Journal of the Korean Chemical Society
2008, *Vol. 52, No. 6*
Printed in the Republic of Korea

확장용매화이론을 사용한 마그네슘 및 코발트이온과 인간성장호르몬과의 상호작용에 대한 열역학적 연구

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(2008. 9. 12 접수)

Using the Extended Solvation theory for Thermodynamic Study on the interaction of Magnesium and Cobalt ions with human growth hormone

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(Received September 12, 2008)

요 약. 등온적정열량계법을 사용하여 27 °C NaCl 용액(50 mM)에서 마그네슘 및 코발트이온(M^{2+})과 인간성장호르몬(hGH)과의 상호작용에 대한 열역학적연구를 수행하였다. 등온적정열량계법을 사용하여 hGH과 M^{2+}간의 결합등온선을 얻었다. 이로부터 코발트 및 마그네슘이온에 각각 동일한 3개 및 2개의 비상호작용위치가 있음을 알았다. 확장용매화이론을 사용하여 여러 금속이온농도에 걸쳐서 hGH과 M^{2+} 간의 상호작용엔탈피를 계산하였다. 용매화모델로부터 얻은 용매화변수들은 금속이온과 상호작용에 의한 hGH의 구조변화에 기인한다. 확장용매화모델을 사용하여 Mg^{2+} and Co^{2+} 결합의 단백질안정에 미치는 영향을 해석하였다.

주제어: 등온적정열량계법, 인간성장호르몬 (hGH), 용매화모델

ABSTRACT. A thermodynamic study on the interaction between magnesium and cobalt ions (M^{2+}), and human growth hormone, hGH, was studied at 27 °C in NaCl solution (50 mM) using the isothermal titration calorimetry. Isothermal titration calorimetry was applied to obtain the binding isotherm for hGH+M^{2+}. The results obtained indicate that there is a set of three identical and noninteracting binding sites for Cobalt ions and a set of two for magnesium. The extended solvation model was used to reproduce the enthalpies of M^{2+}+hGH interactions over the whole metal ions concentrations. The solvation parameters recovered from the solvation model were attributed to the structural change of hGH due to the metal ion interaction. The extended solvation model was applied to elucidate the effect of Mg^{2+} and Co^{2+} binding on the protein stability.

Keywords: Isothermal Titration Calorimetry, Human Growth Hormone (hGH), Solvation Model

INTRODUCTION

Minerals are classified into two main groups: macro elements (>100 mg/day) and micro elements (<100 mg/day). Some of the macro elements are calcium, phosphorus, sodium, potasium, chlorine (as chloride), magnesium and sulphur.[1,2] Magnesium is an essential element that is required for the catalytic activity of numerous metalloenzymes and serves a structural role by stabilizing the conformation of

certain metal dependent protein domains, such as calcium-binding proteins and receptors. It participates in all reactions involving the formation and utilization of ATP and thus has a critical role in the transfer, storage and utilization of energy within the body. Magnesium is also required for protein and nucleic acid synthesis and for a number of mitochondrial reactions.[2] Magnesium deficiencies can have devastating effects leading to muscle paresis and seizures. Moreover, magnesium depletion has been implicated in a variety of vascular diseases ranging from hypertension and cardiac arrhythmias to asthma and migraines.[3] The important micro elements essential for normal body functions are iron, copper, iodine, fluorine, zinc, cobalt, manganese, chromium, molybdenum, selenium etc. Cobalt occurs in small amount in all tissues, higher concentrations occurring in liver and kidneys. Most of the cobalt is present in vitamin B12, which is necessary for red blood cells maturation.[1,2]

Human growth hormone, hGH, as a single domain globular protein containing 191 amino acids, plays an important role in somatic growth through its effects on the metabolism of proteins, carbohydrates, and lipids. hGH is produced recombinantly and is available worldwide for clinical use. It has limited stability in solution. Development has therefore focused on more stable and understanding on its interaction with ligands. The interaction of hGH with some of divalent metal ions (Ca^{2+} and Cu^{2+}) in aqueous solution was studied using different techniques. The binding isotherm for hGH-metal ion was obtained by two techniques of potentiometric, using a metal-selective membrane electrode, and isothermal titration calorimetry. The circular dichroism spectroscopy study on the protein upon interaction with Cu^{2+} does not show any changes on the secondary structure of hGH. However, the stability of the protein decreases due to the binding of copper ions. The importance of metal ions such as Zn^{2+} and Cd^{2+} in determining the stability of proteins is widely reported.[4-11] 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. Reports have shown that some metal ions like Zn^{2+}, Cd^{2+}, Hg^{2+} and Co^{2+} are known to promote hGH reversible dimerization.[12,10] But 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 in solutions.[13,10] In this paper, the interaction between two different classes of minerals Mg^{2+} (macro element) and Co^{2+} (micro element) ions and hGH was studied at 27 °C in NaCl solution using the isothermal titration calorimetry. The solvation parameters recovered from the high performance solvation theory were attributed to the structural change of hGH and its biological activity.

