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Thermodynamic Study of the Binding of Mercury Ion to Human Growth Hormone at Different Temperatures

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Abstract The interaction of human growth hormone (hGH) with the divalent mercury ion was studied by isothermal titration calorimetry at two temperatures of 27 °C and 37 °C in aqueous solutions. We found that there is a set of two identical and non-interacting binding sites for Hg^{2+} ions. The intrinsic dissociation equilibrium constant and the molar enthalpy of binding are 4.2 mmol·L^{-1} and −14.8 kJ·mol^{-1} at 27 °C and 5.1 mmol·L^{-1} and −14.2 kJ·mol^{-1} at 37 °C, respectively. The results obtained indicate that the stability of the protein increases due to the binding of mercury ions using the extended solvation theory.

Keywords Human growth hormone (hGH) · Isothermal titration calorimetry (ITC) · Solvation model · Mercury ion · Metal binding

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1 Introduction

Recently, the isothermal titration calorimetry (ITC) technique has provided a direct route to the complete thermodynamic characterization of non-covalent interactions. Hence, ITC has become the fastest developing technique in biological research [1–3]. Some applications of the ITC technique have been reported for protein folding and molecular recognition [4].

Human growth hormone (hGH) is a single domain globular protein containing 191 amino acids with two disulfide bridges between residues 53–165 (large loop) and residues 182–189 (small loop), which plays an important role in somatic growth through its effects on the metabolism of proteins, carbohydrates and lipids [5–8]. It has a limited stability in aqueous solution. Developments have therefore focused on more stable formulations and on understanding its interaction with ligands. The thermal transition of hGH 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 and extreme salt concentrations [9]. Differential scanning calorimetry (DSC) and circular dichroism spectroscopy (CD) has shown that the thermal unfolding of hGH is reversible only below pH = 3.5, and under these conditions there is a single two-state transition from the native state to a partially folded state [10]. There are some reports on the binding properties and structural changes of hGH due to its interaction with metal ions [11–20].

A 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 on helix IV [14]. There is a set of three identical and noninteracting binding sites for both Ca^{2+} [15, 16] and Mg^{2+} ions [17, 18]. Both Ca^{2+} and Mg^{2+} binding to hGH increase the protein's thermal stability by increasing of the alpha helix content as well as decreasing both the beta and random coil structures [15, 17]. There is a set of four binding sites on hGH for Fe^{3+} [19]. Interaction of the first three iron ions with hGH prevents irreversibility and aggregation. Interaction of Co^{2+} with hGH, at a set of three identical and independent binding sites, prevented protein aggregation by an effect on the hydrophobicity of the macromolecule [20]. Some metal ions like Zn^{2+}, Cd^{2+}, Hg^{2+} and Co^{2+} are known to promote the reversible dimerization of hGH.

The zinc ion has been shown to be important for the function of growth hormones [21, 22]. The presence of Zn^{2+} has been demonstrated to enhance the activity of human growth hormone (hGH) in a cell line based biological assay. Also, it has been previously reported that the interaction of bovine serum albumin (BSA) with Zn^{2+} ions, that was followed by differential scanning calorimetry (DSC), showed an increase in the stability and activity of the BSA protein [23]. It has also been demonstrated that the zinc ion induces dimerization of hGH, and the resulting Zn^{2+}–hGH dimer has been proposed as the major storage form of hGH in vivo. However, in the presence of some other ions like Ca^{2+}, Ba^{2+}, Mg^{2+}, Pb^{2+}, Al^{2+}, Fe^{2+} and Fe^{3+}, there is no significant dimerization of hGH [12].

There are no thermodynamic reports on mercury ion binding to hGH in the literature. The present paper elucidates some previously unknown aspects concerning the interaction of Hg^{2+} with hGH and the thermodynamics of Hg^{2+} binding properties in neutral aqueous solutions. One of the unique aspects of this report is the use of a recently extended solvation model to clarify the stability of the protein. This method is capable of correlating the binding parameters to the effects of metal ions on the stability of a protein.

