

## A CALORIMETRIC STUDY ON THE INTERACTION OF ZINC ION WITH HUMAN GROWTH HORMONE\*

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**Abstract** – A thermodynamic study on the interaction between zinc ion ( $Zn^{2+}$ ) and human growth hormone (hGH) was studied at two temperatures of 27°C and 37°C in aqueous solution using an isothermal titration calorimetry. It was found that there is a set of three identical and non-interacting binding sites for  $Zn^{2+}$  ions. The intrinsic dissociation equilibrium constant and the molar enthalpy of binding are 1.54 mM and  $-17.6$  kJ mol<sup>-1</sup> at 27°C and 1.93 mM and  $-17.1$  kJ mol<sup>-1</sup> at 37°C, respectively. To reproduce the binding parameters of metal ion–hormone interaction over the whole range of  $Zn^{2+}$  concentrations a solvation theory was applied. The binding parameters deduced from the solvation model were attributed to the structural change of hGH and its biological activity due to the metal ion interaction.

**Keywords** – Human growth hormone (hGH), Isothermal titration calorimetry (ITC), Solvation model, Zinc ion, Metal binding, Interaction, Ligand binding, Biothermodynamics, Enthalpy of binding, Intrinsic equilibrium constant

### 1. INTRODUCTION

Human Growth Hormone, hGH, as a single domain globular protein containing 191 amino acids, plays an important role in somatic growth through its effects on the metabolism of proteins, carbohydrates and lipids [1-3]. hGH is produced recombinantly and is available worldwide for clinical use. It has a limited stability in aqueous solution. Development has therefore focused on more stable formulations and understanding on its interaction with ligands. A more stable variant of hGH can improve pharmacokinetics or enhanced shelf-life, or be more amenable to use in alternate delivery systems and formulation. The native state of hGH is stable and does not undergo significant conformational changes between pH 2 and 11 [4]. The protein denaturation occurs when a polypeptide loses its higher level of structure, and leads to aggregation. The most common mechanism of protein aggregation is believed to involve protein denaturation, via hydrophobic interfaces and often results in loss of biological activity [5-9]. The thermal transition is highly sensitive to pH changes, which suggests that the unfolding is coupled to the protonation of carboxyl groups. In most cases, these partially folded conformations are stabilized at acidic pH values, mild concentrations of denaturants or extreme salt concentrations [10]. The thermal denaturation of recombinant human growth hormone (rhGH) is reversible only below pH 3.5, and under these conditions a single two-state transition was observed between 0 °C and 100 °C. The hormone aggregation is prevented in aqueous solutions of alcohols such as n-propanol, 2-propanol, or 1,2-propanediol (propylene glycol), which suggests that the self-association is caused by hydrophobic

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interactions [11]. There are some reports on the binding properties and structural changes of hGH due to its interaction with metal ions [12-21]. The metal-binding site in hGH is located in the hydrophobic core. A well-resolved crystal structure of hGH has been obtained, showing that metal-binding site is likely composed of  $^{18}\text{His}$  and  $^{21}\text{His}$  on helix I and  $^{174}\text{Glu}$  on helix IV [15]. There is a set of three identical and noninteracting binding sites for both  $\text{Ca}^{2+}$  [16-17] and  $\text{Mg}^{2+}$  [18-19] ions. Both  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions binding to hGH increase the protein thermal stability by increasing the alpha helix content as well as decreasing both beta and random coil structures [16, 18]. There is a set of four binding sites on hGH for  $\text{Fe}^{3+}$  [20]. Interaction of the first three iron ions with hGH prevents irreversibility and aggregation. Interaction of  $\text{Co}^{2+}$  with hGH, in a set of three identical and independent binding sites, prevents protein aggregation by an affect on the hydrophobicity of the macromolecule [21]. Some metal ions like  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Hg}^{2+}$  and  $\text{Co}^{2+}$  are known to promote hGH reversible dimerization. But in the presence of some other ions like  $\text{Ca}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Al}^{2+}$ ,  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$ , there is no significant dimerization of hGH in solutions [13].

