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Thermodynamic Study on the Interaction of Copper Ion with Human Growth Hormone

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Abstract A thermodynamic study on the interaction between the Cu^{2+} ion and human growth hormone (hGH) was studied at the temperatures 300.15 and 310.15 K in NaCl solution using isothermal titration calorimetry. The new solvation model was used to reproduce the enthalpies of Cu^{2+} + hGH interaction over the whole range of Cu^{2+} concentrations. It was found that there is a set of three identical and non-interacting binding sites for Cu^{2+} ions. The intrinsic dissociation equilibrium constant and the molar enthalpy of binding are 1313.4 μmol·L^{-1} and −16.80 kJ·mol^{-1} at 300.15 K, and 1648.2 μmol·L^{-1} and −16.40 kJ·mol^{-1} and 310.15 K, respectively. The binding parameters recovered from the new equation are attributed to a structural change of hGH and its biological activity due to metal ion interaction.

Keywords Isothermal titration calorimetry · Human growth hormone (hGH) · Solvation model

1 Introduction

Human growth hormone (hGH) is a polypeptide hormone, essential for normal growth and development, that is secreted by the anterior pituitary gland. Circulating hGH consists of a heterogeneous mixture of proteins including a predominant 22 kDa hGH isoform, and other

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less abundant variants such as the 20 kDa hGH [1, 2]. Therapeutically, hGH is employed in children to treat growth retardation; examples include short stature due to insufficient growth hormone secretion, Turner's syndrome or chronic renal insufficiency. In adults, it is used as a treatment for growth hormone deficiency and for management of HIV-related wasting and cachexia [3, 4].

HGH is produced recombinantly and is available worldwide for clinical use. It has limited stability in solution. Development has therefore focused on more stable formulations and understanding on its interaction with ligands. A more stable variant of hGH could have improved pharmacokinetics or enhanced shelf-life, or be more amenable to use in alternate delivery systems and formulations [4–6]. The well-resolved crystal structure of hGH has been obtained, showing that the metal-binding sites are likely composed of ^{18}His and ^{21}His on helix I and ^{174}Glu and helix IV [7]. For example zinc binding to residues 18 and 174 of hGH constrains the articulation of helices 1 and 4 and stabilizes the hGH structure [5]. The secondary structure of hGH was unperturbed in soluble zinc complexes and zinc-induced precipitates, as measured by infrared and circular dichroism spectroscopy. The soluble zinc complex of recombinant human growth hormone (rhGH) had minor tertiary structural alterations that are is essential for its activity [8, 9].

Metal binding sites in human growth hormone are located in the hydrophobic core. The interaction of hGH with some of divalent metal ions (Ca^{2+} and Cu^{2+}) in aqueous solution has been studied using different techniques. The binding isotherm for hGH + metal ion was obtained by two techniques: potentiometric, using a metal-selective membrane electrode, and isothermal titration calorimetry. The circular dichroism spectroscopy study on the protein interacting with Cu^{2+} did not show any changes on the secondary structure of hGH. However, the stability of the protein decreases due to the binding with copper ions. The importance of metal ions such as Zn^{2+} and Cd^{2+} in determining the stability of proteins has been widely reported. Calcium ion binding increases the protein thermal stability by increasing the alpha helix content as well as decreasing both beta and random coil structures [10–16]. Reports indicate that some metal ions like Zn^{2+}, Cd^{2+}, Hg^{2+} and Co^{2+} promote reversible hGH dimerization [15–17]. Also, other studies have shown that dimerization of hGH is initiated by other metal ions such as Cd^{2+}, Hg^{2+}, Cu^{2+}, Ag^{+}, Au^{3+}, Au^{+}, Pd^{2+}, Ni^{2+}, and Pt^{4+}. In some cases (Hg^{2+}, Ag^{+}, Au^{3+}, and Ni^{2+}) formation of higher oligomers also took place [20]. However, in the presence of some other ions such as Ca^{2+}, Ba^{2+}, Mg^{2+}, Pb^{2+}, Al^{3+}, Fe^{2+} and Fe^{3+}, no significant dimerization of hGH occurs in solutions [6, 16].