MATERIALS

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 mM NaCl using double-distilled water.

Methods

The isothermal titration microcalorimetric experiments were performed with the four channel commercial microcalorimetric system, Thermal Activity Monitor 2277, Thermometric, Sweden. The titration vessel was made from stainless steel. The metal nitrate solutions [2 mM $Mg(NO_3)_2$ and 30 mM $Co(NO_3)_2$] was injected by use of a Hamilton syringe into the calorimetric titration vessel, which contained 1.8 mL hGH (35 μM hGH+2 mM $Mg(NO_3)_2$ and 60 μM hGH+30 mM $Co(NO_3)_2$). Thin (0.15 mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. Injection of metal nitrate solutions into the perfusion vessel was repeated 30 times, with 20 μL per injection. 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 heats of dilu-

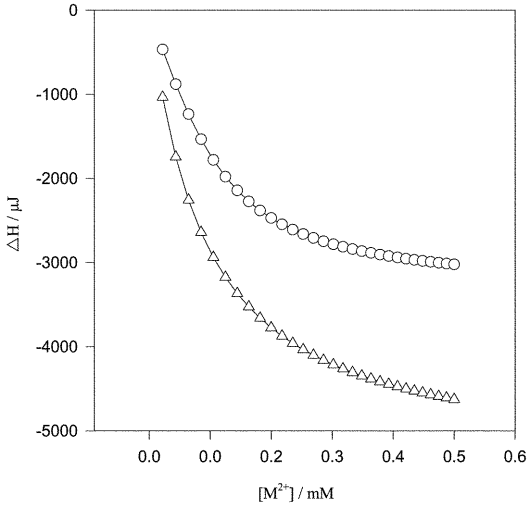

Fig. 1. The heat of Magnesium (O) and Cobalt ions (△) binding with hGH for 30 automatic cumulative injections, each of 20 μl, 2mM of the cations solutions, into sample cell containing 1.8 ml 35 μM hGH solution vs. total concentration of M^{+2} ions (Magnesium or Cobalt ions).

tion of the metal ions solution were measured as described above except 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. The heats of metal ions binding (raw data) with hGH are shown against the metal ion concentrations in *Fig*. 1.

RESULTS AND DISCUSSION

We have shown previously[14-24] that the enthalpies of the ligand+hGH interactions in the aqueous solvent (M^{2+}+water in the present case) system, can be calculated via the following equation:

$$\Delta H = \Delta H_{max} x_B' - \delta_A^{\theta}(x_A' L_A + x_B' L_B) - (\delta_B^{\theta} - \delta_A^{\theta})(x_A' L_A + x_B' L_B) x_B' \quad (1)$$

ΔH are the enthalpies of M^{2+}+hGH interactions and ΔH_{max} represents the heat value upon saturation of all hGH. The parameters δ_A^{θ} and δ_B^{θ} are the indexes of hGH stability in the low and high M^{2+} concentrations respectively. Cooperative binding requires that the macromolecule have more than one binding site, since cooperativity results from the interactions between identical binding sites with the same ligand. If the binding of ligand at one site increases the affinity for ligand at another site, the macromolecule exhibits positive cooperativity. Conversely, if the binding of ligand at one site lowers the affinity for ligand at another site, the protein exhibits negative cooperativity. If the ligand binds at each site independently, the binding is non-cooperative. $p<1$ or $p>1$ indicate positive or negative cooperativity of macromolecule for binding with ligand respectively; $p = 1$ indicates that the binding is non-cooperative. x_B' can be expressed as follow:

$$x_B' = \frac{p x_B}{x_A + p x_B} = \frac{\nu}{g} \quad (2)$$

x_B is the fraction of the metal ion needed for saturation of the binding sites, and $x_A = 1 - x_B$ is the fraction of unbounded M^{2+} ions. Now the model is a simple mass action treatment, with metal ions replacing water molecules, at the binding sites in the present case. We can express x_B fractions, as the total M^{2+} concentrations divided by the maximum concentration of the M^{2+} upon saturation of all hGH as follow:

$$x_B = \frac{[M^{2+}]_T}{[M^{2+}]_{\max}} \quad x_A = 1 - x_B \quad (3)$$

$[M^{2+}]$ is the total concentration of metal ions and $[M^{2+}]_{max}$ is the maximum concentration of the M^{2+} upon saturation of all hGH. In general, there will be "g" sites for binding of M^{2+} per hGH molecule and ν is defined as the average moles of bound M^{2+} per mole of total hGH. L_A and L_B are the relative contributions due to the fractions of unbounded and bounded metal ions in the enthalpies of dilution in the absence of hGH and can be calculated from the enthalpies of dilution of M^{2+} in buffer, ΔH_{dilut}, as follow:

$$L_A = \Delta H_{dilut} + x_B \left(\frac{\partial \Delta H_{dilut}}{\partial x_B} \right),$$

$$L_B = \Delta H_{dilut} + x_A \left(\frac{\partial \Delta H_{dilut}}{\partial x_B} \right) \quad (4)$$

