Structural Studies on Metal–Serum Albumin
I. An Ultraviolet Spectroscopic Study of Copper(II)–Human Serum Albumin Complexes

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Abstract

It has been reported for the first time that the concentration of human serum albumin (HAS) has a notable impact on the structure of Cu(II)–HSA complexes. Two configurations of Cu(II)–HSA have been found in the range of experimental concentrations used and the formation conditions and transformation processes of these two configurations have been studied by ultraviolet spectroscopy. The optical electronegativities of Cu(II) and relevant ligand atoms have also been calculated and discussed.

Introduction

Human serum albumin (HSA) is the most abundant protein in blood plasma or serum. One of its important characteristics is that it is able to bind various molecules and ions. A small copper fraction in blood binds to HSA and only this copper is known to be exchangeable [1, 2]. HSA therefore plays an important role in transport and allocation of copper *in vivo* [3]. As a result, the copper–HSA system has been extensively studied. The early investigations of this interaction were carried out with copper(II)–BSA (bovine serum albumin) and the results of such studies are often cited in connection with the copper(II)–HSA system, especially in structural studies, because the structures of HSA and BSA are very similar [4].

In the 1960s, Peters *et al.* [5–9], Breslow [10] and Gurd *et al.* [11] made intensive studies of Cu(II) binding to BSA. Peters and Blumenstock [6] first proposed a possible binding site and structural model of copper(II) in BSA. They proposed that the Cu^{2+} ion is located at the N-terminal sequence of BSA (Asp–Thr–His–), and a square-planar chelate (Fig. 1(a)) is formed with α-amino, imidazoyl and two peptide nitrogens in the neutral pH range. In addition to this primary site, there are secondary sites for copper(II) binding in BSA [8], but so far they have not been identified.

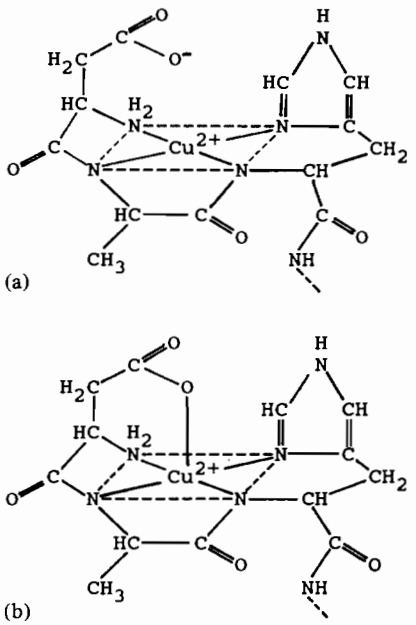

Fig. 1. Proposed structural models of copper(II) in HSA: (a) square-planar structure; (b) square-pyramid structure.

Later, Sarkar *et al.* [12–14], Asaturian *et al.* [15, 16] and Rakhit and Sarkar [17] made a series of more intensive studies of Cu(II)–HSA, Cu(II)–BSA and Cu(II)–peptide models (tripeptides and P_{1-24}) and confirmed the configuration proposed by Peters *et al.* Research on Cu(II) binding to peptide models, however, has shown that in addition to the four nitrogen ligands at the Cu(II) binding site, there is an additional binding site involving a side chain carboxyl group that results in a pentacoordinate structure (Fig. 1(b)) [14, 18, 19]. Nevertheless, this configuration has not been reported to occur in the Cu(II)–albumin system. The present paper reports a UV spectroscopic study on the Cu(II)–HSA system and describes the conditions under which the tetracoordinated complex (a) and the pentacoordinated (b) can be formed. It is suggested

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that the Cu^{2+} ion binding to HSA *in vitro* is mainly of form (a), but may also take form (b) at low concentrations. The optical electronegativities of the Cu(II), carboxyl group and deprotonated peptide nitrogen have been calculated and discussed on the basis of UV spectra.

