the course of the study.

The molecular weight of hGH was taken to be 22 kDa [39–40].

Results and discussion

The raw data obtained from isothermal titration calorimetry of hGH interaction with calcium ion was shown in Fig. 1. Figure 1a is showing the heat of each injection and Fig. 1b is showing 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 nanomole) 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. 2a, or *vs*. total concentration of the protein, Fig. 2b.

Consider a solution containing ligand L, and a biomacromolecule (M_n) that contains n 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 ($M_n \rightarrow nM$) with the same set of dissociation equilibrium constant (K) values. Thus, the reaction under consideration can be written:

$$M+L \Leftrightarrow ML \quad K = [M][L]/[ML] \tag{1}$$

If α 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:

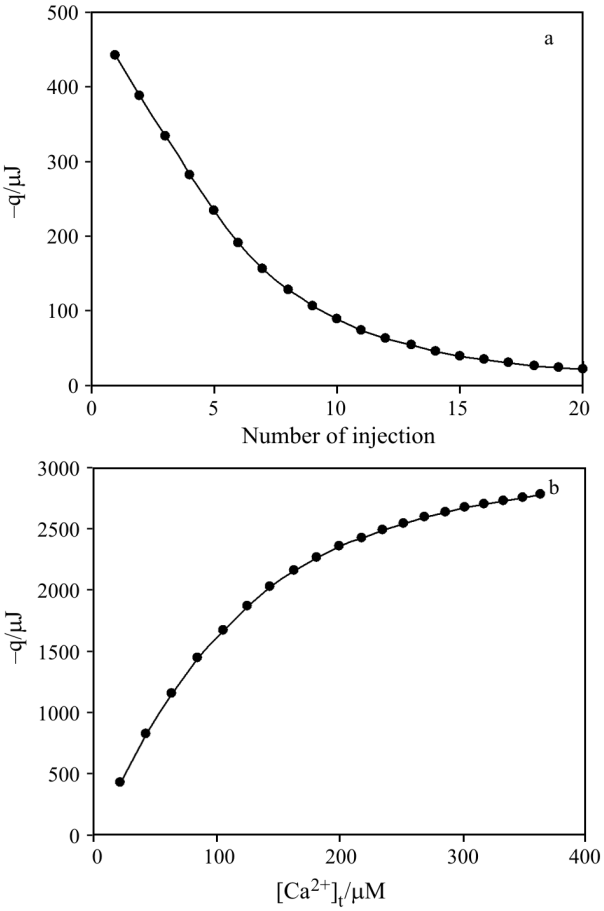

Fig. 1 a – The heat of calcium binding on hGH for 20 automatic cumulative injections, each of 20 μL, of calcium, 2 mM, into the sample cell containing 1.8 mL hGH solution at initial concentration of 35 μM at 27°C; b – the total cumulative heat of binding *vs*. total concentration of calcium ion, calculated from Fig. 1a

$$[ML] = n(1-\alpha)M_0 \tag{2}$$

$$[L] = L_0-[ML] = L_0-n(1-\alpha)M_0 \tag{3}$$

$$[M] = nM_0-[ML] = nM_0-n(1-\alpha)\,M_0 = \alpha nM_0 \tag{4}$$

Substitution of free concentrations of all these components in Eq. (1) gives:

$$K = \left(\frac{\alpha}{1-\alpha}\right)L_0 - \alpha nM_0$$

or

$$\alpha M_0 = \left(\frac{\alpha}{1-\alpha}\right)\frac{1}{n}L_0 - \frac{K}{n} \tag{5}$$

The value of 1–α 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. 2b, using the relationship

