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Equilibrium dialysis of metal-serum albumin [1]. I. Successive stability constants of Zn(II)-serum albumin and the Zn^{2+}-induced cross-linking self-association

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Abstract

The binding of Zn(II) to human serum albumin (HSA) and bovine serum albumin (BSA) was intensively studied by equilibrium dialysis. The successive stability constants were obtained by least-squares fitting. For both the Zn(II)-HSA and Zn(II)-BSA systems, the successive stability constants are basically similar, though such constants for the latter generally slightly larger than those for the former; the order of magnitude of K_1 and K_2 was found to be $\approx 10^5$ M^{-1}. The twelve binding sites found for Zn(II)-HSA and fourteen binding sites for Zn(II)-BSA can be divided into three and two different sets, respectively, but in both systems, there exist approximately three identical strongest binding sites. The binding equilibrium of Zn(II)-HSA markedly depends on the concentration of HSA. The type of Scatchard plots indicates the existence of the Zn^{2+}-induced cross-linking self-association of HSA, which was found from Wyman plots to be the strongest at a concentration of Zn^{2+} in the range $(4-6) \times 10^{-6}$ M. The maximum of the apparent self-association constants k_2 so obtained was approximately 2.6×10^4 M^{-1}. Such a self-association mechanism and the order of magnitude of K_1 and K_2 both to some extent support the inference that the zinc group ions are bound to the cysteinyl sulphur atoms.

Key words: Zn(II)-serum albumin; Equilibrium dialysis; Successive stability constants; Zn^{2+}-induced cross-linking self-association

1. Introduction

Zinc is among the elements required for human growth, development, cell splitting, and synthesis of proteins and DNA [1]. In human serum, 98% of the total exchangeable zinc is bound to

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HSA (human serum albumin) [2]. However, the binding constants reported in the early literature for Zn(II)-serum albumin have usually been obtained from Scatchard equation [3] or its analogues, which are essentially average values of some kind. The binding constants of Zn(II)-HSA and Zn(II)-BSA (bovine serum albumin) systems have been reported to be 10^3–10^4 in order of magnitude [4–9], with those for the latter slightly larger than for the former. All these constants were measured under weakly acidic conditions (pH = 5–6). In discussing such constants, Meyer [4] cited the stability constant 380 (24°C) or 575 (4.5°C) [10] of $ZnIm_4$ (Im = imidazole) and considered them as evidence for the ligation of Zn(II) to the His imidazole group in HSA or BSA.

Our previous studies [11] indicated that the binding sites of the zinc group ions are most probably located at the seven pairs of adjacent disulfide bridges, leading to formation of the tetrahedral MS_4 (M = Zn, Cd, Hg) metal centers. We wish to report here the equilibrium dialysis studies of the Zn(II)-HSA and Zn(II)-BSA systems, which allows us to obtain the successive stability constants for both systems. A full discussion on the results seems to make it clear that such data further support our inference mentioned above about the binding sites of the zinc group ions.

2. Experimental

HSA and BSA, both being electrophoresis grade reagents, were purchased from Beijing Red Cross Blood Center and Tianjin Institute of Blood, respectively. The major impurity was moisture. Contents (in ppm) of other impurities including K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Cd^{2+} and Cu^{2+} ions were respectively determined to be 2 (60), 240 (17), 18 (29), 8.9 (2.9), 1.7 (0.4) and 14 (15) for HSA (BSA). A trace of iron, manganese or nickel was also present, but no mercury was detected. The total contents of all metal ions in both albumins were less than 0.03%. Preliminary tests indicated that, for the equilibrium dialysis of the zinc group ions, when the albumin were used as received or pre-dialyzed against 200 times their volume of deionized water for 48 h prior to use, the difference between the results was within the uncertainty of the experiments. Therefore, the albumins in all measurements were used without further purification. NaCl, $ZnCl_2$ and hydrochloric acid were all of analytical grade. The buffer Tris had a purity of no less than 99.5%. All solutions containing 0.1 M NaCl and 0.1 M Tris-HCl (pH = 7.43 ± 0.02) were prepared with deionized water. The solutions of albumins were freshly prepared, and their concentrations determined spectrophotometrically [12]. The concentration of the $ZnCl_2$ solution was first determined by titration with EDTA, then diluted to be suitable for the dialysis studies and further measured by dithizone photometric method, with the working curve corrected everyday with solutions of known concentration.

