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Metal Ions Binding Study on Human Growth Hormone By Isothermal Titration Calorimetric Method

A.A. Saboury* **and M.S. Atri**

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

ABSTRACT

The interaction of hGH with some metal ions (Ca^{2+}, Mg^{2+}, Cu^{2+} and Fe^{3+}) at 27 °C in NaCl solution, 50 mM was studied using Isothermal titration calorimetry. There is a set of three identical and non-interacting binding sites for binding of all these metal ions, except Fe^{3+}. The intrinsic association equilibrium constants (K_a) are not very different for Mg^{2+} ($K_a = 2.17\times10^4$) and Ca^{2+} ($K_a = 1.92\times10^4$), and also their molar enthalpies of binding ($\Delta H°=-17.7$ kJ/mol for Mg^{2+} and $\Delta H°=-17.4$ kJ/mol for Ca^{2+}) are similar showing same thermodynamical properties for hGH upon interaction with both Mg^{2+} and Ca^{2+}. Thermodynamical properties for hGH upon interaction with Cu^{2+} is completely different from those of Mg^{2+} and Ca^{2+}. The affinity of binding is the highest ($K_a = 11.67\times10^4$), but the molar enthalpy of binding is the lowest ($\Delta H°=-16.7$ kJ/mol) for Cu^{2+}. There is a set of four identical and independent binding sites for Fe^{3+}. The binding process for iron ions is more exothermic ($\Delta H°=-18.7$ kJ/mol) than other three metal ions and with a higher affinity ($K_a = 2.50\times10^4$) respect to Mg^{2+} and Ca^{2+}.

Keywords: Isothermal titration calorimetry; human growth hormone; Calcium; Magnesium;Copper; Iron (III).

* Corresponding author: Tel: +98-21-66956984 Fax+ 98-21-66404680

E-mail: saboury@ut.ac.i

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 [6-23], protein denaturation [24-28], kinetic parameters [29-30], enzyme inhibition [31-35] and material stability [36-41].

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 [42-55]. The principle 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 [45]. 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, 56-58]. A graphical fitting simple method for determination of thermodynamic parameters has also been introduced, which has been applied in inhibitor binding on the enzymes [32, 46, 50]. The Scatchard plot [59-61], as a binding isotherm for ligand-protein interaction in a set of identical and independent binding site, can be easily obtained by carrying out two different ITC experiments [51]. A calorimetric data analysis has been also 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 researches in the field of thermodynamic for protein ligand interaction. Today, drug design and synthesis of new compounds for the enzyme inhibition is one of the most important researches in the chemical science [61-81]. ITC data analysis methods can be used in drug-enzyme interaction.

Human growth hormone (hGH) is a polypeptide hormone, which plays an important role in somatic growth through its effects on the metabolism of proteins, carbohydrates, and lipids [82]. hGH is a globular protein containing 191 amino acids (22 kDa) and two disulfide bridges, including single domain combined from four helices [83-87]. There are some reports on the binding properties and structural changes of hGH due to its interaction with metal ions [88-91].

The binding of calcium and magnesium ions changes the secondary structure of the protein, increasing alpha helix content accompanied with decreasing beta and random coil structures. However, the secondary structural change is not permanent, and hGH returns to its native form in the presence of high concentrations of magnesium ion. Calcium and magnesium binding process leads to inaccessibility of aromatic amino acids residues (tryptophan and tyrosine) [92-95]. The circular dichroism spectroscopy study on the protein upon interaction with Cu^{2+} has not shown any changes on the secondary structure of hGH. However, the stability of the protein decreases due to the

binding of copper ions [96]. Binding of iron ions to hGH macromolecule prevents irreversibility and aggregation. Iron binding affects the hydrophobisity of the macromolecule [97-98].

In this paper, the interaction between calcium, magnesium, copper and ferric ions with hGH have been investigated in neutral aqueous solutions to clarify thermodynamics of metal binding properties.

