Binding equilibrium study between Mn(Ⅱ) and HSA or BSA

LIANG, Hong*,[a](梁宏) TU, Chu-Qiao[a](涂楚桥) ZHANG, Hong-Zhi[a](张宏志)
SHEN, Xing-Can[a](沈星灿) ZHOU, Yong-Qia[b](周永洽) SHEN, Pan-Wen[b](申泮文)
[a] *Research Institute of Bioinorganic Chemistry · Department of Chemistry and Chemical Engineering, Guangxi Normal University, Guilin, Guangxi 541004, China*
[b] *Department of Chemistry, Nankai University, Tianjin 300071, China*

The binding of Mn(Ⅱ) to human serum albumin (HSA) or bovine serum albumin (BSA) has been studied by equilibrium dialysis at physiological pH (7.43). The Scatchard analysis indicates that there are 1.8 and 1.9 strong binding sites of Mn(Ⅱ) in HSA and BSA, respectively. The successive stability constants which are reported for the first time are obtained by non-linear least-squares methods fitting Bjerrum formula. For both Mn(Ⅱ)-HSA and Mn(Ⅱ)-BSA systems, the order of magnitude of K_1 was found to be 10^4. The analyses of Hill plots and free energy coupling show that the positive cooperative effect was found in both Mn(Ⅱ)-HSA and Mn(Ⅱ)-BSA systems. The results of Mn(Ⅱ) competing with Cu(Ⅱ), Zn(Ⅱ), Cd(Ⅱ) or Ca(Ⅱ) to bind to HSA or BSA further support the conjecture that there are two strong binding sites of Mn(Ⅱ) in both HSA and BSA. One is most probably located at the tripeptide segment of *N*-terminal sequence of HSA and BSA molecules involving four groups composed of nitrogen atoms, and the fifth coordination atom is the carboxyl oxygen of Asp[1]. The coordinated atoms of the other are most probably almost all oxygen atoms.

Keywords Mn(Ⅱ)-serum albumin, equilibrium dialysis, successive stability constants, positive cooperative effect, coordination analysis

Manganese is one of the necessary trace elements for growth of living organisms, but its bioinorganic chemistry behavior has rarely been studied. We have reported[1] the structure of metal center in 1:1 Mn(Ⅱ)-HSA and Mn(Ⅱ)-BSA systems at physiological pH by observed LMCT bands in ultraviolet region. Results indicated that the structures of the metal center in Mn(Ⅱ)-HSA and Mn(Ⅱ)-BSA are almost the same. It takes pentacoordinated square-pyramid configuration at lower concentrations, and becomes tetracoordinated square-planar structure at higher concentrations. The binding site is most probably located at the tripeptide segment of *N*-terminal sequence of HSA or BSA involving four groups composed of nitrogen atoms. The fifth coordinated atom is the carboxyl oxygen of Asp[1]. The binding of Mn(Ⅱ) to HSA or BSA at physiological pH is further studied by equilibrium dialysis in this paper. The successive stability constants are obtained by non-linear least-squares methods fitting Bjerrum formula for the first time. The type and number of binding sites and the cooperation among the binding sites are reported. According to the equilibrium dialysis results and the study of competition between Mn(Ⅱ) with Cu(Ⅱ), Zn(Ⅱ), Cd(Ⅱ) or Ca(Ⅱ) binding to HSA or BSA, we also suggest that there are two strong binding sites of Mn(Ⅱ) in both HSA and BSA for the first time.

Experimental

Materials, reagents and methods

HSA and BSA, which were both of electrophoretic purity, were purchased from Sino-American Biotechnology Company, Beijing Branch and mailed freshly. They were used without further purification. $MnCl_2$, $CaCl_2$, $CuCl_2$, $ZnCl_2$, $CdCl_2$, NaCl, HCl, HAc and NaAc were all of analytical grade. Buffer Tris was biochemical

Received April 19, 1999; accepted June 25, 1999.
Project supported by the National Natural Science Foundation of China (No. 29961001), the Natural Science Foundation of Guangxi Universities and the Ten, Hundred or Thousand Distinguished Persons Foundation of Guangxi.

reagent. All of the solutions which were prepared with deionized water contained 0.10 mol/L NaCl and 0.10 mol/L Tris-HCl to maintain the same ionic strength and pH = 7.43 (± 0.02) (except the indicated pH). The concentrations of $MnCl_2$, $CaCl_2$, $CuCl_2$, $ZnCl_2$ and $CdCl_2$ solutions were determined by titration with EDTA. The solutions of HSA and BSA were prepared freshly. The concentrations of HSA and BSA solutions were determined by Hitach UV-3400 spectrophotometer.[1]

