Journal of Thermal Analysis and Calorimetry, Vol. 89 (2007) 3, 921–927

BINDING PROPERTIES AND STRUCTURAL CHANGES OF HUMAN GROWTH HORMONE UPON INTERACTION WITH COBALT ION

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Binding properties and structural changes of human growth hormone (hGH) due to the interaction by cobalt ion (Co^{2+}) were done at 27°C in NaCl solution, 50 mM, using different techniques of UV-Vis spectroscopy, circular dichroism (CD), isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC) techniques. There is a set of three identical and non-interacting binding sites for cobalt ions. The intrinsic association equilibrium constant and the molar enthalpy of binding obtained by ITC are 0.80 mM^{-1} and –16.70 kJ mol^{-1}, respectively. The intrinsic association equilibrium constant obtained by a standard isothermal titration UV-Vis spectrophotometry method is also 0.79 mM^{-1}, which is in good agreement with the value obtained from ITC. The Gibbs free energy and entropy changes due to the binding of cobalt ion on hGH are –16.67 kJ mol^{-1} and –0.1 J K^{-1} mol^{-1}, respectively. Energetic domains analysis by DSC shows that phase transition of hGH in the presence of cobalt occurs at one main transition. Deconvolution of the main transition provides two sub-transitions with different values of the melting point and enthalpy of unfolding (33°C and 164 kJ mol^{-1} for the first and 49°C and 177 kJ mol^{-1} for the second, respectively). Interaction of cobalt ions with hGH prevents aggregation by an affect on the hydrophobicity of the protein macromolecule and provide useful information about its structure due to becoming reversible of protein thermal denaturation.

Keywords: *circular dichroism, cobalt, human growth hormone, protein stability, titration calorimetry*

Introduction

Human growth hormone (hGH) is a single domain globular protein containing 191 amino acids [1]. 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 molecular involving residues 53 and 165 (large loop) and another near the C terminal between residues 182 and 189 (small loop) [7]. More than a half of the polypeptide backbone exists in a right-handed α-helical conformation [6, 8].

There are some reports on the binding properties and structural changes of hGH due to its interaction with metal ions [9–12]. Metal-binding site in hGH is located in the hydrophobic core. Well-resolved crystal structure of hGH has been obtained, showing that metal-binding site is likely composed of ^{18}His and ^{21}His on helix I and ^{174}Glu on helix IV [12]. Some metal ions like Zn^{2+}, Cd^{2+}, Hg^{2+} and Co^{2+} are known to promote hGH reversible dimerization. But in the presence of some other ions like Ca^{2+}, Ba^{2+}, Mg^{2+}, Pb^{2+}, Al^{3+}, Fe^{2+} and Fe^{3+} there is no significant dimerization of hGH in solutions [10].

Since there were not any exact reports on the metal binding for hGH in literature, we have attempted to find binding properties and conformational changes of hGH due to the binding of different metal ions. There is a set of three identical and non-interacting binding sites for both magnesium and calcium ions. The intrinsic dissociation equilibrium constant (K_d) and the molar enthalpy of binding (ΔH) are 46 μM and –17.7 kJ mol^{-1}, respectively, for magnesium [13] and 52 μM and –17.4 kJ mol^{-1}, respectively, for calcium [14, 15] ions. Both magnesium and calcium ions binding increase the protein thermal stability by increasing of the alpha helix content as well as decreasing of both beta and random coil structures [13–15]. There is also a set of three identical and non-interacting binding sites for copper ion with a K_d value of 8.5 μM and a ΔH value of –16.7 kJ mol^{-1} [16]. 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 [16]. However, a set of four binding sites has been found on hGH for Fe^{3+}; K_d=40 μM and ΔH= –18.7 kJ mol^{-1} [17]. Interaction

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1388–6150/$20.00

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of three iron ions with hGH prevents irreversibility and aggregation in thermal denaturation of the hormone. In the presence of iron, there are at least two main transitions corresponding with the two groups of helices [17]. In this paper, the interaction between Co^{2+} and hGH has been investigated in neutral aqueous solution to clarify thermodynamics of metal binding properties as well as structural change of the protein due to its interaction with cobalt ion.