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. Mercuric nitrate was purchased from Merck Co. (Darmstadt, Germany). 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 four-channel commercial microcalorimetric system, Thermal Activity Monitor 2277, Thermometric (Sweden). The titration vessel was made from stainless steel. The mercuric nitrate solution (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 mercury nitrate solution into the perfusion vessel was repeated 30 times, with 20 μL used per injection. We calculated the heat of each injection with the "Thermometric Digitam 3" software program. The enthalpy of dilution of the Hg^{2+} solution was measured as described above except that hGH was excluded. The microcalorimeter was frequently calibrated electrically during the course of the study. The molecular weight of hGH was taken to be 22 kDa [6, 7]. The heats of hGH + Hg^{2+} interactions (in μJ) are reported in Tables 1 and 2.

3 Results and Discussion

The raw data obtained from ITC of hGH interaction with mercury ions at temperatures of 27 °C and 37 °C are shown in Fig. 1. Figure 1a shows the heat change from each injection and Fig. 1b shows the cumulative heat change at each total concentration of mercury ion, $[Hg^{2+}]_T$. A plot of $(\Delta q/q_{max})M_0$ versus $(\Delta q/q)L_0$ should be linear with a slope of $1/g$ and vertical-intercept of K_d/g, from which g and K_d can be obtained [17, 24]:

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

where g is the number of binding sites, K is the dissociation equilibrium constant, M_0 and L_0 are total concentrations of biomacromolecule and metal ion, respectively, $\Delta q = q_{max} - q$, and q represents the heat value at a certain L_0, and q_{max} represents the heat value upon saturation of all biomacromolecules.

The linearity of the plot of $(\Delta q/q_{max})M_0$ versus $(\Delta q/q)L_0$ for the binding of Hg^{2+} ions by hGH has been examined by using different estimated values for q_{max}, in order to find the best value for the correlation coefficient (nearest to one). The best linear plot with the correlation coefficient (R^2) value nearest to one was obtained using −3197 μJ and −3067 μJ (equal to −29.6 kJ·mol^{-1} and −28.4 kJ·mol^{-1}) at 27 °C and 37 °C, respectively, for q_{max} (Fig. 2). The value of g is 2, which was calculated from the slope and values of K, was obtained from the vertical-intercept plot. For a set of identical and independent binding sites, the values are 4.2 mmol·L^{-1} and 5.1 mmol·L^{-1} at 27 °C and 37 °C, respectively. Dividing the q_{max} values of −29.6 kJ·mol^{-1} and −28.4 kJ·mol^{-1} by $g = 2$, therefore, gives $\Delta H = -14.8$ kJ·mol^{-1} and −14.2 kJ·mol^{-1} at 27 °C and 37 °C, respectively (Table 3).

For a set of identical and independent binding sites, we have before shown [24–27] that:

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

Table 1 Enthalpies of Hg^{2+} + hGH interaction at $T = 300$ K in 50 mmol·L^{-1} NaCl solutions

q (μJ)	q_{dilut} (μJ)	[hGH] (mmol·L^{-1})	[Hg^{2+}] (mmol·L^{-1})
−651.3	−327.0	59.34	1098.9
−1077.1	−608.5	58.69	2173.9
−1376.4	−839.0	58.06	3225.8
−1597.9	−1034.9	57.45	4255.3
−1768.4	−1203.1	56.84	5263.2
−1903.6	−1339.8	56.25	6250.0
−2013.4	−1459.3	55.67	7216.5
−2104.3	−1562.1	55.10	8163.3
−2180.8	−1648.3	54.54	9090.9
−2246.1	−1718.5	54.00	10000
−2302.4	−1783.5	53.46	10891
−2351.5	−1840.1	52.94	11765
−2394.7	−1891.2	52.43	12621
−2433.0	−1933.0	51.92	13462
−2467.2	−1972.2	51.43	14286
−2497.9	−2005.4	50.94	15094
−2525.6	−2036.0	50.47	15889
−2550.7	−2064.4	50.00	16667
−2573.6	−2090.2	49.54	17431
−2594.6	−2113.1	49.09	18182
−2613.9	−2133.4	48.65	18919
−2631.7	−2151.4	48.21	19643
−2648.1	−2168.0	47.79	20354
−2663.3	−2183.2	47.37	21053
−2677.5	−2197.5	46.96	21739
−2690.7	−2209.6	46.55	22414
−2703.0	−2220.6	46.15	23077
−2714.6	−2229.9	45.76	23729
−2725.5	−2238.5	45.38	24370
−2735.7	−2245.8	45.00	25000