The tubular dialysis membrane was purchased from Allied Carbide Corporation (USA) and treated as described in Ref.2. The dialysis was performed for 48 h at 20 ± 0.5℃ . During the experiments, the concentration of $MnCl_2$ solution varied in a range of 10^{-5}—10^{-3} mol/L, and about 20 different concentrations were involved in each set of the measurements, each point is parallel operated at least twice. The concentration of free Mn^{2+} ion was determined with WFX-IF_2 atomic absorption spectrophotometer after dialysis.

Binding of Mn(Ⅱ) *to* HSA *or* BSA

The experiments were carried out at the conditions stated above, corresponding to the serum albumin concentrations of 1.0, 2.0, 3.0, 4.0×10^{-4} mol/L, respectively.[2]

Binding of Mn(Ⅱ) *to* HSA *or* BSA *at* pH 5.0

All of the solutions contained 0.10 mol/L NaCl and 0.10 mol/L HOAc-NaOAc to maintain the same ionic strength and pH = 5.0 (± 0.02). The concentrations of HSA and BSA solutions were both 1.0×10^{-4} mol/L. The others were the same as those stated above.

Competition between Mn(Ⅱ) *and* Cu(Ⅱ)、Zn(Ⅱ)、Cd(Ⅱ) or Ca(Ⅱ) *binding to* HSA *or* BSA

Take the competition experiments between Mn(Ⅱ) and Cu(Ⅱ) binding to HSA or BSA as an example. The concentrations of HSA and BSA were both 1.0×10^{-4} mol/L. Before dialysis, the same amount Cu(Ⅱ) was added into a set of Mn(Ⅱ) solutions, then equilibrium dialysis was carried out. After equilibrium, the concentrations of the free Mn(Ⅱ) were measured. The others were the same as those stated above. The experiments of competition between Mn(Ⅱ) and Zn(Ⅱ)、Cd(Ⅱ) or Ca(Ⅱ) binding to HSA or BSA were analogous to those stated above.

Results and discussion

Type and number of binding sites and the successive stability constants of Mn(Ⅱ)-HSA *and* Mn(Ⅱ)-BSA

Fig.1 (a) and (b) are the results of equilibrium dialysis of Mn(Ⅱ)-HSA and Mn(Ⅱ)-BSA, respectively. $\bar{n}_c$ is Bjerrum function,[3] which shows the average number of metal ions binding to a serum albumin molecule.

According to Ref. 2 the successive stability constants of Mn(Ⅱ)-HSA or Mn(Ⅱ)-BSA are obtained by non-linear least-squares methods fitting Bjerrum formula and summarized in Table 1. The degree of fitting experi-

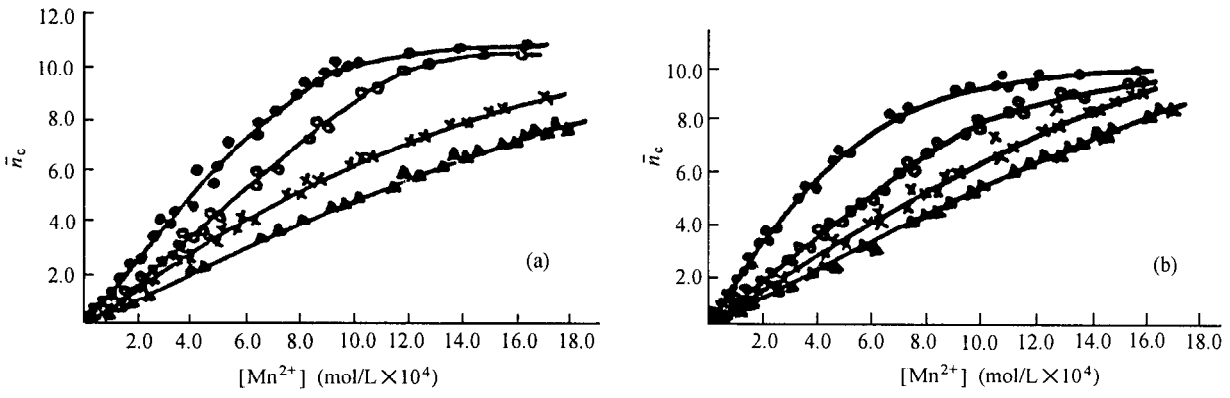

Fig. 1 $\bar{n}_c$ vs [Mn^{2+}] (a) Mn(Ⅱ)-HSA; (b) Mn(Ⅱ)-BSA. The concentrations of HSA or BSA: ● 1.0, ○ 2.0, × 3.0, ▲ 4.0×10^{-4} mol/L. The solid curve results from the nonlinear least squares fitting.

