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Thermodynamics of Binding Copper(II) Ion by Human Growth Hormone

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The interaction of human growth hormone (hGH) with divalent copper ions in aqueous solution was studied using different methods. The binding isotherm for hGH-Cu^{2+} was obtained by two techniques of potentiometric, using a Cu^{2+}-selective membrane electrode and isothermal titration calorimetry. There is a set of three identical and non-interacting binding sites. The intrinsic association equilibrium constant is 117 mM^{-1} and the molar enthalpy of binding is –16.7 kJ/mol. Although the binding process of copper ions to the surface of protein do not change the secondary structure of hGH significantly, however, thermodynamic stability of the protein decreases considerably due to the binding of metal ions.

Key Words: Human growth hormone, Copper, Isothermal titration calorimetry, Potentiometry, Circular dichroism.

INTRODUCTION

Human growth hormone (hGH) is a polypeptide hormone that is synthesized by the somatotropic cells of the anterior pituitary. It plays an important role in somatic growth through its effects on the metabolism of proteins, carbohydrates and lipids. hGH is currently used for the treatment of pediatric hypopituitary dwarfism and in children suffering from low levels of hGH[1].

hGH is a single domain globular protein containing 191 amino acids. The molecular mass is approximately 22 kDa, with pI 5.3[2–6]. There are two disulfide bridges present in the protein: one connecting distant parts of the molecule involving residues 53 and 165 (large loop) and another near the C terminal between residues 182 and 189 (small loop)[7]. Approximately 55% of the polypeptide backbone exists in a right-handed α-helical conformation[6, 8].

hGH is produced recombinantly and is available worldwide for clinical use. It has limited stability in solution (for *ca.* 2 weeks at 2–8°C) and is commonly stored in freeze-dried form[6]. Development has, therefore, focused on more stable or sustained-release formulation and alternatives to injectable delivery that would increase bioavailability and make it easier for patients to use.

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There are some reports on the binding properties and structural changes of hGH due to its interaction with metal ions[9, 10]. Some transition metal ions form a 1 : 1 complex with hGH, while two globules of such a complex then may associate slowly to form a dimer in alkaline buffer at high concentration of metal ions[10]. In this paper, copper(II) ion, interaction with hGH has been investigated in neutral aqueous solution to clarify thermodynamics of metal binding properties as well as the stability and structural change of the protein due to its interaction with copper(II) ions.

EXPERIMENTAL

Highly purified preparations of human growth hormone (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. An $E^{1\%}$ (277 nm) = 9.3 was used as reported by Bewley *et al.*[11] Copper nitrate was purchased from Merck Co. All other materials and reagents were of analytical grade and solutions were made in NaCl 50 mM using double-distilled water.

A highly selective and sensitive membrane sensor for copper(II) ions was made as reported before[12]. A titration of 8 mL NaCl solution, 50 mM, by a solution of $Cu(NO_3)$, 1 mM, was done to obtain a calibration curve of potential against logarithm of copper ion concentration according to the Nernst equation. A linear plot for this calibration curve due to the range of copper ion concentration, as shown in Fig. 1, was observed, which shows an appropriate response for the electrode. Then, to obtain the binding isotherm for copper ion binding to hGH by

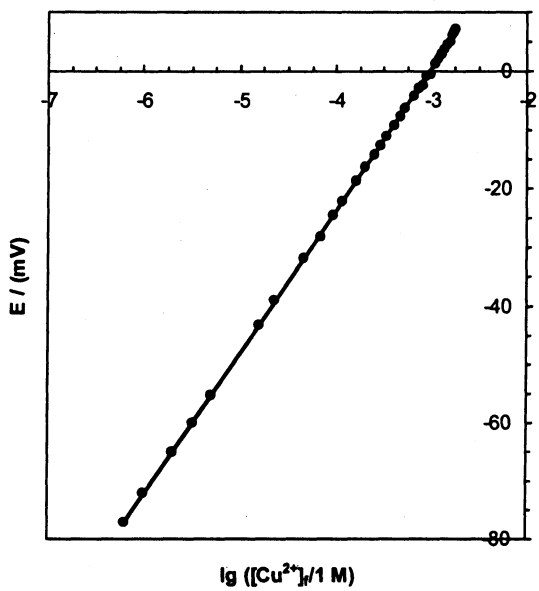

Fig. 1. The calibration curve for Cu^{2+}-selective membrane electrode related to a titration of an aqueous solution including NaCl, 50 mM, by a solution of copper nitrate, 10 mM at room temperature (25°C)