where A_i, B_i, and C_i are constants for each injection i, which are defined as follows:

$$A_i = V_i/2Q_i, \qquad B_i = gM_i + L_i, \quad \text{and} \quad C_i = 4gM_iL_i \tag{3}$$

where V_i is the volume of the reaction solution in the calorimetric sample cell at each injection step. M_i is the total hGH concentration, and L_i is the total Hg^{2+} concentration in the calorimetric sample cell at that injection step. Equation 2 contains two unknown parameters, K and ΔH. A series of reasonable values for K are inserted into Eq. 2 and the corresponding amounts for ΔH are calculated, and then the graph ΔH versus K is constructed. Curves of all titration steps will intersect at one point, which corresponds to the true values of ΔH and K. The plots of ΔH versus K, according to Eq. 2, are shown in Fig. 3 for all injections. The intrinsic dissociation equilibrium constant and the molar enthalpy of binding were found to be 4200 μmol·L^{-1}, −14.8 kJ·mol^{-1} and 5100 μmol·L^{-1}, −14.2 kJ·mol^{-1} at 27 °C and

Table 2 Enthalpies of Hg^{2+} + hGH interaction at $T = 310$ K in 50 mmol·L^{-1} NaCl solutions

q (μJ)	q_{dilut} (μJ)	[hGH] (mmol·L^{-1})	[Hg^{2+}] (mmol·L^{-1})
−535.3	−286.1	59.34	1098.9
−906.4	−532.4	58.69	2173.9
−1178.3	−734.2	58.06	3225.8
−1385.8	−905.8	57.44	4255.3
−1549.3	−1053.1	56.84	5263.2
−1681.4	−1173.0	56.25	6250.0
−1790.3	−1277.9	55.67	7216.5
−1881.7	−1368.1	55.10	8163.3
−1959.4	−1444.0	54.54	9090.9
−2026.3	−1505.5	54.00	10000
−2084.5	−1562.7	53.46	10891
−2135.6	−1612.6	52.94	11765
−2180.8	−1657.5	52.42	12621
−2221.1	−1694.4	51.92	13462
−2257.2	−1728.8	51.42	14286
−2289.7	−1758.0	50.94	15094
−2319.2	−1785.0	50.46	15888
−2346.1	−1810.0	50.00	16667
−2370.7	−1832.9	49.54	17431
−2393.2	−1853.2	49.09	18182
−2414.0	−1871.2	48.64	18919
−2433.2	−1887.2	48.21	19643
−2451.0	−1902.0	47.78	20354
−2467.5	−1915.6	47.36	21053
−2482.9	−1928.1	46.95	21739
−2497.3	−1938.9	46.55	22414
−2510.8	−1948.8	46.15	23077
−2523.4	−1957.1	45.76	23729
−2535.3	−1964.2	45.38	24370
−2546.5	−1970.7	45.00	25000

37 °C, respectively (Fig. 3). The calorimetric data analysis method using this equation has been applied to studies of enzyme inhibition [26–29] and metal binding to proteins [15, 17, 19, 30–32].

It has been shown previously [33–40] that the heat of interaction of biopolymers with ligands (hGH and Hg^{2+} in this case) in aqueous solvent mixtures can be reproduced via the following equation:

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

where q is the heat of hGH + Hg^{2+} interaction and q_{max} represents the heat value upon saturation of all of the hGH. The parameters δ^{θ}_A and δ^{θ}_B are indexes of hGH stability in the low and high Hg^{2+} concentration regions, respectively. If the binding of ligand at one site increases the affinity for the ligand at another site, then the macromolecule exhibits positive