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.* [18]. Cobalt(II) nitrate, $Co(NO_3)_2 \cdot 6H_2O$, was purchased from Merck Co. All other materials and reagents were of analytical grades, and solutions were made in NaCl 50 mM (as a solvent) using double-distilled water.

Methods

Isothermal titration UV-Vis spectrophotometry

UV-Vis spectrophotometric titration curve was obtained by measuring the maximum absorbance (278 and 525 nm) of the 2.5 mL solution containing 30 μM hGH *vs.* 2.5 mL solvent solution using a Shimadzu model UV-3100 spectrophotometer and 3-cm cuvettes thermostated to maintain the temperature at 27.0±0.1°C. Appropriate volumes of 0.2 M cobalt nitrate solution were cumulatively added to the both sample and reference cells. All measurements were made after hGH and cobalt ions have been incubated for over 3 min, after which time the absorbance did not change.

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. Cobalt solution (100 mM) was injected by use of a Hamilton syringe into the calorimetric titration vessel including a stirrer, 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 cobalt solution into the perfusion vessel was repeated 30 times, and each injection included 20 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 cobalt 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 cobalt ion, was injected to the protein solution in the sample cell. The enthalpies of cobalt and protein solutions dilution were subtracted from the enthalpy of hGH – cobalt interaction. The microcalorimeter was frequently calibrated electrically during the course of the study. The molecular mass of hGH was taken to be 22 kDa [4, 6].

Obtaining further information on thermodynamics of binding cobalt ion on hGH, it is possible to calculate the standard Gibbs free energy change of binding (ΔG^0) using the association binding constant (K_a) in the equation $\Delta G^0 = -RT\ln K_a$, where R is the gas constant, and T is the absolute temperature (300 K). Finally the standard entropy change (ΔS^0) can be calculated using the equation $\Delta S^0 = (\Delta H^0 - \Delta G^0)/T$, which the value of standard molar enthalpy of binding (ΔH^0) is obtained from ITC experiment.

Circular dichroism experiments

Circular dichroism (CD) spectra were recorded on an Aviv-215 spectropolarimeter (USA). The results were expressed as ellipticity [θ (° cm^2 $dmol^{-1}$)] considering a mean amino acid residues mass (MRW) of 116 and mass of 22 kDa for hGH [4, 6]. The molar ellipticity was determined as $[\theta]_\lambda = (100 \cdot MRW \cdot \theta_{obs}/cl)$, where θ_{obs} is the observed ellipticity in degrees at a given wavelength, c is the protein concentration in mg mL^{-1} and l is the length of the light path in cm. The instrument was calibrated with (+)-10-camphorsulfonic acid, assuming $[\theta]_{291}$=7820° cm^2 $dmol^{-1}$ and standard nonhygroscopic ammonium, (+)-10-camphorsulfonate assuming $[\theta]_{290.5}$=7910° cm^2 $dmol^{-1}$ [19]. The noise in the data was smoothed by using the software included in the spectropolarimeter apparatus. This software uses the fast Fourier-transform noise reduction routine that allows decrease of most noisy spectra without distorting their peak shapes. The software was used to predict the secondary structure of the protein according to the statistical method [20, 21]. Far-UV CD was carried out in the presence of 0.20 mg mL^{-1} (9.1 μM) of hGH.

Fluorescence measurements

Fluorescence intensity measurements were carried out on a Hitachi Spectrofluorimeter model MPF-4. The excitation wavelength was adjusted at 278 nm and the emission spectra were recorded for all of the samples in

the range of 290–400 nm. Fluorescence measurements were carried out in the hGH concentration of 7.0 μM at different fixed concentrations of Co^{2+} by applying a 1-cm path length fluorescence cuvette.

