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Binding properties and conformational change of human growth hormone upon interaction with Fe^{3+}

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Abstract

Binding properties and conformational change of human growth hormone (hGH) upon interaction with Fe^{3+} were investigated at 27 °C in NaCl solution, 50 mM, by calorimetry and spectroscopy. UV spectroscopy indicates that thermal denaturation of hGH is an irreversible process and is accompanied by aggregation. At an optimum concentration of iron thermal denaturation of hGH becomes reversible. Results from equilibrium dialysis and isothermal titration calorimetry indicate a set of four binding sites on hGH for Fe^{3+}. Interaction of three iron ions with hGH prevents irreversibility and aggregation. Differential scanning calorimetry confirms the UV spectroscopic finding. Domain analysis by DSC shows that in the presence of iron, there are at least two main transitions corresponding with the two groups of helices. Deconvolution of the main transitions provides two sub-transitions each, the first pair is similar, but the second pair is considerably different in the enthalpy change of unfolding. Interaction of iron ions with hGH prevents aggregation by an effect on the hydrophobicity and provides information about its structure and thermal denaturation.

Keywords: Human growth hormone (hGH); Fe^{3+}; Conformational change; Metal binding; Isothermal titration calorimetry (ITC); Differential scanning calorimetry (DSC)

1. Introduction

Human growth hormone (hGH, somatotropin) is a single chain polypeptide hormone consisting of 191 residues with two disulfide bonds [1]. The major structural feature of the hGH molecule is a four-helical bundle. The NH_2- and COOH-terminal helices (helices 1 and 4) are longer than the other two (26 and 30 residues compared to 21 and 23 residues). In addition to the four helices in the core, three much shorter segments of helix are found in the connecting loops [2]. The molecular mass of hGH is ~22 kDa, with p*I* near 5.3. Approximately 55% of the polypeptide backbone exists in a right-handed alpha-helical conformation [3].

Metal binding site in human growth hormone is located in the hydrophobic core. A well-resolved crystal structure of hGH has been obtained, showing that the metal binding site is likely composed of ^{18}His and ^{21}His on helix 1 and ^{174}Glu on helix 4 [4]. Zn^{2+}, Cd^{2+}, Hg^{2+} and Co^{2+} are known to promote hGH reversible dimerization. But in the presence of Ca^{2+}, Ba^{2+}, Mg^{2+}, Pb^{2+}, Al^{3+}, Fe^{2+} and Fe^{3+} there is no significant dimerization of hGH in solution [5]. Zinc binding to residues 18 and 174 of hGH confines the articulation of helices 1 and 4 and stabilizes hGH structure [6]. 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 [7].

The native state of hGH is stable and does not undergo significant conformational changes between pH 2 and 11 [8]. The hormone is fully unfolded above 5 M guanidine hydrochloride. The thermal transition is highly sensitive to pH changes, which suggests that the unfolding is coupled to the protonation of carboxyl groups. The thermal unfolding is reversible below pH 3.5, and under these conditions a single two-state transition was

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observed. The magnitudes of the ΔH and ΔC_p of this transition indicate that it corresponds to a partial unfolding of rhGH. This is also supported by CD data, which show that significant secondary structure remains after the unfolding [9]. In most cases, these partially folded conformations are stabilized at acidic pH values, mild concentrations of denaturants or extreme salt concentrations [10]. Above pH 3.5 the thermal denaturation is irreversible due to the aggregation of rhGH upon unfolding. This 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 interactions [9].

There are a few reports on the metal binding for hGH in literature. This study detemined the binding properties and conformational changes of hGH due to the binding of Fe^{3+} 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 Fe^{3+}.

2. Materials and methods

2.1. 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. [10]. Visking membrane dialysis tubing (MW cut-off 10,000–14,000) was obtained from Scientific Instrument Center Ltd. (SIC, Eastleigh, Hampshire, England). Ferric chloride was purchased from Merck Co. All other materials and reagents were of analytical grades, and solutions were made in 50 mM NaCl with double-distilled water.

2.2. Methods

2.2.1. Equilibrium dialysis

Experiments were carried out at 300 K, with 32 μM hGH. Two millilitres of aliquots was placed in dialysis bags and equilibrated with 2 ml of Fe^{3+} solutions (12.8–640 μM) for over 96 h. The free Fe^{3+} concentrations in equilibrium with complexes of protein–iron were assayed by atomic absorption (Perkin-Elmer, Model 603) method.