The tubular dialysis membrane (6 mm in diameter) was purchased from the Allied Carbide Corporation (USA), which was cut into pieces ready to use, i.e. dialysis bags, each being ≈ 25 cm long. The dialysis bag was soaked initially in deionized water for 48 h, then in turn boiled for 15 min in deionized water, in 5% $NaHCO_3$ solution and in 0.05–0.005 M EDTA solution (pH 6–8). Finally, the dialysis bag was resoaked in deionized water for 48 h with changes in deionized water every 4 h, after which it was prepared for the experimental runs.

Equilibrium dialysis was carried out in a half-moon-shaped tubular glass chamber (inside diameter: 12 mm). Dialysis bag was placed into the tubular chamber and its two ends being just outside the chamber. The albumin solution (≈ 5 ml) and $ZnCl_2$ solution (≈ 10–15 ml) were respectively inside and outside the bag, and the level of both solutions was maintained to be the same. The solution volume should be precisely measured. In order to reduce the solvent loss due to evaporation, both the dialysis bag and the half-moon-shaped chamber were properly closed. The half-moon-shaped chambers were then placed in a water bath of 20 ± 1°C and the dialysis was performed for 24 h, during which the solutions were swirled from time to time. In fact, the dialysis basically reached equilibrium within 12 h. The concentrations of the $ZnCl_2$ solution before

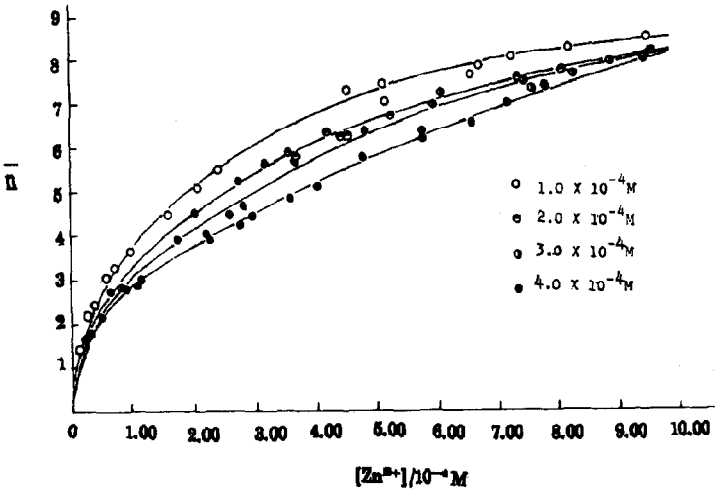

Fig. 1. $\bar{n}$ versus $[Zn^{2+}]$ correlation for Zn(II)-HSA under different concentrations of HSA (the solid curves result from the non-linear least squares fitting).

and after the dialysis were determined. The adsorption of Zn(II) by the dialysis bag was corrected through the experiment in which the $ZnCl_2$ solution of the same concentration was input inside or outside the dialysis bag and let it stand for 24 h, then the change in its concentration was measured.

