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# Stopped-flow Studies on Binding of $Cu^{2+}$ Ion to Human Serum Albumin

Naohisa KURE, Thoru NAKATSUKA, Takayuki SANO,* and Tatsuya YASUNAGA
*Department of Materials Science, Faculty of Science, Hiroshima University, Naka-ku, Higashisenda-machi, Hiroshima 730*
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Both static and kinetic investigations have been carried out regarding the interaction between $Cu^{2+}$ and human serum albumin (HSA). The static experiment, using a UV spectrophotometric technique, shows the presence of one primary binding site and other weaker binding sites at pH 5.2. The kinetic experiment, using stopped-flow apparatus, revealed a reaction process on the order of several seconds and a rapid absorbance change within the dead-time of the apparatus. These results are discussed from the viewpoint of the substitution effect of the second residue of the N-terminal position in the protein with reference to the previously reported results for a $Cu^{2+}$-bovine serum albumin (BSA) system.

The bindig of the $Cu^{2+}$ ion to bovine serum albumin (BSA) and/or human serum albumin (HSA) has been widely studied. Extensive investigations by Peters,[1-5] Breslow,[6] Gurd,[5,7] and Sarker[8-10] revealed that a specific binding site of $Cu^{2+}$ is located at the N-terminal sequence of both albumins (Asp-Thr-His- for BSA and Asp-Ala-His- for HSA), and that the chelation of a $Cu^{2+}$ ion formed at this site involves coordination by α-amino, imidazolyl, and two peptide nitrogens in the neutral pH range. Different types of bound species were also evident at low pH from visible and circular dichroism spectroscopies.[2,6,10] In previous studies of the kinetics of a $Cu^{2+}$-BSA system,[11] we revealed that one low-pH type complex is also formed at the N-terminal sequence of BSA in competition with a chelation complex. This present report is concerned with a further kinetic study on the binding of a $Cu^{2+}$ ion to serum albumin by using HSA from the viewpoint of the substitution effects of alanin for threonin at the second residue of the N-terminal position in serum albumin.

## Experimental

*Materials.* Human serum albumin (essentially fatty acid-free) was purchased from Sigma Chem. Co. and fractionated on a Sephadex G-150 column to obtain a monomeric form. The concentration of the HSA monomer was determined spectrophotometrically.[12] Solutions of $Cu^{2+}$ ions were produced by direct weighing of anhydrous $CuSO_4$. The pH values of solutions were adjusted by $H_2SO_4$ and/or KOH, and no pH buffer was added. All measurements were made in a 1-mM† solution of $K_2SO_4$. Analyticalgrade reagents were used throughout.

*Equilibrium Dialysis.* The extent of binding was measured using the equilibrium dialysis technique with a dialysis cell (Sanko Plastics Co. type 5044-05) having ten separate chambers. After 70 to 80 h of dialysis at 25 °C, concentration of $Cu^{2+}$ in the dialyzed solutions were determined with a Perkin-Elmer Model 5000 atomic absorption spectrophotometer. The detection limit of this method for the $Cu^{2+}$ concentration was $1.5\times10^{-7}$ M. The pH values of the dialyzed solutions were 5.02±0.07.

*Spectrophotometry.* The difference spectra of HSA solutions in the presence and absence of $Cu^{2+}$ ions were measured in the ultraviolet region using a Union Giken SM 401 spectrophotometer at 25±0.2 °C.

*Stopped-flow Experiments.* The kinetic measurements were carried out using a Union Giken Stopped-flow spectrophotometer RA 401. The dead-time of the apparatus is 5 ms. For the case of very small signal amplitudes, its relaxation time was obtained by signal averaging 2—10 measurements using the averaging capabilities of a wave-memory apparatus. All kinetic measurements were made at 25±0.2 °C.

† 1 M = 1 mol dm$^{-3}$.