Differential scanning calorimetry (DSC)

DSC measurements were carried out on a Scal-1 calorimeter (Russia), the heating rate was fixed at 2 K min^{-1}. The DSC sample volume was 0.4 mL. An extra pressure of 1.5 atm was maintained during all DSC runs to prevent possible degassing of the solutions on heating. The deconvolution analysis and fitting were done based on Privalov and Potekhin theory [22], which was installed as DOS program in software package (named Scal-2) and supplied by Scal (Russia). This program enables the deconvolution of excess heat capacity (C_p^{excess}) profile into the corresponding subpeaks. The best fitting is selected as a best deconvolution (in this work the best fitting error equaled 0.5%). The baseline was run with NaCl+Co^{2+} in both sample and reference cells. The protein concentration was 1 mg mL^{-1}. The experiment was repeated three times. All experiments were nearly equal so one was selected for deconvolution. Reversibility was checked by cooling the heated sample and repeating the experiment.

Results and discussion

The raw data obtained from ITC 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 cobalt ion, $[Co^{2+}]_t$. For a set of identical and independent binding sites, we have before shown three different methods of ITC data analysis. According to the recently data analysis method, using Eq. (1), a plot of $\Delta q_i/q_{max})M_i$ *vs.* $\Delta q_i/q_i)L_i$ should be a linear plot by a slope of $1/g$ and the vertical-intercept of K_d/g, which g and K_d can be obtained [13, 15].

$$\frac{\Delta q_i}{q_{max}} M_0 = \left(\frac{\Delta q_i}{q_i}\right) L_0 \frac{1}{g} - \frac{K_d}{g} \qquad (1)$$

where g is the number of binding sites, K_d is the dissociation equilibrium constant, M_i and L_i are total concentrations of biomacromolecule and ligand, respectively, $\Delta q_i=q_{max}-q_i$, q_i represents the heat value at a certain L_i and q_{max} represents the heat value upon saturation of all biomacromolecule. If q_i and q_{max} are calculated per mole of biomacromolecule then the standard molar enthalpy of binding for each binding site (ΔH^0) will be $\Delta H^0=q_{max}/g$. The related plot for the binding of cobalt ions by hGH is shown in Fig. 2a. 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 value of 0.99 was obtained using amount of –901.8 μJ (equal to –50.1 kJ mol^{-1}) for q_{max}. The amounts of g and K_d, obtained from the slope and vertical-intercept plot, are 3 and 1.25 mM, respectively. The value for K_a was 0.8 mM^{-1}. Dividing the q_{max} amount of –50.1 kJ mol^{-1} by g=3, therefore, gives ΔH^0= –16.7 kJ mol^{-1}.

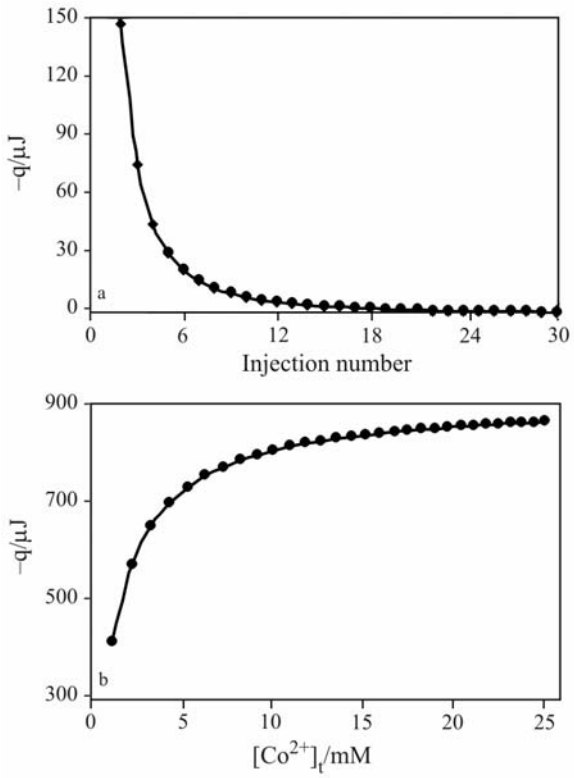

Fig. 1 a – The heat of cobalt binding on hGH for 30 automatic cumulative injections, each of 20 μL, of 0.2 M calcium, into the sample cell containing 1.8 mL hGH solution at initial concentration of 10 μM at 300 K. The heat of first injection was –419.1 μJ. b – The total cumulative heat of binding *vs.* total concentration of cobalt ion, calculated from Fig. 1a

In the second ITC data analysis method, a simple linear plot of q_i/L_i *vs.* q_i can also be used for determination of the association equilibrium constant (K_a), $K_a=1/K_d$, and the molar enthalpy of binding by using equation [23, 24]:

$$q_i/L_i=K_a(\Delta H^0-q_i) \qquad (2)$$

This linear plot was shown in Fig. 2b. The values of K_d and ΔH obtained from the axis intercept and slope are K_d=1.25 mM and ΔH^0= –16.7 kJ mol^{-1}. The results obtained from two methods are identical.