The binding parameters derived by using the extended solvation theory were attributed to a structural change of hGH and its biological activity. There are some reports on the binding properties and structural changes of hGH due to its interaction with metal ions [14–17]. In this paper, the interaction between Cu^{2+} and hGH has been investigated in neutral aqueous solution to clarify the thermodynamic values for metal binding. The extended solvation method is able to correlate the solvation parameters to the effect of metal ions on protein stability in a very simple way. The calorimetric method described recently allows evaluation the number of binding sites (g), the molar enthalpy of binding (ΔH), and the association equilibrium constant (K_a) for a set of biomacromolecule binding sites.

2 Materials and Method

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. All other

materials and reagents were of analytical grade, and solutions were made in 50 mmol·L^{-1} NaCl using double-distilled water. The isothermal titration microcalorimetric experiments were performed with a commercial four-channel microcalorimetric system, Thermal Activity Monitor 2277, Thermometric, Sweden. The titration vessel was made from stainless steel. A $Cu(NO_3)_2$(aq) solution of concentration 100 mmol·L^{-1} was injected by use of a Hamilton syringe into the calorimetric titration vessel which contained 1.8 mL hGH (60 μmol·L^{-1}). Thin (0.15 mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. Injection of samples of the Cu^{2+} solution into the perfusion vessel was repeated 30 times, with 20 μL per injection. The calorimetric signal was measured with 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 $Cu(NO_3)_2$ solution was measured as described above except that hGH was not present. The microcalorimeter was frequently calibrated electrically during the course of the study. The molecular weight of hGH was taken to be 22 kDa.

3 Results and Discussion

Consider a solution containing a ligand (Cu^{2+}) and a macromolecule (hGH_g) that contains "g" sites capable of binding the ligand. If the multiple binding sites on a macromolecule are identical and independent, the ligand binding sites can be represented by a model system of monovalent molecules ($hGH_g \rightarrow g$hGH) with the same set of dissociation equilibrium constant (K_d) values. Thus, the reaction under consideration can be written as:

$$M + L \rightleftarrows ML, \qquad K_d = \frac{[ML]}{[M][L]} \tag{1}$$

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 monomeric molecule [M] and ligand [L], as well as the concentration of bound ligand [ML], can be deduced as follows:

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

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

$$[M] = gM_0 - [ML] = gM_0 - g(1-\alpha)M_0 = \alpha g M_0 \tag{4}$$

Substitution of free concentrations of all these components into Eq. 1 gives:

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

or

$$\alpha M_0 = \left(\frac{\alpha}{1-\alpha}\right)\frac{1}{g}L_0 - \frac{K}{g} \tag{6}$$

The value of $1-\alpha$ is the fraction of occupied binding sites on the biomacromolecule:

$$1-\alpha = \frac{q}{q_{max}} \tag{7}$$

where q represents the heat value at a certain L_0, whereas q_{max} represents the heat value upon saturation of all biomacromolecules. If q and q_{max} are calculated per mole of biomacromolecule, then the molar enthalpy of binding for each binding site (ΔH) will be:

$$\Delta H_{max} = \frac{q_{max}}{g}$$

$$\Delta H_{max}(T = 300\ \text{K}) = -(5443/3)\ \mu\text{J}$$
$$= -1814.3\ \mu\text{J}\cdot\text{mol}^{-1}$$
$$= -16.79\ \text{kJ}\cdot\text{mol}^{-1}$$

$$\Delta H_{max}(T = 310\ \text{K}) = -(5314/3)\ \mu\text{J}$$
$$= -1771.3\ \mu\text{J}\cdot\text{mol}^{-1}$$
$$= -16.40\ \text{kJ}\cdot\text{mol}^{-1}$$

Combining Eqs. 6 and 7 yields:

$$\frac{\Delta q}{q_{max}} M_0 = \left(\frac{\Delta q}{q}\right) L_0 \frac{1}{g} - \frac{K}{g} \tag{8}$$

where $\Delta q = q_{max} - q$. Therefore, a plot of $(\Delta q/q_{max})M_0$ versus $(\Delta q/q_{max})L_0$ should be linear with a slope of $\frac{1}{g}$ and a vertical intercept of $\frac{K}{g}$.