mental data is expressed by Hamilton R-factor (Table 1).[2]

Table 1 shows that the successive stability constants are basically independent of the concentrations of albumin. The values of K_1 and K_2 are significantly larger than the following ones except for individual concentra-

Table 1 Successive stability constants of Mn(Ⅱ)-HSA and Mn(Ⅱ)-BSA

System		Mn(Ⅱ)-HSA			
Albumin concentration (mol/L)		1.0×10^{-4}	2.0×10^{-4}	3.0×10^{-4}	4.0×10^{-4}
Successive stability constants	K_1	1.22×10^{4}	1.33×10^{4}	1.28×10^{4}	1.23×10^{4}
	K_2	7.40×10^{3}	6.05×10^{3}	5.94×10^{3}	4.98×10^{3}
	K_3	6.15×10^{3}	2.60×10^{3}	1.16×10^{3}	3.81×10^{3}
	K_4	3.15×10^{3}	1.98×10^{3}	1.17×10^{3}	3.55×10^{3}
	K_5	1.32×10^{3}	1.38×10^{3}	1.65×10^{3}	1.36×10^{3}
	K_6	9.38×10^{2}	1.11×10^{3}	1.37×10^{3}	5.21×10^{2}
	K_7	8.12×10^{2}	1.03×10^{3}	8.47×10^{2}	4.76×10^{2}
	K_8	7.12×10^{2}	9.54×10^{2}	4.85×10^{2}	4.75×10^{2}
	K_9	6.36×10^{2}	8.87×10^{2}	4.66×10^{2}	4.64×10^{2}
	K_{10}	5.94×10^{2}	8.05×10^{2}	4.61×10^{2}	4.54×10^{2}
	K_{11}	5.29×10^{2}	7.15×10^{2}	4.59×10^{2}	4.73×10^{2}
	K_{12}	4.86×10^{2}	6.55×10^{2}	4.79×10^{2}	4.79×10^{2}
	K_{13}	4.55×10^{2}	5.99×10^{2}	4.40×10^{2}	4.58×10^{2}
	K_{14}	4.27×10^{2}	5.32×10^{2}	4.90×10^{2}	4.01×10^{2}
	K_{15}	4.20×10^{2}	4.95×10^{2}	4.37×10^{2}	3.72×10^{2}
	K_{16}	4.10×10^{2}	4.59×10^{2}	3.70×10^{2}	3.28×10^{2}
	K_{17}	3.96×10^{2}	4.33×10^{2}	3.64×10^{2}	3.26×10^{2}
	K_{18}	3.98×10^{2}	4.19×10^{2}	3.21×10^{2}	3.23×10^{2}
	K_{19}	3.78×10^{2}	4.03×10^{2}	3.01×10^{2}	3.12×10^{2}
	K_{20}	3.66×10^{2}	3.81×10^{2}	2.92×10^{2}	2.98×10^{2}
R-factor		0.097	0.073	0.064	0.064