The enthalpies of M^{2+}+hGH interactions, ΔH, were fitted to Eq. 1 across the whole M^{2+} compositions. In the procedure the only adjustable parameter (p) was changed until the best agreement between the experimental and calculated data was approached. δ_A^θ and δ_B^θ parameters have been also optimized to fit 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. 1. The small relative standard coefficient errors and the high r^2 values (0.99999) support the method. The agreement between the calculated and experimental results (*Figs*. 2 and 3) is striking, and gives considerable support to the use of Eq. 1.

Φ is the fraction of hGH molecule undergoing complexation with M^{2+} ions which can be expressed as follow:

$$\Phi = \frac{\Delta H}{\Delta H_{\max}} \tag{5}$$

The appearance association equilibrium constant values, K_a, as a function of M^{2+} concentration can

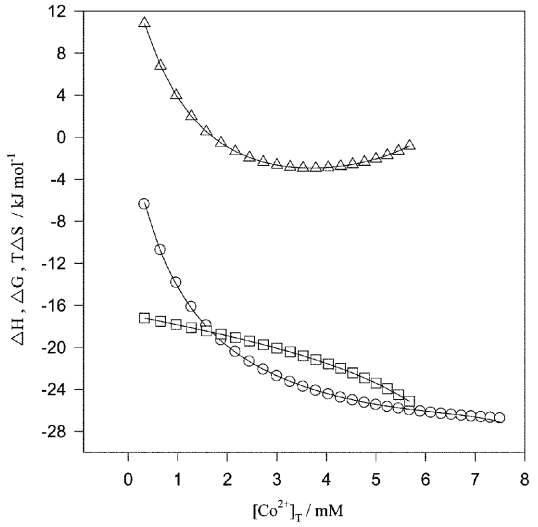

Fig. 2. Comparison between the experimental H (O), G (□) and TS values (△) for hGH+ Co^{2+} interactions, and their calculated data (lines) via Eqs. 1 and 7. $[Co^{2+}]_T$ is the total concentration of Co^{2+} in mM. As it is clear, the Co^{2+}-induced structural changes of hGH in the enthalpies and entropies of interactions, have canceled each other exactly in the free energies of interaction. The compensation of the structural change in the free energy is another support for Eqs. 1 and 7.

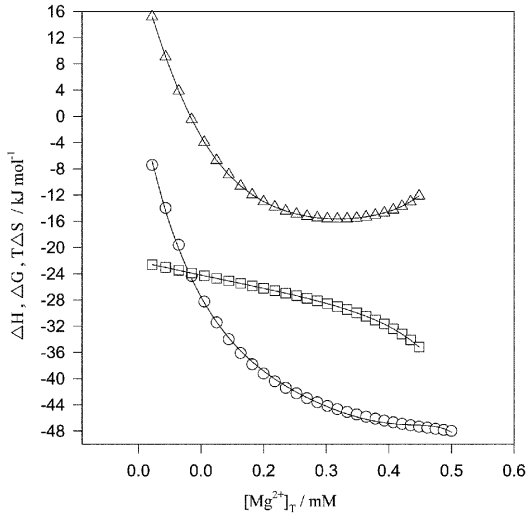

Fig. 3. Comparison between the experimental H (O), G (□) and TS values (△) for hGH+ Mg^{2+} interactions, and their calculated data (lines) via Eqs. 1 and 7. $[Mg^{2+}]_T$ is the total concentration of Mg^{2+} in mM. As it is clear, the Mg^{2+}-induced structural changes of hGH in the enthalpies and entropies of interactions, have canceled each other exactly in the free energies of interaction. The compensation of the structural change in the free energy is another support for Eqs. 1 and 7.

be calculated as follow:

$$k_a = \frac{\Phi}{(1-\Phi)[M^{2+}]_F} \tag{6}$$

$[M^{2+}]_F = (1-x_B)[M^{2+}]_T$ are the unbounded or the free M^{2+} ion concentrations. The variable Φ represents the fraction of binding sites that are occupied on the peptide molecule. Therefore, 1-Φ represents the fraction of binding sites that are not occupied. The appearance association equilibrium constants, K_a, for successive replacement of water molecules by M^{2+} ions are as follow:

$$K_a = x_A^g - \sum_{i=1}^{g} K_i \frac{x_B^i}{x_A^{i-B}} \tag{7}$$

Where the K_i's are the macroscopic association equilibrium constants which are the equilibrium constants for every successive replacement of water molecules by M^{2+} ions in the equilibria:

$$hGH(H_2O)_g + i(M^{2+}) \Leftrightarrow hGH(H_2O)_{g-i}(M^{2+})_i \quad \text{(E1)}$$