electrochemical method, a titration of 8 mL hormone solution, 23 μM, in NaCl solution, 50 mM, by a solution of $Cu(NO_3)$, 1 mM, was done for recording potential and finding the free concentration of Cu^{2+} according to the calibration curve. Titrations were done in triplicate at 300 K in the same conditions and the average data has been reported.

Temperature scanning spectroscopy: Absorbance profiles, which describe the thermal denaturation of hGH, were obtained from a UV-Visible spectrophotometer CARY-100-Bio model fitted with a temperature programmer, which controls the speed of temperature change in melting experiments. The cuvette holder can accommodate 2 samples: one as a reference solution and the other one for a sample experimental determination. Both reference and samples cells had identical concentrations of copper. The concentration of hGH in the sample cells was 10 μM for copper interaction. The recording chart reads the temperature, reference line (from the reference cuvette) and the absorbance change at 278 nm for the sample in the cuvette.

Isothermal titration calorimetry: 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. Copper solution (0.8 mM) was injected by use of a Hamilton syringe into the calorimetric stirred titration vessel, which contained 1.8 mL hGH (10 μM). Thin (0.15 mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. Injection of copper(II) solution into the perfusion vessel was repeated 40 times and each injection included 25 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 copper 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 that the aqueous solution, without copper ion, was injected to the protein solution in the sample cell. The enthalpies of copper and protein solution dilution were subtracted from the enthalpy of hGH-copper interaction. The microcalorimeter was frequently calibrated electrically during the course of the study.

Circular dichroism experiments: Circular dichroism (CD) spectra were recorded on a JASCO J-715 spectrophotometer (Japan). The results were expressed as ellipticity [θ (deg cm^2 $dmol^{-1}$)] considering a mean amino acid residue weight (MRW) of 115 and weight of 22 kDa for hGH[4, 6]. The molar ellipticity was determined as $[\theta]_\lambda = (100 \times MRW \times \theta_{obs}/cl)$, where θ_{obs} is the observed ellipticity in degrees at a given wavelength, c is the protein concentration in mg/mL and *l* is the length of the light path in cm. The instrument was calibrated with (+)-10-camphor sulfonic acid, assuming $[\theta]_{291} = 7820$ deg cm^2 $dmol^{-1}$ and JASCO standard non-hygroscopic ammonium, (+)-10-camphor sulfonate assuming $[\theta]_{290.5} = 7910$ deg cm^2 $dmol^{-1}$.[13] The noise in the data was smoothed by using the JASCO J-715 software. This software uses the fast Fourier-transform noise reduction routine that allows decrease of most noisy spectra without distorting

their peak shapes. The JASCO J-715 software was used to predict the secondary structure of the protein according to the statistical method[14, 15]. Far-UV CD was carried out in the presence of 0.20 mg/mL of hGH.

RESULTS AND DISCUSSION

The concentration of copper(II) ions bound to the protein during the potential titration can be obtained by subtracting the free concentration of copper(II) ions measured by the sensitive electrode from the total concentration of copper ion at equilibrium. Then, the average number of bound copper to one macromolecule of hGH, ν, can be calculated by dividing the bound concentration of copper ion to the total concentration of the protein. The binding isotherm has been plotted as ν *vs.* ln $[Cu^{2+}]_f$, where $[Cu^{2+}]_f$ is the free concentration of copper(II) ions, as shown in Fig. 2a. In this case, the Scatchard plot[16] is linear, as shown in Fig. 2b. The shapes of the Scatchard plots are clearly characteristic of different types of