EXPERIMENTAL

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 1-cm quartz cuvettes. An $E^{1\%}$(277 nm)=9.3 was used as reported by Bewley et al [99]. Calcium chloride, magnesium chloride, copper nitrate and ferric chloride were 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 from stainless steel. Metal ion solution was injected by use of a Hamilton syringe into the calorimetric stirred titration vessel, which contained 1.8 ml hGH. Thin (0.15 mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. Injection of metal ion solution into the perfusion vessel was repeated several times, and each injection included specific volume of reagent. The concentration of hGH in the sample cell was 35 μM for both of Mg^{2+} and Ca^{2+} interaction; however, the initial protein concentrations were 10 and 60 μM for Cu^{2+} and 2 and 80 μM for Fe^{3+} in two separate experiments. The volume injected for both of Mg^{2+} (2 mM) and Ca^{2+} (2 mM) was 20 μl and the injection number was 20 for Ca^{2+} and 30 for Mg^{2+}. The volume injected for Cu^{2+} (0.8 mM) was 25 μl and the injection number was 40. The volume injected for Fe^{3+} (1 or 2 mM) was 20 μl and the injection number was 20. The calorimetric signal was measured by a digital voltmeter that was a 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 metal ion 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 metal ion, was injected to the protein solution in the sample cell. The enthalpies of metal ion and protein solutions dilution were subtracted from the enthalpy of hGH-metal ion interaction. The microcalorimeter was frequently calibrated electrically during the course of the study.

RESULTS AND DISCUSSION

Calcium

The raw data obtained from isothermal titration calorimetry of hGH interaction with calcium ion is shown in Fig. 1. Fig. 1a is showing the heat of each injection and Fig. 1b is showing the total cumulative heat 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 versus total concentration of Ca^{2+}, Fig. 2a, or versus total concentration of the protein, Fig. 2b.

For a biomacromolecule that contains g sites capable of binding the ligand with the same dissociation equilibrium constant (K) value, it has been shown [93, 100]:

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

where M_0 is the total biomacromolecule concentration, L_0 is the total ligand concentration, q represents the heat value at a certain L_0, q_{max} represents the heat value upon saturation of all biomacromolecule and $\Delta q = q_{max} - q$. Therefore, the plot of $(\Delta q/q_{max})M_0$ versus $(\Delta q/q)L_0$ 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 related plot for the binding of calcium ions by hGH is showing in Fig. 3. The linearly 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) for q_{max}. The amounts of g and K, obtained from the slope and vertical-intercept plot, are 3 and 52 μM, respectively. If q and q_{max} are calculated per mole of biomacromolecule then the standard molar enthalpy of binding for each binding site (ΔH°) will be $\Delta H° = q_{max}/g$. therefore, ΔH°= −17.4 kJ/mol.

For a set of identical and independent binding sites, we have before shown [46, 50]:

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

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

$$A_i = V_i/2q_i \quad B_i = nM_0 + L_0 \quad C_i = 4nM_0 L_0 \quad (3)$$

where V_i and q_i are the volume of the reaction solution and total cumulative heat (by kJ/mol, 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 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 (2) contains two unknowns, K and ΔH°. A series of reasonable value for K is inserted into equation (2) and corresponding values for ΔH° are calculated and the graph ΔH° versus 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 versus K, according to equation (2), for all injections are shown in Fig. 4. The intersection of curves gives K = 52 μM and ΔH° = −17.4 kJ/mol.

Magnesium

The raw data obtained from isothermal titration calorimetry of hGH interaction with magnesium ion is shown in Fig. 5. Fig. 5 is showing the heat of binding of magnesium ions per mole of hGH versus total concentration of Mg^{2+}, Fig. 5a, or versus total concentration of the protein, Fig. 5b.

The plot of $(\Delta q/q_{max})M_0$ versus $(\Delta q/q)L_0$ for the binding of magnesium ions by hGH is showing in Fig. 6. according to equation (1),The amounts of g and K, obtained from the slope and vertical-intercept plot, are 3 and 46 μM, respectively. Dividing the q_{max} amount of −53.1 kJ/mol by g=3, therefore, gives ΔH°= −17.7 kJ/mol. The plots of ΔH° versus K, according to equation (2), for all injections are shown in Fig. 7. The intersection of curves gives: K = 46 μM and ΔH° = −17.7 kJ/mol.

Copper

The raw data obtained from isothermal titration calorimetry of hGH interaction with copper ion in two different concentrations of the protein is shown in Fig. 8. Fig. 8a is showing the heat of each injection and Fig. 8b 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 hGH ($\Delta H°$) *versus* total concentration of copper ions, Fig. 9a, or *versus* total concentration of the protein, Fig. 9b.