The linearity of the plot has been examined by testing different estimated values for q_{max} to find the best value for the correlation coefficient (which should be unity for perfect data). The best linear plot with a correlation coefficient (r^2) value near to one was obtained by using $q_{max} = -5443$ μJ and $q_{max} = -5314$ μJ (equal to -16.79 kJ·mol^{-1} and -16.40 kJ·mol^{-1}, at 300.15 and 310.15 K, respectively). The values of g and K_d, obtained from the slope and vertical-intercept plot, are 3 and 1313.4 μmol·L^{-1} K and 1648.2 μmol·L^{-1} at 310.15 K, respectively. The absence of a suitable value for q_{max} to obtain a completely linear plot of $(\Delta q/q_{max})M_0$ versus $(\Delta q/q)L_0$ may be related to the existence of non-identical binding sites or the interaction between them. Using this method also showed that there are three identical and non-interacting binding sites for Cu^{2+} ions.

We have shown previously [18–38] that the enthalpies of ligand + hGH interactions in an aqueous solvent (M^{2+} + water in the present case) system, can be calculated via the following equation:

$$q = q_{max}x'_B - \delta^{\theta}_A(x'_A L_A + x'_B L_B) - (\delta^{\theta}_B - \delta^{\theta}_A)(x'_A L_A + x'_B L_B)x'_B \tag{9}$$

where q are the enthalpies of Cu^{2+} + hGH interactions and q_{max} represents the heat value upon saturation of all hGH. The parameters δ^{θ}_A and δ^{θ}_B, respectively, are the measures of hGH stability at low and high M^{2+} concentrations. If the ligand binds at each site independently, then the binding is non-cooperative. Values of $p < 1$ or $p > 1$ indicate negative or positive cooperativity of macromolecule for binding with ligand, respectively, whereas $p = 1$ indicates that the binding is non-cooperative. The quantity x'_B can be expressed as follows:

$$x'_B = \frac{px_B}{x_A + px_B} \tag{10}$$

whereas x_B is the fraction of the metal ion needed for saturation of the binding sites, and x'_B is the fraction of unbound Cu^{2+} ions. Thus the model is a simple mass action treatment, with

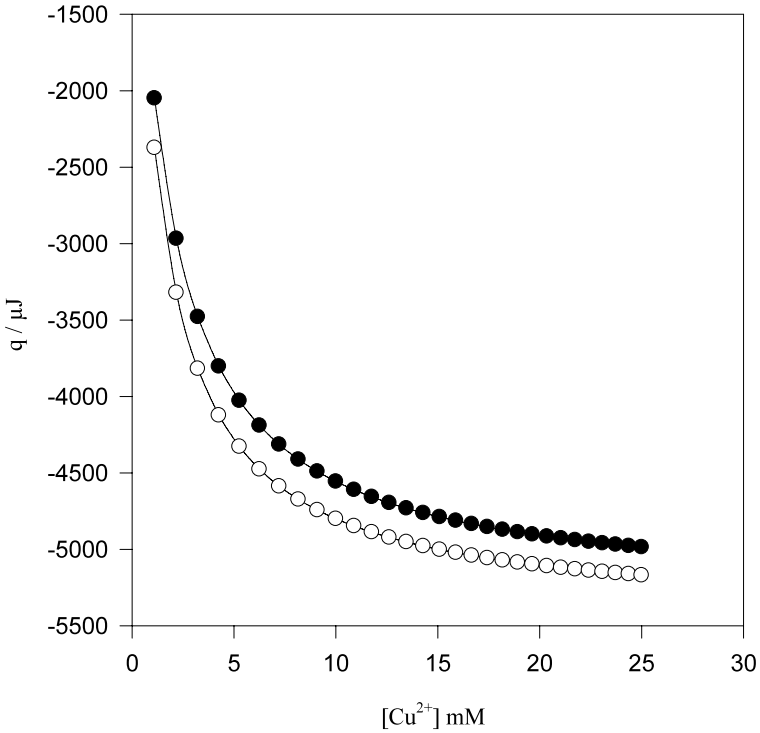

Fig. 1 The heat of Cu^{2+} ions binding with hGH at 300.15 K (○) and 310.15 K (●), for 30 cumulative automatic injections of 100 mmol·L^{-1} $Cu(NO_3)_2$(aq), each of 20 μL, into a sample cell containing 1.8 mL (60 μmol·L^{-1}) of hGH solution, plotted versus the total concentration of Cu^{2+}