During the experiments, four sets of measurements for both HSA and BSA were carried out, corresponding to the albumin concentrations of

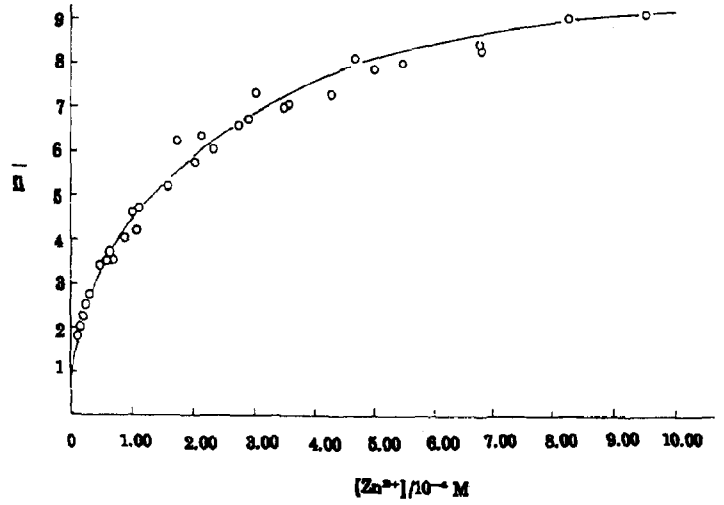

Fig. 2. $\bar{n}$ versus $[Zn^{2+}]$ correlation for Zn(II)-BSA under 1.0×10^{-4} M of BSA (the solid curve results from the non-linear least squares fitting).

1.0, 2.0, 3.0, and 4.0×10^{-4} M, respectively. The concentrations of the $ZnCl_2$ solution varied in the range 10^{-3}–10^{-5} M, and about 20 different such concentrations were involved in each set of the measurements.

3. Results and discussion

3.1. Multistep binding equilibrium

Figs. 1 and 2 show the results from equilibrium dialysis of the Zn(II)-HSA and Zn(II)-BSA systems, respectively. The horizontal coordinate represents the equilibrium concentrations of the free zinc(II) ions $[Zn^{2+}]$, and the vertical one the formation function $\bar{n}$ [13], which is usually called the average binding number and defined as follows:

$$\bar{n} = \frac{\text{total bound ligand}}{\text{total protein}} = \frac{[Zn^{2+}]_T - [Zn^{2+}]}{[P]_T},$$

P represents protein, and the subscript T represents the total concentration. $\bar{n}$ can be calculated according to the solution volumes inside and outside the dialysis bag and experimentally found $[Zn^{2+}]$ on an assumption that the equilibrium concentrations (or, actually, the chemical potentials) of the free zinc ions inside and outside the dialysis bag are equal to each other.

It can be seen from Figs. 1 and 2 that the experimentally found $\bar{n}$ values for both systems are larger than 8–9. However, there are some remarkable difference between them. For the HSA system, four different $\bar{n}$ versus $[Zn^{2+}]$ curves were obtained under four different HSA concentrations, whereas the corresponding curve for the BSA system is actually independent of the BSA concentrations. The curve shown in Fig. 2 corresponds to 1.0×10^{-4} M.

The multistep equilibrium for both systems should follow the following equation:

$$\bar{n} = \frac{K_1[M] + 2K_1K_2[M]^2 + \ldots + nK_1K_2 \ldots K_n[M]^n}{1 + K_1[M] + K_1K_2[M]^2 + \ldots + K_1K_2 \ldots K_n[M]^n},$$

where k_i $(i = 1, 2, \ldots n)$ is the successive stability constant (stoichiometric binding constants), [M] is the equilibrium concentration of the metal ion. For data processing, we used a computer program based on the standard non-linear least