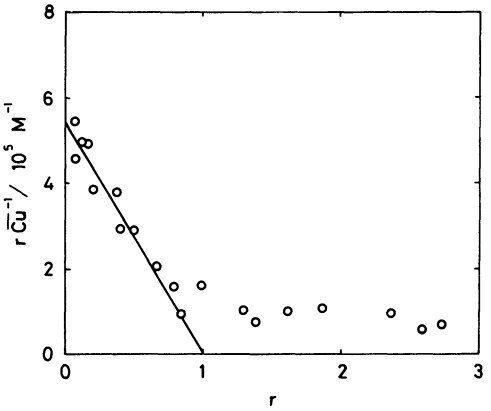



Fig. 1. Scatchard plot of the binding of $Cu^{2+}$ ion to HSA in 1 mM $K_2SO_4$ solution at pH 5.02±0.07 and 25±0.2 °C. The slope of the solid line yields $K_p=(5.3\pm0.5)\times10^5$ $M^{-1}$ for the binding constant of the primary site.

## Results

*Static Measurements.* Figure 1 illustrates a Scatchard plot of the binding of the $Cu^{2+}$ ion to HSA monomer obtained by an equilibrium dialysis technique at pH 5.02±0.07. The shape of the isotherm clearly demonstrates the presence of one primary binding site with high affinity and number of secondary sites with low affinities. From the slope of this isotherm in the range of $r<1$ ($r$ is binding ratio of bound $Cu^{2+}$ to protein), the binding constant for the primary site was estimated to be $K_p=(5.3\pm0.5)\times10^5$ $M^{-1}$; where subscript p refers to the value for the primary site.

The binding of $Cu^{2+}$ to HSA was also examined spectrophotometrically at pH 5.0 by monitoring the absorbance change at a wave length 255 nm which is specific to a charge-transfer transition from amino,

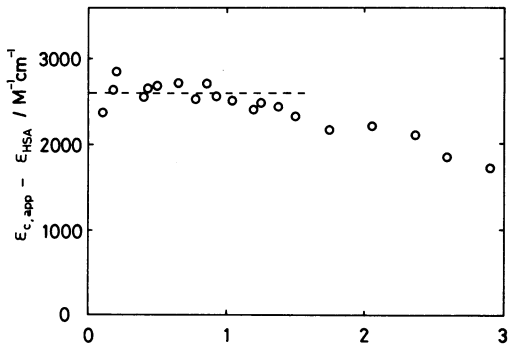



Fig. 2. A plot of $\varepsilon_{c,app}-\varepsilon_{HSA}$ *vs.* binding ratio r. The data were obtained from the spectrophotometric titration of HSA ($2.0\times10^{-5}$ M) solutions with various amounts of $Cu^{2+}$ ions at 255 nm, pH 5.0±0.02, and 25±0.2°C.

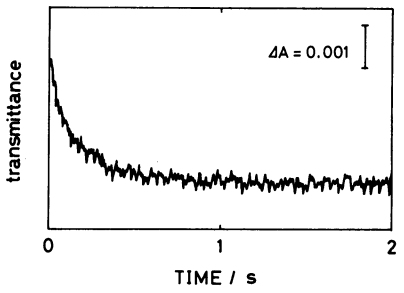



Fig. 3. A typical oscillographic trace of the stopped-flow experiments of the reaction between $Cu^{2+}$ ion ($4.0\times10^{-6}$ M) and HSA ($2.0\times10^{-5}$ M) monitored by the change in the transmittance intensity (increasing upward) at 255 nm, pH 4.6 and 25±0.2°C in 1 mM $K_2SO_4$ solution. The magnitude of the absorbance change is indicated by the bar in the upper right-hand corner.

carboxylato, imidazolyl groups, and ionized peptide nitrogens to bound $Cu^{2+}$.[11] Since the contribution from the free $Cu^{2+}$ ion to the absorbance is negligibly small at the present wave length of 255 nm, the observed change in absorbance ($\Delta A^{255}$) may be written as

$$\Delta A^{255}/\text{cm} = (\varepsilon_{c,app}-\varepsilon_{HSA})\cdot\bar{C}, \quad (1)$$