metal ions replacing water molecules at the binding sites in the present case. We can express the x_B fractions as the total Cu^{2+} concentration divided by the maximum concentration of Cu^{2+} needed for saturation of all hGH binding sites, as follows:

$$x_B = \frac{[Cu^{2+}]_T}{[Cu^{2+}]_{max}}, \qquad x_A = 1 - x_B \tag{11}$$

$[Cu^{2+}]$ is the total concentration of Cu^{2+} ions and $[Cu^{2+}]_{max}$ is the maximum concentration of Cu^{2+} upon saturation of all binding sites on hGH. In general, there will be "g" sites for binding of Cu^{2+} per hGH molecule. L_A and L_B are the relative contributions of unbound and bound metal ions to the enthalpies of dilution in the absence of hGH, and can be calculated from the enthalpies of dilution of Cu^{2+} in the buffer, q_{dilut}, as follows:

$$L_A = q_{dilut} + x_B \frac{\partial q_{dilut}}{\partial x_B} \quad \text{and} \quad L_B = q_{dilut} + x_A \frac{\partial q_{dilut}}{\partial x_B} \tag{12}$$

The enthalpies of Cu^{2+}+ hGH interactions, q, were fitted to Eq. 9 over the whole range of Cu^{2+} concentrations. In this procedure the only adjustable parameter (p) was changed until the best agreement between the experimental and calculated data was achieved. The δ_A^θ and δ_B^θ parameters also have been optimized to fit the data. The optimized δ_A^θ and δ_B^θ values are

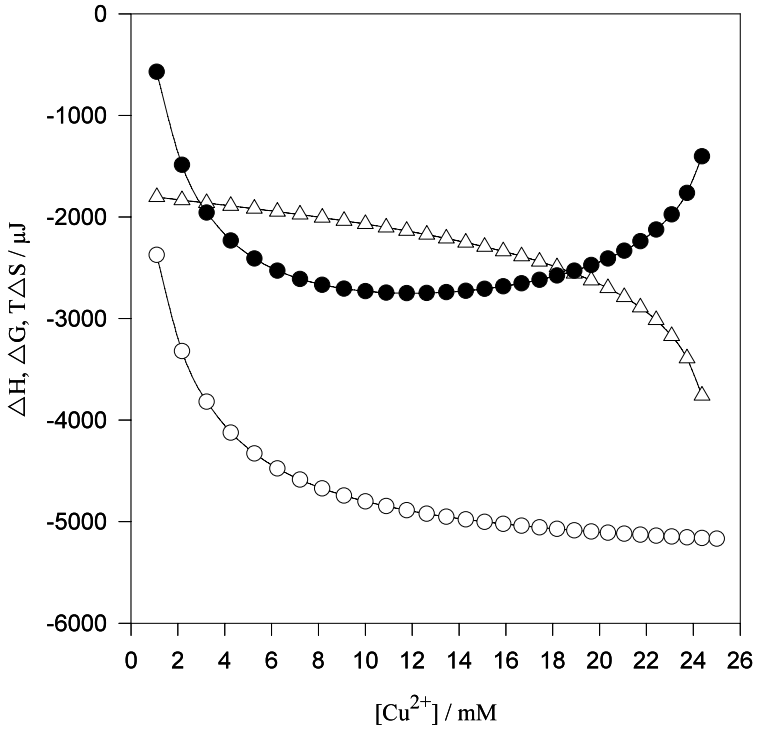

Fig. 2 Comparison among the experimental ΔH (○), ΔG (△) and $T\Delta S$ values (●) for hGH + Cu^{2+} interaction at $T = 300.15$ K

recovered from the coefficients of the second and third terms of Eq. 9. The small relative standard coefficient errors and the high r^2 values (0.99999) support the use of this method.