There are more reports on the zinc ion interaction than on other metal ions. Zinc ion has been shown to be important for the function of growth hormones [22-23]. For example,  $\text{Zn}^{2+}$  has been demonstrated to enhance the activity of human growth hormone (hGH) in a cell line based biological assay. Zinc ion has also been demonstrated to induce dimerization of hGH, and the resulting  $\text{Zn}^{2+}$ -hGH dimer has been proposed as the major storage form of hGH in vivo. Mutational analysis indicated that  $\text{His}^{18}$ ,  $\text{His}^{21}$ , and  $\text{Glu}^{174}$  participate in coordinating  $\text{Zn}^{2+}$  and promoting formation of the hormone dimer [14]. Moreover, zinc-protein precipitates may be useful for protein purification, storage, and formulation [14]. Precipitation of hGH by zinc does not alter the secondary structure of hGH, and the process is fully reversible [14]. Zinc binding induces only minor tertiary structural changes to the protein. Zinc ions binding to hGH leads to increasing the hormone stability [22]. However, there is no exact report on the binding of  $\text{Zn}^{2+}$  to hGH quantitatively. In this paper, the interaction between  $\text{Zn}^{2+}$  and hGH has been investigated in neutral aqueous solution to clarify the thermodynamics of  $\text{Zn}^{2+}$  binding properties. One of the unique aspects of this report is the use of a new solvation model to attempt to clarify the stability of protein. The extended solvation method is able to correlate the binding parameters to the effect of metals on the stability of protein in a very simple way.

## 2. MATERIALS AND METHODS

Highly purified preparations of hGH were provided by the National Research Center of Genetic Engineering and Biotechnology (NRCGEB), Tehran, Iran. Protein concentrations were determined from absorbance measurements at 277 nm in 1 cm quartz cuvettes. Zinc nitrate was purchased from Merck Co. (Darmstadt, Germany). All other materials and reagents were of analytical grade, and solutions were made in 50 mM NaCl using double-distilled water. The isothermal titration microcalorimetric experiments were performed with the four channel commercial microcalorimetric system, Thermal Activity Monitor 2277, Thermometric, Sweden. The titration vessel was made from stainless steel. Zinc solution (100 mM) was injected by use of a Hamilton syringe into the calorimetric titration vessel, which contained 1.8 mL hGH (60  $\mu\text{M}$ ). Thin (0.15 mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. Injection of Zinc solution into the perfusion vessel was repeated 30 times, with 20  $\mu\text{L}$  per injection. 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 ( $q_{\text{dilut}}$ ) of the  $\text{Zn}^{2+}$  solution was measured as described above, except hGH was excluded. All measured heat values have been given in Table 1 and 2. The microcalorimeter was frequently calibrated electrically during the course of the study. The molecular weight of hGH was taken to be 22 kDa [23].

Table 1. Enthalpies of Zn<sup>2+</sup>+hGH interaction at T=300 K in 50 mM NaCl solution

q / $\mu\text{J}$	q <sub>dilut</sub> / $\mu\text{J}$	[hGH] / $\mu\text{M}$	[Zn <sup>2+</sup> ] / $\mu\text{M}$
-2282.3	-564.2	59.340	1098.901
-3271.8	-1050.3	58.695	2173.913
-3813.6	-1447.6	58.064	3225.806
-4153.6	-1786.2	57.446	4255.319
-4386.3	-2076.5	56.842	5263.158
-4555.3	-2312.6	56.25	6250
-4683.6	-2519.1	55.670	7216.495
-4784.3	-2696.7	55.102	8163.265
-4865.4	-2845.3	54.545	9090.909
-4932.1	-2966.4	54	10000
-4987.9	-3078	53.465	10891.09
-5035.3	-3175.8	52.941	11764.71
-5076.1	-3264	52.427	12621.36
-5111.5	-3336.4	51.923	13461.54
-5142.6	-3403.3	51.428	14285.71
-5170.1	-3460.8	50.943	15094.34
-5194.6	-3514.3	50.467	15887.85
-5216.5	-3562.9	50	16666.67
-5236.2	-3607.5	49.541	17431.19
-5254.1	-3647	49.090	18181.82
-5270.4	-3682	48.648	18918.92
-5285.3	-3712.9	48.214	19642.86
-5299	-3741.5	47.787	20353.98
-5311.6	-3768.2	47.368	21052.63
-5323.2	-3792.5	46.956	21739.13
-5334	-3813.68	46.551	22413.79
-5344	-3832.78	46.153	23076.92
-5353.3	-3848.68	45.762	23728.81
-5362	-3863.58	45.378	24369.75
-5370.1	-3876.48	45	25000