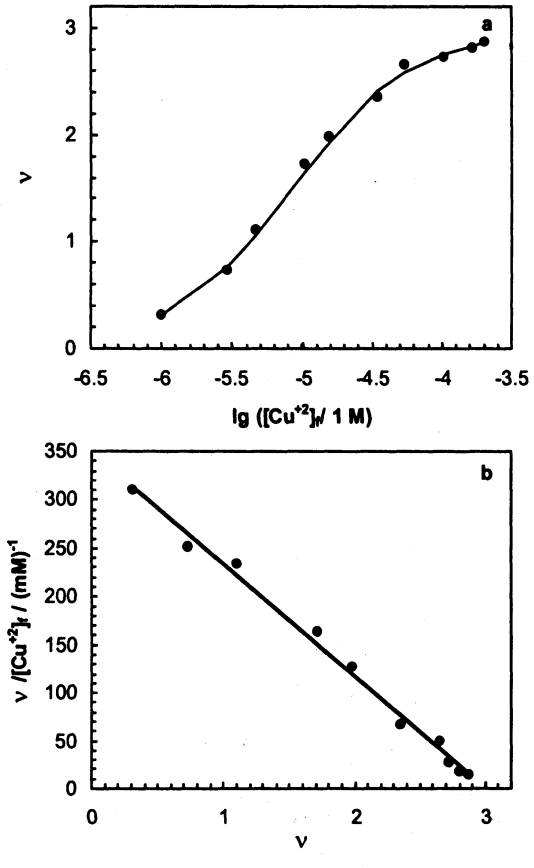

Fig. 2. The binding isotherm (a) and the Scatchard plot (b) of binding Cu^{2+} by hGH at 25°C according to the Emf measurements. The best-fit curve of the experimental binding data was transformed to both binding isotherm and the Scatchard plot using the Scatchard equation ($\nu = gK[Cu^{2+}]_f/(1 + K[Cu^{2+}]_f)$) with g = 3 and K = 116.5 mM^{-1}

cooperativity[17]. A linear plot, as shown in Fig. 2b, describes a system with non-cooperativity. According to the Scatchard equation, $\nu/[Cu^{2+}]_f = K(g - \nu)$, it might be possible to find binding parameters (K and g) from intercepts and the slope of the Scatchard plot, where K and g are the association binding constant and the number of binding sites, respectively. The results are: g = 3 and K = 116.5 mM^{-1}.

The raw data obtained from isothermal titration calorimetry of hGH interaction with copper(II) ions in two different concentrations of the protein was shown in Fig. 3. Fig. 3a is showing the heat of each injection and Fig. 3b is showing the heat 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(II) ions per mole of hGH (ΔH) *vs.* total concentration of copper ions, Fig. 4a, or *vs.* total concentration of the protein, Fig. 4b.

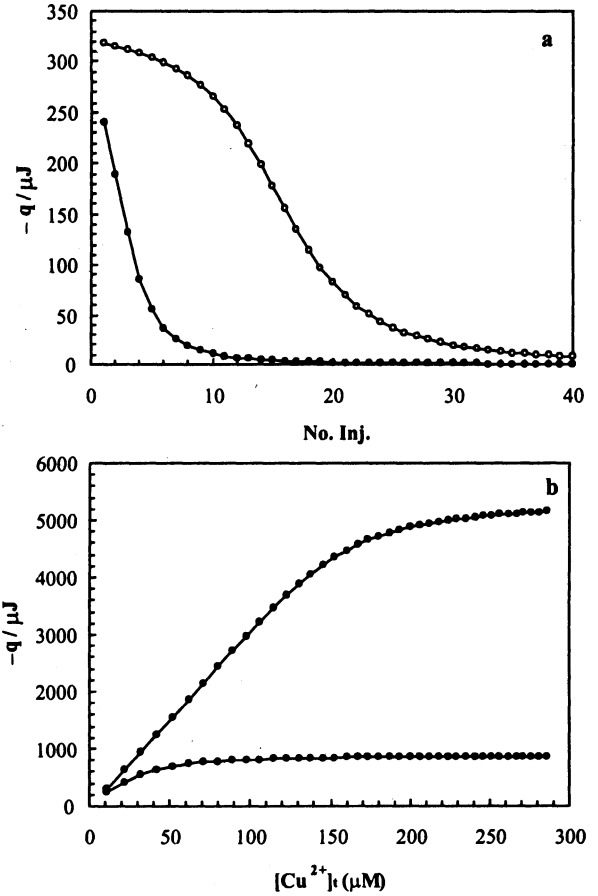

Fig. 3. (a) The heat of Cu^{2+} binding on hGH for 40 automatic cumulative injections, each of 25 μL, of Cu^{2+}, 0.8 mM, into the sample cell containing 1.8 mL hGH solution at two initial concentrations of 10 μM (•) and 60 μM (o). (b) The heat of binding *vs.* total concentration of copper ion, calculated from Fig. 3a