Experimental

Materials and Instruments

HSA, purity not less 95%, was purchased from Beijing Blood Center of the Red Cross and used without further purification. The purity of tris-(hydroxymethyl)aminomethane (tris) was greater than 99.5%. Sodium chloride, copper(II) chloride and hydrochloric acid were all analytical grade reagents. Deionized water was used throughout.

Spectrophotometer: Shimadzu UV-240 Electronic Absorption Spectrograph. pH meter: pHs-2-type Acidimeter.

Experiment

The concentration of HSA (molecular weight 67 500 [21]) was determined spectrophotometrically [20]. The concentration of $CuCl_2$ in solution was determined by titration with EDTA.

1:1 Cu(II)–HSA solutions (molar ratio) of various concentrations were freshly prepared by mixing appropriate amounts of copper(II) chloride and HSA solutions. The reference solution was the HSA solution in which all conditions were the same as the corresponding Cu(II)–HSA solution except for the Cu^{2+} ion. The UV spectra were recorded at 25.0 ± 0.2 °C. All solutions contained 0.1 M NaCl and were buffered with 0.1 M tris–HCl (pH 7.43 ± 0.02).

Results and Discussion

UV Spectra

The UV difference spectra data of the Cu(II)–HSA systems of various concentrations are listed in Table 1 and some typical spectra are shown in Fig. 2. When the HSA concentration is 5.0×10^{-5} M, there is only one broad band at 244.0 nm (Fig. 2(a)). As the concentration increases over 1.0×10^{-4} M, two bands appear. The first one, whose λ_{max} occurs at about 248 nm, consists of several peaks as evidenced in the first order derivative spectra (not shown). The second one seems to be a single peak at 289 nm. No new bands occur at higher concentrations. The continuous reduction in the number of absorptions at the low energy side of the first band is obtained from the first order derivative spectra. Furthermore, the first band shows two peaks which are well matched in their intensities

TABLE 1. UV spectra of 1:1 Cu(II)–HSA systems

No.	Concentration ($\times 10^4$ M)	λ_{max} (nm)		
1	0.50	244.0(0.16)[a]		
2	1.00	248.0(0.38		289.0(0.12)
3	1.50	(i) 250.5(0.34)	(iii)	291.0(0.12)
		(ii) 253.0(0.33)		
4	2.00	(i) 252.0(0.12)	(iii)	292.8(0.105)
		(ii) 253.5(0.12)		
5	2.50			293.8(0.18)
6	3.00			294.8(0.205)

[a]Values given in parentheses are the absorbances.

at 2.0×10^{-4} M (Fig. 2(b)). If the HSA concentration is about 3.0×10^{-4} M, the first band vanishes (Fig. 2(c)). These phenomena suggest that when the molar ratio of Cu(II) to HSA in the Cu(II)–HSA system is fixed, the change of the apparent HSA concentration leads to certain changes in the structure of the metal center.

The Structure of Complexes and the Assignment of UV Spectra

HSA can coordinate freely at low concentrations and it is assumed the complex takes configuration (b) under these condition. Thus, there are the negative oxygen ion of the carboxyl group and deionized peptide nitrogen ions among the ligand atoms. As a result of the chelate effect the bonds between the peptide nitrogen ions and the Cu^{2+} ion may be mainly covalent, but the binding of the negative oxygen ion to Cu^{2+} is weaker and together they can form an 'associated ionic pair' involving a charge-transfer transition which can be affected by thermal motion [22]. This makes the charge-transfer absorption become a broad band. As the concentration increases, the 'associated ionic pair' becomes more stable. Then, two separate bands can be seen in the UV region. The first band may be attributed to the chromophore CuN_4. As the concentration increases, there is a reduction in the number of absorption peaks in this band for two reasons. On the one hand, as the concentration constantly increases the Cu–N tends to typical covalent bonding and multipeaks, emerged owing to the difference of associated strength, amalgamate progressively. The chromophore CuN_4 would have finished the transition to covalent coordination at about 2.0×10^{-4} M. On the other hand, because there are some secondary binding sites of Cu(II) in HSA, the absorptions from these secondary binding sites may lose their activities owing to dissociation of the Cu^{2+} ion under stereochemical effects and finally vanish from the spectra. In this range of concentration, the two stable peaks in the first band, together with the single peak in the second band,