The Scatchard plot as shown in Fig. 10, can be obtained using a simple method of analyzing data, which has previously been used [52-53]. The base of this simple method is that at any constant value of $\Delta H°$, ν, the average number of irons bound to one macromolecule, and $[Cu^{2+}]_f$, the free concentration of iron ion, are also constant at equilibrium. This forms the basis by which one can calculate ν as a function of $[Cu^{2+}]_f$ from a minimum of two titrations. From titrations curves, Fig. 9, performed at two different total concentrations of protein (M_1 and M_2), one determine the set of values of the total ligand concentration (L_1 and L_2) for which $\Delta H°$ is constant. This is done by drawing a horizontal line, defining a constant $\Delta H°$ that intersects both titration curves (hGH concentrations M_1 and M_2), and determining the values of L_1 and L_2 at the points of intersection. One can then calculate ν from equation $\nu=(L_2-L_1)/(M_2-M_1)$. In this way, one can obtain a binding isotherm or the Scatchard plot, $\nu/[Cu^{2+}]_f$ *versus* ν, as shown in Fig. 10. The Scatchard plot according to the equation [59-61]

$$\frac{\nu}{[L]_f} = \frac{1}{K}(g - \nu) \qquad (4)$$

also shows g=3 and K=8.6 μM. Moreover, values of ΔH in different values of ν (obtained from Fig. 9) give the molar enthalpies of binding –16.7 kJ/mol in each binding sites.

Iron (III)

The raw data obtained from isothermal titration calorimetry at two different concentrations of the protein are shown in Fig. 11. Fig. 11a shows the heat of each injection and Fig. 11b shows the cumulative heat at each total concentration of iron ion, $[Fe^{3+}]_t$. The Scatchard plot, $\nu/[Fe^{3+}]_f$ versus ν, as shown in Fig 12, was obtained by the method described previously [52-53]. This Scatchard plot shows g=4 and K=40 μM. A series of reasonable values for K was also inserted into equation (2) and corresponding values for $\Delta H°$ were calculated and the graph $\Delta H°$ versus K was constructed. Curves of all titration steps will intersect in one point, which represents the true value for $\Delta H°$ and K. The plots of $\Delta H°$ versus K for 15 injections are shown in Fig. 13. The K values obtained from two ITC methods agree.

To compare all thermodynamic parameters in metal binding process for hGH, the change in standard Gibbs free energy ($\Delta G°$) should be calculated according to the equation (5), which its value can use in equation (6) for calculating the change in standard entropy ($\Delta S°$) of binding process.

$$\Delta G° = -RT \ln K_a \qquad (5)$$

$$\Delta G° = \Delta H° - T\Delta S° \qquad (6)$$

Where K_a is the association binding constant (the inverse of the dissociation binding constant, K). All thermodynamic parameters for the interaction between hGH and metal ions studied are summarized in Table 2.

Overall, it can be concluded that interaction of calcium and magnesium ions with hGH are very similar; both bind exothermically in a set of three identical and independent binding sites on the surface of hGH. The dissociation equilibrium constants are not very different for Ca^{+2} and Mg^{+2}, and also their standard molar enthalpies of binding are similar showing same thermodynamical properties for hGH upon interaction with both Ca^{+2} and Mg^{+2}. Copper ions bind in a set of three identical and independent binding

sites on the surface of hGH. The binding process is exothermic with the highest affinity for the binding ($K=1.167 \times 10^5$). There is a set of four identical and independent binding sites for iron ions, which corresponds to the number of helices of hGH. The binding process is more exothermic than other metal ions with relatively high affinity ($K=40$ μM) respect to magnesium and calcium ions.

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Table1. Thermodynamic parameters of binding for metal ions to hGH obtained by ITC.

Metal ion	g	K (μM)	K_a (M^{-1})	ΔH° (kJ/mol)	ΔG° (kJ/mol)	ΔS° (J/mol K)
Ca^{2+}	3	52.0	1.92×10^4	−17.4	−24.6	24.0
Mg^{2+}	3	46.0	2.17×10^4	−17.7	−24.9	24.0
Cu^{2+}	3	8.6	11.67×10^4	−16.7	−29.1	41.3
Fe^{3+}	4	40.0	2.50×10^4	−18.7	−25.3	22.0