Table 2. Enthalpies of Zn<sup>2+</sup>+hGH interaction at T=310 K in 50 mM NaCl solution

q / $\mu\text{J}$	q <sub>dilut</sub> / $\mu\text{J}$	[hGH] / $\mu\text{M}$	[Zn <sup>2+</sup> ] / $\mu\text{M}$
-1936.1	-506.4	59.340	1098.901
-2875.5	-941.7	58.695	2173.913
-3422.2	-1297.7	58.064	3225.806
-3778.1	-1600.7	57.446	4255.319
-4027.7	-1860.4	56.842	5263.158
-4212.3	-2071.7	56.250	6250
-4354.2	-2256.5	55.670	7216.495
-4466.7	-2415.4	55.102	8163.265
-4558	-2549.3	54.545	9090.909
-4633.6	-2657.9	54.000	10000
-4697.3	-2757.4	53.465	10891.09
-4751.6	-2844.9	52.941	11764.71
-4798.5	-2923.8	52.427	12621.36
-4839.4	-2988.6	51.923	13461.54
-4875.3	-3048.2	51.428	14285.71
-4907.2	-3099.7	50.943	15094.34
-4935.6	-3147.6	50.467	15887.85
-4961.1	-3191.1	50.000	16666.67
-4984.1	-3231	49.541	17431.19
-5005	-3266.1	49.090	18181.82
-5024.1	-3297.4	48.648	18918.92

Table 2. (Continued)

q / $\mu\text{J}$	$q_{\text{dilut}} / \mu\text{J}$	[hGH] / $\mu\text{M}$	$[\text{Zn}^{2+}] / \mu\text{M}$
-5041.5	-3325.1	48.214	19642.86
-5057.5	-3350.8	47.787	20353.98
-5072.3	-3593.9	47.368	21052.63
-5086	-3615.6	46.956	21739.13
-5098.7	-3634.5	46.551	22413.79
-5110.5	-3651.6	46.153	23076.92
-5121.5	-3666	45.762	23728.81
-5131.8	-3679.3	45.378	24369.75
-5141.4	-3690.9	45.000	25000

### 3. RESULTS AND DISCUSSION

The raw data obtained from ITC at two temperatures of 27°C and 37°C are shown in Fig. 1. Figure 1a shows the heat of each injection and Fig. 1b shows the cumulative heat at each total concentration of zinc ion,  $[\text{Zn}^{2+}]_t$ . For a set of identical and independent binding sites, three different methods of ITC data analysis have previously been shown [24]. According to the recent data analysis method, using Eq. (1), a 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  $K_d/g$ , in which  $g$  and  $K_d$  can be obtained [18, 24].