In third ITC data analysis method, for a set of identical and independent binding sites, we have before shown [24–26]:

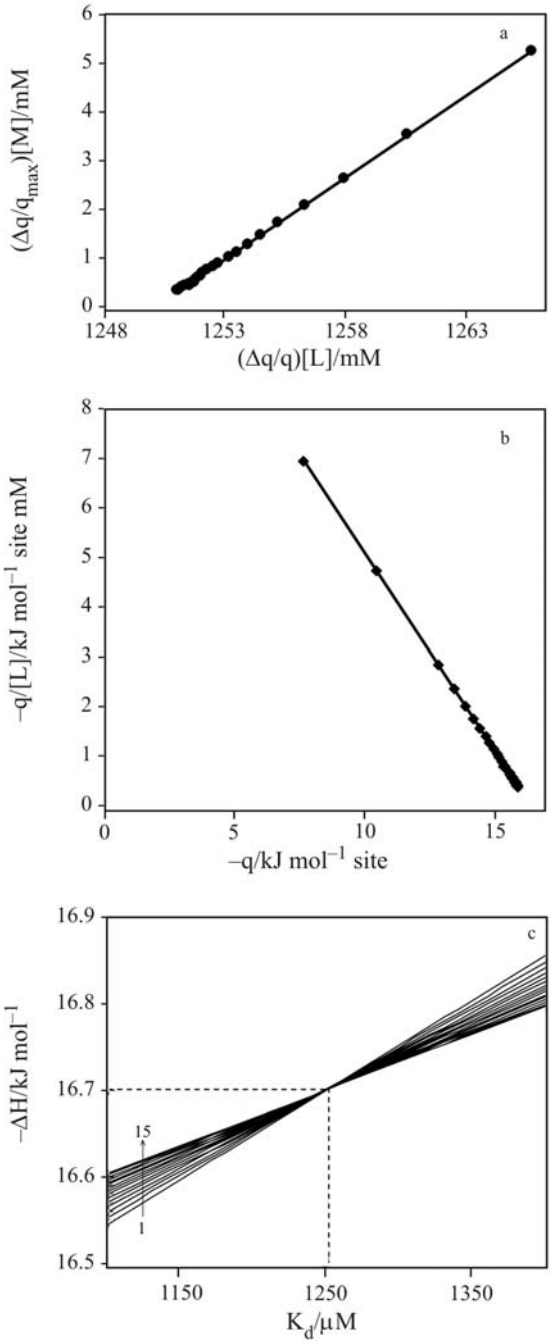

Fig. 2 a – The best linear plot of $(\Delta q/q_{max})M$ *vs.* $(\Delta q/q)L$, according to the Eq. (1), b – the linear plot of q/L *vs.* q, according to the Eq. (2), using data shown in Fig. 1b and c – ΔH *vs.* K_d for the first 15 injections (injection numbers 1 to 15), according to Eq. (3), using data in Fig. 1b

$$\Delta H^0 = 1/A_i[(B_i + K_d) - \sqrt{(B_i + K_d)^2 - C_i}] \quad (3)$$

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

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

where V_i is the volume of the reaction solution in the calorimetric sample cell in each injection step. Equation (3) contains two unknown parameters, K_d and ΔH^0. A series of reasonable amount for K_d is inserted into Eq. (3) and corresponding amounts for ΔH calculated and the graph ΔH^0 *vs.* K_d is constructed. Curves of all titration steps will intersect in one point, which represents true amounts for ΔH^0 and K_d. Actually, this method represents a simple graphical non-linear fitting method. The plots of ΔH^0 *vs.* K_d, according to Eq. (3), for the first 15 injections are shown in Fig. 2c. The intersection of curves gives:

$$K_d = 1.25 \text{ mM} \quad \Delta H^0 = -16.7 \text{ kJ mol}^{-1}$$

The conformity for amounts of K and ΔH^0 obtained from three calorimetric methods are observed.