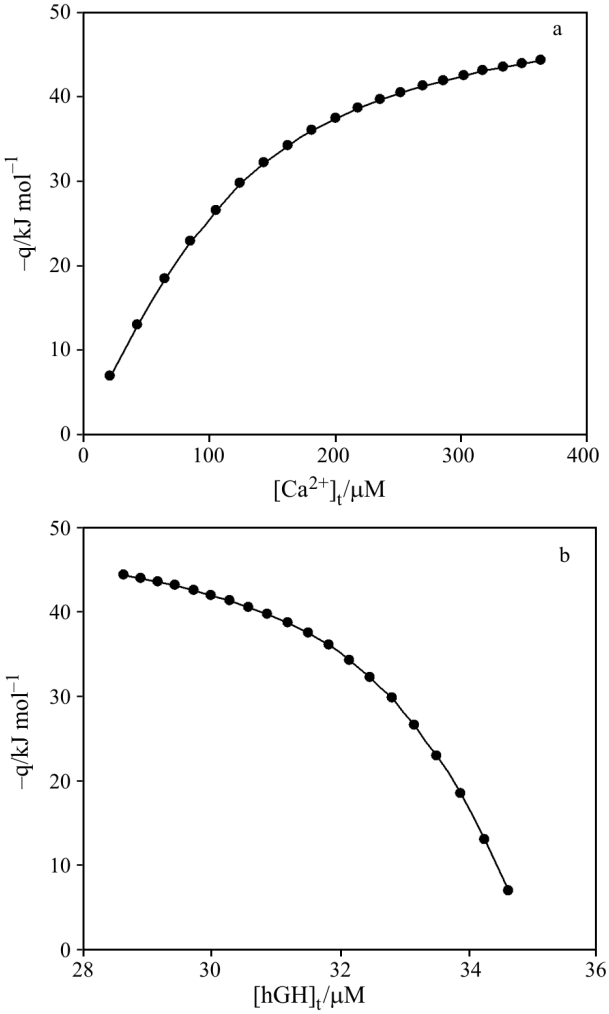

Fig. 2 a – The heat of binding calcium ions per mole of hGH *vs*. total concentration of calcium ions, calculated from Fig. 1b; b – the heat of binding calcium ions per mole of hGH *vs*. total concentration of the protein. The initial concentration of hGH was 35 μM

$$1-\alpha=\frac{q}{q_{max}} \tag{6}$$

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 (ΔH) will be $\Delta H=q_{max}/n$. Combination of Eqs (5) and (6) yields:

$$\frac{\Delta q}{q_{max}}M_0=\left(\frac{\Delta q}{q}\right)L_0\frac{1}{n}-\frac{K}{n} \tag{7}$$

where $\Delta q=q_{max}-q$. Therefore, the plot of $(\Delta q/q_{max})M_0$ *vs*. $(\Delta q/q)L_0$ should be a linear plot by a slope of $1/n$ and the vertical-intercept of K/n, which n and K can be obtained. The related plot for the binding of calcium ions by hGH is showing in Fig. 3. The linearity

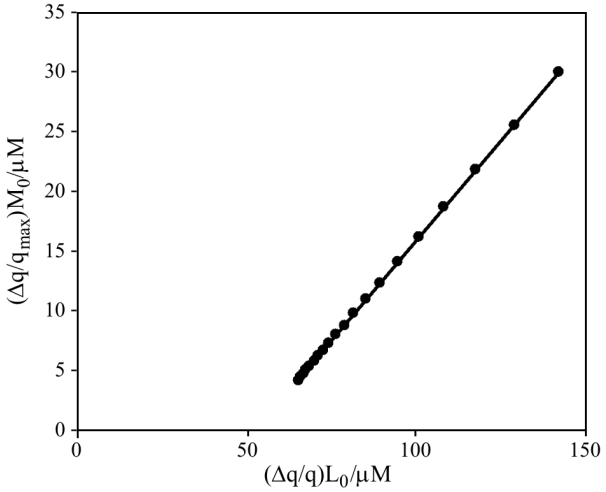

Fig. 3 The best linear plot of $(\Delta q/q_{max})M_0$ *vs*. $(\Delta q/q)L_0$, according to the Eq. (7), using a value of –3290 μJ (equal to –52.2 kJ mol^{-1}) for q_{max} to obtain the best correlation coefficient value (R^2=0.99). Values of n and K can be obtained from the slope and the vertical-intercepts, respectively