2.2.2. Isothermal titration calorimetric method

The isothermal titration calorimetric experiments were performed with a four-channel Thermal Activity Monitor 2277 (Thermometric, Sweden). The insertion vessel was made from stainless steel. Iron solution (1 mM) was injected by a Hamilton syringe into a stirred vessel, which contained 1.8 ml hGH (2 μM). In another experiment, iron solution (2 mM) was injected into the titration vessel, which contained 1.8 ml hGH (80 μM). Thin (0.15 mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. Injection of iron solution into the perfusion vessel was repeated 30 times, each injection was 20 μl. The heat of each injection was calculated by the "Thermometric Digitam 3" software program. The heat of dilution of the iron solution was measured as described above except hGH was excluded. The heat of dilution of the protein solution was measured as described above except the NaCl solution was injected into the protein solution in the sample cell. The enthalpies of dilution for iron and hGH were subtracted from the enthalpy of protein–iron interaction. The calorimeter was frequently calibrated electrically during the course of the study.

2.2.3. Fluorescence measurements

Fluorescence intensity measurements were carried out on a Hitachi spectrofluorimeter model MPF-4. The binding of a hydrophobic fluorescent probe, 1-anilino naphthalene-8-sulfonate (ANS) to hGH was monitored by exciting the ANS at 350 nm and recording the emission spectra in the range of 400–600 nm.

2.2.4. UV spectrophotometry

Experiments were performed with a Shimadzu model UV-3100, Japan. The sample cell contained 0.8 ml of human growth hormone solution at 0.3 mg/ml (13.8 μM) at 300 K and different fixed concentrations of Fe^{3+} ranging from 0.0 to 72 μM. The reference cell contained NaCl and the same concentration of Fe^{3+} as in the sample cell. Absorbance was recorded at 260–640 nm. In temperature-scanning spectroscopy, absorbance profiles, which describe the thermal denaturation of hGH, were obtained with a CARY-100-Bio spectrophotometer fitted with a temperature programmer. The sample cells contained 30 μM hGH and 0–16.6 mM Fe^{3+}, the reference cuvettes which contained NaCl and the same concentration of Fe^{3+} as in sample cell. The absorbance change at 278 nm was recorded.

2.2.5. Differential scanning calorimetry (DSC)

DSC measurements were carried out on a Scal-1 calorimeter (Russia), the heating rate was fixed at 2 K/min. 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 [11], 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 + Fe^{3+} in both sample and reference cells. The protein concentration was 1 mg/ml. 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.

3. Results and discussion

Equilibrium dialysis data are plotted as $\nu/[Fe^{3+}]_f$ versus ν, the Scatchard plot (Fig. 1). ν is the average number of irons bound to one macromolecule of hGH and $[Fe^{3+}]_f$ is the free concentration of iron ion. The Scatchard plot is linear. The number of binding

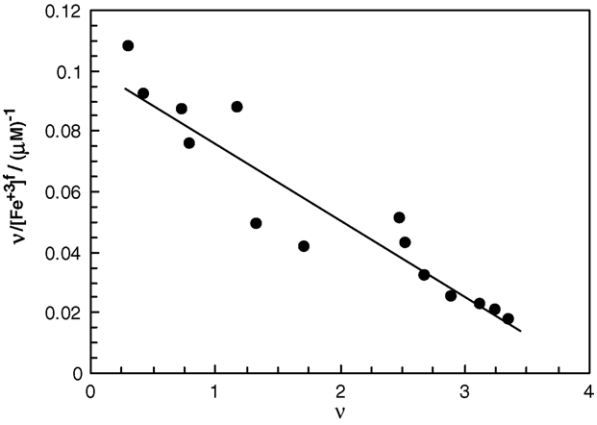

Fig. 1. The Scatchard plots for Fe^{3+} ion on interaction with hGH at NaCl solution (50 mM) and temperatures of 27 °C.

sites (g) and the intrinsic dissociation equilibrium constant (K) are obtained from the abscissa intercept and the slope of the Scatchard plot, respectively, according to:

$$\frac{\nu}{[Fe^{3+}]} = \frac{1}{K}(g - \nu) \tag{1}$$

The equilibrium study shows there is a set of four identical and independent binding sites for Fe^{3+} on the surface of hGH with an intrinsic dissociation equilibrium constant value of 40 μM.