All thermodynamics parameters (K_d, K_a, ΔG^0, ΔH^0 and ΔS^0) were summarized in Table 1. The binding process of cobalt ion on the hormone is spontaneous ($\Delta G^0 < 0$). The binding process of Co^{2+} is enthalpy driven ($\Delta H^0 < 0$). The electrostatic interaction can be important for cobalt ion interaction due to the enthalpically driven of binding process. So, cobalt ions bind to the more accessible amino acid residues with negative charge on the surface of hGH.

Table 1 Thermodynamic parameters of binding cobalt ion on human growth hormone (hGH) at 27°C obtained by isothermal titration calorimetry

K_d/ mM	K_a/ M^{-1}	ΔH^0/ kJ mol^{-1}	ΔG^0/ kJ mol^{-1}	$T\Delta S^0$/ kJ mol^{-1}
1.25	800	–16.70	–16.67	–0.03

Figure 3 shows the increase in absorbance at both 278 and 525 nm upon titration of a limiting concentration of hGH (30 μM) with increasing aliquotes of a fixed stock concentration of cobalt ion (0.2 M). Note that as the aliquotes of cobalt ion are increased in the titration mixture, the absorption at 278 nm first starts increasing due to the increasing concentration of hGH–Co^{2+} complex, attains a maximum value, and then starts decreasing due to the dilution effect. This behavior is consistent with the prediction of volume (aliquot)-dependence changes in the spectroscopic signals due to protein-ligand interactions proposed by Wang–Kumar–Srivastava [27]. Their equation for calculation of the dissociation constant of ligand–protein complex (K_d) is:

$$K_d = \frac{([L]_0 V_c - [P]_0 V_0)^2}{(V_0^2 - V_c^2)([L]_0 - [P]_0)} \quad (5)$$

where $[P]_0$ and V_0 are initial concentration and volume of the protein solution, respectively, $[L]_0$ is the stock ligand concentration, and V_c is the volume of the ligand solution added to attain the maximum ob-

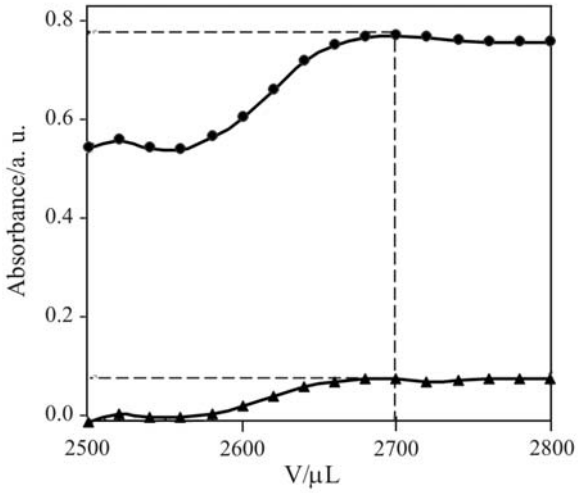

Fig. 3 The change in the absorbance at both • – 278 and ▲ – 525 nm upon titration of a 2.5 mL hGH solution (30 μM) with increasing aliquotes of a fixed stock concentration of cobalt ion (0.2 M) at 27°C

served changes in the spectroscopic signal. Equation (5) is sufficient to calculate the dissociation constant of a protein-ligand complex when the stoichiometry of protein ligand reaction is 1:1. For three identical and independent binding sites on hGH, the concentration of the protein should be multiplied by three to apply Eq. (5). Using Eq. (5) by V_c equal to 200 μL give: K_d=1.27 mM (K_a=0.79 mM^{-1}). The result is approximately consistent with the result obtained by calorimetric method.