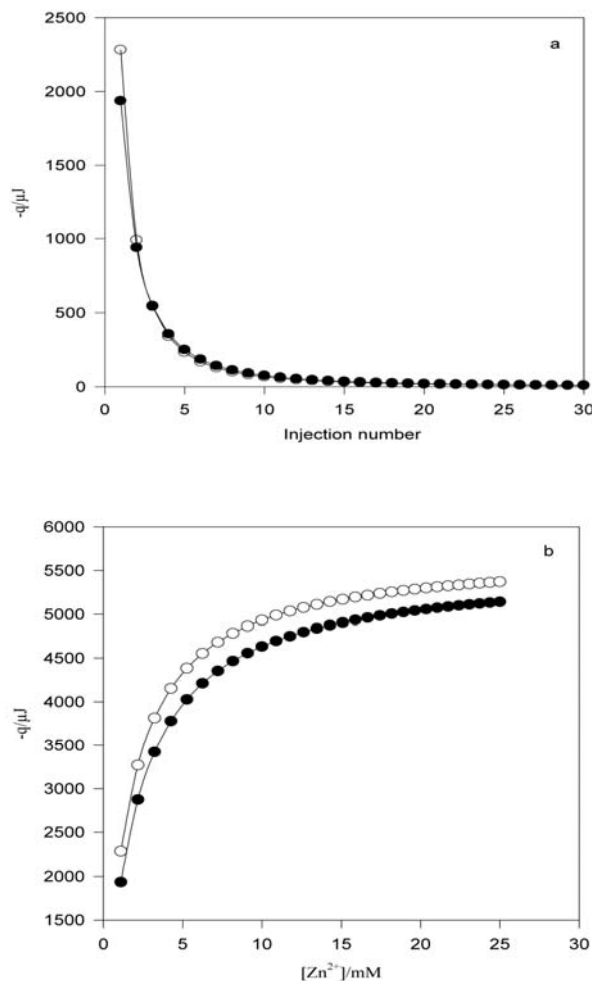


Fig. 1. a–The heat of zinc binding on hGH for 30 automatic cumulative injections, each of 20  $\mu\text{l}$ , 100 mM of the cation solutions, into a sample cell containing 1.8 ml 60 $\mu\text{M}$  hGH solution at 300K (○) and 310K (●).  
 b–The total cumulative heat of binding vs. total concentration of zinc ion, calculated from Fig. 1a

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

where  $g$  is the number of binding sites,  $K$  is the dissociation equilibrium constant,  $M_0$  and  $L_0$  are total concentrations of the biomacromolecule and metal ion, respectively,  $\Delta q = q_{\max} - q$ ,  $q$  represents the heat value at a certain  $L_0$  and  $q_{\max}$  represents the heat value upon saturation of all biomacromolecules. The related plot for the binding of  $Zn^{2+}$  ions by hGH is shown in Fig. 2. The linearity 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 ( $R^2$ ) value (near to one) was obtained using  $-5702 \mu J$  and  $-5540 \mu J$  (equal to  $-52.8 \text{ kJ/mol}$  and  $-51.3 \text{ kJ/mol}$ ) at  $27^\circ C$  and  $37^\circ C$ , respectively, for  $q_{\max}$

(Fig. 2). The lack of a suitable value for  $q_{\max}$  to obtain a linear plot of  $\left(\frac{\Delta q}{q_{\max}}\right) M_0$  vs.  $\left(\frac{\Delta q}{q}\right) L_0$  may be

related to the existence of non-identical binding sites or the interaction between them. The value of  $g$  is 3, obtained from the slope and values of  $K$ , obtained from the vertical-intercept plot for a set of identical and independent binding sites,  $1.54 \text{ mM}$  and  $1.93 \text{ mM}$  at  $27^\circ C$  and  $37^\circ C$ , respectively. Dividing the  $q_{\max}$  amount of  $-52.8 \text{ kJ/mol}$  and  $-51.3 \text{ kJ/mol}$  by  $g=3$ , therefore, gives  $\Delta H = -17.6 \text{ kJ/mol}$  and  $-17.1 \text{ kJ/mol}$  at  $27^\circ C$  and  $37^\circ C$ , respectively. Another calorimetric method described recently allows the number of binding sites ( $g$ ), the molar enthalpy of the binding site ( $\Delta H$ ), and the dissociation equilibrium constant ( $K$ ) for a set of biomacromolecule binding sites to be obtained via a simple graphical nonlinear fitting method. For a set of identical and independent binding sites, we have shown before [24-27].