System		Mn(Ⅱ)-BSA			
Albumin concentration (mol/L)		1.0×10^{-4}	2.0×10^{-4}	3.0×10^{-4}	4.0×10^{-4}
Successive stability constants	K_1	1.52×10^{4}	1.42×10^{4}	1.46×10^{4}	1.22×10^{4}
	K_2	8.05×10^{3}	5.38×10^{3}	5.73×10^{3}	4.73×10^{3}
	K_3	2.94×10^{3}	1.76×10^{3}	1.83×10^{3}	1.37×10^{3}
	K_4	2.66×10^{3}	1.48×10^{3}	1.25×10^{3}	1.35×10^{3}
	K_5	2.63×10^{3}	1.02×10^{3}	7.31×10^{2}	1.29×10^{3}
	K_6	2.66×10^{2}	8.61×10^{2}	6.28×10^{2}	1.12×10^{3}
	K_7	2.75×10^{2}	8.78×10^{2}	6.40×10^{2}	8.38×10^{2}
	K_8	2.73×10^{2}	9.38×10^{2}	7.44×10^{2}	4.62×10^{2}
	K_9	1.84×10^{2}	9.53×10^{2}	8.79×10^{2}	4.54×10^{2}
	K_{10}	1.53×10^{2}	8.47×10^{2}	9.22×10^{2}	4.26×10^{2}
	K_{11}	1.03×10^{2}	6.16×10^{2}	7.87×10^{2}	3.87×10^{2}
	K_{12}	9.73×10^{2}	4.69×10^{2}	6.37×10^{2}	3.54×10^{2}
	K_{13}	9.42×10^{2}	3.33×10^{2}	5.63×10^{2}	3.39×10^{2}
	K_{14}	9.27×10^{2}	3.18×10^{2}	4.73×10^{2}	2.92×10^{2}
	K_{15}	8.77×10^{2}	3.18×10^{2}	2.18×10^{2}	1.46×10^{2}
	K_{16}	8.64×10^{2}	3.15×10^{2}	1.05×10^{2}	1.05×10^{2}
R-factor		0.080	0.065	0.062	0.073

tions of albumin, which indicates the importance of K_1 and K_2 . By comparison , the stability constants of Mn(Ⅱ)-HSA or Mn (Ⅱ)-BSA are less than those of Cu(Ⅱ),[2] Zn(Ⅱ)[3] or Ni(Ⅱ)[4] binding to HSA or BSA at the similar condition. It is probably because the radius of Mn^{2+} ion is relatively big and there is zero ligand-field stabilization energy in its complex.[5]

The statistical effect is defined as: $K_n/K_{n+1} = [(N-n+1)(n+1)]/[(N-n)\cdot n]$, $N = n_t$, n_t is the total number of binding sites. According to the results from Fig.2, for HSA, $n_t = 20$; for BSA, $n_t = 16$. For the two systems, K_1/K_2 is almost in accordance with the statistical effect, but the following K_n/K_{n+1} deviate far from it, indicating that the binding of the first Mn^{2+} ion exerts little coordinate effect. However, the following binding Mn^{2+} ions can cause the allosteric effect of serum albumin. As a result, relatively stronger coordination inclination can be found for the following binding of Mn^{2+} ions.

From the Scatchard plots, we obtain the type and the number of binding sites of Mn(Ⅱ) in HSA and BSA, as shown in Fig.2. There are two types of binding sites inboth Mn(Ⅱ)-HSA and Mn(Ⅱ)-BSA systems: there are 1.8 strong binding sites and 18 weak binding sites in Mn(Ⅱ)-HSA, and 1.9 strong binding sites and 14 weak binding sites in Mn(Ⅱ)-BSA.

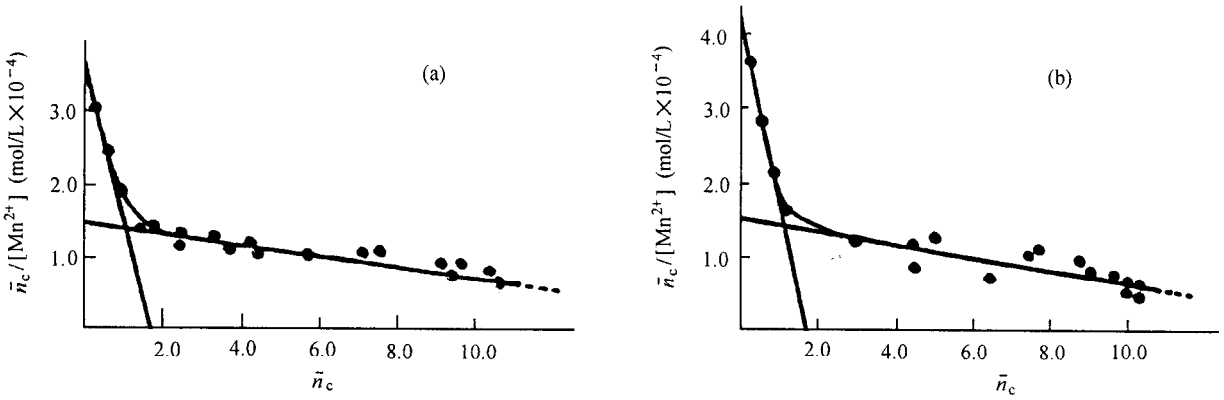

Fig. 2 Scatchard plots: (a) Mn(Ⅱ)-HSA , (b) Mn(Ⅱ)-BSA. The concentrations of HSA and BSA are both 1.0×10^{-4} mol/L.