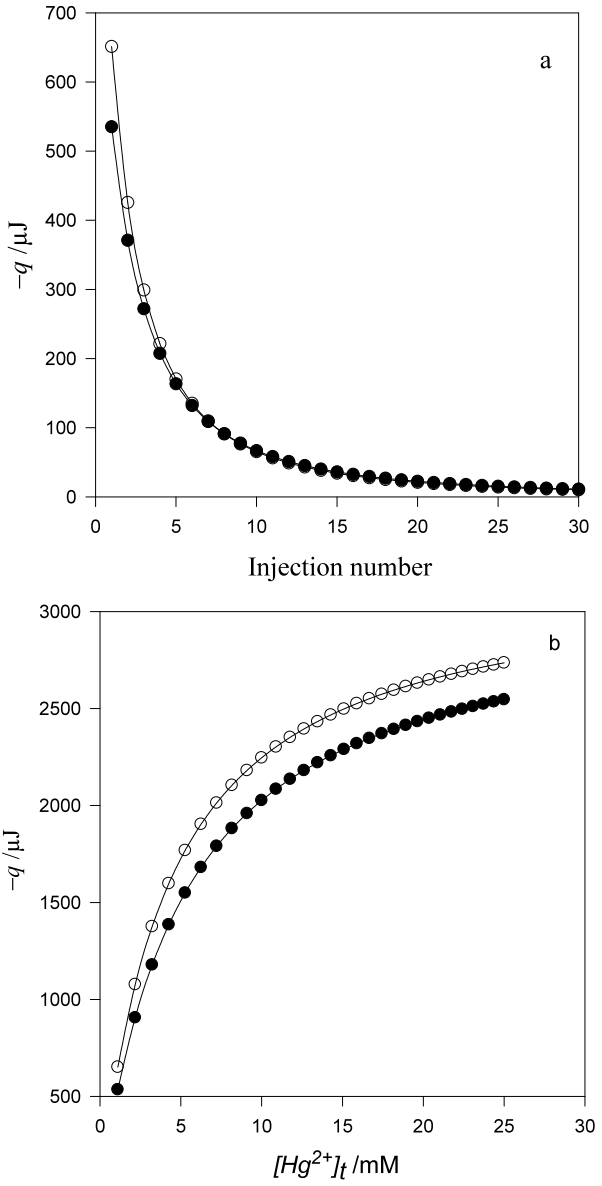

Fig. 1 (**a**) The heat of mercury binding to hGH for 30 automatic cumulative injections. Each injection was of 20 μL of 100 mmol·L^{-1} of cation solution into the sample cell containing 1.8 mL of 60 μmol·L^{-1} hGH solution, at 300 K (○) and 310 K (●); (**b**) the total cumulative heat of binding versus total concentration of mercury ion, calculated from Fig. 1a

cooperativity. Conversely, if the binding of ligand at one site lowers the attraction for the ligand at another site, then the protein exhibits negative cooperativity. If the ligand binds at each site independently, then the binding is non-cooperative. Values of $p > 1$ or $p < 1$ reflect positive or negative cooperativity, respectively, of the macromolecule for binding with the ligand, whereads $p = 1$ indicates that the binding is non-cooperative.

The quantity x'_{B} can be expressed as follows [41–43]:

$$x'_{\mathrm{B}} = \frac{p x_{\mathrm{B}}}{x_{\mathrm{A}} + p x_{\mathrm{B}}} \tag{5}$$