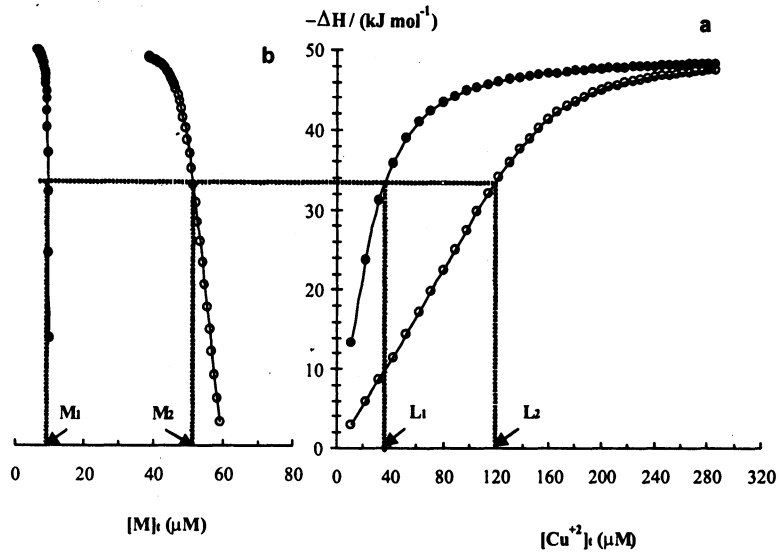

Fig. 4. (a) The heat of binding copper ion per mole of hGH (ΔH) *vs.* total concentration of copper ion, calculated from Fig. 3b. (b) The heat of binding copper ion per mole of hGH (ΔH) *vs.* total concentration of hGH. The initial concentration of hGH was 10 μM (•) and 60 μM (o)

The Scatchard plot, as shown in Fig. 5, can be obtained using a simple method of analyzing data, which has previously been used[18, 19]. The base of this simple method is that at any constant value of ΔH, ν and $[Cu^{2+}]_f$ 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 titration curves, Figs. 4, performed at two different total concentrations of protein (M_1 and M_2), one can determine the set of values of the total ligand concentration (L_1 and L_2) for which ΔH is constant. This is done by drawing a horizontal line, defining a constant ΔH

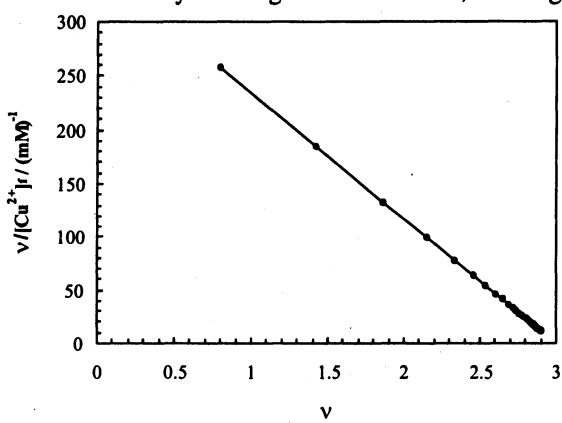

Fig. 5. The Scatchard plot for binding of Cu^{2+} by hGH at 25°C based on the isothermal titration calorimetric data. The best-fit curve of the experimental binding data was transformed to the Scatchard plot using the Scatchard equation ($\nu = gK[Cu^{2+}]_f/(1+K[Cu^{2+}]_f)$) with g = 3 and K = 117.0 mM^{-1}

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, $\nu/[Cu^{2+}]_f$ *vs.* ν, as shown in Fig. 5. This Scatchard plot also shows g = 3 and K = 117.0 mM^{-1}, while there is a very good conformity between both results of titration potentiometry and titration calorimetry. Moreover, values of ΔH in different values of ν (obtained from Fig. 4) give the molar enthalpies of binding −16.7 kJ mol^{-1} in each binding site.