Cooperativity of binding sites in Mn (Ⅱ) - HSA *or* Mn(Ⅱ)-BSA

The cooperativity among binding sites in the molecule involving more than one binding site can be analyzed quantitatively by Hill plots,[6] Hill equation is defined as:

$$\log(\frac{Y}{1-Y}) = h\log[Mn^{2+}] - \log K$$

where Y is fraction of HSA or BSA which is saturated by Mn^{2+} ions, $Y = \bar{n}_c/n_t$, and n_t is the totle number binding sites. According to the results shown in Fig. 2, for HSA, $n_t = 20$; for BSA, $n_t = 16$. h is Hill coefficient, the measurement of cooperativity among binding sites. When $h = 1$, there is no cooperativity; $h < 1$, there is negative cooperativity; $h > 1$, these is positive cooperativity. The larger the h is, the stronger the positive cooperativity is, and the maximum value of h is n_t.[6]

Fig . 3 is the Hill plots of Mn (Ⅱ) - HSA and Mn(Ⅱ)-BSA systems. The analysis of the divided curve indicates that h is slightly more than 1 at relatively low concentrations of Mn^{2+} ions, which shows that the weak positive cooperativity is produced by binding to strong binding sites and a few of weak binding sites. With the increasing concentration of Mn^{2+} ions, h is slightly increasing. In Mn(Ⅱ)-HSA, $h_{max} \approx 1.3$; in Mn(Ⅱ)-BAS, $h_{max} \approx 1.2$, which indicates that there is certain position cooperativity effect in both systems.

The cooperativity effect of binding ligands can also be described by free energy coupling. The formula is:

$\triangle G_{xx} = \triangle G^0(p-px_n) - n\triangle G^0(p-px)$,

$\triangle G^0 = -RT\ln K$,

$\triangle G_{xx}$ is the free energy coupling of ligand x binding to serum albumin, when $\triangle G_{xx} < 0$, the binding is cooperative; $\triangle G_{xx} > 0$, the binding is negative cooperative; n

is the number of x; $\triangle G^0$ is the standard free energy; K is the stability constants. The average free energy coupling of several ligands can be evaluated by the formula.

$$\triangle G_{xx} = -RT(\ln f_1 K_1 K_2 - 2\ln K_1) = -0.586 \text{ kJ/mol} < 0$$

which shows that a slightly strong cooperativity can be produced as the first Mn^{2+} ion binds to HSA or BSA, it may favor the binding of the second one.

Considering the statistical factor stated above, $K_n/K_{n+1} = f_n$, so $f_n K_{n+1}$ can be used to analyze the free energy coupling. According to Table 1, and $T = 293$ K, we can obtain:

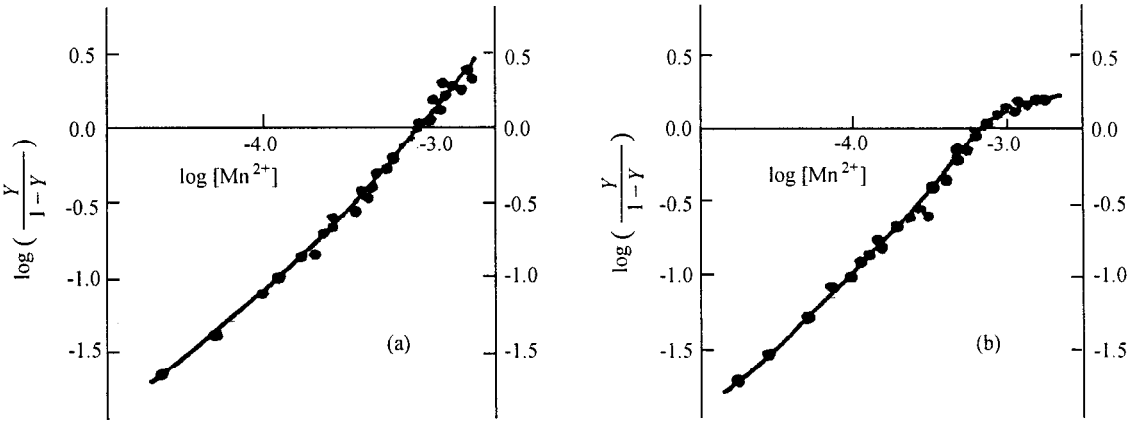

Fig. 3 Hill plots of (a) Mn(Ⅱ)-HSA and (b) Mn(Ⅱ)-BSA. The concentrations of HSA and BSA are both 1.0×10^{-4} mol/L.