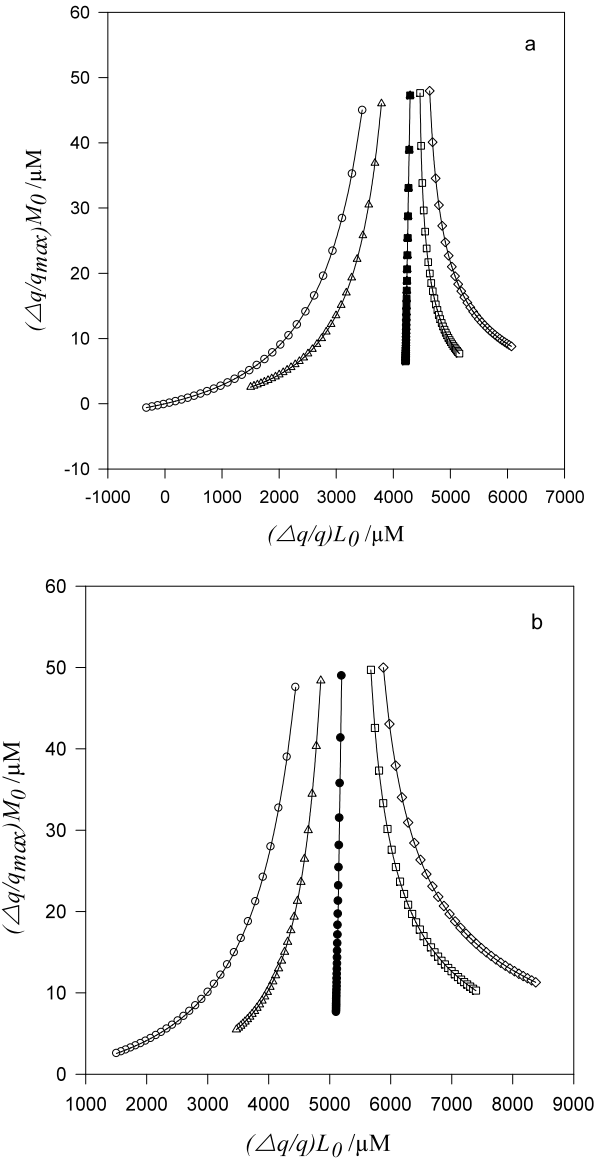

Fig. 2 The best linear plot of $(\frac{\Delta q}{q_{\max}})M_0$ versus $(\frac{\Delta q}{q})L_0$, according to Eq. 1, using $q_{\max}$ values of −3400 (◊), −3300 (□), −2700 (○), −2900 (Δ), −3197 (■), and −3067 μJ (●), at 300 K (**a**) and 310 K (**b**), to obtain the best correlation coefficient value ($R^2 = 0.999$) for linear plots at 300 K (−3197 μJ) and 310 K (−3067 μJ)

where x'_{B} is the fraction of Hg^{2+} bound to the binding sites on hGH, and $x'_{\mathrm{A}} = 1 - x'_{\mathrm{B}}$ is the fraction of unbound Hg^{2+}. We can express x_{B} as follows:

$$x_{\mathrm{B}} = \frac{[\mathrm{Hg}^{2+}]_t}{[\mathrm{Hg}^{2+}]_{\max}}, \qquad x_{\mathrm{A}} = 1 - x_{\mathrm{B}} \tag{6}$$

where $[Hg^{2+}]_{\mathrm{T}}$ is the total concentration of mercury ions and $[Hg^{2+}]_{\max}$ is the maximum concentration of Hg^{2+} upon saturation of all hGH. L_{A} and L_{B} are the relative contributions to the fractions of unbound and bound mercury ions from the heats of dilution in the absence of hGH and can be calculated from the heats of dilution of Hg^{2+} in the buffer solution, q_{dilut},

Table 3 Binding parameters for Hg^{2+} + hGH interaction in 50 mmol·L^{-1} NaCl solution

Parameters	hGH + Hg^{2+} ($T = 300$ K)	hGH + Hg^{2+} ($T = 310$ K)
δ_A^θ	-0.47 ± 0.02	-0.64 ± 0.05
δ_B^θ	2.60 ± 0.03	1.87 ± 0.02
K (mmol·L^{-1})	4.2 ± 0.1	5.1 ± 0.1
g	2	2
p	1	1
ΔH_{max} (kJ·mol^{-1})	-14.82 ± 0.02	-14.21 ± 0.03

as follows:

$$L_A = q_{dilut} + x_B\left(\frac{\partial q_{dilut}}{\partial x_B}\right), \qquad \left(L_B = q_{dilut} + x_A\frac{\partial q_{dilut}}{\partial x_B}\right) \tag{7}$$