Table 1
Successive stability constants for Zn(II)-HSA and Zn(II)-BSA systems

	System				
	Zn(II)-HSA albumin conc. ($\times 10^4$ M)				Zn(II)-BSA albumin conc. ($\times 10^{-4}$ M)
	1.0	2.0	3.0	4.0	1.0
Successive stability constants					
K_1	2.00×10^5	2.00×10^5	2.00×10^5	2.00×10^5	2.00×10^5
K_2	1.01×10^5	1.00×10^5	1.00×10^5	1.00×10^5	2.01×10^5
K_3	1.34×10^4	1.03×10^4	0.99×10^4	0.90×10^4	2.40×10^4
K_4	9.67×10^3	6.84×10^3	6.34×10^3	5.36×10^3	1.56×10^4
K_5	5.48×10^3	3.79×10^3	2.09×10^3	2.02×10^3	7.57×10^3
K_6	5.09×10^3	4.81×10^3	3.55×10^3	3.42×10^3	8.02×10^3
K_7	1.95×10^3	1.35×10^3	1.67×10^3	0.44×10^3	3.13×10^3
K_8	2.55×10^3	1.43×10^3	1.69×10^3	1.41×10^3	3.38×10^3
K_9	2.23×10^3	1.65×10^3	1.71×10^3	2.08×10^3	1.25×10^3
K_{10}	0.15×10^3	0.35×10^3	0.03×10^3	1.08×10^3	0.55×10^3
K_{11}	0.22×10^2	0.25×10^3	0.14×10^2	0.56×10^3	0.61×10^3
K_{12}	0.12×10^2	0.77×10^2	0.12	0.29	0.71×10^3
K_{13}					0.59×10^3
K_{14}					0.29×10^3
R factor	0.034	0.035	0.035	0.032	0.041

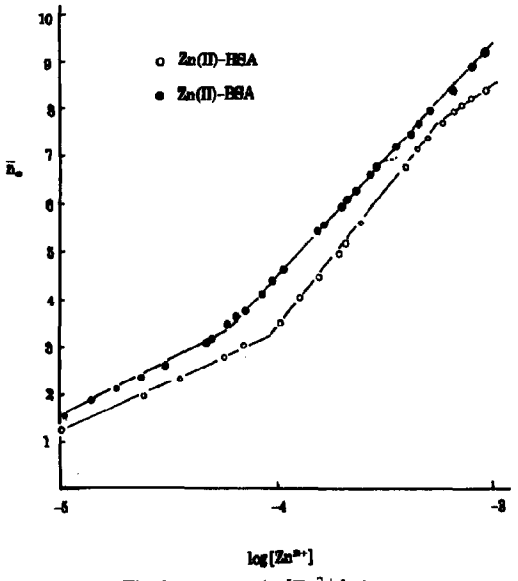

Fig. 3. $\bar{n}_c$ versus $\log[Zn^{2+}]$ plots.

squares method [14], with the deviation estimated by the Hamilton R-factor [15] defined as

$$R = \left(\sum_{i=1}^{i=n} (\bar{n}_p - \bar{n}_c)^2 \Big/ \sum_{i=1}^{i=n} \bar{n}_p^2 \right)^{1/2},$$

where the subscripts p and c represent the experimental and calculated values, respectively. The results are summarized in Table 1. It should be noted that the solid curves in both Figs. 1 and 2 are versus $\bar{n}_c$.

Obviously, it is impossible that the twelve binding sites of Zn(II)-HSA or the fourteen binding sites of Zn(II)-BSA resulting from the least-squares fitting are all identical. Using a common method [16], we obtained $\bar{n}_c$ versus $\log[Zn^{2+}]$ plots (Fig. 3) for both HSA and BSA systems when $[P]_T$ equals to 1.0×10^{-4} M, and found that the binding sites seem to be classified into three different sets for Zn(II)-HSA and two different sets for Zn(II)-BSA. In both systems, the three strongest binding sites can be considered to be identical. The 4th up to 8th and the 9th up to 12th binding sites of Zn(II)-HSA constitute the other two different sets, respectively. For Zn(II)-BSA, all the other binding sites seem to be identical.

