Thermodynamic Studies on The Interaction of Nickel With Human Serum Albumin

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Abstract The interaction of human serum albumin with divalent nickel ion was studied by equilibrium dialysis, isothermal titration calorimetry (ITC), differential scanning calorimetry (DSC) and circular dichroism (CD) in 30 mmol/L Tris buffer, pH = 7.0. There is a set of 8 identical binding sites for nickel binding on the protein at two temperatures of 300 K and 310 K. The cooperativity in the binding is observed at 310 K. The Hill coefficients at 300 K and 310 K are 0.97 and 1.25, respectively. The interaction between nickel ions and HSA is exothermic. A value of −36.5 kJ for enthalpy of interaction (1∶1 stoichiometry) was obtained. The secondary structure of HSA dose not show any change during the binding nickel ions process. However, the tertiary structure of the protein changes, which shows the existence of two natives like states.

Key words serum albumin, nickel, isothermal titration calorimetry, differential scanning calorimetry, circular dichroism

1 Introduction

Serum albumin proteins are among the most highly studied and applied in biochemistry[1~4]. Albumin is the most abundant protein in blood plasma and one of its main functions is based on a unique ability to bind numerous endogenous and exogenous compounds. Due to its ligand binding properties albumin serves as a circulating depot of some metabolites. This depot effect is often made use of in drug therapy.

Human serum albumin (HSA) is a single peptide chain consisting of 585 amino acids (66.5 ku) as determined by amino acid sequence studies[5] and as deduced from the nucleotide sequence of cloned cDNA[6]. The three-dimensional structure of HSA has been determined crystallographically[7]. It comprises three homologous domains that repeat in the molecule (denoted Ⅰ, Ⅱ and Ⅲ). Each domain is formed by two smaller subdomains, A and B[3,7,8]. The principal function of HSA is to contribute to colloidal osmotic blood pressure and to many transport and regulatory processes[9]. This protein binds a wide variety of substrates, ranging from metals such as calcium[10], zinc[10], copper[11] and cobalt[12] to fatty acids[13], amino acids[14], hormones[15], and an impressive spectrum of therapeutic drugs[16~20]. Although albumin usually interacts with ligands in a reversible manner, it is also able to interact covalently with several compounds. For example, Ag^+, Hg^{2+}[21], cysteine, glutathine[22,23] and lysine vasopressin[24] bind covalently to the sole, free cysteine residue of albumin-in position 34. Two other very important examples of covalent binding are those of D-glucose[25,26] and D-galactose[27]. The ability of albumin to interact reversibly and with a high affinity with different ligands is also of methodological interest. Determination of the concentration of albumin and purification procedures for the protein have been developed on the basis of that ability[28]. For this reason, the interaction of any ligand with this protein must be understood if a suitable comprehension of its pharmacokinetics and pharmacodynamics is desired.

After the discovery of nickel enzyme, the importance in biochemistry of the element nickel has been further confirmed in recent years. The interaction between Ni^{2+} ion and HSA was usually considered to be physiologically inactive, leading to the discovery of a series of novel behaviors in this system. Dixon and Sarkar[29] deduced that there is one prior binding site at N-terminal residue 1 ~ 3 in the binding of HSA with Ni^{2+} ions. A group of China discovered that there are actually two closely neighbouring identical binding sites[30]. They suggested after the binding of the first nickel ion, an induced slow conformational transition happened which leads to the binding of the second nickel ion.

In the present investigation, the interaction of human serum albumin with divalent nickel ions has been studied by equilibrium dialysis, calorimetry and circular dichroism (CD) techniques. Changes in the secondary and tertiary structures of HSA during the binding process of nickel ions to the protein are discussed.

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2 Materials and methods

2.1 Materials

Human serum albumin (HSA) and Tris-HCl were obtained from Sigma Chemical Co. Visking membrane dialysis tubing (MW cut-off 10 000 ~ 14 000) was obtained from Scientific Instrument Center Ltd. (SIC, Eastleigh, Hampshine, England). Nickel nitrate hexahydrate was purchased from Riedel-dehaen Co. All other materials and reagents were of analytical grades, and solutions were made in double-distilled water. Tris-HCl solution with 30 mmol/L concentration, pH = 7.0, was used as a buffer.