The Gibbs energies as a function of the M^{2+} concentrations can be obtained as follows:

$$\Delta G = -RT \ln K_a \tag{13}$$

where K_a is the association equilibrium constant ($= 1/K_d$) as a function of the Cu^{2+} concentration. Gibbs energies, ΔG, calculated from Eq. 13 are shown graphically in Figs. 2 and 3. The corresponding ΔS values were calculated using the ΔG values and are also shown in Figs. 2 and 3. The δ_A^θ values for hGH + Cu^{2+} interactions are negative (Table 2), indicating that at low copper ion concentrations the hGH structure was destabilized, resulting in a decrease in its biological activity. The values of δ_B^θ for hGH + Cu^{2+} interactions are positive, indicating that at high copper ion concentrations the hGH structure was stabilized.

The analyses via Eq. 9 indicate that no denaturation of hGH results from interaction with Cu^{2+}. Protein denaturation occurs when a polypeptide loses its higher level of structure, which leads to aggregation. The most common mechanism of protein aggregation is believed to involve protein denaturation via hydrophobic interfaces, and often results in a loss of biological activity [37]. 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 dimers that exhibit unexpected stability against denaturation and maintain the activity of hGH for long periods at temperatures up to and beyond 300.15

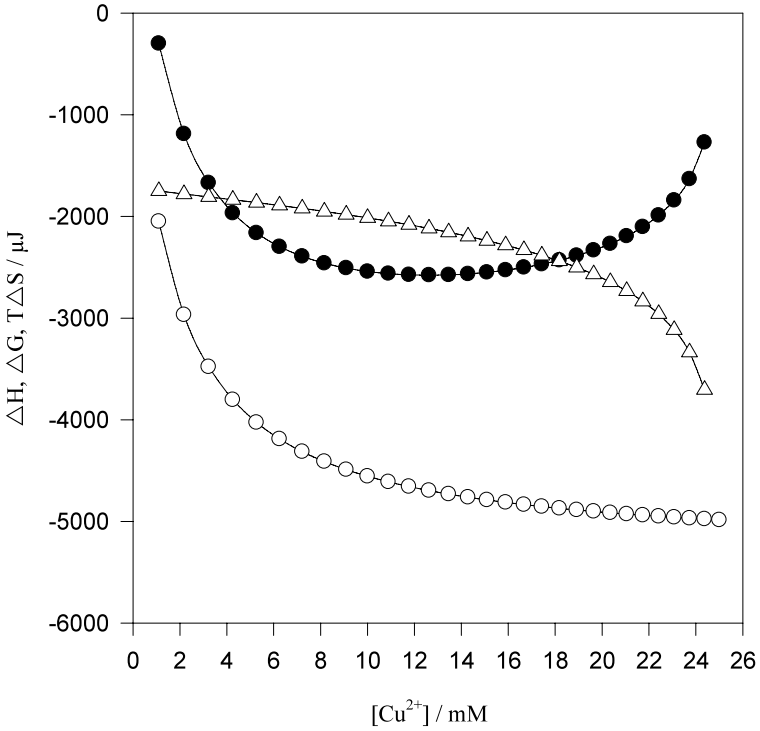

Fig. 3 Comparison among the experimental ΔH (○), ΔG (△) and $T\Delta S$ values (●) for hGH + Cu^{2+} interaction at $T = 310.15$ K

to 310.15 K. Our results are in very good agreement with previous studies that show that Ca^{2+} binding increases the thermal stability of hGH by increasing of the α-helix content as well as decreasing both the beta and random coil structures. Other investigations using differential scanning calorimetry (DSC) revealed that the interaction of three iron ions with hGH prevents irreversibility and aggregation by acting on the hydrophobicity, and there are at least two main transitions corresponding to the two groups of helices.

Using Eq. 9, the calculated δ_A^θ values for hGH + Cu^{2+} interactions are negative (Table 3), indicating that at low Cu^{2+} concentrations the hGH structure is destabilized thereby resulting in an decrease in its biological activity. Destabilization by a ligand indicates that the ligand binds preferentially (either at more sites or with higher affinity) to the unfolded (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 ligand concentration and available solvent-exposed protein surface area that is increased through unfolding events. The determined p values are less than one ($p = 0.676$ and $p = 0.68$) at the two temperatures, indicating that copper ions bind preferentially to the partially unfolded intermediate forms of hGH. Values of δ_B^θ are positive, indicating that the hGH structure is stabilized by the addition of Cu^{2+} (Table 3), which is in agreement with the formation of stable Cu^{2+} + hGH dimers. The Cu^{2+}−hGH dimer is significantly more stable to denaturation than monomeric hGH. Analyses via Eq. 9 indicate that no denaturation of hGH occurs due