The raw data obtained from isothermal titration calorimetry at two different concentrations of the protein are shown in Fig. 2. Fig. 2a shows the heat of each injection and Fig. 2b shows the cumulative heat at each total concentration of iron ion $[Fe^{3+}]_t$. The Scatchard plot, $\nu/[Fe^{3+}]_f$ versus ν, as shown in Fig. 3, was obtained by the method described previously [12,13]. The Scatchard plot is similar to results obtained by equilibrium dialysis.

For a set of identical and independent binding sites, we have before shown [14–16]:

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

ΔH is the molar enthalpy of binding for Fe^{3+} and A_i, B_i and C_i are constants in each injection i, defined as:

$$A_i = \frac{V_i}{2q_i}, \qquad B_i = gM_i + L_i, \qquad C_i = 4gM_iL_i \tag{3}$$

where V_i is the volume of the solution in the calorimetric sample cell at each injection step, M_i is the total hGH concentration and L_i is the total Fe^{3+} concentration in the sample cell at each injection step. Eq. (2) contains two unknown parameters, K and ΔH. A series of reasonable values for K is inserted into Eq. (2) and corresponding values for ΔH are calculated and the graph ΔH versus K is constructed. Curves of all titration steps will intersect in one point, which represents the true value for ΔH and K. The plots of ΔH versus K for 15 injections are shown in Fig. 4. The intersection of curves gives: $K = 40$ μM and $\Delta H = -18.7$ kJ/mol. The equilibrium constant reported here is actually conditional

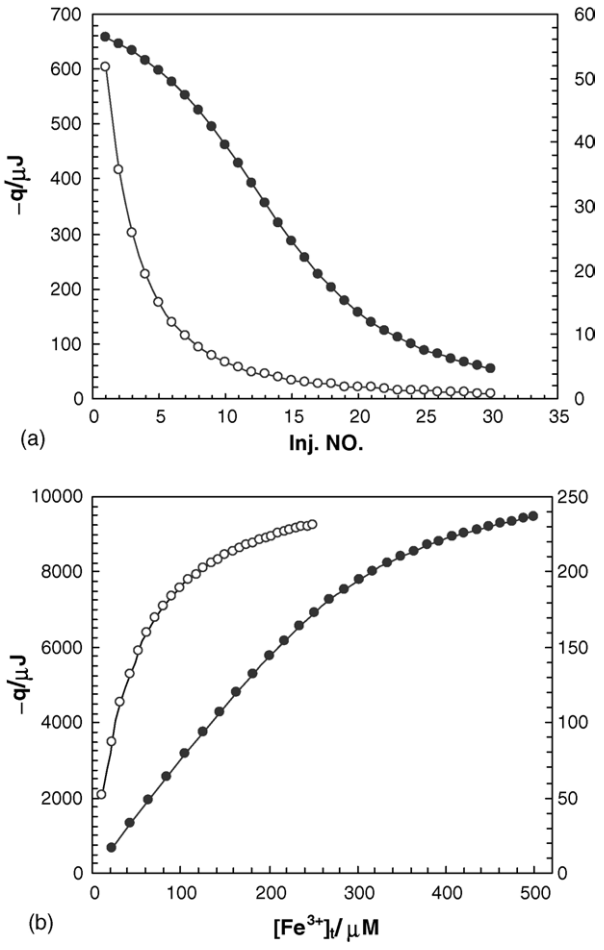

Fig. 2. (a) The heat of Fe^{3+} binding on hGH for 30 automatic cumulative injections, each of 20 μl, of iron, 1 or 2 mM, into the sample cell containing 1.8 ml hGH solution at two initial concentrations of 1 μM (◯) and 80 μM (●). (b) The cumulative heat of binding vs. total concentration of Fe^{3+}, calculated from (a). The left vertical axis is for (●) and the right vertical axis is for (◯).