where $\varepsilon_{HSA}$, $\varepsilon_{c,app}$, and $\bar{C}$ denote the molar extinction coefficient of HSA at 255 nm, the apparent molar extinction coefficient of the complex and its equilibrium concentration, respectively. The variation of $\varepsilon_{c,app}-\varepsilon_{HSA}$, which is given by calculating $\Delta A^{255}/\bar{C}$ using the binding data obtained above, was represented as a function of the binding ratio $r$ in Fig. 2. The constancy of the value of $(\varepsilon_{c,app}-\varepsilon_{HSA})$ up to an $r$ value of unity gives further evidence for the presence of one primary site. Since the highest affinity site on HSA for the $Cu^{2+}$ ion has been identified to be the three N-terminal peptides (Asp-Ala-His),[8-10] the primary site found in the present study is most likely attributable to this sequence. The apparent molar extinction coefficient of the complex formed at this site ($\varepsilon_{c,app}$) was estimated to be 22100 $M^{-1}cm^{-1}$ by using a value of 19500 $M^{-1}cm^{-1}$ for $\varepsilon_{HSA}$.

*Kinetic Measurements.* In order to detect a reaction between the $Cu^{2+}$ ion and the primary site of HSA, stopped-flow experiments were carried out at a small *M/P* (metal to protein ratio) of 0.2 by using a change in the absorbance at 255 nm. As shown in Fig. 3, a single relaxation process was observed in the sec range under various pH and concentration values. Other reaction processes were not discernible within the time resolution of the apparatus (5 ms—100 s). The spectra of the solutions after mixing were quite stable over a period of 2 d.

The reciprocal relaxation time ($\tau^{-1}$) was determined by a semilogarithmic plot of the reaction curve near the equilibrium point. The pH and concentration dependences of the obtained $\tau^{-1}$ are represented in Figs.

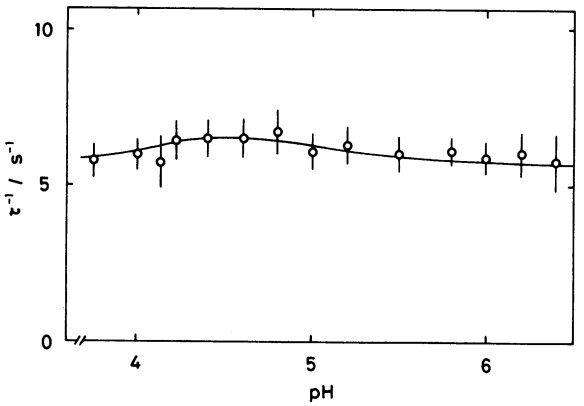



Fig. 4. The pH dependence of $\tau^{-1}$ at a fixed concentrations of $Cu^{2+}$ ($4.0\times10^{-6}$ M) and HSA ($2.0\times10^{-5}$ M).

4 and 5, respectively. The behaviors for $\tau^{-1}$ (shown in these figures) are quite similar to a previous observation in a $Cu^{2+}$-BSA system,[11] but the present $\tau^{-1}$ is several times larger.

On the other hand, the absorbance changes ($\Delta A_{SF}$) at 255 nm (estimated directly from the reaction traces observed by the stopped-flow measurements) are represented as a function of pH in Fig. 6, together with the total absorbance changes ($\Delta A_{total}$) measured during static spectrophotometrical experiments at the same wavelength. Disagreement between $\Delta A_{SF}$ and $\Delta A_{total}$ implies the presence of at least one more reaction process within the dead-time of the stopped-flow apparatus accompanied by the absorbance changes ($\Delta A_{DT}$) corresponding to the difference between $\Delta A_{total}$ and $\Delta A_{SF}$. As judged from the pH dependence of $\Delta A_{DT}$ and $\Delta A_{SF}$, a complex formed within the dead-time of the apparatus predominates above pH 5.5, and the other complex yielded by the observed reaction process around pH 4.5. These features in the complexation of $Cu^{2+}$ ion with HSA are also quite similar to those previously observed for a $Cu^{2+}$-BSA system.[11]