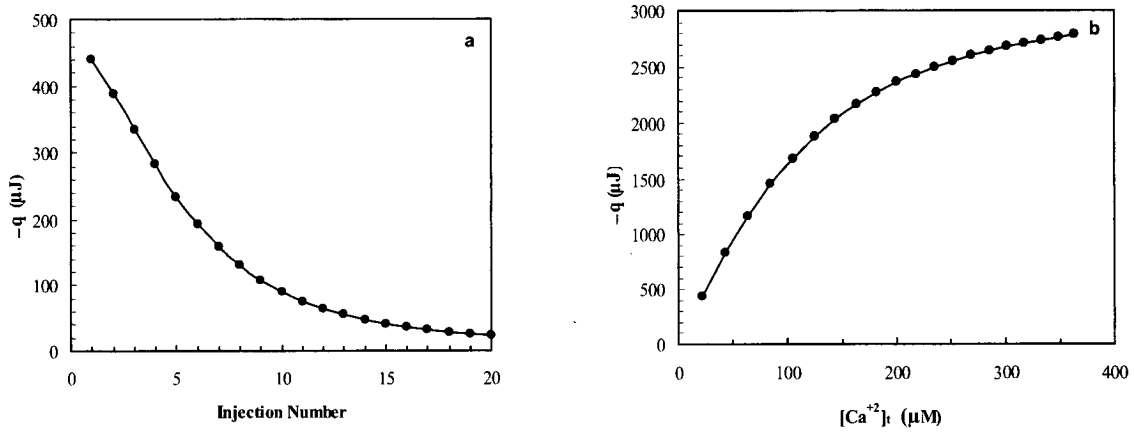

Fig. 1. (a) The heat of calcium binding on hGH for 20 automatic cumulative injections, each of 20 μl, of calcium, 2 mM, into the sample cell containing 1.8 ml hGH solution at initial concentration of 35 μM at 27°C. (b) The total cumulative heat of binding versus total concentration of calcium ion, calculated from Fig. 1a.

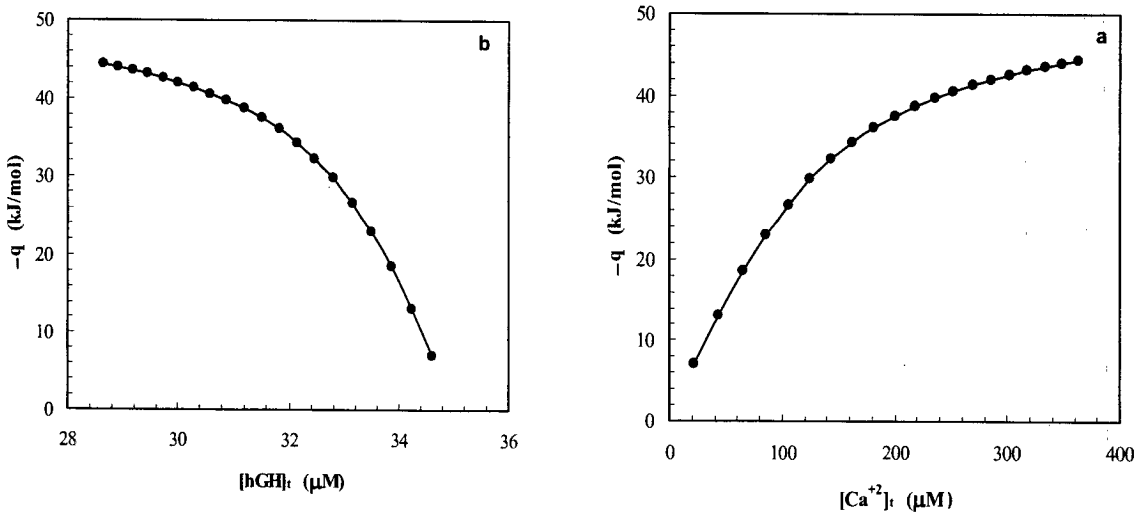

Fig. 2. (a) The heat of binding calcium ions per mole of hGH versus total concentration of calcium ions, calculated from Fig. 1b. (b) The heat of binding calcium ions per mole of hGH versus total concentration of the protein. The initial concentration of hGH was 35 μM.