Table 1 Enthalpies of Cu^{2+} + hGH interaction at $T = 300.15$ K in 30 mmol·L^{-1} Tris buffer, at pH = 7

q/μJ	q_{dilut}/μJ	[hGH]/μmol·L^{-1}	[Cu^{2+}]/μmol·L^{-1}
−2374.3	−467.2	59.340	1098.9
−3321.5	−869.3	58.695	2173.9
−3818.9	−1198.4	58.064	3225.8
−4123.5	−1478.2	57.446	4255.3
−4327.8	−1718.4	56.842	5263.2
−4476.2	−1913.8	56.25	6250.0
−4587.3	−2084.5	55.670	7216.5
−4673.9	−2231.4	55.102	8163.3
−4743.2	−2354.6	54.545	9090.
−4800.2	−2454.9	54	10000
−4847.7	−2547.8	53.465	10891
−4887.9	−2628.7	52.941	11765
−4922.4	−2701.7	52.427	12621
−4952.3	−2761.5	51.923	13462
−4978.5	−2817.5	51.428	14286
−5001.6	−2865	50.943	15094
−5022.1	−2908.7	50.467	15888
−5040.5	−2949.2	50	16667
−5057.1	−2986.1	49.541	17431
−5072.1	−3018.9	49.090	18182
−5085.7	−3047.9	48.648	18919
−5098.1	−3073.6	48.214	19643
−5109.5	−3097.3	47.787	20354
−5120	−3119.1	47.368	21053
−5129.7	−3139.5	46.956	21739
−5138.7	−3156.8	46.551	22414
−5147	−3172.6	46.153	23077
−5154.8	−3185.9	45.762	23729
−5162	−3198.2	45.378	24370
−5168.8	−3208.6	45	25000

to interaction with Cu^{2+} ions, which is consistent with the formation of the $(Cu^{2+})(hGH)_2$ complex.

4 Conclusion

Operationally, it has been confirmed that the extended coordination model, Eq. 9, satisfactorily reproduces the enthalpies of Cu^{2+} + hGH interaction. Analysis of these results in terms of the new extended coordination model confirms the model's reliability. Prediction of biological activity of proteins, structural changes of macromolecules, along with binding enthalpies and associated binding constant, make this theory a most powerful one.