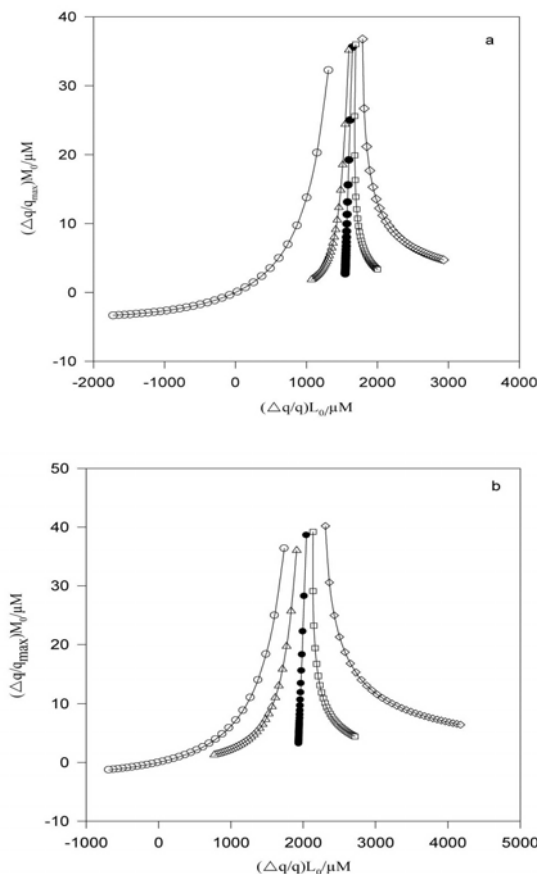


Fig. 2. The best linear plot of  $\left(\frac{\Delta q}{q_{\max}}\right) M_0$  vs.  $\left(\frac{\Delta q}{q}\right) L_0$ , according to Eq. (1), using values of  $-6000 \mu J$  ( $\diamond$ ),  $-5800 \mu J$  ( $\square$ ),  $-5000 \mu J$  ( $\circ$ ),  $-5400 \mu J$  ( $\Delta$ ),  $-5702 \mu J$  and  $-5540 \mu J$  ( $\bullet$ ), at  $300K$  (a) and  $310K$  (b), respectively for  $q_{\max}$  to obtain the best correlation coefficient value ( $R^2=0.999$ ) for a linear plot

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

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

$$A_i = V_i / 2Q_i \quad B_i = gM_i + L_i \quad C_i = 4gM_iL_i \quad (3)$$

where  $V_i$  is the volume of the reaction solution in the calorimetric sample cell in each injection step.  $M_i$  is the total hGH concentration, and  $L_i$  is the total  $Zn^{2+}$  concentration in the calorimetric sample cell in each injection step. Equation (2) contains two unknown parameters,  $K$  and  $\Delta H$ . A series of reasonable values for  $K$  is inserted into equation (2) and corresponding amounts for  $\Delta H$  are calculated and the graph  $\Delta H$  versus  $K$  is constructed. Curves of all titration steps will intersect in one point, which represents true amounts for  $\Delta H$  and  $K$ . The plots of  $\Delta H$  versus  $K$ , according to Eq. (2) for all injections are shown in Fig. 3. The intrinsic dissociation equilibrium constant and the molar enthalpy of binding are  $1540 \mu M$ ,  $-17.6 \text{ kJmol}^{-1}$  and  $1930 \mu M$ ,  $-17.1 \text{ kJmol}^{-1}$  at  $27^\circ C$  and  $37^\circ C$ , respectively (Fig. 3).