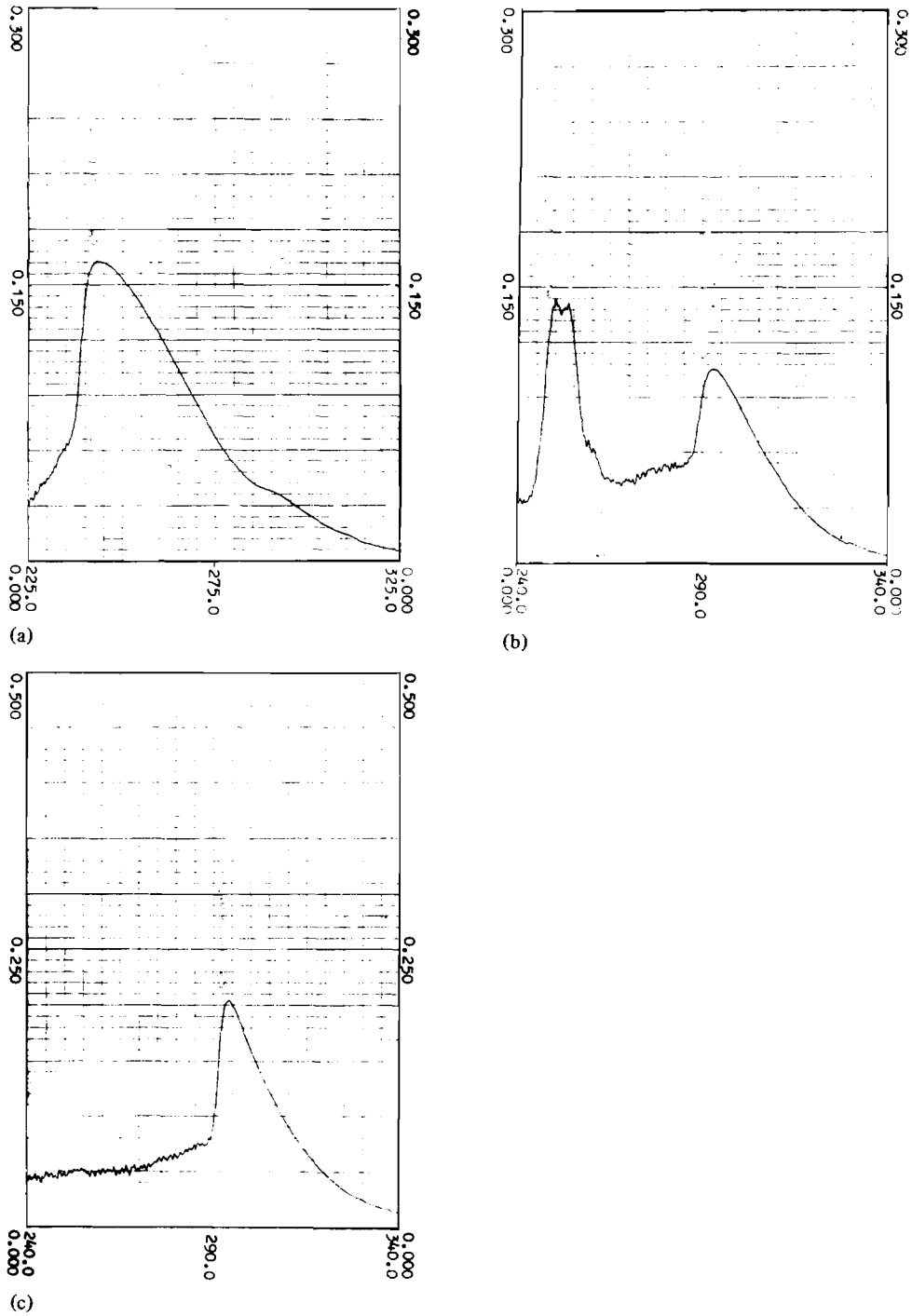

Fig. 2. UV absorption spectra of 1:1 Cu(II)–HSA complexes at different HSA concentrations in 0.1 M NaCl and pH 7.43 buffer solutions: (a) $c_{HSA} = 0.5 \times 10^{-4}$ M, (b) $c_{HSA} = 2.0 \times 10^{-4}$ M, (c) $c_{HSA} = 3.0 \times 10^{-4}$ M.

can be assigned on the basis of the common molecular orbital scheme of the chromophore MN_4O at local symmetry C_{4v} (Fig. 3). There are three allowed transitions, namely