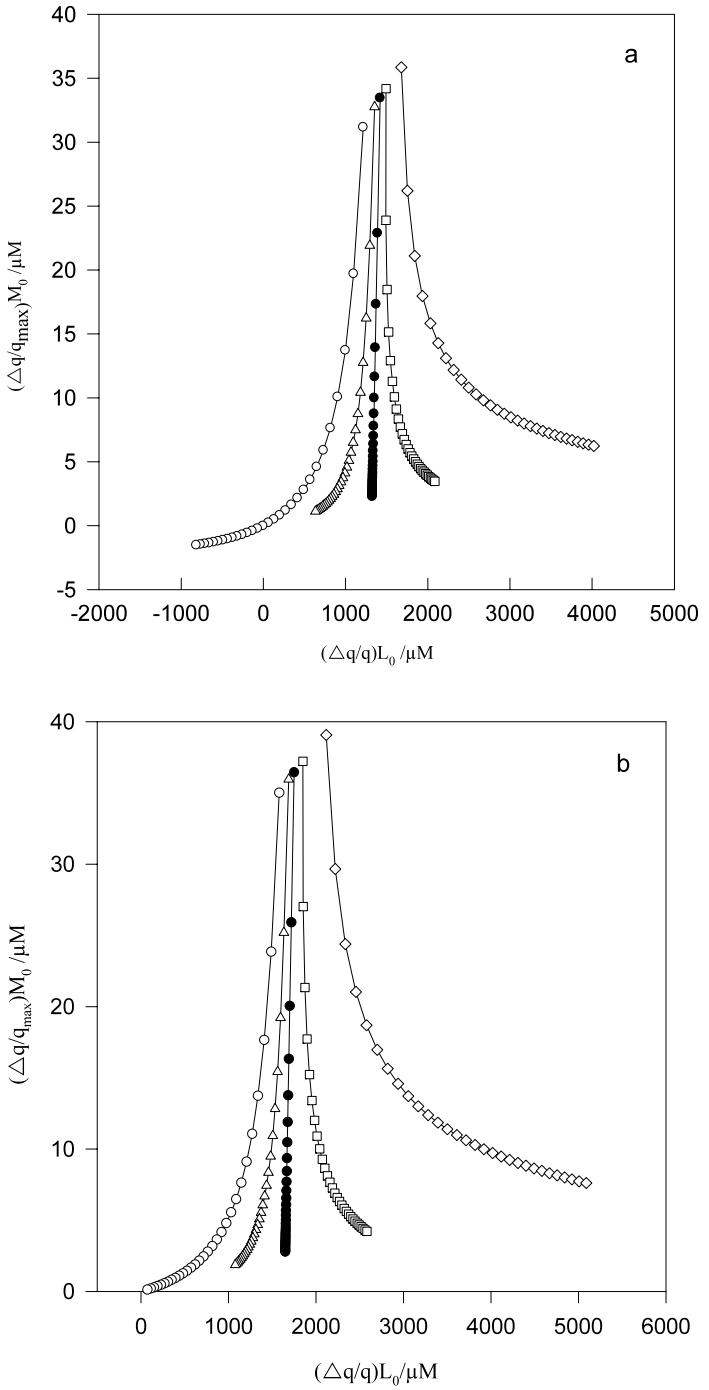

Fig. 4 The best linear plot of $(\frac{\Delta q}{q_{\max}})M_0$ *versus* $(\frac{\Delta q}{q})L_0$, according to Eq. 8, using values of −6000 μJ (◇), −5600 μJ (□), −5000 μJ (○), −5300 μJ (△), −5443 μJ and −5314 μJ (●), at $T = 300.15$ K (**a**) and $T = 310.15$ K (**b**), respectively, for $q_{\max}$, in order to obtain the best correlation coefficient value ($r^2 = 0.999$)

Table 2 Enthalpies of Cu^{2+} + hGH interaction at $T = 310.15$ K in 100 mmol·L^{-1} $Cu(NO_3)_2$ solution

q/μJ	q_{dilut}/μJ	[hGH]/μmol·L^{-1}	[Cu^{2+}]/μmol·L^{-1}
−2050.1	−408.8	59.340	1098.9
−2968.3	−760.7	58.695	2173.9
−3479.7	−1049	58.064	3225.8
−3803.9	−1294.2	57.446	4255.3
−4027.2	−1504.6	56.842	5263.2
−4190.2	−1675.9	56.25	6250.0
−4314.4	−1825.6	55.670	7216.5
−4412.1	−1954.5	55.102	8163.3
−4491	−2063	54.545	9090.9
−4556	−2150.9	54	10000
−5610.5	−2232.6	53.465	10891
−4656.8	−2303.9	52.941	11765
−4696.7	−2368.1	52.427	12621
−4731.4	−2420.8	51.923	13462
−4761.8	−2470	51.428	14286
−4788.7	−2511.7	50.943	15094
−4812.7	−2550.3	50.467	15888
−4834.2	−2586.1	50	16667
−4853.6	−2618.8	49.541	17431
−4871.2	−2647.8	49.090	18182
−4887.2	−2673.6	48.648	18919
−4901.8	−2696.5	48.214	19643
−4915.2	−2717.6	47.787	20354
−4927.6	−2737	47.368	21053
−4939	−2754.9	46.956	21739
−4949.6	−2770.4	46.551	22414
−4959.4	−2784.6	46.153	23077
−4968.6	−2796.5	45.762	23729
−4977.2	−2806.6	45.378	24370
−4985.2	−2815.9	45	25000

Table 3 Binding parameters for the Cu^{2+} + hGH interaction in 100 mmol·L^{-1} $Cu(NO_3)_2$ solution

Parameters	hGH + Cu^{2+} ($T = 300.15$ K)	hGH + Cu^{2+} ($T = 310.15$ K)
δ_A^θ	−0.621	−0.738
δ_B^θ	6.281	6.668
K_d/μmol·L^{-1}	1313.4	1648.2
g	3	3
p	0.68	0.70
ΔH_{bin}/kJ·mol^{-1}	−16.79	−16.40

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