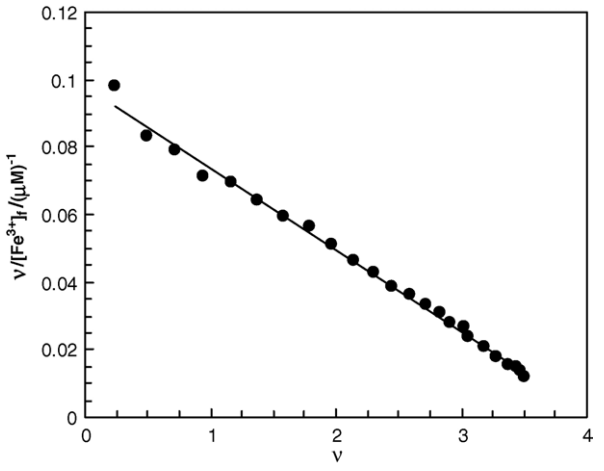

Fig. 3. The Scatchard plot of binding iron ion by hGH at 27 °C using ITC data analysis. The best-fit curve of the experimental binding data was transformed to the Scatchard plot using Eq. (1) with $g = 4$ and $K = 40$ μM.

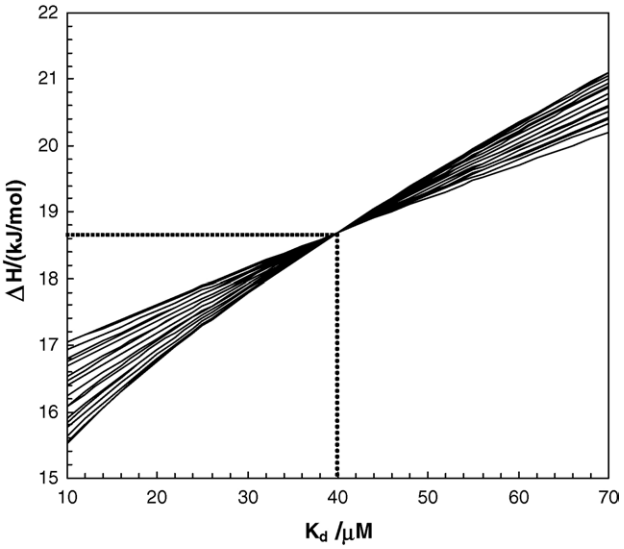

Fig. 4. ΔH vs. K for all 15 injections in the reasonable values of K, according to Eq. (2), using data in Fig. 2 in the initial concentration of 80 μM (●) for hGH. The coordinates of intersection point of curves give true value for ΔH and K.

constant, which is valid only at the same conditions as the measurements were made. The K values obtained from equilibrium dialysis and the two ITC methods agree.

Fig. 5 represents the UV absorbance change of hGH at the wavelength of 278 nm as a function of temperature (25–90 °C) in the presence of various concentrations of Fe^{3+}. Turbidity increased when the temperature is increased. Thermal denaturation of hGH is irreversible and the turbidity of the protein solution is observed in the absence and presence of all concentrations of Fe^{3+}, except at the molar ratio 6.8, mol of iron/mol of protein. The UV spectra of a hGH solution in different concentrations of Fe^{3+} (see Fig. 6) also shows no turbidity at the molar

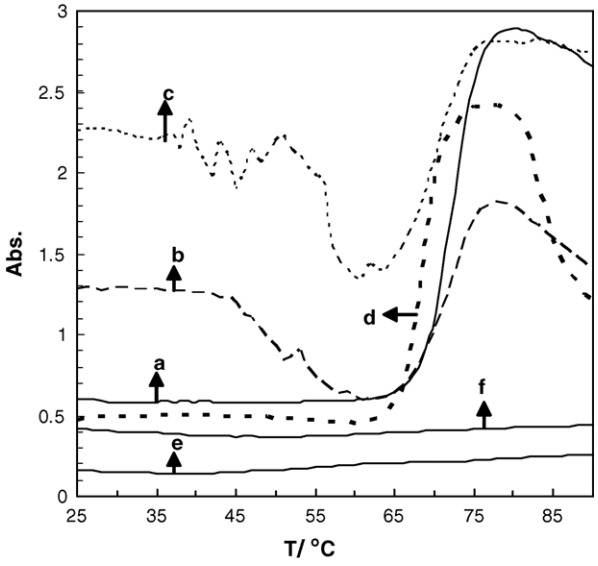

Fig. 5. Profiles of thermal denaturation of hGH in 50 mM NaCl, in the absence of Fe^{3+}: (a), and in the presence Fe^{3+} with different molar ratios ("mol of iron/mol of hGH") of 0.7 (b), 1.4 (c), 2.7 (d), 4.8 (e) and 6.8 (f), detected by UV–vis spectrophotometry at 278 nm.