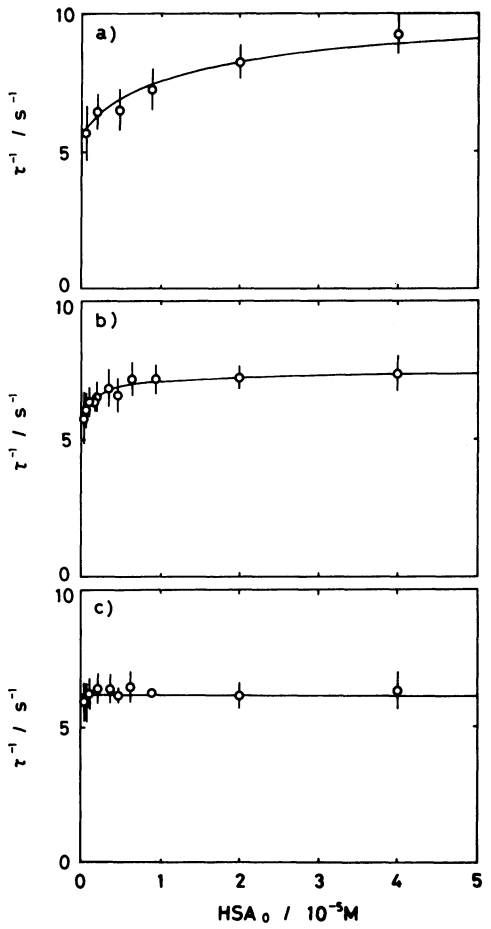



Fig. 5. The variation of $\tau^{-1}$ with the total concentration of HSA ($HSA_0$) at a fixed $M/P$ of 0.2: (a), pH 4.2; (b), pH 4.6; (c), pH 5.2.

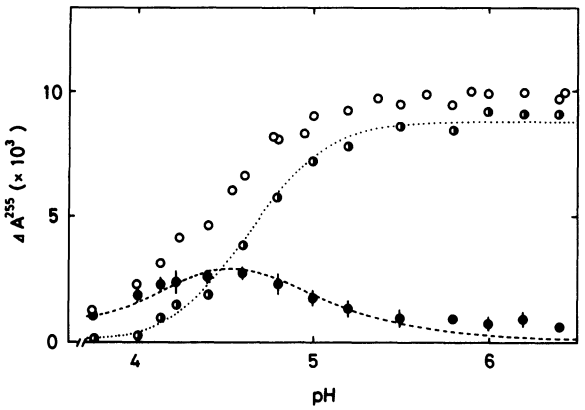
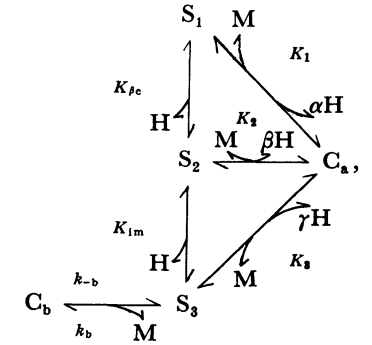



Fig. 6. The pH dependence of absorbance changes at 255 nm due to the reaction between $Cu^{2+}$ ($4.0\times10^{-6}$ M) and HSA ($2.0\times10^{-5}$ M): ○, the total absorbance change ($\Delta A_{total}$) measured spectrophotometrically; ●, the absorbance change ($\Delta A_{SF}$) observed by stopped-flow method; ○, the absorbance change within the dead-time of the stopped-flow apparatus ($\Delta A_{DT}$) determined from $\Delta A_{total}-\Delta A_{SF}$ at each pH value.

*Examination of Reaction Mechanism.* Looking for close simillarities regarding the kinetic behaviors for $Cu^{2+}$-BSA and $Cu^{2+}$-HSA systems, the following reaction mechanism is proposed for the binding of a $Cu^{2+}$ ion to the three N-terminal peptides of BSA. This is also being applied to the present system.




(I)