$$a_1(O) \longrightarrow a_1^*(3d) \quad \text{(i)}$$

$$e(N) \longrightarrow a_1^*(3d) \quad \text{(ii)}$$

$$a_1(N) \longrightarrow a_1^*(3d) \quad \text{(iii)}$$

They are all ligand to metal charge-transfer (LMCT) transitions. Analysis of the data in Table 1 shows the

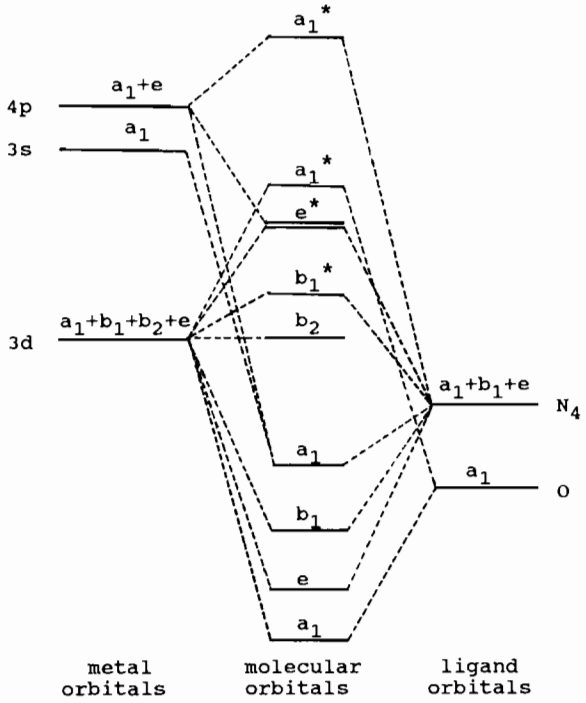

Fig. 3. A molecular orbital energy diagram for square-pyramid complexes assuming C_{4v} symmetry.

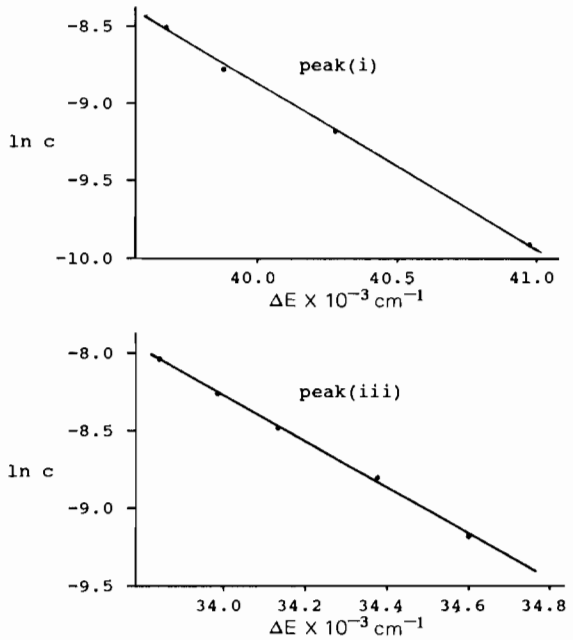

Fig. 4. Correlation of ln c vs. ΔE.