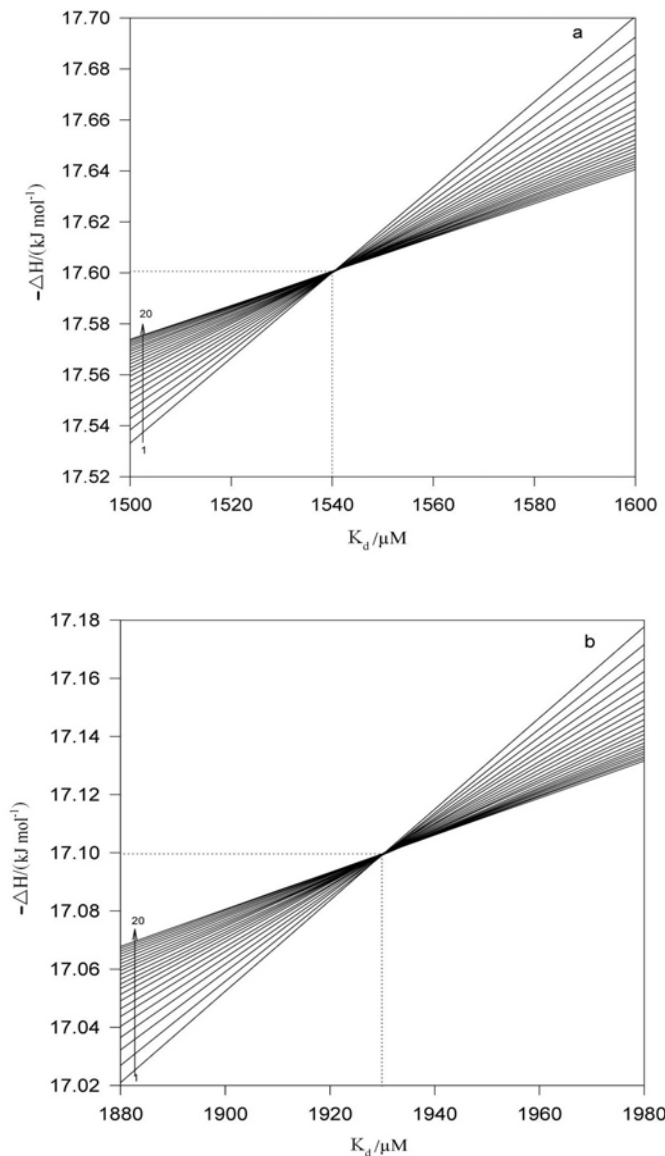


Fig. 3.  $\Delta H$  versus  $K$  for the first 20 injections (injection numbers 1 to 20), at 300 K (a) and 310 K (b) using data in Fig.1b. The coordinates of the intersection point of the curves give the true values for  $\Delta H$  and  $K$

It has been shown previously [28-35] that the enthalpies of interactions of biopolymers with ligands (hGH and  $Zn^{2+}$  in this case) in the aqueous solvent mixtures, can be reproduced via the following equation:

$$q = q_{max}x'_B - \delta'_A(x'_A L_A + x'_B L_B) - (\delta'_B - \delta'_A)(x'_A L_A + x'_B L_B)x'_B \quad (4)$$

$q$  is the heat of  $Zn^{2+}$ +hGH interaction and  $q_{max}$  represents the heat value upon saturation of all hGH. The parameters  $\delta'_A$  and  $\delta'_B$  are the indexes of hGH stability in the low and high  $Zn^{2+}$  concentrations, respectively.  $x'_B$  can be expressed as follows [36-38]:

$$x'_B = \frac{px_B}{x_A + px_B} = \frac{v}{g} \quad (5)$$

$x_B$  is the fraction of the metal ion needed for saturation of the binding sites, and  $x_A = 1 - x_B$  is the fraction of unbounded  $Zn^{2+}$ . The parameter value of  $p$  is related to the solvation process [36].  $p < 1$  or  $p > 1$  indicate a preferential solvation of hGH or  $Zn^{2+}$ , respectively;  $p = 1$  indicates random solvation. The solvation parameters recovered from Eq. 4 are shown in Table 3. Now the model is a simple mass action treatment, with metal ions replacing water molecules at the binding sites in the present case. We can express  $x_B$  fractions, as the total  $Zn^{2+}$  concentrations divided by the maximum concentration of the  $Zn^{2+}$  upon saturation of all hGH as follows:

Table 3. Binding parameters for  $Zn^{2+}$ +hGH interaction in 50 mM NaCl solution