To understand the structural changes of hGH related to the interaction with copper(II) ions, it would be valuable to take a look at the CD spectra of the hormone in the absence and presence of different concentrations of Cu^{2+} (Fig. 6). Detailed secondary structural specifications of hGH can be calculated from the CD spectra in Far region[20]. The results show only a maximum of 0.6 per cent increasing in alpha structure and 1.2 per cent decreasing of beta structure due to the binding of copper(II) ions, which means that the secondary structure of the hormone is not changed considerably.

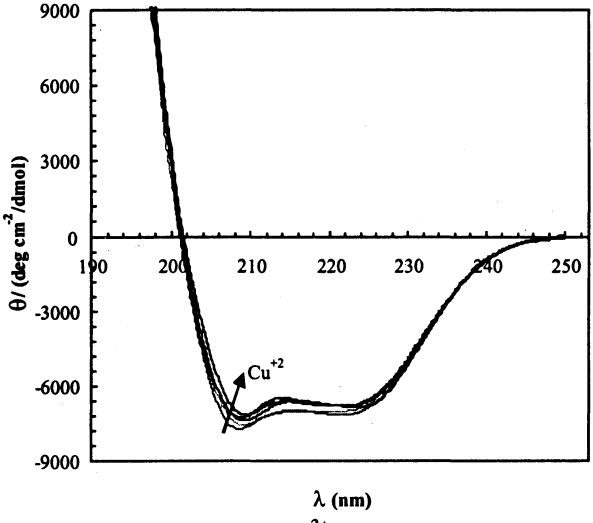

Fig. 6. Far-UV CD spectra of hGH in different Cu^{2+}: hGH ratios of 0, 1 : 1, 2 : 1, 4 : 1 and 6 : 1 in aqueous solution containing NaCl 50 mM at 25°C

To obtain information about the effect of copper(II) ions binding on the stability of the protein, the thermal denaturation curves for hGH were obtained in different concentrations of copper(II) ions (Fig. 7a). The transition temperature (T_m) of the protein does not change significantly by increasing the concentration of Cu^{2+}. However, the absorbance change from the native state to denatured state of the protein at 278 nm is decreased due to the increasing of the concentration of Cu^{2+} which means copper(II) ions binding leads to inaccessibility of aromatic chromophores (tryptophan and tyrosine residues) in denatured state of the protein. The amount of ΔG°_{25} (the standard Gibbs free energy of protein denaturation at 25°C) for different concentrations of copper(II) ions can be obtained from Pace analysis[21] of thermal denaturation curves (Fig. 7b). Determination of the standard

Gibbs free energy of denaturation ($\Delta G°$), as a criterion of conformational stability of a globular protein, is based on two state theory as follows:

$$\text{Native (N)} \rightleftharpoons \text{Denatured (D)}$$

This process was described as a single denaturant-dependent step according to the two-step theory[21,22]. By assuming two-state mechanism for protein denaturation by temperature, one can determine the process by monitoring the