first and third absorptions have a regular red shift depending on the increase of the concentration. These two absorptions are the charge-transfer bands of the 'associated ionic pair' Cu–O and Cu–N, respectively. These data are dealt with using the empirical equation derived in ref. 22

$$\ln c = \ln c_\infty - \frac{\Delta E}{bRT}$$

where c is the 'mean geometrical molar concentration', ΔE is the transition energy, R is the gas constant, T is the Kelvin temperature, b is an empirical constant, and c_∞ is a parameter whose physical meaning is the concentration when $\Delta E = 0$. The results are illustrated in Fig. 4. The well-seen linear correlation means that the covalent coordination between Cu(II) and HSA is not fulfilled in a single step, but that it undergoes the transition in the form of the 'associated ionic pair'.

It has been reported that the centers of the charge-transfer bands of copper(II)–L-α-amino acid complexes in weak basic solution appear in the region of 250–270 nm [23]. Moreover, the band occurring at 250 nm in $Cu(Gly)_2$ (Gly = glycinate anion) has been assigned to the $\sigma(O) \rightarrow \sigma^*(3d)$ transition [24], and in the Cu(II)–L-argine complex the $\sigma(O) \rightarrow \sigma^*(3d)$ transition occurs at 230 nm, but $\sigma(N) \rightarrow \sigma^*(3d)$ occurs at a lower energy, 255 nm [25]. Referring to these data, we assign the first absorption of the Cu(II)–HSA complex to transition (i) and hold that the electron of transition (ii) principally comes from $-NH_2$ [25, 26], but the electron of transition (iii) comes from the deprotonated peptide nitrogen [27]. Furthermore, transition (iii) does not occur in the copper(II)–L-α-amino acid complexes.

When the concentration of HSA is higher than 3.0×10^{-4} M, the conformation of HSA in solution is restricted. Owing to the steric hindrance, the axial carboxyl oxygen is no longer able to coordinate. As a result, the complex transforms into the square-planar structure (Fig. 1(a)); the local symmetry of chromophore CuN_4 transforms into D_{4h} and the allowed transition, based on the common molecular orbital scheme illustrated in Fig. 5, is only one, namely

$$e_u(N) \longrightarrow b_{1g}^*(3d) \qquad \text{(iii)}$$

Thus, we may assign the single band at about 294 nm to this transition; the electron mainly comes from the deprotonated peptide nitrogen [24, 27]. Actually, this transition is transition (iii) in the square-pyramid configuration.

Optical Electronegativity Studies

Optical electronegativity is primarily used to study charge-transfer spectra [28]. For LMCT,

$$E(\text{cm}^{-1}, \text{LMCT}) = 30000[\chi_{opt}(L) - \chi_{opt}(M)] + \Delta$$

where $\chi_{opt}(L)$ and $\chi_{opt}(M)$ are the optical electronegativities of the donor and acceptor orbitals on the ligand and metal, respectively. E(LMCT) and Δ are the transition energy and the ligand field

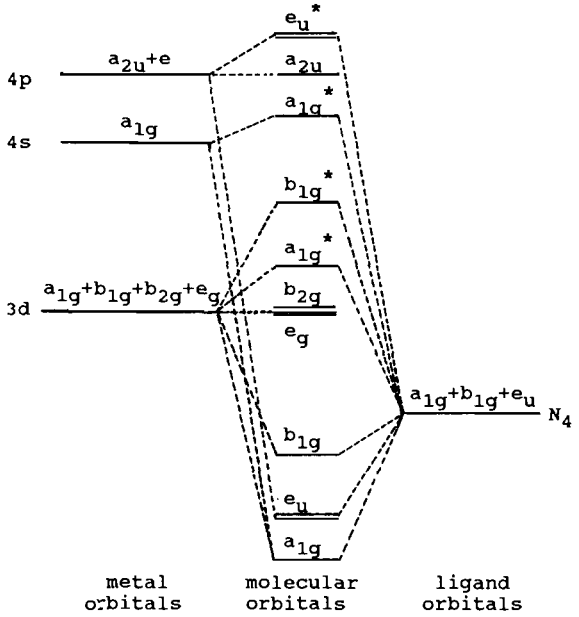

Fig. 5. A molecular orbital energy diagram for square-planar complexes assuming D_{4h} symmetry.

splitting energy of the d orbitals of the central atom, respectively.