with

$$K_b = \frac{k_b}{k_{-b}}, \tag{2}$$

$K_i$ ($i$=1, 2, 3, $b$), $k_i$ ($i$=$b$, -$b$), $H$, $M$, $C_a$, and $C_b$ denote binding constants, rate constants, protons, free $Cu^{2+}$ ions, complex formed from the rapid reaction, and the other complex formed during the observed reaction, respectively. $\alpha$, $\beta$, and $\gamma$ are the number of protons released in each step and $S_i$ ($i$=1, 2, 3) refers to the possible protonated states of the three N-terminal peptides of HSA, defined as $S_1$: Asp(protonated $\beta$-carboxyl)-Ala-His(protonated imidazolyl), $S_2$: Asp(deprotonated $\beta$-carboxyl)-Ala-His(protonated imidazolyl), $S_3$: Asp(deprotonated $\beta$-carboxyl)-Ala-His(deprotonated imidazolyl). The equilibria among these states are characterized by two acid dissociation constants of $K_{\beta c}$ (for $\beta$-carboxyl group) and $K_{im}$ (for imidazolyl group), since the $\alpha$-amino group of the terminal aspartyl residue ($pK_a$=7.73[10]) is almost acidic under the present pH conditions of 3.7—6.4. The values of $K_{\beta c}$ and $K_{im}$ were calculated using $pK_a$ values (2.98 for $\beta$-carboxyl group and 6.55 for imidazolyl group) obtained from titration studies of a synthetic tripeptide of HSA primary site by Iyer *et al.*[10]

Firstly, the observed absorbance changes ($\Delta A_{DT}$) within the dead-time of the apparatus were analyzed on the basis of the rapid equilibria represented in the right-hand side of Mechanism I. The cyclic nature of the scheme requires

$$K_1 = K_{\beta c}K_2 = K_{\beta c}K_{im}K_3, \tag{3}$$

$$\alpha = \beta + 1 = \gamma + 2. \tag{4}$$

By use of the molar extinction coefficients, $\varepsilon$ of $C_a$ and HSA, the equation of a net absorbance change resulting from the formation of $C_a$ is given by

$$\Delta A_{DT}/\text{cm} = (\varepsilon_{C_a}-\varepsilon_{HSA})\cdot\bar{C}_{a,r}, \tag{5}$$

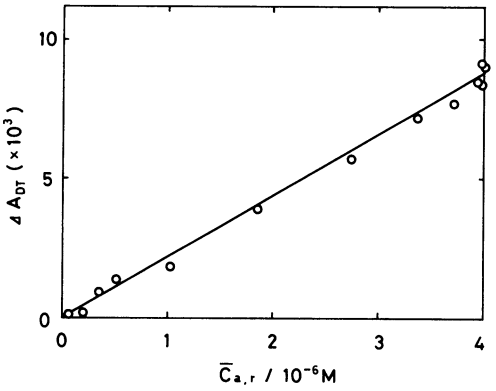



Fig. 7. A plot of $\Delta A_{DT}$ *vs.* $\bar{C}_{a,r}$ calculated for $\gamma=1$ and $K_3=105$.

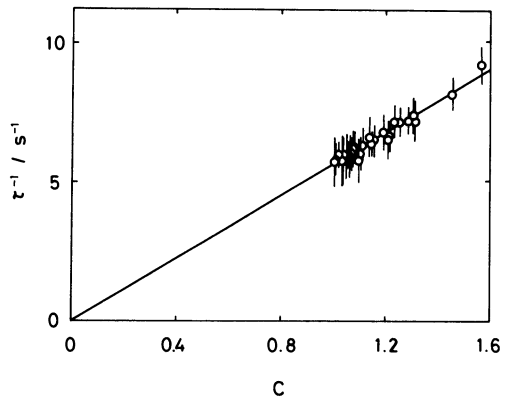



Fig. 8. A plot of $\tau^{-1}$ obtained at various pH and concentrations *vs.* the concentration term in Eq. 9, designated by $C$ in this figure and calculated for $K_b=1.9\times10^{-6}\,M^{-1}$.

with

$$\bar{C}_{a,r}=\frac{1}{2A}\{A(M_0+S_0)+B-\sqrt{A^2(M_0-S_0)+2AB(M_0+S_0)+B^2}\}, \quad (6)$$

$$A=K_1/\bar{H}_r^{r+2}, \quad (7)$$

$$B=1+K_{\beta c}/\bar{H}_r+K_{\beta c}K_{1m}/\bar{H}_r^2, \quad (8)$$

where the bar with subscripts $r$ and 0 refer to the equilibrium concentration after rapid equilibria and the total concentration of each species, respectively. In the present analyses, the equilibrium proton concentrations were approximated by the final concentrations since pH changes in solutions after mixing were within ±0.03 of the initial pH values. As shown in Fig. 7, a plot of experimental values of $\Delta A_{DT}$ *vs.* $\bar{C}_{a,r}$ calculated using $\gamma=1$ and $K_3=105\pm8$ gives an excellent straight line through the origin. From the slope of this line, the value of $\varepsilon_{C_a}$ was estimated to be 21700 $M^{-1}cm^{-1}$. As can be seen from Fig. 6, data regarding $\Delta A_{DT}$ fall on the theoretical curve (dotted line) calculated from Eqs. 5—8 while using this value. This demonstrates the plausibility of a reaction scheme involving a rapid equilibria.