To understand the structural changes of hGH related to the interaction with cobalt 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 Co^{2+} (Fig. 4). Secondary structural analysis of hGH can be calculated from the CD spectra in far region [28]. A slight increase in the alpha helix content (2%) and a slight decrease in both beta and random coil structures are resulted due to the presence of cobalt ion in molar ratio of 1:1 respect to the hGH, without any other change by increasing the concentration of Co^{2+}. It means that only the first binding sites effects on the secondary structure of the hormone slightly.

Figure 5 represents the effect of the addition of Co^{2+} concentration on the intrinsic fluorescence of hGH at 27°C. It shows that addition of Co^{2+} induces a significant increase (approximately a maximum of 6.5%) on the emission of native hGH. Hence, increasing the concentration of Co^{2+} has a minor effect on the position of the Trps of the hGH, in which accessibility of Trps are somewhat achieved. On the other words, cobalt ion causes effects on the tertiary structure of hGH. The maximum accessibility of Trps is achieved in the molar ratio of 1:1 for Co^{2+}:hGH. Increasing the molar ratio by increasing the concentration of cobalt

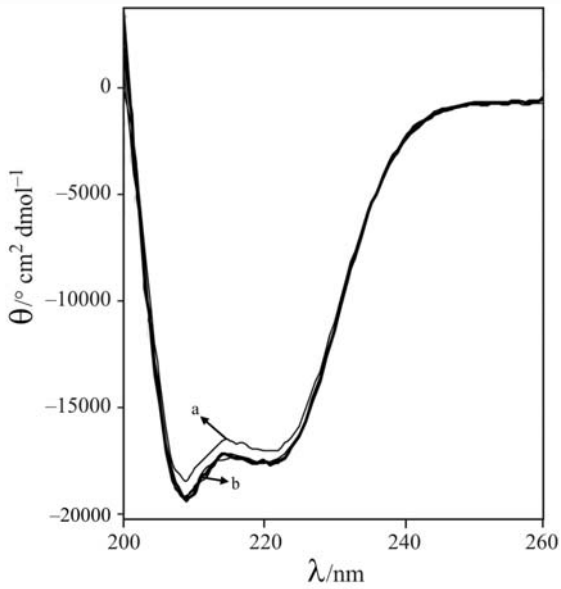

Fig. 4 Far-UV CD spectra of the hGH (9.1 μM) in different Co^{2+}:hGH ratios of a – 0 and b – 2:1, 5:1, 10:1 and 20:1 in aqueous solution containing 50 mM NaCl at 27°C

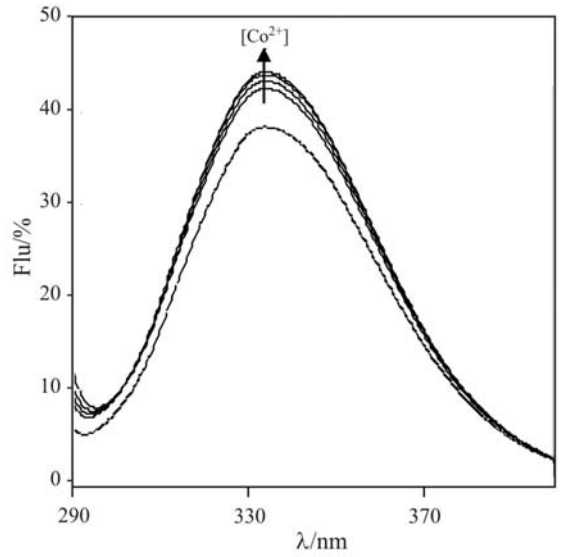

Fig. 5 The effect of increasing concentration of Co^{2+} on the intrinsic fluorescence of hGH (7 μM) in aqueous solution containing NaCl 50 mM at 27°C. The bottom curve (· · ·) is for hGH in the absence of Co^{2+} and other curves are for Co^{2+}:hGH ratios of 2:1, 5:1, 10:1 and 20:1 from bottom to top

does not show any considerable fluorescence change. It is in coincidence with Fig. 4.