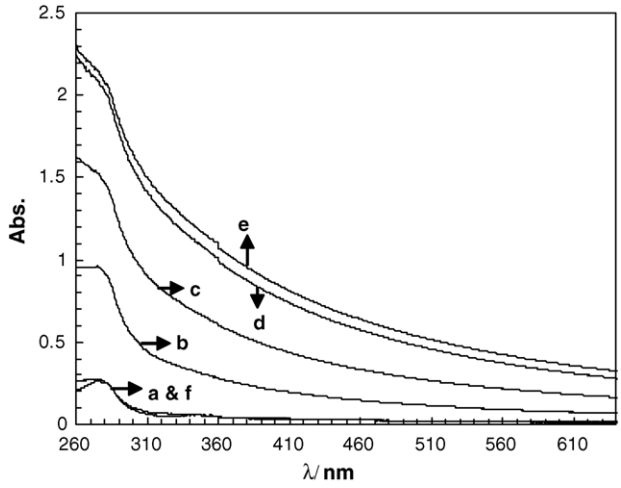

Fig. 6. UV–vis spectra of hGH, in the absence of Fe^{3+}: (a), and in the presence Fe^{3+} with different molar ratios ("mol of iron/mol of hGH") of 0.5 (b), 1 (c), 2 (d), 3 (e) and 6.8 (f) at 27 °C.

ratio of 6.8. According to the binding isotherm, the molar ratio of 6.8 is corresponds with $\nu = 3$. It can be concluded that three iron ions binding prevents aggregation and irreversibility.

The role of Fe^{3+} in the prevention of aggregation may be an effect on the hydrophobicity. Fluorescence is a suitable method to detect hydrophobicity changes [17] with 1-anilo-8-naphtaline sulfonate (ANS). The emission for ANS bound with the hydrophobic sites of the protein increases considerably [18]. The fluorescence spectra of hGH and hGH incubated with iron at the molar ratio 6.8 at 27 °C in the presence of ANS are illustrated in the Fig. 7. The results indicate that the iron ions have considerable effect on the hydrophobicity of hGH since the spectrum of hGH in the presence of iron has higher emission than the spectrum in the absence of iron.

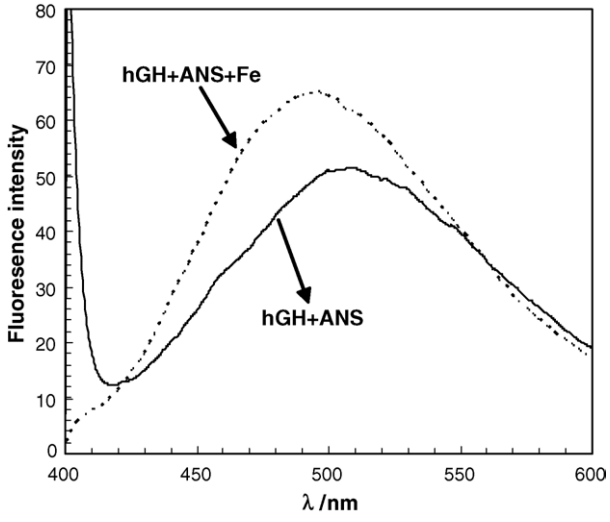

Fig. 7. The fluorescence emission spectra of ANS+ hGH in the absence (—) and in the presence of iron ion with molar ratio of 6.8 as "mol of iron/mol of hGH" (- - -). The concentrations of ANS and hGH are 10 and 5 μM, respsctively. The excitation wavelength was 350 nm. Experiments were done in 50 mM NaCl at 27 °C.