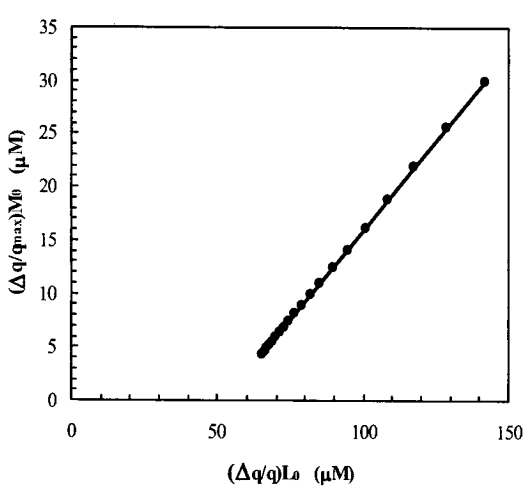

Fig. 3. The best linear plot of $(\Delta q/q_{max})\ M_0$ versus $(\Delta q/q)L_0$ for the binding of calcium ions by hGH, according to the equation (1), using a value of −52.2 kJ/mol for q_{max} to obtain the best correlation coefficient value (R^2=0.99).

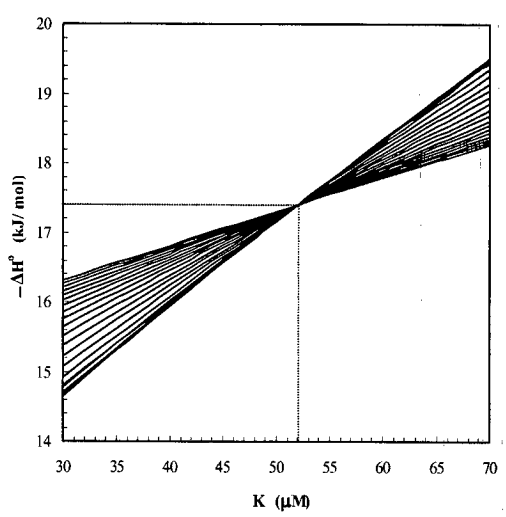

Fig. 4. ΔH° versus K of calcium binding on hGH for all 20 injections in the reasonable values of K, according to equation (2), using data in Fig. 2 . The coordinates of intersection point of curves give true value for ΔH and K

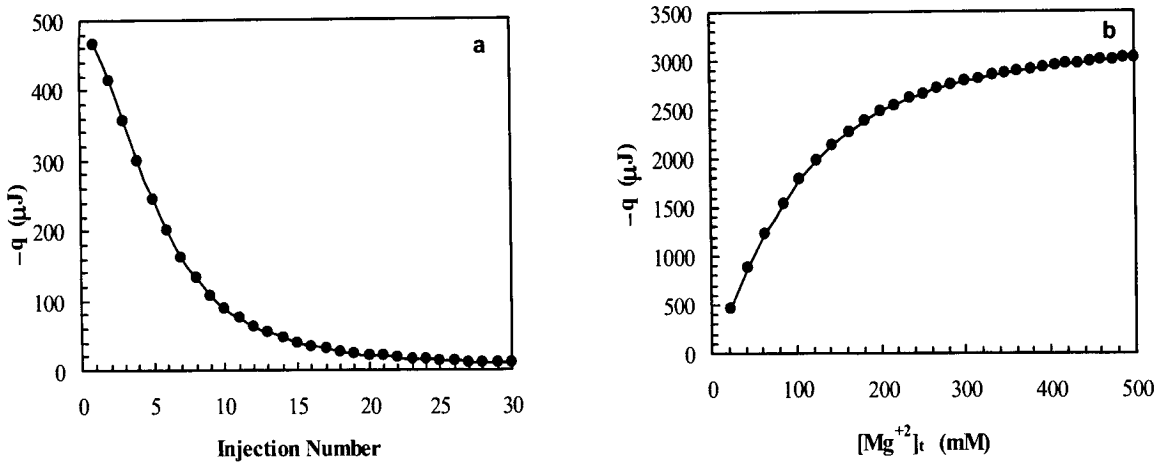

Fig. 5. (a) The heat of binding magnesium ions per mole of hGH versus total concentration of magnesium ions. (b) The heat of binding magnesium ions per mole of hGH versus total concentration of the protein. The initial concentration of hGH was 35 μM. The heat of magnesium binding on hGH obtained for 30 automatic cumulative injections, each of 20 μl, 2 mM, into the sample cell containing 1.8 ml hGH solution at initial concentration of 35 μM at 27°C.