2.2 Methods

2.2.1 Equilibrium dialysis: Experiments were carried out at two temperatures of 300 K and 310 K, using an HSA solution with a concentration of 1.0 μmol/L, of which 2 ml aliquots were placed in dialysis bags and equilibrated with 2 ml of the nickel solution, covering the required concentrations range for over 96 h. Corrections for inequalities arising from Donnan effects were negligible at the ionic strength used. The free nickel concentrations in equilibrium with complexes of protein-nickel were assayed by the atomic absorption (Perkin Elmer, model 603) method. The molecular mass of HSA was taken to be 66.5 ku[5].

2.2.2 Isothermal titration calorimetric method: The isothermal titration microcalorimetric experiments were performed with the 4-channel commercial microcalorimetric system, Thermal Activity Monitor 2277, Thermometric, Sweden. Each channel is a twin heat-conduction calorimeter where the heat-flow sensor is a semiconducting thermopile (multi-junction thermocouple plates) positioned between the vessel holders and the surrounding heat sink. The insertion vessel was made from stainless steel. Nickel solution (50 μmol/L) was injected by use of a Hamilton syringe into the calorimetric stirred titration vessel, which contained 1.5 ml HSA, 1.0 μmol/L. Thin (0.15 mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. Injection of nickel solution into the perfusion vessel was repeated 32 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. The heat of dilution of the nickel solution was measured as described above except HSA was excluded. The heat of dilution of the protein solution was measured as described above except the buffer solution was injected to the protein solution in the sample cell. The enthalpies of dilution for nickel and HSA were subtracted from the enthalpy of protein-nickel interaction. The microcalorimeter was frequently calibrated electrically during the course of the study.

2.2.3 Differential scanning calorimetric method: Differential scanning calorimetric (DSC) experiments were carried out on a Scal-1 microcalorimeter (Russian), the heating rate was fixed at 1 K/min. An additional pressure of 2.02×10^5 Pa was applied during all DSC runs; in order to prevent any possible degassing of the solutions during heating. A dos-based software package (Scal-2) was also supplied and used for data analysis. All experiments were repeated three times and the results were identical. The concentration of HSA solution was 1 g/L.

2.2.4 Circular dichroism: Circular dichroism (CD) spectra were recorded on a JASCO J-715 spectropolarimeter (Japan). Near and far UV-CD were used to measure changes in the tertiary and secondary structure of HSA, respectively. Measurements were conducted at 300 K; path length 1.0 cm. The results are expressed as molar ellipticity $[\theta]$ (deg · cm^2 · dmol^{-1}), based on a mean amino acid residues weight (MRW). $[\theta]$ is defined as $[\theta] = 100 \times \theta_{obs}/(l\rho)$, where θ_{obs} is the observed ellipticity in degrees, ρ is the protein concentration in g/L, and l is the length of the light path in cm. The instrument was calibrated with (+)-10-camphorsulfonic acid, assuming $[\theta]_{291} = 7\,820$ deg · cm^2 · dmol^{-1}[31], and with JASCO standard nonhygroscopic ammonium (+)-10-camphorsulfonate, assuming $[\theta]_{290.5} = 7\,910$ deg · cm^2 · dmol^{-1}[32,33]. HSA was diluted in Tris buffer to concentrations of 0.5 and 1.5 g/L for the far UV (190 ~ 250 nm) and near UV (250 ~ 350 nm) measurements, respectively. The data was smoothed by applying the Jasco-J-715 software, including the fast Fourier-transform noise reduction routine, which allows the enhancement of most noisy spectra without distorting their peak shapes.

3 Results and discussion

The binding isotherm has been plotted as the average number of bound nickels to one macromolecule of HSA, υ vs. $\log[Ni^{2+}]_f$, where $[Ni^{2+}]_f$ is the free concentration of nickel ion, as shown in Figure 1a. In this case, the Scatchard plot[34] is approximately linear at 300 K. However, it is not linear at 310 K, as shown in Figure 1b. Therefore, the binding of nickel ion is cooperative[35,36] at 310 K. The number of binding sites (g), the apparent equilibrium constant (K) and the Hill coefficient (n) can be obtained by fitting of experimental data to the Hill equation[37,38]:

$$\upsilon = \frac{g(K[Ni^{2+}]_f)^n}{1 + (K[Ni^{2+}]_f)^n} \quad (1)$$

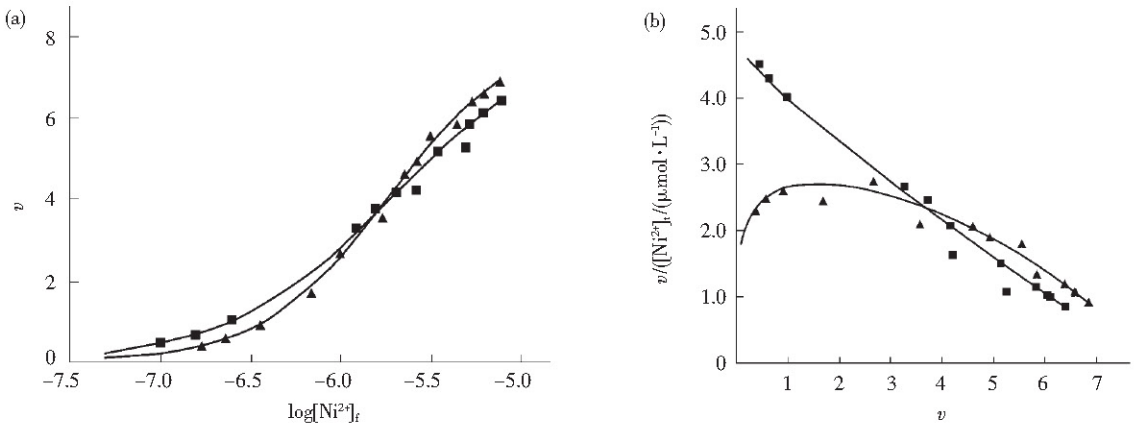

Fig. 1　Binding isotherms (a) and the Scatchard plots (b) for divalent nickel ion on interaction with HSA at pH = 7.0 and two temperatures of 27℃ and 37℃

Experiments were carried out using an HSA solution with a concentration of 1.0 μmol/L, of which 2 ml aliquots were placed in dialysis bags and equilibrated with 2 ml of the nickel solution, covering the required concentrations range for over 96 h. ■——■: 27℃; ▲——▲: 37℃.

The binding data for the binding of nickel ions to HSA have been fitted to Hill equation using a computer program for nonlinear least-square fitting[39]. The results are tabulated in Table 1.

Table 1　Values of binding parameters in the Hill equation for interaction between Ni^{2+} and HSA in 30 mmol/L Tris buffer, pH = 7.0

Parameter	t = 27℃	t = 37℃
g	8	8
K/(L·mmol^{-1})	537	556
n	0.97	1.25

The maximum error on experimental values of v, respect to those values obtained by using these results in equation (1) is ±0.7.

The maximum error on experimental values of v, respect to those values obtained by using these results (Table 1) in equation (1), is ±0.7. There is a set of 8 identical binding sites for nickel binding on the protein at two temperatures of 300 K and 310 K. The Hill coefficients at 300 K and 310 K are 0.97 and 1.25, respectively. So, the positive cooperativity in the binding is observed at 310 K. A relationship between v and total concentration of nickel ion, $[Ni^{2+}]_t$, can be obtained using the following equation:

$$[Ni^{2+}]_t = [Ni^{2+}]_f + v[HSA]_t \quad (2)$$

Which $[HSA]_t$ is the total concentration of protein. According to equation (1) and result tabulated in Table 1, the relationship between v and $[Ni^{2+}]_t$ (by μmol/L) can be obtained as follow:

$$v = -4.5642 + 0.9018[Ni^{2+}]_t - 0.0404[Ni^{2+}]_t^2 + 0.0006[Ni^{2+}]_t^3 \quad (3)$$

The data obtained from isothermal titration microcalorimetry of HSA interaction with nickel ions is shown in Figure 2. Figure 2a shows the heat of each injection and Figure 2b shows the heat related to each total concentration of nickel. The plot of ΔH versus nickel ion concentrations is shown in Figure 3a, which can lead to the plot of ΔH versus v according to