Subsequently, the reaction given by the left-hand side of the proposed mechanism was examined on the basis of data for both $\tau^{-1}$ and $\Delta A_{SF}$ obtained from the present stopped-flow measurements. By coupling with a rapid equilibria, the equations for $\tau^{-1}$ and $\Delta A_{SF}$ in the present process can be written as

$$\tau^{-1}=k_{-b}\left[K_b\frac{\bar{S}_3(F-K_3\bar{M}/\bar{H})}{F(1+K_3\bar{S}_3/\bar{H})}+\frac{\bar{M}}{F}+1\right], \quad (9)$$

with

$$F=(1+\bar{H}/K_{\beta c})(\bar{H}+K_3\bar{S})/K_{1m}+K_3(\bar{M}+\bar{S}_3)/\bar{H}+1, \quad (10)$$

and

$$\Delta A_{SF}/cm=(\varepsilon_{C_a}-\varepsilon_{HSA})(\bar{C}_a-\bar{C}_{a,r})+(\varepsilon_{C_b}-\varepsilon_{BSA})C_b, \quad (11)$$

respectively. The overbar and $\varepsilon_{C_b}$ denote the equilibrium concentration of each reacting species and the molar extinction coefficient of $C_b$, respectively. The concentration term in brackets in Eq. 9 was calculated for various values of $K_b$. As shown in Fig. 8, a plot of $\tau^{-1}$ obtained at various pH values and concentrations *vs.* the concentration term calculated by using $K_b=1.9\times10^6\,M^{-1}$ gives a good proportionality through the origin. From the slope of this line, $k_{-b}$ was determined to be 5.1 $s^{-1}$ and $k_b$ was calculated to be $9.7\times10\ M^{-1}s^{-1}$. All the data of $\tau^{-1}$ fall on the theoretical curves calculated using these values, as can be seen from Figs. 4 and 5. Furthermore, the theoretical curve of $\Delta A_{SF}$, drawn with a dashed line in Fig. 6, was calculated from Eq. 11 by using $\varepsilon_{C_b}=24000\ M^{-1}cm^{-1}$ and gives a good fit with the data at each pH value. This good agreement between the theoretical curves and the experimental data confirms the validity of Mechanism I.

The apparent association constant $K_{app}$, defined as $(C_a+C_b)/(\bar{S}_1+\bar{S}_2+\bar{S}_3)\cdot M$, and the apparent molar extinction coefficient $\varepsilon_{c,app}$ can be calculated from the following relations:

$$K_{app}=\frac{K_1/\bar{H}+K_{\beta c}K_{1m}K_b/\bar{H}^2}{1+K_{\beta c}/\bar{H}+K_{\beta c}K_{1m}\bar{H}^2}, \quad (12)$$

and

$$\varepsilon_{c,app}=\frac{\varepsilon_{C_a}\bar{C}_a+\varepsilon_{C_b}\bar{C}_b}{\bar{C}_a+\bar{C}_b}. \quad (13)$$

Calculations using these equations yield $K_{app}=3.1\times10^5\ M^{-1}$ and $\varepsilon_{c,app}=22200\ M^{-1}cm^{-1}$ for pH 5.0. Good agreement with the values ($K_p=5.3\times10^5\,M^{-1}$ and $\varepsilon_{c,app}=22100\ M^{-1}cm^{-1}$) obtained statically also strongly supports the present assignments.