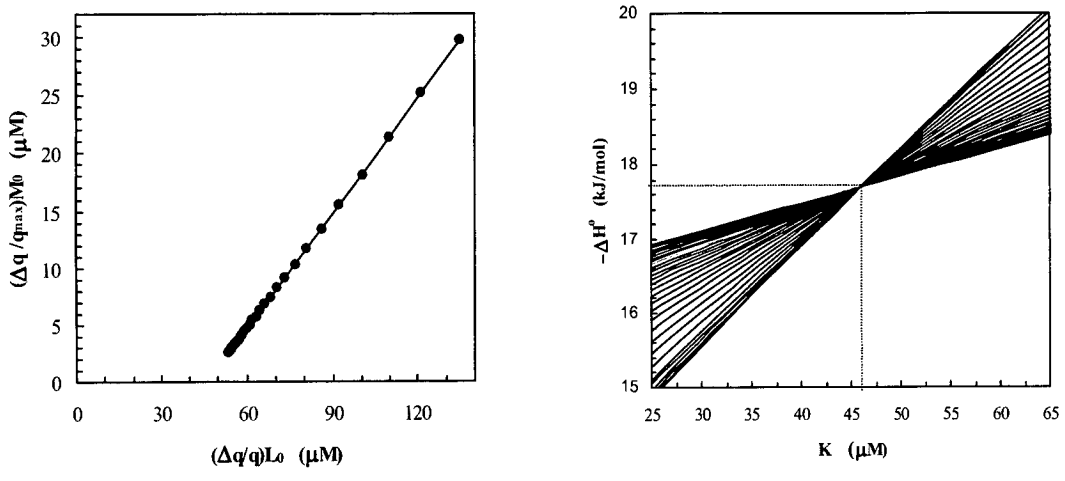

Fig. 6. The best linear plot of $(\Delta q/q_{max})M_0$ versus $(\Delta q/q)L_0$, for the binding of magnesium ions by hGH according to the equation (1), using a value of −3345 μJ (equal to −53.1 kJ/mol) for q_{max} to obtain the best correlation coefficient value (R^2=0.99). Values of g and K can be obtained from the slope and the vertical-intercepts, respectively.

Fig. 7. ΔH° versus K for magnesium binding on hGH for all 30 injections in the reasonable values of K, according to equation (2), using data in Fig. 5. The coordinates of intersection point of curves give true value for ΔH° and K

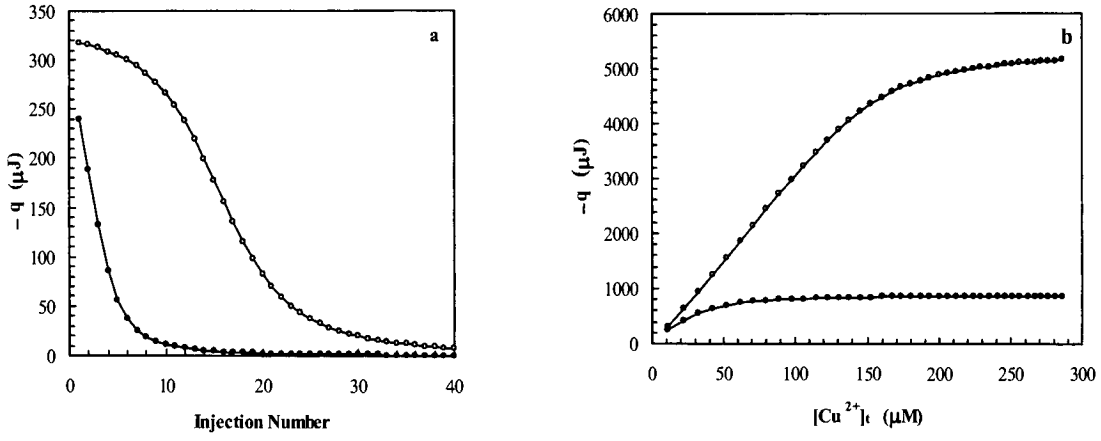

Figure 8. (a) The heat of Cu^{2+} binding on hGH for 40 automatic cumulative injections, each of 25 μl, of Cu^{2+}, 0.8 mM, into the sample cell containing 1.8 ml hGH solution at two initial concentrations of 10 μM (●) and 60 μM (O). (b) The heat of binding *versus* total concentration of copper ion, calculated from Fig. 8a.