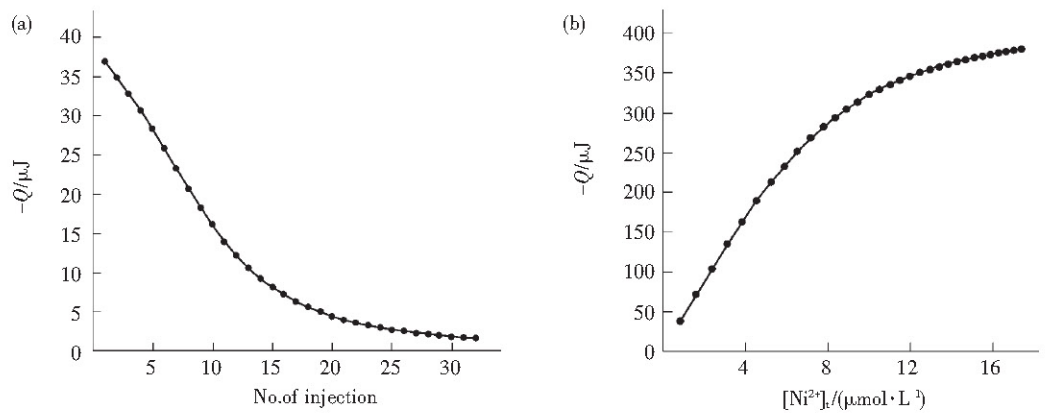

Fig. 2

(a) The heat of nickel binding on HSA for 32 automatic cumulative injections, each of 25 μl, of Ni^{2+} solution 50 μmol/L, into the sample cell containing 1.5 ml HSA solution at a concentration of 1.0 μmol/L at pH = 7.0 and 27℃. (b) The cumulative heat related to each total concentration of nickel.

equation(3) (Figure 3b). From Figure 3b, the enthalpies of interaction between nickel ions and HSA for each of binding sites ($v = 1$ to $v = 8$) can be concluded at 300 K. The enthalpies of interaction for all binding sites are approximately identical with a value of −36.5 kJ per mole of protein and per mole of binding site. So assumed a set of 8 identical binding sites for nickel ions on HSA could be true.

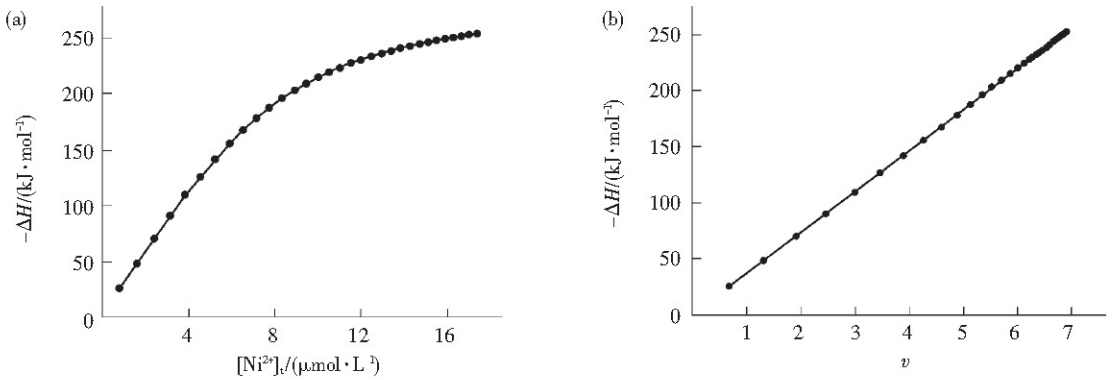

Fig. 3 Plots of ΔH versus nickel ion concentration (a) and ΔH versus v according to equation 3 (b)
Obtained from data shown in **Fig. 2.**

Structural analysis of HSA is studied by differential scanning calorimetry in the absence and in the presence of various concentration of Ni^{2+}. Thermal profile of HSA (Figure 4a) shows two transitions including minor(transition Ⅰ) and major (transition Ⅱ) transitions. The minor transition is reversible but the