With simple modification of Eq. 4, it is possible to use this equation to reproduce the enthalpies of metal–macromolecule interactions. q is the heat of hGH + Hg^{2+} interaction and q_{max} represents the heat value upon saturation of all hGH. The heats of hGH + Hg^{2+} interactions were fitted to Eq. 4 over the whole Hg^{2+} concentration range (Fig. 4). In the procedure, the only adjustable parameter p was changed until the best agreement was achieved between the experimental and calculated data. The δ_A^θ and δ_B^θ parameters have also been optimized by fitting to the data. The optimized δ_A^θ and δ_B^θ values are recovered from the coefficients of the second and third terms of Eq. 4. The agreement between calculated and experimental results (Fig. 1) is excellent, which gives considerable support to the use of Eq. 4.

The Gibbs energy change as a function of the Hg^{2+} concentration can be obtained as follows:

$$\Delta G = -RT \ln K_a \tag{8}$$

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 the Hg^{2+} concentration. The Gibbs energies, ΔG, were calculated from Eq. 8. Also, estimated entropy changes ΔS from the q and ΔG values, are shown graphically in Fig. 4.

The δ_A^θ value for hGH + Hg^{2+} interaction is negative (Table 3), indicating that at low concentration of mercury ion the hGH structure 1s destabilized, resulting in a decrease in its biological activity. Destabilization by a ligand indicates that the ligand preferentially binds to the denatured protein or to a partially folded intermediate form of the protein. Such effects are characteristic of non-specific interactions, in that the non-specific 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 (which is increased through unfolding events), and agrees with results of microcalorimetric technique. The dissociation equilibrium constants are rather small, implying that there is a non-specific interaction.

The δ_B^θ value for hGH + Hg^{2+} interaction is positive (Table 3), indicating that at high concentrations of the mercury ion the hGH structure is stabilized. Also, previous reports indicate that the inclusion of a divalent metal ion such as zinc, cobalt or copper into an hGH formulation results in the formation of stable dimers that exhibit unexpected stability to denaturation and maintain the biological activity of hGH for long periods at temperatures up to and beyond 25–37 °C. Our results are in good agreement with previous investigations that showed for some metal ions, binding increases the thermal stability of hGH by increasing

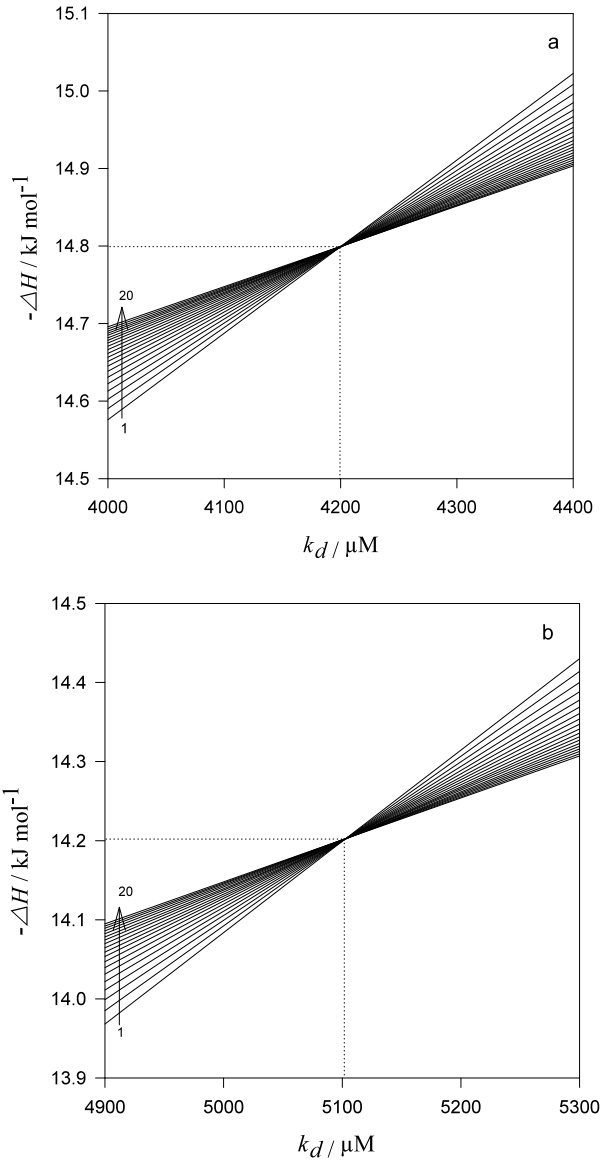

Fig. 3 ΔH versus K for the first 20 injections (injection numbers 1 to 20), at 300 K (**a**) and 310 K (**b**), using the data shown in Fig. 1b. The coordinates of the intersection point of the curves give the true values for ΔH and K