K_a values obtained from Eq. 6, have been fitted to Eq. 7 using a computer program for nonlinear least-square fitting. Therefore, we can approach to "g" value and the macroscopic association equilibrium constants in the first, K_1, the second, K_2, and the third, K_2, binding site, which are corresponded to ν=1, ν=2 and ν=3 respectively. ν values can be calculated at any concentration of M^{2+} via Eq. 2. The binding parameters obtained form this method are listed in the *Table* 1. The Gibbs energies as a function of M^{2+} concentrations can be obtained as follow:

$$\Delta G = -RTLnK_a \quad (8)$$

Gibbs energies, ΔG, calculated from Eq. 8 have shown graphically in *Figs*. 2 and 3. ΔS values were calculated using ΔG values and have shown in *Figs*. 2 and 3. The less negative Gibbs free energies in the low M^{2+} concentrations (*Figs*. 2 and 3) indicate the lower affinity in this region.

Previous investigations have shown that metal-binding site in hGH is located in the hydrophobic core and likely composed of His18 and His21 on helix I and Glu174 on helix IV. For example Zinc binding to residues 18 and 174 of hGH confines the articulation of helices 1 and 4 and stabilizes hGH structure.[10] It is possible to introduce a correlation between change in δ_A^θ and increase in the stability of proteins. The δ_A^θ value reflects the hydrophobic property of hGH, leading to the enhancement of water structure. The greater the extent of this enhancement, the greater the stabilization of the hGH structure and the greater the value of δ_A^θ. δ_A^θ value (*Table* 1) for hGH+M^{2+}interactions are small and positive (*Table* 1), indicating that in the low concentration of the metal ions the hGH structure is stabilized, resulting in an increase in its biological activity. The previously reported results indicates 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 to denaturation and maintain the activity of hGH long periods at temperatures up to and beyond 37 °C.[25] The positive values of δ_A^θ and δ_B^θ indicate that Co^{2+} ions stabilize hGH srtucture (*Table* 1), which is in agreement with the formation of stable cobalt-hGH dimers. The Co^{2+}-hGH dimer is significantly more stable to denaturation than monomeric hGH. The analyses via Eq. 1 indicates no denaturation of hGH due to interaction with Co^{2+} ions which is consistent with the formation of $(Co^{2+}\text{-hGH})_2$ complex.

Table 1. Binding parameters for hGH + Co^{2+} and hGH + Mg^{2+} interactions via Eqs. 1 and 7. p> 1 indicates that there is positive cooperativity which is in agreement with $K_3>K_2>K_1$

Parameters	hGH+Co^{2+}	hGH+Mg^{2+}
δ_A^o	1.594	1.757
δ_{Bq}^o	0.809	-0.075
K_1/mM^{-1}	5.650	22.601
K_2/mM^{-1}	10.061	146.733
K_3/mM^{-1}	18.240	
ΔH_{max}/kJ mol^{-1}	-26.891	-48.823
g	3	2
p	8.285	6.650

Our results have very good agreement with previous studies that represented calcium ions binding increase the hGH thermal stability by increasing of the alpha helix content as well as decreasing of both beta and random coil structures. Other investigations with Differential scanning calorimetry (DSC) revealed that interaction of three iron ions with hGH prevent irreversibility and aggregation by an effect on the hydrophobicity and there are at least two main transitions corresponding with the two groups of helices.[10]

A value of p=1 would mean that $K_1=K_2$, indicates that the binding of the second site occurs non-cooperatively as compared to binding of the second site, whereas a value of p>1 would indicate that binding of the second site is facilitated and a value of p<1 indicates that binding of the second site is inhibited (anticooperativity or negative cooperativity).

p values are more than one (p=8.582 and p= 6.655), indicating that there are a set of three identical binding sites for hGH+Co^{2+} with positive cooperativity and a set of two identical binding sites for hGH+Mg^{2+} with positive cooperativity. We will arrive to the same conclusion via Eq. 7 because the mac-

roscopic association equilibrium constants recovered from this equation are increased from K_1 to K_3 (*Table* 1). We can calculate all thermodynamic functions, cooperativity parameters, equilibrium constants and stability prediction as a result of ligand interaction with a biopolymer, just using Eqs. 1 and 7 and it is a revolution in the ligand + macromolecule interactions.

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