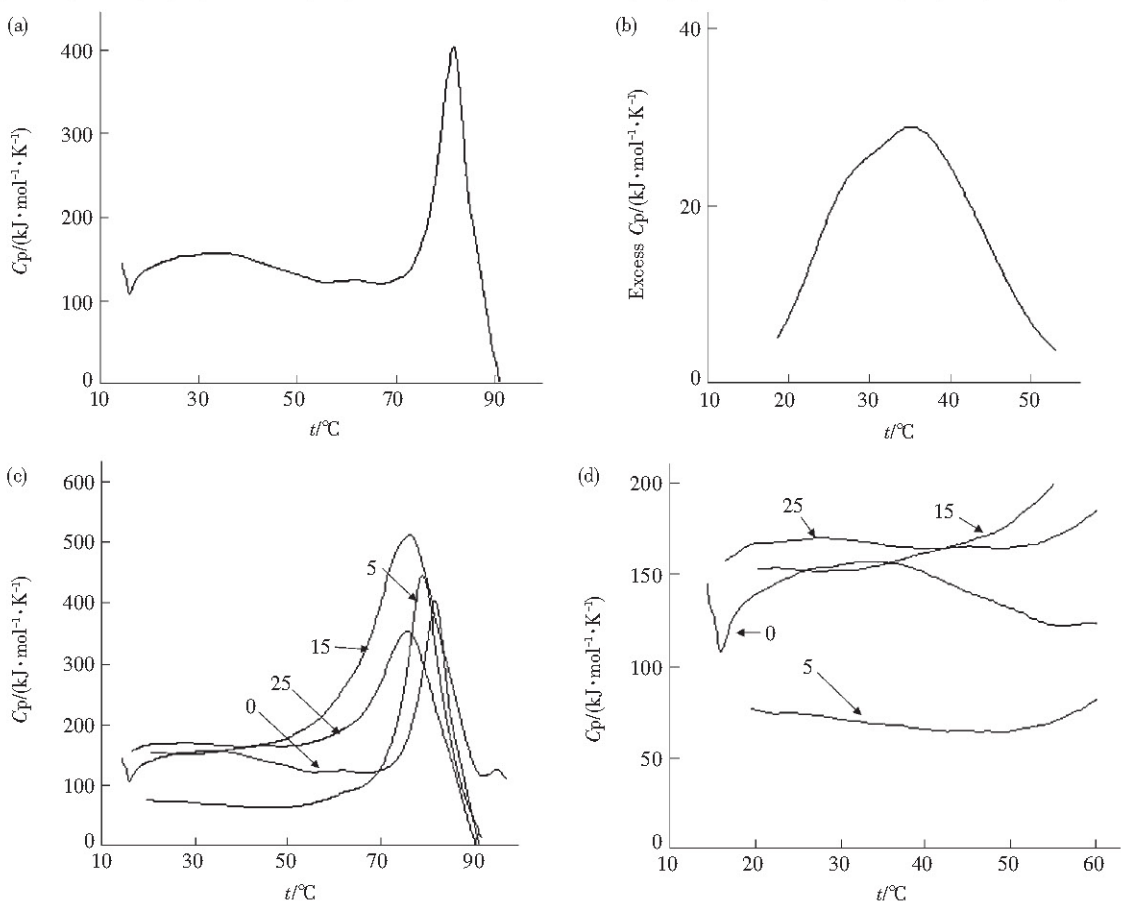

Fig. 4 Thermal profile of HSA
Thermal profile of HSA including two transitions, Ⅰ and Ⅱ(a). Excess heat capacity for transition Ⅰ is represented in (b) to obtain the amount of ΔH_U equal to (662.5 ± 9) kJ/mol. Thermal profiles of HSA in the presence of nickel ion molar ratios 5, 15 and 25 indicate that HSA is unfolded via single transition in the presence of various nickel ion molar ratios (c). The minor transition (Ⅰ) is deleted due to the effect of nickel ion on the HSA (d).

other one is irreversible transition. The amounts of melting point (t_m) for transitions Ⅰ and Ⅱ are 34℃ and 83℃, respectively. Excess heat capacity for transition Ⅰ is represented in the Figure 4b, and the amount of ΔH_U for this transition is equal to (662.5 ± 9) kJ/mol. It was reported that the normal (N) form of bovine serum albumin which occurs at pH 4.3, at higher pHs (8 ~ 9) transforms in a reversible manner into the basic (B) form[40]. Perhaps the reversible transition Ⅰ indicates to the conformational change in HSA. Thermal profiles of HSA in the presence of nickel ion with molar ratios 5, 15 and 25 for $[Ni^{2+}]_t/[HSA]_t$ are represented in the Figure 4c. These profiles indicate that HSA is unfolded via single transition in the presence of various nickel ion molar ratios. The results show that the minor transition is deleted due to the effect of nickel ion on the HSA (Figure 4d). It can be concluded that nickel ion prevents of induction of an intermediate during thermal denaturation of HSA. The amounts of apparent t_m for irreversible thermal denaturation of HSA in the presence of nickel ion molar ratios 5, 15 and 25 are equal to 79℃, 77℃ and 77℃, respectively (Figure 4c). It seems that nickel ion has a mild effect in the stabilization of HSA due to alteration on aggregation of HSA and this effect is appeared in 2 steps. Step 1 is according to the molar ratio of 5 and the second step is consisted to the molar ratios of 15 and 25. The peak width in the DSC profile for phase change region is an index for cooperativity of denaturation[41], the results indicate that presence of nickel ions lead to reduction of cooperativity for thermal denaturation of HSA (Figure 4c). DSC curve in the presence of molar ratio 5 nickel shows mild reduction of cooperativity, while the effect of nickel ion in the molar ratios 15 and 25 on the reduction of cooperativity is a gross effect. It was reported that electrostatic interaction induces new conformational structure for BSA[42]. DSC results indicating that nickel ion in the various concentration induces at least two native-like structures in the HSA.