of the plot has been examined by different estimated values for q_{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 value of –3290 μJ (equal to –52.2 kJ mol^{-1}) for q_{max}. The amounts of n and K, obtained from the slope and vertical-intercept plot, are 3 and 52 μM, respectively. Dividing the q_{max} value of –52.2 kJ mol^{-1} by n=3, therefore, gives ΔH= –17.4 kJ mol^{-1}.

For a set of identical and independent binding sites, we have before shown [19, 30]:

$$\Delta H=1/A_i\{(B_i+K)-[(B_i+K)^2-C_i]^{1/2}\} \tag{8}$$

A_i, B_i and C_i are constants in each injection i, which have been defined as follow:

$$A_i=V_i/2q_i\ B_i=nM_0+L_0\quad C_i=4nM_0L_0 \tag{9}$$

where V_i and q_i are the volume of the reaction solution and total cumulative heat (by kJ mol^{-1}, which can be obtained from Fig. 2) in the calorimetric sample cell in each injection step, respectively. According to data shown in Fig. 2, the total cumulative heats respect to kJ mol^{-1} are known in any different values of M_0 and L_0; therefore, A_i, B_i and C_i are known in all titration steps. Equation (8) contains two unknowns, K and ΔH. A series of reasonable value for K is inserted into Eq. (8) and corresponding values for ΔH are calculated and the graph ΔH *vs*. K is constructed. Curves of all titration steps will intersect in one point, which represents the true value for ΔH and K. Actually, this method represents a simple graphical non-linear fitting method. The plots of ΔH *vs*. K, according to Eq. (8), for all injections are shown in Fig. 4. The intersection of curves gives:

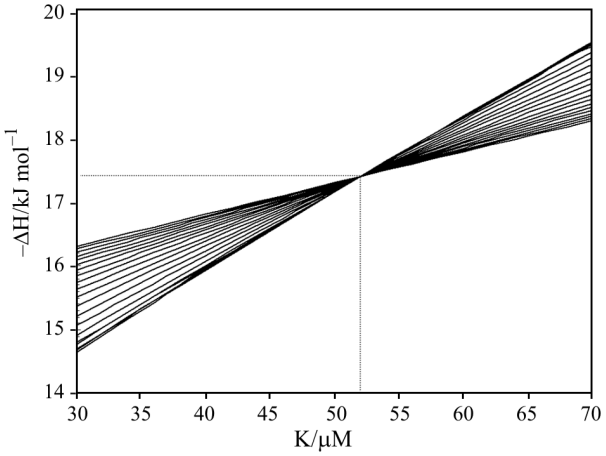

Fig. 4 Δ*H vs*. *K* for all 20 injections in the reasonable values of *K*, according to Eq. (8), using data in Fig. 2. The coordinates of intersection point of curves give true value for Δ*H* and *K*

$$K = 52\ \mu M \quad \Delta H = -17.4\ \text{kJ mol}^{-1}$$

The conformity of *K* and Δ*H* values obtained from two methods are observed.

Some metal ions like Zn^{2+}, Cd^{2+}, Hg^{2+} and Co^{2+} are known to promote hGH reversible dimerization. But in the presence of Ca^{2+} there is no significant dimerization of hGH in solutions [41]. There is not any precise and complete report on the thermodynamics of metal ions binding by hGH in the literature. Now we found that there is a set of three identical and independent binding sites for calcium ion binding on the surface of hGH. The intrinsic dissociation equilibrium constant and the molar enthalpy of binding for calcium ions are 52 μM and –17.4 kJ mol^{-1}, respectively. Calcium ions binding to the surface of the protein may cause some modification to prevent dimerization of hGH.

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

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