Binding of Mn(Ⅱ) *to* HSA *or* BSA *at* pH 5.0

The number of strong binding sites at pH 5.0 is nearly the same as that at the pH 7.43 in Mn(Ⅱ)-HSA or Mn(Ⅱ)-BSA, both are 2 (figures are not shown). The results indicate that carboxyl is also the ligand of Mn(Ⅱ)[1,7] besides imidazdyls. At pH 5.0, in Mn(Ⅱ)-HSA, $K_1 = 1.1 \times 10^4$, $K_2 = 6.8 \times 10^3$; and in Mn(Ⅱ)-BSA, $K_1 = 1.5 \times 10^4$, $K_2 = 4.9 \times 10^3$, both of them are reduced slightly compared with those at pH 7.43. The reason is probably that with increasing acidity, protons have stronger competition for carboxyl, which decreases the trendency of Mn^{2+} ions binding to carboxyl, and induces the decrease of stability constants of Mn(Ⅱ)-serum albumin.

Binding sites and ligands analysis in Mn(Ⅱ)-HSA *or* Mn(Ⅱ)-BSA

Fig.4 is the binding curve when existing sufficient Cu(Ⅱ)、Zn(Ⅱ)、Cd(Ⅱ) or Ca(Ⅱ) in Mn(Ⅱ)-HSA or Mn(Ⅱ)-BSA systems. The results of competition between Mn(Ⅱ) and these metal ions are summarized in Table 2.

Our results show that when existing sufficient Cu(Ⅱ), there is only one strong binding site of Mn(Ⅱ) in HSA and BSA. It is because when the first transition system metal ions binding to the same ligands, their stability constants are in accordance with Irving-Williams order. Acoording to this order, the binding ability of Cu(Ⅱ) is stronger than that of Mn(Ⅱ) if they compete for the same ligands. Combined with the results of this paper and Ref.2, we can infer that in HSA and BSA, it is the same strong binding site that Mn(Ⅱ) and Cu(Ⅱ) compete for. At present, it is believed that there is only one strong binding site in Cu(Ⅱ)-HSA or Cu(Ⅱ)-BSA and it locates at the *N*-terminal tripeptide sequence in protein molecule involving four groups composed of nitrogen atoms and a carboxyloxygen atom of Asp[1].[2,7-9] So we can infer that the site is also one of strong binding sites of Mn(Ⅱ) in HSA and BSA. But considering the ability of coordination, Mn(Ⅱ) is weaker than that of Cu(Ⅱ), when Cu(Ⅱ)、Mn(Ⅱ) exist in the same system at the same time and compete the same site, it is Cu(Ⅱ) that ultimately binds to the site, so only one strong binding site appears in Mn(Ⅱ)-serum albumin.

We have reported[10] that in HSA molecules the *N*-

terminal tripeptide segment is probably located in IA subdomain which comprises six main helixes h1 ~ h6. IA subdomain connects with h1 and is located in the outer-sphere of the protein. Although the radius of Mn^{2+} ion is relatively big, considering it has certain affinity for O and N atoms, it can bind to the site. Combining with the results from Ref. 1, the suggestion that one of strong binding sites of Mn(Ⅱ) in HSA and BSA is most probably located at the tripeptide segment of *N*-terminal sequence is sound.

Sadler *et al*.[8] demonstrated the existence of two strong binding sites (site A and site B) in both Cd(Ⅱ)-HSA and Cd(Ⅱ)-BSA by equilibrium dialysis and ^{113}Cd-NMR. The site A is located at internal of albumin molecule which involves three nitrogen atoms and one or more oxygen atoms. The site B is considered to probably include all oxygen atoms, and the site A is also the strong binding site of Zn(Ⅱ) in HSA and BSA. Our recent study[11] by fluorescence EXAFS also suggests that the metal center which is formed by interaction between Zn(Ⅱ) in HSA or BSA is probably coordinated by four nitrogen atoms, but the possibility that oxygen atom joins coordination cannot be excluded. At present it is considered that there is only one strong binding site of Zn(Ⅱ) in HSA and BSA molecules.[7,12]

When existing sufficient Zn(Ⅱ), there are still two strong binding sites of Mn(Ⅱ) in HSA and BSA molecules . In accordance with Irving - williamsorder , Zn(Ⅱ) has a stronger affinity than Mn(Ⅱ) when they bind to the same ligands. Combined with the results in this paper and Ref. 3, we can conclude that the other strong binding site of Mn(Ⅱ) is not site A in serum albumin. When existing sufficient Cd(Ⅱ), there exists only one strong binding site of Mn(Ⅱ), which suggests that site B may be the common strong binding site of Mn(Ⅱ) and Cd(Ⅱ) in HSA and BSA molecules.