Figure 5 depicts far-UV CD spectra for HSA in different molar ratios of 0, 5, 15 and 25 for nickel ion respect to the protein. The spectra do not show any significant changes in the secondary structure of HSA.

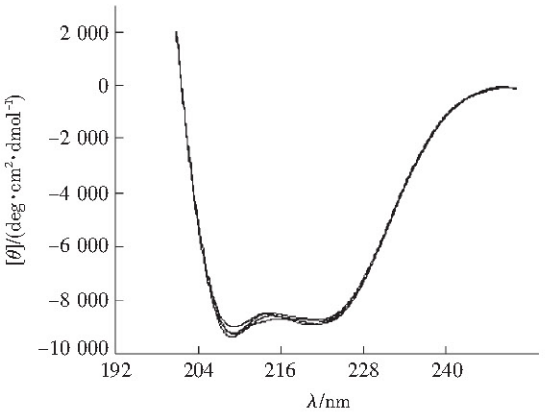

Fig. 5 Far-UV CD spectra of HSA

Far-UV CD spectra (elipticity, [θ]) of HSA in different molar ratios of 0, 5, 15 and 25 for nickel ion respect to the protein at pH = 7.0. The concentration of HSA was 0.5 g/L. Measurements were conducted at 300 K; path length 1.0 cm.

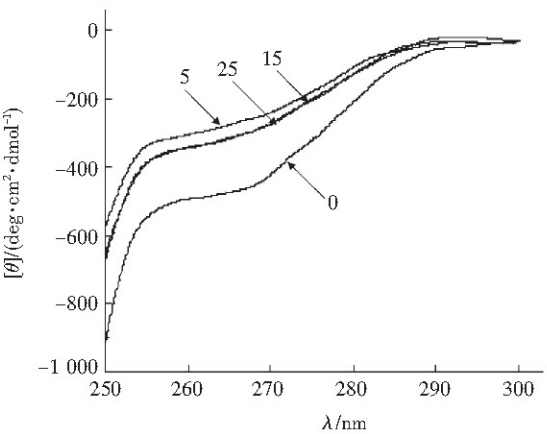

Fig. 6 Near-UV CD spectra of HSA

Near-UV CD spectra (elipticity, [θ]) of HSA in different molar ratios of 0, 5, 15 and 25 for nickel ion respect to the protein at pH = 7.0. The concentration of HSA was 1.5 g/L. Measurements were conducted at 300 K; path length 1.0 cm.

Table 2 The percentage of the secondary structures of HSA in different molar ratios of nickel ions respect to the protein in 30 mmol/L Tris buffer, pH = 7.0

Component	$[Ni^{2+}]/[HSA] = 0$	$[Ni^{2+}]/[HSA] = 5$	$[Ni^{2+}]/[HSA] = 15$	$[Ni^{2+}]/[HSA] = 25$
α-helix	33.0	33.0	32.7	33.2
β-sheet	13.8	14.6	13.1	15.0
turn	37.6	36.5	37.5	36.8
random coil	15.6	15.8	16.7	16.0

The maximum error on these percentage values was ±0.2.

The amount of α-helix, β-sheets, random coil and turn are tabulated in Table 2. It seems that the overall percentage of secondary structure components have not changed dramatically due to the binding of nickel ions to HSA. Figure 6 shows near-UV CD spectra for HSA in different molar ratios of 0, 5, 15 and 25 for nickel ion respect to the protein. Decrease in the tertiary structure of HSA is observed due to the binding of

nickel ions to the protein. But decrease in the tertiary structure of HSA for molar ratios of 15 and 25 are identical to and smaller than for molar ratio of 5. This is similar to the results obtained by DSC, which the effect of molar ratio 5 is different from molar ratios 15 and 25. However, binding of nickel ions to the protein cause decreasing tertiary structure without changing of the secondary structure in HSA. It seems a molten globule native-like state of HSA may be formed.