the alpha helix content as well as decreasing both the beta and random coil structures [15, 17, 20, 21].

The $p = 1$ values at both temperatures show the overall non-cooperativity for the interaction of Hg^{2+} ions with hGH. There is a set of two identical and non-interacting binding sites for mercury ion on the surface of hGH. In the current study, it can be concluded that the mercury ion bonding process increases the protein stability of hGH. The analyses via Eq. 4 indicates there is no denaturation of hGH due to interaction with Hg^{2+} ions, which is consistent with the formation of the $(Hg^{2+}–hGH)_2$ complex [11]. Analysis of these in terms of the new extended coordination model confirms the model's ability to represent

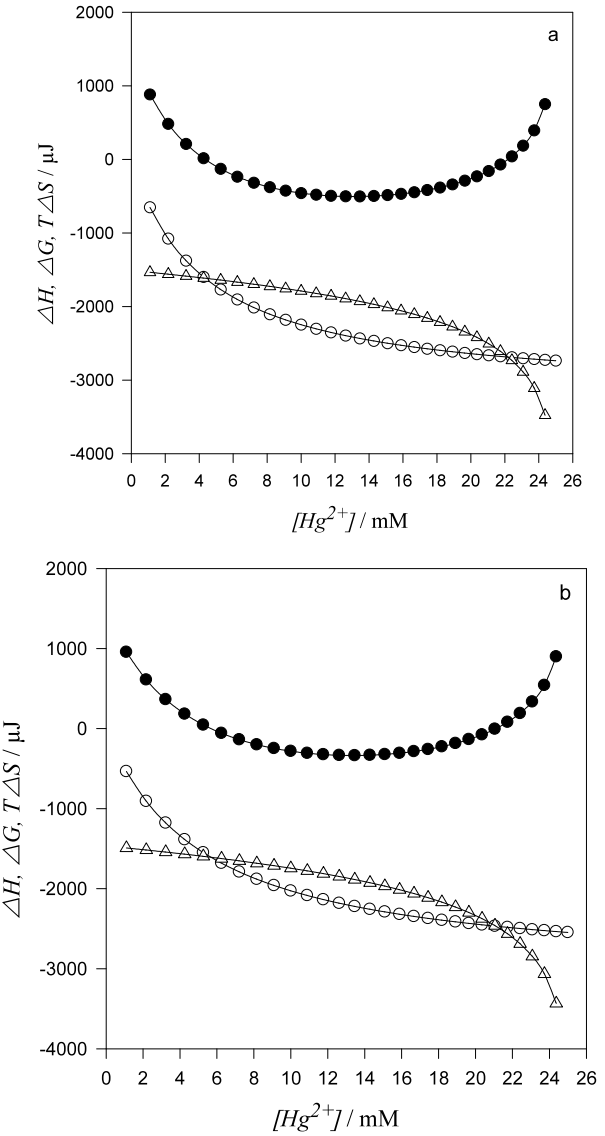

Fig. 4 Comparison among the experimental ΔH(○), ΔG(△), and $T\Delta S$ values (●) for hGH + Hg^{2+} interaction and their calculated data (*lines*) at 300 K (**a**) and 310 K (**b**). The Gibbs energy curves are roughly linear, indicating that the structural changes of hGH compensate each other in the heats of hGH + Hg^{2+} interaction (Fig. 1) and $T\Delta S$ (enthalpy–entropy compensation), which is another support for Eq. 4

these data. Predictions of the biological activity of proteins, structural changes of macromolecules, along with binding enthalpies and their associated binding constants, make this theory a most powerful one.

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