The thermal profile of hGH in the presence of 10 of mole of Co^{2+}/mole of protein is illustrated in Fig. 6. Conformational change in the hGH structure occurs in two steps. The phase transitions are reversible in the presence of cobalt ion at 10 of mole of Co^{2+}/mole of protein. Deconvolution of the thermal transition, as

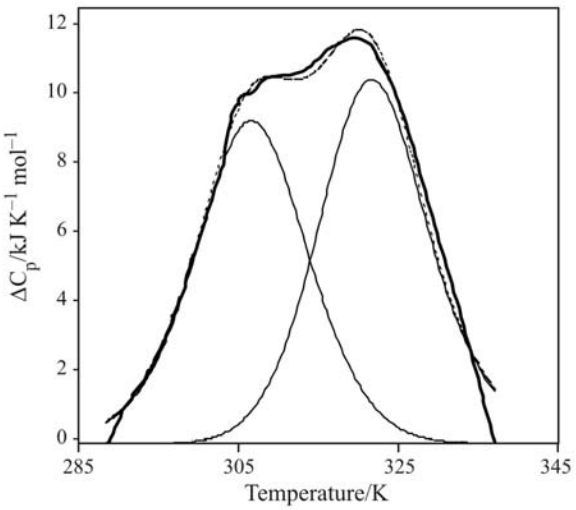

Fig. 6 Partial molar heat capacity of hGH in the presence of cobalt ion with molar ratio of 10 as 'mole of cobalt/mole of hGH' in 50 mM NaCl. Subpeaks were obtained by deconvolution of C_p^{excess} profile. The dotted curve is the sum of two subpeaks

depicted in Fig. 6, shows two sub-peaks for the hGH phase transition. T_m and enthalpy change for the first sub-transition is 306 K and 164 kJ mol^{-1} and for the second sub-transition is 322 K and 177 kJ mol^{-1}, respectively. The structure of hGH is composed of four α-helices in two categories two by two [4]. The DSC findings indicate that the phase transition of the hGH helices in the presence of cobalt ion is a sequential process. It can be concluded that cobalt ion bind to the two parts of the hGH and protect them from aggregation and irreversible process. Increasing hydrophobicity in the core of a protein structure is accompanied with increasing enthalpy of unfolding [29]. The enthalpy of unfolding for the second sub-transition is 13 kJ mol^{-1} higher from the first sub-transition. The forth helix in hGH is the more hydrophobic one [4, 17]. These finding indicate that the last sub-transition belongs to the forth helix in hGH. It has been reported that first and forth helices of hGH are combined together [12, 30], therefore the first sub-transition is related to the second and third helices and the second sub-transition is related to the first and forth helices. The thermal profile of hGH in the absence of cobalt ion did not show reversibility.

Overall, it can be concluded that cobalt ions bind to a set of three identical and independent binding sites on the surface of the hGH molecule. The binding process is exothermic (ΔH=–16.7 kJ mol^{-1}) with relatively high affinity (K=800 M^{-1}). The binding of cobalt ions induces a small change in the secondary structure of the protein, accompanied by a slight change in the tertiary structure of hGH via the accessibility of aromatic amino acids residues (tryptophan and tyrosine). The binding of cobalt ion causes reversible thermal denaturation of the hormone in a sequential process in which the second and third helices are shown phase transition in the first following by two other helices (first and fourth), which need more energy for transition.

Acknowledgements

The financial support of the Research Council of University of Tehran, the Iran National Science Foundation (INSF) and the National Research Center of Genetic Engineering and Biotechnology (NRCGEB), Tehran, are gratefully acknowledged.