Existence of at least two native-like structures for HSA is consisted with high ability of this molecule for activity in the various conditions or multi function of protein as a powerful carrier in the serum.

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References

1 Peters T Jr. Serum albumin. Adv Prot Chem, 1985, **37**: 161 ~ 245
2 Kragh-Hansen U. Molecular aspects of ligand binding to serum albumin. Pharmacol Rev, 1981, **33**: 17 ~ 53
3 Carter D C, He X M, Munson S H, *et al.* Three-dimensional structure of human serum albumin. Science, 1989, **244**: 1195 ~ 1198
4 Foster J F. Some aspects of the structure and conformational properties of serum albumin. In: Rosenoer V M, Orats M, Rothschild M A, eds. Albumin Structure, Function, and Uses, Oxford: Pergamon, 1977. 53 ~ 84.
5 Meloun B, Moravek L, Kostka V. Complete amino acid sequence of human serum albumin. FEBS Letters, 1975, **58**: 134 ~ 137
6 Dugiaczyk A, Law S M, Dennison O E. Nucleotide sequence and the encoded amino acids of human serum albumin mRNA. Proc Natl Acad Sci USA, 1982, **79**: 71 ~ 75
7 He X M, Carter D C. Atomic structure and chemistry of human serum albumin. Nature, 1992, **358**: 209 ~ 215
8 Carter D C, He X M. Structure of human serum albumin. Science, 1990, **249**: 302 ~ 303
9 Figge J, Rossing T H, Fenel V. The role of serum proteins in acid-base equilibria. J Lab Clin Med, 1991, **117**: 453 ~ 467
10 Kragh-Hansen U, Brennan S O, Minchiotti L, *et al.* Modified high-affinity binding of Ni^{2+}, Ca^{2+} and Zn^{2+} to natural mutants of human serum albumin and proalbumin. Biochem J, 1994, **301**: 217 ~ 223
11 Laussac J P, Sarkar B. Characterization of the copper (Ⅱ) - and nickel (Ⅱ) -transport site of human serum albumin. Studies of copper (Ⅱ) and nickel (Ⅱ) binding to peptide 1 ~ 24 of human serum albumin by ^{13}C and ^{1}H NMR spectroscopy. Biochemistry, 1984, **23**: 2832 ~ 2838
12 Liang H, Huang J, Tu C Q, *et al.* The subsequent effect of interaction between Co^{2+} and human serum albumin or bovine serum albumin. J Inorg Biochem, 2001, **85**: 167 ~ 171
13 Vorum H, Fisker K, Honore B. Palmitate and stearate binding to human serum albumin. Determination of relative binding constants. J Pep Res, 1997, **49**: 347 ~ 354
14 Jenkins B G, Lanffer R B. Detection of site-specific binding and co-binding of ligands to human serum albumin using ^{19}F NMR. Mol Pharmacol, 1990, **37**: 111 ~ 118
15 Finotti P, Pagetta A. Heparin-induced structural modifications and oxidative cleavage of human serum albumin in the absence and presence of glucose——implications for transcapillary leakage of albumin in hyperglycaemia. Eur J Biochem, 1997, **247**: 1000 ~ 1008
16 Kasai-Morita S, Horie T, Awazu S. Influence of the N-B transition of human serum albumin on the structure of the warfarin-binding site. Biochim Biophys Acta, 1987, **915**: 277 ~ 283
17 Bos O J, Remijn J P, Fischer M J, *et al.* Location and characterization of the warfarin binding site of human serum albumin. A comparative study of two large fragments? Biochem Pharmacol, 1988, **37**: 3904 ~ 3909
18 Bos O J, Fischer M J, Wilting J *et al.* Mechanism by which warfarin binds to human serum albumin. Stopped-flow kinetic experiments with two large fragments of albumin. Biochem Pharmacol, 1989, **38**: 1979 ~ 1984
19 Porter D J. Binding of 2-acetylpyridine-5- [2-chloroanilino) thiocarbonyl] thiocarbonohydrazone (BW348U87) to human serum albumin. Biochem Pharmacol, 1992, **44**: 1417 ~ 1429