3.2. Ligand-induced cross-linking self-association of HSA [17]

We have rebuilt the four solid curves in Fig. 1 into the Scatchard plots ($\bar{n}/[Zn^{2+}]$ versus $\bar{n}$) as shown in Fig. 4. All these curves are concave upward and intersect with each other at $\bar{n}$ of one, clearly indicating that the dependence of the binding equilibrium of Zn(II) to HSA on the concentration of HSA can be ascribed to the presence of the ligand-induced cross-linking self-association of HSA. Despite the fact that all the current theoretical models for such self-association involve the case where each macromolecule binds one ligand only, i.e. excluding the multiligand binding, if we consider only the case under physiological concentrations, which are usually very low, these models should be fully applicable.

In such self-association, the ligand bridges two macromolecules, with each macromonomer has two ligand-binding sites, as depicted below:

$$A + X = AX,\ k_1,$$

$$AX + A = AXA,\ k_1,$$

$$AXA + X = AXAX,\ k_1,$$

where A represents a macromolecule, and X the ligand. If k_2 is defined as the apparent self-association constant, the following equation can be obtained:

$$k_2 = \frac{k_1^2[Zn^{2+}]}{\left(1 + k_1[Zn^{2+}]\right)^2}.$$

On substituting k_1 with K_1 shown in Table 1 and plotting $\ln k_2$ against $\ln[Zn^{2+}]$ with $[Zn^{2+}]$ in the range 10^{-5}–10^{-7} M, we can obtain Fig. 5 [18]. It is obvious from this figure that k_2 has the largest value (about 2.6×10^4 M^{-1}) at a certain $[Zn^{2+}]$ in the range $(4–6) \times 10^{-6}$ M but decreases rapidly at other $[Zn^{2+}]$. Hughes also reported a similar association of human mercaptalbumin induced by mercury chloride [19]. The apparent association constant obtained by him has an order of magnitude similar to that of the corresponding constant found for our Zn^{2+}-induced case [20].

Worthy to note is that Hughes has given some evidence [21] to show the mercury salt-induced association of human mercaptalbumin was

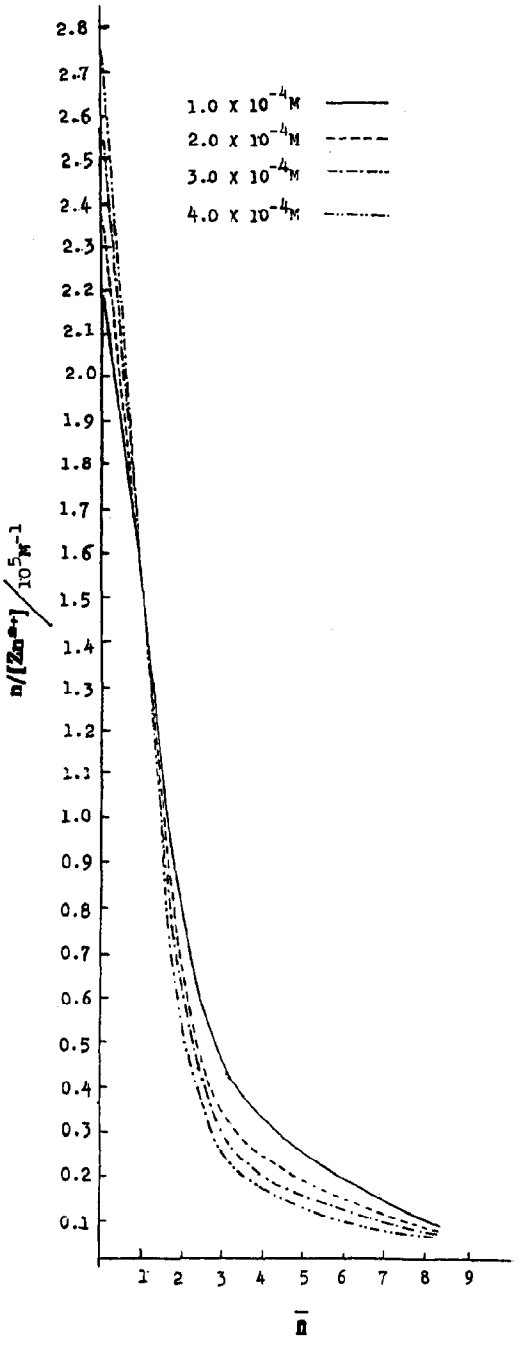

Fig. 4. Scatchard plots of the Zn^{2+}-induced cross-linking self-association of HSA.