Parameters	hGH+ $Zn^{2+}$ (T=300 K)	hGH+ $Zn^{2+}$ (T=310 K)
$\delta'_A$	-0.982	-1.006
$\delta'_B$	1.827	0.619
$K$ /mM	1.54	1.93
$g$	3	3
$p$	0.56	0.38
$\Delta H_{max}$ /kJ mol <sup>-1</sup>	-17.6	-17.1

$$x_B = \frac{[Zn^{2+}]_T}{[Zn^{2+}]_{max}}, \quad x_A = 1 - x_B \quad (6)$$

$[Zn^{2+}]_T$  is the total concentration of metal ions and  $[Zn^{2+}]_{max}$  is the maximum concentration of the  $Zn^{2+}$  upon saturation of all hGH. In general, there will be "g" sites for binding  $Zn^{2+}$  per hGH molecule and  $v$  is defined as the average moles of bound  $Zn^{2+}$  per mole of total hGH.  $L_A$  and  $L_B$  are the relative contributions due to the fractions of unbounded and bounded metal ions in the enthalpies of dilution in the absence of hGH and can be calculated from the enthalpies of dilution of  $Zn^{2+}$  in buffer,  $q_{dilat}$ , as follows:

$$L_A = q_{dilat} + x_B \left( \frac{\partial q_{dilat}}{\partial x_B} \right), \quad L_B = q_{dilat} + x_A \left( \frac{\partial q_{dilat}}{\partial x_B} \right) \quad (7)$$

With simple modification of Eq. 4, it is possible to use this equation to reproduce the enthalpies of metal-macromolecules interactions.  $\Delta H = q$ , is the enthalpy of  $Zn^{2+}$ -hGH interaction and  $\Delta H_{max} = q_{max}$ , represents the enthalpy value upon saturation of all hGH. The enthalpies of  $Zn^{2+}$ +hGH interactions,  $\Delta H$ , were fitted

to Eq. 4 across the whole  $Zn^{2+}$  composition (Fig. 4). This procedure, the only adjustable parameter ( $p$ ) was changed until the best agreement between the experimental and calculated data was achieved.  $\delta_A^\theta$  and  $\delta_B^\theta$  parameters have also been optimized to fit the data. The optimized  $\delta_A^\theta$  and  $\delta_B^\theta$  values are recovered from the coefficients of the second and third terms of Eq. 4. The small relative standard coefficient errors and the high  $R^2$  values (0.99999) support the method. The Gibbs energies, as a function of  $Zn^{2+}$  concentrations, can be obtained as follows:

$$\Delta G^\circ = -RTL \ln K_a \tag{8}$$

Where  $R$  is the universal gas constant,  $T$  is the absolute temperature and  $K_a$  is the association equilibrium constant ( $1/K$ ) as a function of  $Zn^{2+}$  concentrations. Gibbs energies,  $\Delta G^\circ$ , calculated from Eq. 8 have been shown graphically in Fig. 4.  $\Delta S^\circ$  values were calculated using  $\Delta G^\circ$  values (Eq. 8) and  $\Delta H^\circ$  values (Eq. 4) using equation (9):

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \tag{9}$$

The less negative Gibbs free energies in the low  $Zn^{2+}$  concentrations (Fig. 4) indicate the lower affinity in this region. In Fig. 4,  $[Zn]^{2+}$  is the total concentration of  $Zn^{2+}$  in mM. As it is obvious, the  $Zn^{2+}$  induced structural changes of hGH in the enthalpies and entropies of the interactions, have canceled each other exactly in the free energies of interaction. The compensation of the structural change in the free energy is another support for Eq. 4.