20 Saboury A A, Shamsaei A A, Moosavi-Movahedi A A, *et al.* Thermodynamics of binding 2, 2′-bipyridineglycinato palladium (Ⅱ) chloride on human serum albumin. J Chin Chem Soc, 1999, **46**: 917 ~ 922
21 Peters T Jr, Feldhoff R C. Fragments of bovine serum albumin produced by limited proteolysis. Isolation and characterization of tryptic fragments. Biochemistry, 1975, **14**: 3384 ~ 3391
22 King T P. On the sulfhydryl group of human plasma albumin. J Biol Chem, 1961, **236**: PC 5
23 Anderson L Q. The heterogeneity of bovine serum albumin. Biochem Biophys Acta, 1966, **117**: 115 ~ 133
24 Edward F B, Rombauer R B, Campbell B J. Thioldisulfide interchange reactions between serum albumin and disulfides. Biochem Biophys Acta, 1969, **194**: 234 ~ 245
25 Day J F, Thorpe S R, Baynes J W. Non-enzymatically glucosylated albumin. *In vitro* preparation and isolation from normal human serum. J Biol Chem, 1979, **254**: 595 ~ 597
26 Dolhofer R, Wieland O H. Glycosylation of serum albumin: elevated glycosyl-albumin in diabetic patients. FEBS Letters, 1979, **103**: 282 ~ 286
27 Urbanowski J C, Cohenfold M A, Dain J A. Non-enzymatic galactosylation of human serum albumin. *In vitro* preparation. J Biol Chem, 1982, **257**: 111 ~ 115
28 Hill P G. The measurement of albumin in serum and plasma. Ann Clin Biochem, 1985, **22**: 565 ~ 578
29 Dixon J W, Sarkar B. Isolation, amino acid sequence and copper (Ⅱ) -binding properties of peptide (1 ~ 24) of dog serum albumin. J Biol Chem, 1974, **249**: 5872 ~ 5877
30 Yongqia Z, Xuyimg H, Ouyang D, *et al.* The novel behavior of interactions between Ni^{2+} ion and human or bovine serum albumin. Biochem J, 1994, **304**: 23 ~ 26
31 Schippers P H, Dekkers H P J M. Direct determination of absolute circular dichroism data and calibration of commercial instrument. Anal Chem, 1981, **53**: 778 ~ 788
32 Takakuwa T, Konno T, Meguro H. A new standard substance for calibration of circular dichroism: Ammonium d-10-camphorsulfonate. Anal Sci, 1985, **1**: 215 ~ 218
33 Protasevich I, Ranjbar B, Labachov V, *et al.* Conformation and thermal denaturation of apocalmodulin: role of electrostatic mutations. Biochemistry, 1997, **36**: 2017 ~ 2024
34 Scatchard G. The attractions of proteins for small molecules and ions. Ann N Y Acad. Sci, 1949, **51**: 660 ~ 672
35 Saboury A A, Moosavi-Movahedi A A. Evaluation of Hill coefficient from Scatchard and Klotz plots. Biochem Educ, 1994, **22**: 48 ~ 49
36 Bordbar A K, Saboury A A, Moosavi-Movahedi A A. The shapes of Scatchard plots for systems with two sets of binding sites. Biochem Educ, 1996, **24**: 172 ~ 175
37 Hill A V. The possible effect of aggregation of the molecules of haemoglobin on its dissociation curves. J Physiol, 1910, **40**: Ⅳ ~ Ⅶ
38 Saboury A A, Bordbar A K, Moosavi-Movahedi A A. Resolution of two sets of binding sites for cationic surfactant-urease interaction. Bull Chem Soc Jp, 1996, **69**: 3031 ~ 3035
39 James M L, Smith G M, Wolford J C. Applied Numerical Methods for Digital Computer. 3rd. New York: Harper and Row Publisher, 1985.
40 Giancola C, Sena C D, Fessas D, *et al.* DSC studies on bovine serum albumin denaturation. Effects of ionic strength and SDS concentration. Int J Biol Macromol, 1997, **20**: 193 ~ 204
41 Elhaney R N MC. Differential scanning calorimetric studies of lipid-protein interaction in model membrane systems. Biophys Biochem Acta, 1986, **864**: 361 ~ 421
42 Yamasaki M, Yano H, Aoki K. Differential scanning calorimetric studies on bovine serum albumin: Ⅲ. Effect of sodium dodecyl sulphate. Int J Biol Macromol, 1992, **14**: 305 ~ 312