## Discussion

Both the static and kinetic studies in the present

TABLE 1. STOICHIOMETRIC AND KINETIC CONSTANTS FOR BINDING OF $Cu^{2+}$ TO SERUM ALBUMIN AT 25 °C AND MOLAR EXTINCTION COEFFICIENTS OF COMPLEXES AT 255 nm

| Source | $\gamma$[c] | $K_3$[d] | $K_b$ | $k_b$ | $k_{-b}$ | $\varepsilon_{C_a}(\Delta\varepsilon_{C_a})$ | $\varepsilon_{C_b}(\Delta\varepsilon_{C_b})$ |
|---|---|---|---|---|---|---|---|
| | | | $10^6\,M^{-1}$ | $10^6\,M^{-1}s^{-1}$ | $s^{-1}$ | $M^{-1}cm^{-1}$ | $M^{-1}cm^{-1}$ |
| HSA[a] | 1 | 105±8 | 1.9±0.3 | 9.7±1.8 | 5.1±0.3 | 21700 (2200) | 24000 (4500) |
| BSA[b] | 1 | 60±10 | 3.9±0.8 | 5.2±1.3 | 1.3±0.1 | 23700 (2300) | 26200 (4800) |

a) This work. b) Previous work.[11] c) Values of $\alpha$ and $\beta$ can be calculated from Eq. 3. d) Values of $K_1$ and $K_2$ can be calculated from Eq. 4.

work clearly showed that two types of $Cu^{2+}$ complexs ($C_a$ and $C_b$) are formed at the three N-terminal peptides of HSA (Asp-Ala-His) by the same mechanism proposed for the binding of a $Cu^{2+}$ ion to BSA terminal peptides (Asp-Thr-His-). In previous work regarding a $Cu^{2+}$-BSA system,[11] we revealed that $C_a$ is a chelated complex with a square-planar configuration involving $\alpha$-amino, imidazolyl and two peptide nitrogens. This was originally proposed by Peters and Blumenstock.[2] We also proposed that $C_b$ is a low-pH type complex with a ring structure made by coordinations with imidazolyl nitrogen and $\beta$-carboxylato oxygen. In order to see the substitition effect on the complexation, the stoichiometric constants, kinetic constants, and the molar extinction coefficients of the complexes were determined for both albumins and are summarized in Table 1. The molar extinction coefficients in this table involve the absorption of the albumin molecule itself (at 255 nm), which arises mainly from the aromatic groups in the protein. Thus, the net changes in the molar extinction coefficients due to complexation ($\Delta\varepsilon_{C_a}$ and $\Delta\varepsilon_{C_b}$) were estimated by the substraction of $\varepsilon_{HSA}$ or $\varepsilon_{BSA}$ from the corresponding $\varepsilon_{C_a}$ and $\varepsilon_{C_b}$, and are given within parentheses in Table 1. Good agreement between the obtained values for HSA ($\Delta\varepsilon_{C_a}$=2200, $\Delta\varepsilon_{C_b}$=4500) and those for BSA ($\Delta\varepsilon_{C_a}$=2300, $\Delta\varepsilon_{C_b}$=4800) confirm the formation of the same types of complexes at the terminal sites of both albumins. However, the equilibrium and rate constants are somewhat different for each albumin beyond the experimental uncertainties. Especially, the value of $k_{-b}$ obtained for BSA ($1.3\,s^{-1}$) is about one-fourth of that for HSA ($5.1\,s^{-1}$). This result can be explained on the basis of the ring structure of $C_b$. The construction of molecular models for this complex showed that the side chain of the second residue ($\alpha$-hydroxyethyl group for BSA, methyl group for HSA) is always outside of the peptide ring. In such a configuration, a bulky and hydrophilic 1-hydroxyethyl group would not only interfere with the binding of a peptide chain to the outside, but would participate in the ordering of water molecules around the complex. These functions of this group are most likely responsible for the stabilization of an already formed ring structure of $C_b$. This is consistent with the observed $k_{-b}$ reduction for a BSA system.

Finally, it is worth noting that the complexation of $Cu^{2+}$ with the N-terminal site of albumin can be easily understood without any consideration of the N-F transition of albumin occurring in the range of pH 3.5—4.5. This is presumably because the N-terminal domain of albumin is maintained in a compact form through the N-F transition, in contrast to the disordered C-terminal domain.[13]

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