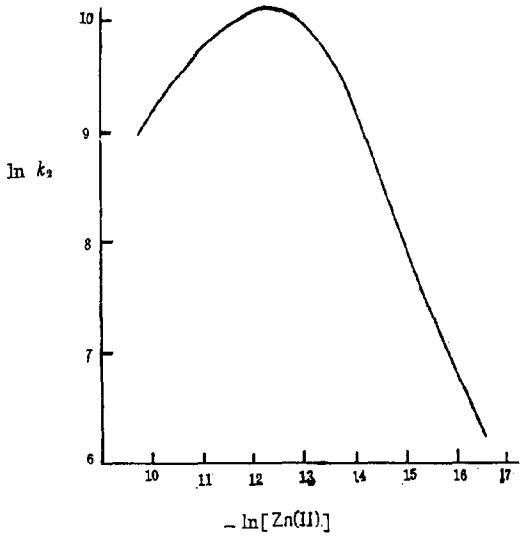

Fig. 5. Wyman plot of the ligand-induced self-association constant [19].

achieved by cysteinyl sulphur atoms, leading to a cross-linking –S–Hg–S– structure. This is consistent with our previous inference [11] that the zinc group ions should be coordinated with sulphur atoms in both HSA and BSA systems. In this work, BSA failed to show the self-association similar to that of HSA, which cannot be fully elucidated here but perhaps has to do with the following fact. Thus, there exist much more alanyl residues in the vicinity of the seven pairs of adjacent disulphide bridges in HSA than those in BSA [22]. Such smallest aminoacids should favor the cross-linking self-association of HSA at these positions.

Interestingly, a calculation using the data in Table 1 reveals that, in the range of $[Zn^{2+}]$ where k_2 reaches maximum value, there exists mainly the species PZn_1 in which only one Zn^{2+} ion is bound to a protein molecule. This indicates that the first bound Zn^{2+} ion plays an important role in inducing the cross-linking self-association of HSA, a fact that may help understand why the K_1 values are nearly the same for both Zn(II)-HSA and Zn(II)-BSA whereas all the K_i ($i > 1$) values

for Zn(II)-BSA are slightly larger than the corresponding ones for Zn(II)-HSA. A possible reason for this may be that the protein association weakens its binding towards the zinc ion.

4. Conclusions

Both the successive stability constants K_1 and K_2 for Zn(II)-HSA and Zn(II)-BSA systems were found to have an order of magnitude of 10^5, in good agreement with the corresponding constant reported very recently by Sarkar and co-workers [23] for the Zn(II)-HSA system, but substantially larger than the stability constants for $ZnIm_4$ reported in the literature. This appears to challenge the inference that the high-affinity Zn(II) binding sites of serum albumins are located at the sites involving histidyl imidazole groups. On the other hand, the results from this work that both systems have similar successive stability constants (those for BSA are only slightly larger than for HSA), and the first three strongest binding sites are identical, and, especially, the Zn^{2+}-induced cross-linking self-association of HSA, support to some extent our previous inference that the strongest binding sites of the zinc group ions are most likely located at the seven pairs of adjacent disulfide bridges.

In the vicinity of physiological concentration of Zn(II) (10^{-6} M), HSA shows the Zn(II)-induced cross-linking self-association, with the maximum apparent self-association constant being 2.6×10^4 M^{-1} corresponding to a $\approx 6 \times 10^{-6}$ M of $[Zn^{2+}]$. These results should be important to medical chemical studies.

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