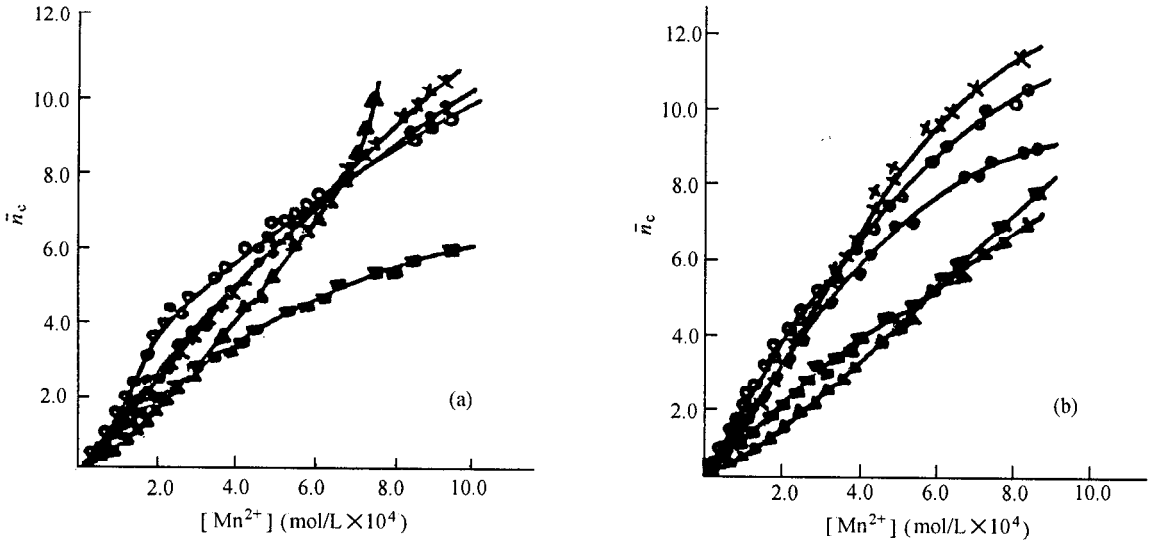

Fig. 4 $\bar{n}_c$ vs [Mn^{2+}] correlation for (a) Mn(Ⅱ)-HSA and (b) Mn(Ⅱ)-BSA when existing sufficient Cu^{2+}, Zn^{2+}, Cd^{+2} or Ca^{2+} ions in systems ● Mn^{2+}, × Mn^{2+} (+ Cu^{2+}), ○ Mn^{2+} (+ Zn^{2+}), ▲ Mn^{2+} (+ Cd^{2+}), ■ Mn^{2+} (+ Ca^{2+}). The concentrations of HSA and BSA are both 1.0×10^{-4} mol /L.

The results of competition between Mn (Ⅱ) and Ca(Ⅱ) show that there left one strong binding site of Mn(Ⅱ) in both HSA and BSA. Sadler *et al*.[8] has reported that the order of magnitude of *K* of Ca(Ⅱ) binding to HSA was found to be 10^4, which is as the same magnitude as that of Mn(Ⅱ)-HSA reported in this paper. So the competition between Ca(Ⅱ) and Mn(Ⅱ) must be very intense. In living systems, if the coordinated atoms are all oxygen atoms, the competition ability of Ca(Ⅱ) increases,[13] so Ca(Ⅱ) still probably occupies the site B. Combining the results of competition between Mn(Ⅱ) and Ca(Ⅱ) or Cd(Ⅱ), site B is most probably the other strong binding site of Mn(Ⅱ) in both HSA and BSA besides the tripeptide segment of *N*-terminal sequence.