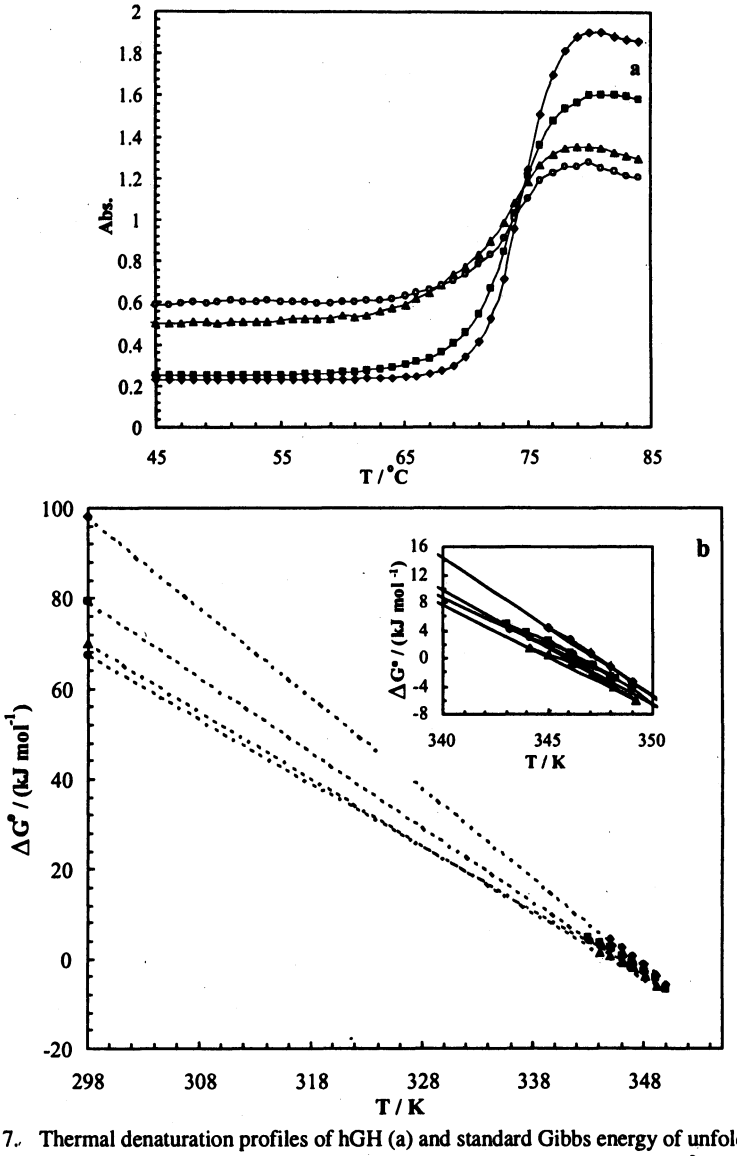

Fig. 7. Thermal denaturation profiles of hGH (a) and standard Gibbs energy of unfolding plots (b) in the absence of copper ion (♦) and in the presence of different Cu^{2+} : hGH ratios of 1 : 1 (■), 2 : 1 (▲) and 3 : 1 (o). There are not any changes in the absorbance of each solution due to the increasing temperature from 25°C to 45°C

changes in the absorbance[22–24] and hence calculate the denatured fraction of protein (F_d) as well as determination of the equilibrium constant (K).

$$F_d = \frac{(Y_N - Y_{obs})}{(Y_N - Y_D)} \tag{2}$$

$$K = \frac{F_d}{(1 - F_d)} = \frac{(Y_N - Y_{obs})}{(Y_{obs} - Y_D)} \tag{3}$$

where Y_{obs} is the observed variable parameter (*e.g.*, absorbance) and Y_N and Y_D are the values of Y characteristics of a fully native and denatured conformation, respectively. The standard Gibbs free energy change ($\Delta G°$) for protein denaturation is given by the following equation:

$$\Delta G° = -RT \ln K \tag{4}$$

where R is the universal gas constant and T is the absolute temperature. $\Delta G°$ varies linearly with T over a limited region. The simplest method of estimating the conformational stability in the room temperature, $\Delta G°_{25}$, is to assume that linear dependence continues to 25°C and to use a least-square analysis. $\Delta G°_{25}$ was estimated to be 98, 79, 70 and 67 kJ mol^{-1} in the Cu^{2+} : hGH molar ratios of 0, 1 : 1, 2 : 1, 3 : 1, respectively. Hence, the copper(II) ions binding process decreases the protein stability of hGH.

In an overall view, it can be concluded that copper(II) ions bind in a set of three identical and independent binding sites on the surface of hGH. The binding process is exothermic ($\Delta H = -16.7$ kJ mol^{-1}) with relatively high affinity for the binding ($K = 1.17 \times 10^5$ M^{-1}). The binding of copper(II) ions does not change the secondary structure of the protein considerably. But this binding process leads to inaccessibility of aromatic amino acid residues (tryptophan and tyrosine). The thermodynamic stability of the protein decreases considerably due to the binding of copper(II) ions on the surface of hGH.

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