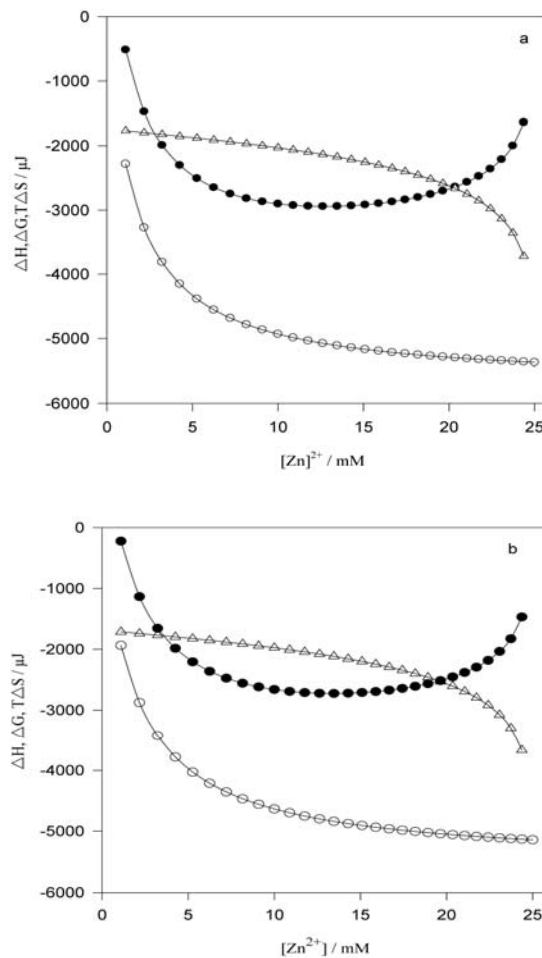


Fig. 4. Comparison between the experimental  $\Delta H$  ( $\circ$ ),  $\Delta G$  ( $\triangle$ ) and  $T\Delta S$  values ( $\bullet$ ) for hGH+  $Zn^{2+}$  interaction and their calculated data (lines) via Eq. 4. at 300 K(a) and 310 K(b)



The  $\delta_A^\theta$  value for hGH+Zn<sup>2+</sup> interaction is negative via Eq. 4, (Table 3), indicating that in the low concentration of the zinc ion the hGH structure was destabilized, resulting in a decrease in its biological activity. Destabilization by a ligand indicates that the ligand binds preferentially denatured protein or to a partially folded intermediate form of the protein. Such effects are characteristic of nonspecific interactions, in that the nonspecific ligand binds weakly to many different groups at the protein/water interface, so that binding becomes a function of the ligand concentration and the available solvent-exposed protein surface area, which is increased through unfolding events and agrees with the results of the microcalorimetric technique that has been shown, The intrinsic dissociation equilibrium constants are rather small, implying that there is a non-specific interaction.  $\delta_B^\theta$  value for hGH+Zn<sup>2+</sup> interaction is positive (Table 3), indicating that in the high concentration of the zinc ion the hGH structure was stabilized. Also, previous reports indicate that the inclusion of a divalent metal ion such as zinc, cobalt or copper, preferably zinc, into an hGH formulation results in the formation of stable zinc+hGH dimmers that exhibit unexpected stability to denaturation and maintain the activity of hGH long periods at temperatures up to and beyond 25°C-37°C. Our results have very good agreement with previous studies that indicate some metal ions binding increase the hGH thermal stability by increasing the alpha helix content as well as decreasing both beta and random coil structures [16, 18, 21-22].

*p* values are less than one (*p*=0.56 and *p*=0.38) at two temperatures, indicating that zinc ions bind preferentially to hGH. There is a report that the Zn<sup>2+</sup>+hGH dimer is significantly more stable to denaturation than monomeric hGH [12]. However, in high concentrations of metal ion each biomacromolecule receives three zinc ion (*g*=3).

In conclusion, there is a set of three identical and non-interacting binding sites for zinc ion to hGH. Operationally it has been confirmed that the extended coordination model, via equation 4 will satisfactorily reproduce the enthalpies of Zn<sup>2+</sup>-hGH interaction. Analysis of these in terms of the new extended coordination model confirms the model's ability. Prediction of biological activity of proteins, structural changes of macromolecules along with binding enthalpies and the associated binding constant, make this theory the most powerful one.

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