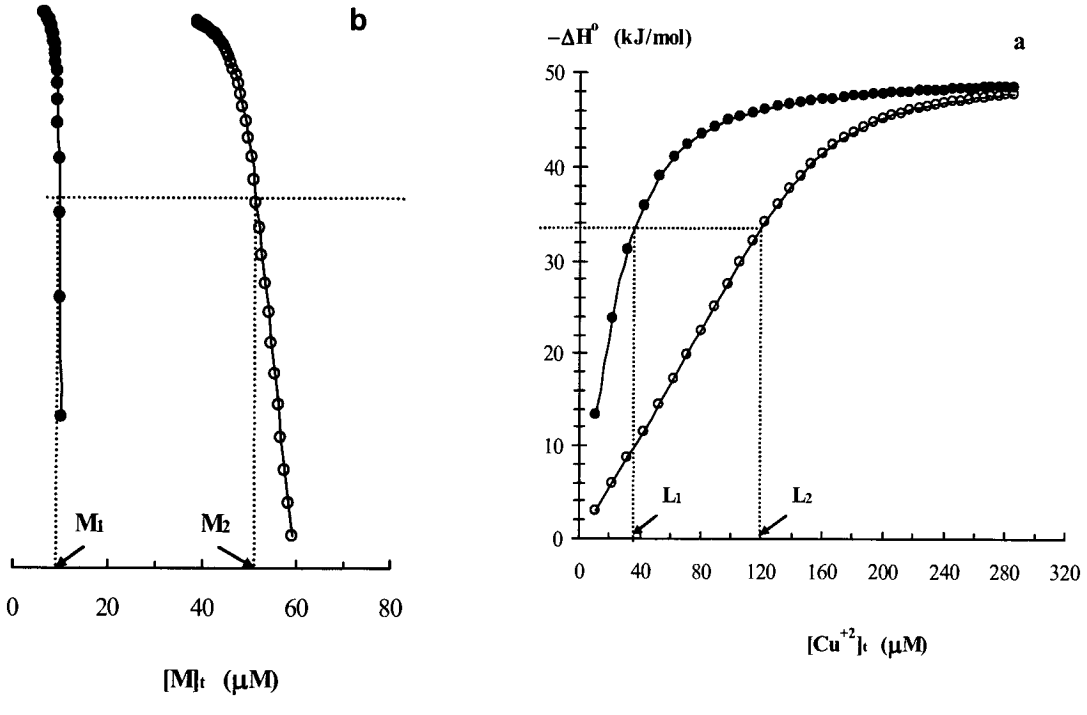

Figure 9. (a) The heat of binding copper ion per mole of hGH (ΔH°) *versus* total concentration of copper ion, calculated from Fig. 8b. (b) The heat of binding copper ion per mole of hGH (ΔH°) *versus* total concentration of hGH. The initial concentration of hGH was 10 μM (●) and 60 μM (O).

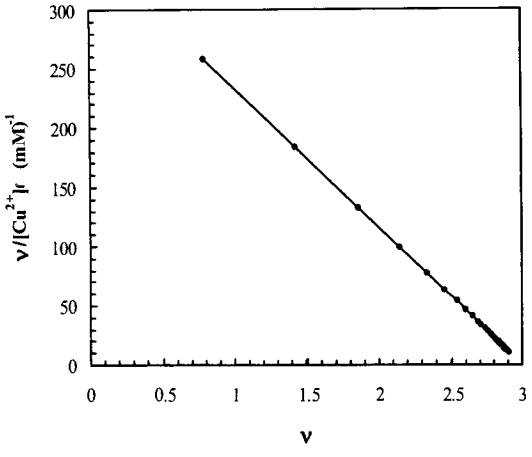

Figure 10. The Scatchard plot for binding of Cu^{2+} by hGH at 27°C based on the isothermal titration calorimetric data. The best-fit curve of the experimental binding data was transformed to the Scatchard plot using the Scatchard equation ($\nu = gK[Cu^{2+}]_f/(1+K_a[Cu^{2+}]_f)$) with g=3 and $K_a=11.63 \times 10^4\ M^{-1}$.