The results from Fig . 4 show the existence of Cu(Ⅱ) and Zn(Ⅱ) which mainly bind to nitrogen atoms[2,7,12] has only slight influence on the binding curve of Mn (Ⅱ) - serum albumin , but with the existence of Ca(Ⅱ) which mainly binds to oxygen atoms[8,13] intensely

influence it. It suggests that Mn(Ⅱ) mainly binds to oxygen atoms in HSA or BSA molecules. The behavior of Cd(Ⅱ) is strange: in BSA it intensely influences the binding curve of Mn(Ⅱ)-serum albumin, and the impacting degree is even bigger than that of Ca(Ⅱ); in HSA, it has a relatively stronger influence, but with the increase of Cd(Ⅱ) concentration, this influence reduces rapidly, at last it is even in favor of Mn(Ⅱ) binding to HSA. Since the main binding atoms and binding way of Cd(Ⅱ) to HSA or BSA have not beenvery clear by now, the explanation for this interesting phenomenon still needs further study.

Table 2 Mn^{2+} ions binding to HSA or BSA under the existence of sufficient Cu^{2+}、Zn^{2+}、Cd^{2+} or Ca^{2+} ions

System	Mn^{2+} + HSA	Mn^{2+} + HSA (+ Cu^{2+})	Mn^{2+} + HSA (+ Zn^{2+})	Mn^{2+} + HAS (+ Cd^{2+})	Mn^{2+} + HSA (+ Ca^{2+})
The number of strong binding sites of Mn(Ⅱ)	1.8	1.3	1.9	1.0	1.3
Stability K_1	1.2×10^4	1.6×10^4	1.6×10^4	1.3×10^4	1.9×10^4
contants K_2	7.4×10^3	8.0×10^3	6.0×10^3	2.4×10^3	9.6×10^3
System	Mn^{2+} + BSA	Mn^{2+} + BSA (+ Cu^{2+})	Mn^{2+} + BSA (+ Zn^{2+})	Mn^{2+} + BSA (+ Cd^{2+})	Mn^{2+} + BSA (+ Ca^{2+})
The number of strong binding sites of Mn(Ⅱ)	1.9	1.2	2.1	1.0	0.9
Stability K_1	1.5×10^4	9.0×10^3	9.6×10^3	9.7×10^3	7.1×10^3
contants K_2	8.0×10^3	6.3×10^3	9.0×10^3	2.2×10^3	5.2×10^3

References

1. Liang, H.; Zhou, Y.Q.; Shen, P.W., *Chinese Sci. Bull.* **39**, 1452(1994).
2. Liang, H.; Xin, B.G.; Wang, X.J.; Yuan, Y.Z.; Zhou, Y.Q.; Shen, B.W. *Chinese Sci. Bull.* **43**, 404 (1998).
3. Zhou, Y.Q.; Wang, Y.W.; Hu, X.Y.; Huang, J.S.; Hao, Y.Q.; Liu, H.; Shen, P.W., *Biophys. Chem*, **51**, 81(1994).
4. Hu, X.Y.; Song, Z.R.; Su, X.D.; Ouyang, D.; Huang, J.S., *Science in China* (Series B)(in Chinese), **27**, 75(1997).
5. Zhao, J.Z., *Advanced Inorganic Chemistry*, Beijing, Beijing Normal University Press, 1982, p. 387.
6. Fersht, A., *Enzyme structure and Mechanism* (in Chinese), Translated by Du, J.Z.; Ru, B.G.; Wei, X. C., Beijing, Beijing University Press, 1991, p. 292.
7. Masuoka, J.; Hegenauer, J.; van Dyke, B. R.; Saltman, P.J. *Biol. Chem.*, **268**, 21533(1993).
8. Sadler, P.J.; Viles, J.H., *Inorg. Chem.* **35**, 4490 (1996).
9. Shen, P.W.; Zhou,Y.Q.; Wang, S.Y.; Che, Y. X., *Inorg. Chim. Acta*, **169**, 161(1990).
10. Liang, H.; Ouyang, D.; Hu, X.Y.; Tai, J.Z.; He, J. T.; Zhou, Y.Q., *Acta Chim. Sin.*, **56**, 62(1998).
11. Zhou, Y.Q.; Zhang, X.Q.; He, J.T.; Liang, X.Z., Hu, T.D.; Xie, Y.L., *Chem. J. Chinese Univ.* (in Chinese), **18**, 851(1997).
12. Tu, C.Q.; Sun, H.L.; Zeng, J.Q.; Liang, H., *Guangxi Sciences* (in Chinese), to be published.
13. Yatsinirskii, K.B., *Elementary Course of Bioinorganic Chemistry* (in Chinese), Beijing, Science Press, 1984.

(JIANG, X.H.; DONG, L.J.)