When the concentration of HSA in the Cu(II)–HSA system is higher than 3.0×10^{-4} M, the Δ value of the complex, which takes the square planar structure, is equal to 19048 cm^{-1} on the ground of the d–d spectra [29]. Although the complex changes into a square-pyramid configuration at lower concentrations, the ligand field splitting energy of the Cu(II) d-orbitals in this structure still takes the above Δ value owing to the fact that the interaction of the axial carboxyl oxygen with Cu(II), predominantly being an electrostatic one, makes little impact on the splitting of the Cu(II) d-orbitals. On the other hand, though the optical electronegativity of Cu(II) has been reported [28], it is not suitable to use this value directly because metals in the organism or binding to biomacromolecules often have a special redox behavior. Considering the nitrogen in the R-NH_2 group, as a donor on the ligand, mainly forms the coordinate covalent bond and its optical electronegativity has been reported, the optical electronegativities of relevant atoms with the aid of the above assignment to those LMCT bands have been calculated (see Table 2). From the data listed in Table 2, the optical electronegativity of Cu(II) is seen to be 0.1–0.2 times larger than the value reported in ref. 28. This, besides being attributed to the characteristic of the bio-macromolecule, may be related to the fact that the data in ref. 28 comes from a tetrahedron as against a square-planar or square-pyramid in this case. Structural differences in complexes can cause the optical electronegativity of the central atom to vary [30]. The optical electronegativity of the carboxyl group evaluated here is lower than the value reported in ref. 31; the principal cause is probably that the interaction of the carboxyl group with Cu(II) is mainly electrostatic in this system, but of strong bonding in ref. 31. The optical electronegativity of the deprotonated peptide nitrogen is reported here for the first time.

Conclusions

According to the results described it seems that the concentration of HSA plays a crucial role in the configuration of Cu(II)–HSA complexes. In the range of the experimental concentrations, the structure of the Cu(II)–HSA complexes is either square-pyramidal (Fig. 1(b)) or square-planar (Fig. 1(a)). At concentrations greater than 2.5×10^{-4} M, the complex is in the form of structure (a). The reason why researchers who study the configuration of Cu(II)–HSA complexes only report the square-planar complex not the square-pyramid complex is that the HSA concentration used in their studies is higher than 2.5×10^{-4} M, so it is impossible to form the square-pyramid complex. When peptide models, however, are utilized to mimic the copper(II) binding site of HSA, the pentacoordinated complex has been reported because the small peptides do not bring about the steric hindrance to the coordination of the carboxyl group.

The normal range of serum albumin is about $(5.2–7.4) \times 10^{-4}$ M in human plasma [32], so we conclude that the square-planar complex predominates *in vivo*.

That the optical electronegativity of Cu(II), binding to HSA, is slightly larger than the documented

TABLE 2. Optical electronegativities

	Cu(II)	R-NH_2	R-COO^-	Deprotonated peptide N
E (cm^{-1}, LMCT)		39525	39920	34364
χ_{opt}	2.52		3.22	3.03
	2.3–2.4[a]	3.2[a]	3.57[b]	

[a]From ref. 28. [b]From ref. 31.

value suggests that the redox of Cu(II) has somehow changed. This is an interesting problem still to be solved.

The multi-peak structure of the first band revealed in the first order derivative spectra indicates there are many binding sites of Cu(II) in HSA. But that the band tends to become simple along with the increase of the HSA concentration suggests the secondary binding sites may be utilized or keep their spectroscopic activities only when the protein takes a favourable conformation. With regard to the primary binding sites, their number is unlikely to be large and they must be very similar.

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