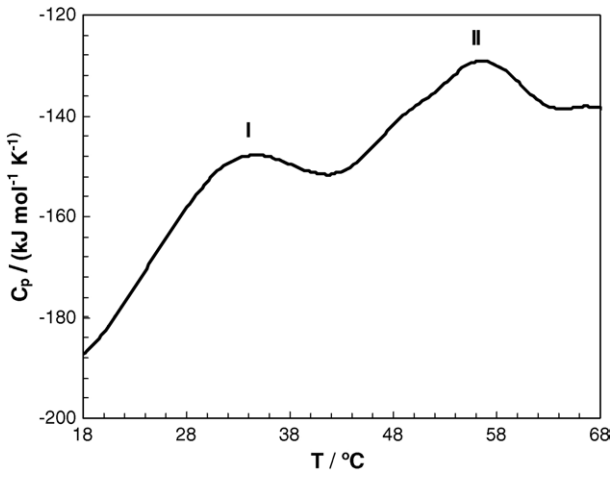

Fig. 8. Partial molar heat capacity of hGH in the presence of iron with molar ratio of 6.8 as "mol of iron/mol of hGH".

Table 1
$T_m s$ and enthalpy changes for the four sub-transitions of hGH as shown in Fig. 9 (i–iv)

Transition	ΔH (kJ mol^{-1})	T_m (°C)
i	229.3	29
ii	233.2	36
iii	230.4	48
iv	265.2	57

The thermal profile of hGH in the presence of 6.8, mol of iron/mol of protein, is illustrated in Fig. 8. Conformational change in the hGH structure occurs in two steps. The phase transitions are reversible in the presence of iron at 6.8, mol of iron/mol of protein. Deconvolution of the transitions I and II, as depicted in the Fig. 9, shows four sub-peaks for the hGH phase transitions (sub-transitions i–iv). $T_m s$ and enthalpy changes for these sub-transitions are tabulated in the Table 1. The structure of hGH is composed of four α-helices in two categories two-by-two. One helix is more hydrophobic and longer than the other three helices [2]. The DSC findings indicate that the phase transition of the helices is a sequential process. It can be concluded that three iron ions 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 [19]. As summarized in Table 1, the enthalpies of unfolding for the first three sub-transitions are approximately equal but differ (about 35 kJ/mol) from the last sub-transition. These finding indicate that the last sub-transition belongs to the forth helix in hGH. It is reported that first and forth helixes of hGH are combined together [4,20], therefore sub-transition (iii) that is related to the sub-transition (iv) correspond to the first helix of hGH.

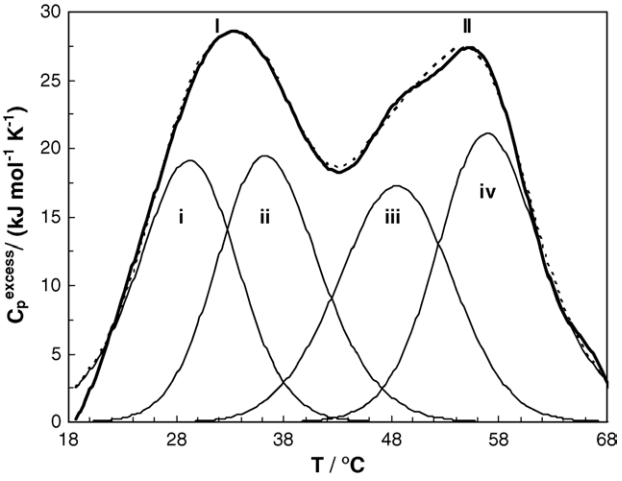

Fig. 9. Thermogram of hGH in the presence of iron with molar ratio of 6.8 as "mol of iron/mol of hGH", in 50 mM NaCl. Subpeaks were obtained by deconvolution of C_p^{excess} profile.

4. Conclusion

There is a set of four identical and independent binding sites for iron ions, which corresponds to the number of helices of hGH. The binding process is exothermic ($\Delta H = -18.7$ kJ/mol) with relatively high affinity (K =40 μM). Binding of three iron ions to one hGH macromolecule prevents irreversibility and aggregation. In the presence of iron at a molar ratio of 6.8, mol of iron/mol of protein ($\nu = 3$), the phase transition of hGH is a reversible and sequential process. Iron binding affects the hydrophobisity of the macromolecule. If the structural changes of hGH in the presence of iron do not affect the function, iron is a suitable candidate for preservation and storage of hGH.

Acknowledgements

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