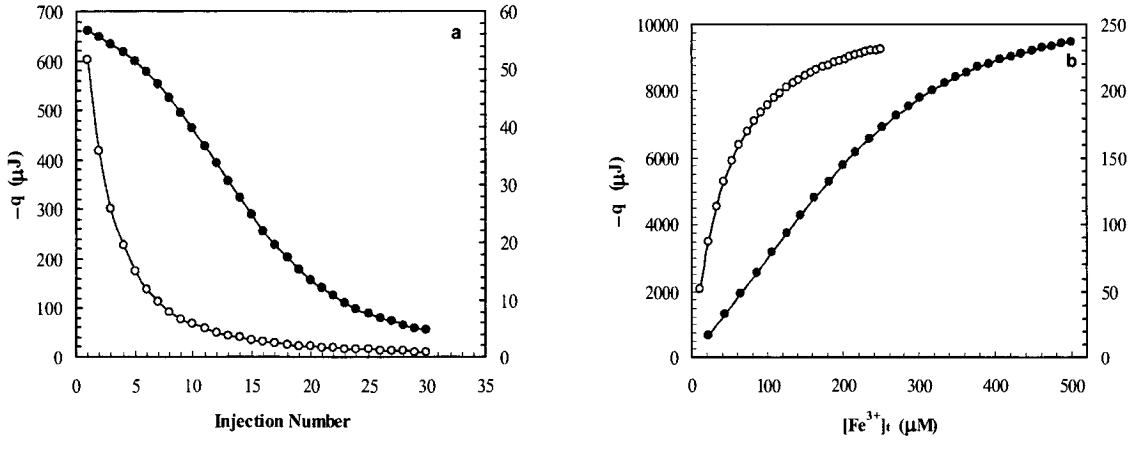

Figure 11. (a) The heat of Fe^{3+} binding on hGH for 30 automatic cumulative injections, each of 20 μl, of iron, 1 or 2 mM, into the sample cell containing 1.8 ml hGH solution at two initial concentrations of 1 μM (O) and 80 μM (●). (b) The cumulative heat of binding *versus* total concentration of Fe^{3+}, calculated from Fig. 2a. The left vertical axis is for (●) and the right vertical axis is for (O). The left vertical axis is for 1 μM (O) and right is for 80 μM (●)

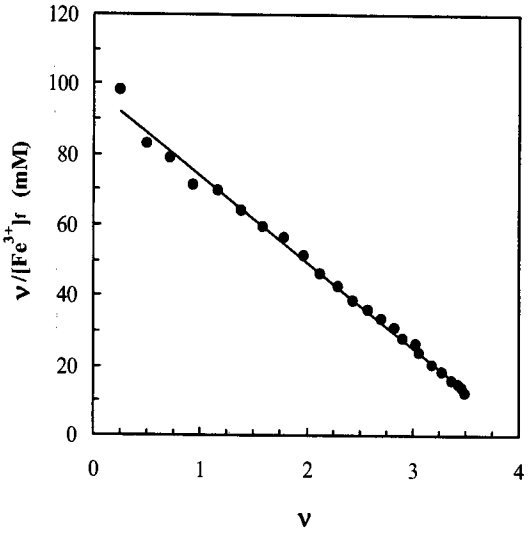

Figure 12. The Scatchard plot of binding iron ion by hGH at 27 °C using ITC data analysis. The best-fit curve of the experimental binding data was transformed to the Scatchard plot using Eq. (4) with g=4 and K=40 μM.

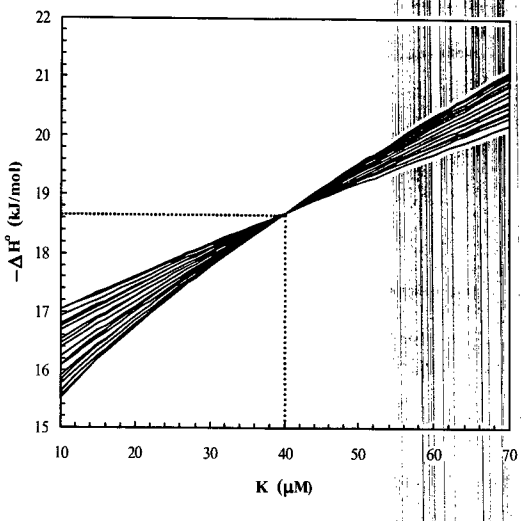

Figure 13. ΔH° versus K for all 15 injections in the reasonable values of K, according to equation (2), using data in Fig. 3 in the initial concentration of 80 μM (●) for hGH. The coordinates of intersection point of curves give true value for ΔH° and K