References

1 P. C. Hindmarsh and C. G. Brook, Br. Med. J. (Clin. Res. Ed.), 295 (1987) 573.
2 S. Raiti, Advance in human growth hormone research, Department of Health, Education and Welfare (DHEW), Publication No. (NIH) 74-612, Washington DC 1974, pp. 409–433.
3 M. Wallis, S. L. Howell and K. W. Taylor, In the biochemistry of the polypeptide hormone, John Wiley & Sons, New York 1985, pp. 185–222.
4 A. M. de Voc, M. Ultsch and A. A. Kossiakoff, Science, 255 (1992) 306.
5 M. R. Kasimova, S. M. Kristensen, P. W. A. Howe, T. Christensen, F. Matthiesen, J. Petersen, H. H. Sørensen and J. J. Led, J. Mol. Biol., 318 (2002) 679.
6 A. V. Filikov, R. J. Hayes, P. Luo, D. M. Stark, C. Chan, A. Kundu and B. I. Dahiyat, Protein Sci., 11 (2002) 1452.
7 K. M. Youngman, D. B. Spencer, D. N. Brems and M. R. DeFelippis, J. Biol. Chem., 270 (1995) 19816.
8 D. N. Brems, P. L. Brown and G. W. Becker, J. Biol. Chem., 265 (1990) 5504.
9 B. C. Cunningham, M. G. Mulkerrin and J. A. Wells, Science, 253 (1991) 545.
10 G. Dienys, J. Sereikaite, V. Luksa, O. Jarutiene, E. Mistiniene and V.-A. Bumelis, Bioconj. Chem., 11 (2000) 646.
11 T. H. Yang, J. L. Cleland, X. Lam, J. D. Meyer, L. S. Jones, T. W. Randolph, M. C. Manning and J. F. Carpenter, J. Pharm. Sci., 89 (2000) 1480.
12 S. W. Hovorka, T. D. Williams and C. Schöneich, Anal. Biochem., 300 (2002) 206.
13 A. A. Saboury, M. S. Atri, M. H. Sanati, A. A. Moosavi-Movahedi, G. H. Hakimelahi and M. Sadeghi, Biopolymers, 81 (2006) 120.
14 A. A. Saboury, M. S. Atri, M. H. Sanati, A. A. Moosavi-Movahedi and K. Haghbeen, Int. J. Biol. Macromol., 36 (2005) 305.
15 A. A. Saboury, M. S. Atri, M. H. Sanati and M. Sadeghi, J. Therm. Anal. Cal., 83 (2006) 175.
16 A. A. Saboury, M. Kordbacheh, M. H. Sanati, F. Mizani, M. Shamsipur, M. B. Yakhchali and A. A. Moosavi-Movahedi, Asian J. Chem., 17 (2005) 2773.

17 M. S. Atri, A. A. Saboury, M. Rezaei-Tavirani, M. H. Sanati, A. A. Moosavi-Movahedi, M. Sadeghi, H. Mansuri-Torshizi and N. Khodabandeh, Thermochim. Acta, 438 (2005) 178.
18 T. A. Bewley, J. Brovetto-Cruz and C. H. Li, Biochemistry, 8 (1969) 4701.
19 T. Takakuwa, T. Konno and H. Meguro, Anal. Sci., 1 (1985) 215.
20 J. T. Yang, C. S. C. Wu and H. M. Martinez, Methods Enzymol., 130 (1986) 208.
21 P. Marthasarathy and W. C. Jr. Johnson, Anal. Biochem., 167 (1987) 76.
22 P. L. Privalov and S. A. Potekhin, Methods Enzymol., 131 (1986) 4.
23 A. A. Saboury and A. A. Moosavi-Movahedi, J. Enzym. Inhib., 12 (1997) 273.
24 A. A. Saboury, J. Therm. Anal. Cal., 72 (2003) 93.
25 M. Ghadermarzi, A. A. Saboury and A. A. Moosavi-Movahedi, Polish J. Chem., 72 (1998) 2024.
26 A. A. Saboury, A. Divsalar, G. Ataie, A. A. Moosavi-Movahedi, M. R. Housaindokht and G. H. Hakimelahi, J. Biochem. Mol. Biol., 35 (2002) 302.
27 Z. Wang, N. R. Kumar and K. Srivastava, Anal. Biochim., 206 (1992) 376.
28 S. M. Kelly and N. C. Price, Biochem. Biophys. Acta, 1338 (1997) 161.
29 V. V. Loladze, D. N. Ermolenko and G. I. Makhatadze, Protein Sci., 10 (2001) 1343.
30 F. Zhao, E. Ghezzo-Schneich, G. I. Aced, J. Hong, T. Milby and C. Schneich, J. Biol. Chem., 272 (1997) 9019.

Received: February 13, 2006
Accepted: June 16, 2006
OnlineFirst: October 20, 2006

DOI: 10.1007/s10973-006-7533-2