STUDIES OF METALLOBLEOMYCINS BY ELECTRONIC SPECTROSCOPY, ELECTRON SPIN RESONANCE SPECTROSCOPY, AND POTENTIOMETRIC TITRATION

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The 1:1 bleomycin-A_2-Cu(II) complex shows an absorption maximum at 595 nm (ϵ 120), circular dichroism extrema at 555 nm ($\Delta\epsilon$ +1.21) and 665 nm (−0.61), and electron spin resonance (ESR) signal with $g_{\parallel}=2.211$, $g_{\perp}=2.055$, and $A_{\parallel}=178\times10^{-4}$ cm^{-1}. The formation constant (log K=12.630) and deprotonation constant ($pK_e=3.585$) of the 1:1 bleomycin-Cu(II) complex were determined by computer analysis of potentiometric data. The results of potentiometric titration also indicate that the stability of bleomycin-metal complexes is in the order Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II) and that these divalent metal complexes have a similar coordination environment. The bleomycin-Cu(II) complex has substantially a square-pyramidal configuration in which the secondary amine nitrogen, pyrimidine(N-1) ring nitrogen, deprotonated peptide nitrogen of histidine residue, and histidine imidazole(N-1) nitrogen coordinate to Cu(II) as planar ligand donors, and the α-amino nitrogen as axial donor. The specific Cu(II)-binding site of bleomycin has been compared with that of human serum albumin.

Bleomycin has been extensively used in the chemothrapy and radiodiagnosis of cancer. One of its most important chemical properties is the ability to coordinate a variety of metals. Bleomycin is isolated as the equimolar Cu(II) complex from the fermentation broth of *Streptomyces verticillus*[1,2]. The copper of naturally occurring bleomycin-Cu(II) complex originates in cupric salt added to the medium, and the production of bleomycin is markedly reduced in the absence of cupric ion[3]. Both copper-free and copper-coordinated bleomycins inhibit growth of microorganisms and mammalian cells. However, DNA strand scission *in vitro* is caused only by the metal-free bleomycin, not by the copper-chelated one[4]. The natural Cu(II)-coordinated bleomycin is easily reconstituted by treatment of the metal-free bleomycin with an excess amount of cupric ion in neutral solution followed by chromatographic separation on CM-Sephadex. Only the natural Cu(II) complex species is regenerated almost quantitatively, though bleomycin has many potential binding sites in the molecule[5]. The earlier investigation has revealed that the α-amino, carbamoyl, imidazole, and pyrimidine groups coordinate to Cu(II)[6,7,8]. Recently, a square-planar geometry has been suggested for the bleomycin-Cu(II) complex[9]. TAKITA *et al.* have proposed a square-pyramidal or tetragonal environment[5]. The recent X-ray crystallographic analysis of the 1:1 P-3A-Cu(II) complex indicated a distorted square-pyramidal structure which involves the secondary amine, pyrimidine, deprotonated peptide, histidine imidazole, and α-amino groups[10]. The four Cu-N distances of the basal plane range from 1.86 to 2.12 Å, and the fifth axial Cu-N distance is 2.28 Å. The ligand, P-3A, is an intermediate compound of bleomycin biosynthesis, lacking the sugar and bithiazole portions of bleomycin.

On the other hand, the bleomycin complexes containing a gamma-emitting metal radionuclide

such as ^{57}Co, ^{99m}Tc, and ^{111}In, have been investigated as a means of visualising tumors[11]. For their diagnostic use, the metal-binding to bleomycin is essential to carry the radioactive metals to the tumor tissue. In addition, it has been pointed out that bleomycin-metal complex, in particular iron complex, participates in the degradation of DNA by bleomycin[5]. Therefore, metal-coordination by bleomycin is closely related to the biochemical properties of bleomycin. In this paper, the divalent metal complexes of bleomycin-A_2 have been investigated by electronic and electron spin resonance (ESR) spectroscopy, and by potentiometric titration.

Experimental

Purified bleomycin-A_2 was kindly supplied by Nippon Kayaku Co. Ltd., and glycyl-L-histidine and glycylglycyl-L-histidine were purchased from Sigma. The standard Cu(II) and other divalent metal solutions were prepared from reagent grade material and standardized complexometrically with EDTA. Carbonate-free potassium hydroxide solution was prepared by the procedure described by ARMSTRONG[12], and was standardized by titration with potassium hydrogen phthalate. Deionized water was used throughout the experiments. All other reagents used were of commercial reagent grade.

Electronic and circular dichroism (CD) spectra were measured in aqueous solution (pH 6.3) at 20°C using a Shimadzu recording spectrophotometer (Model Double 40R) and a Jasco J-20 spectropolarimeter, respectively. The potentiometric titration was carried out with 0.1 N carbonate-free potassium hydroxide solution under a nitrogen atmosphere. To the titration vessel, metal nitrate (2.0 mM), bleomycin-A_2 (2.0 mM), potassium nitrate (0.1 M), and nitric acid (4.0 mM), were added, and the total volume was adjusted to 20 ml by addition of deionized water. Potentiometric pH measurements were made at 20±0.1°C on an Orion ion meter (model 801). The complexation reactions occurring among the species, M, H, and L can be represented by the following equilibrium reaction

$$pM + qH + rL \rightleftharpoons M_pH_qL_r$$

Formation constants ($\beta_{pqr}=[M_pH_qL_r]/[M]^p[H]^q[L]^r$) were refined by a non-linear, least-squares computer program[13] on a FACOM M-190 computer, Kyoto University. The values of $pK_w=14.063$ and $f_{\pm}=0.85$ were obtained from the titration data for 0.0020 M HNO_3 (I=0.1 (KNO_3) and t=20°C) and used in the calculations. X- and Q-Band ESR spectra were obtained with a JES-FE-3X spectrometer operating with 100 KHz magnetic field modulation. ESR operating frequencies were measured with a Takeda-Riken microwave frequency counter. The g values were determined taking Li-TCNQ (g=2.0026) as a standard, and the magnetic fields were calibrated by the splitting of Mn(II) in MgO ($\Delta H_{3-4}=86.9$ G). Magnetic susceptibility was measured in the continuous temperature range from 77 to 293K using a Shimadzu MB-2 magnetic torsion balance, equipped with a low temperature cryostat. Manganese Tutton salt was used for the calibration of the thermometers and field gradient.

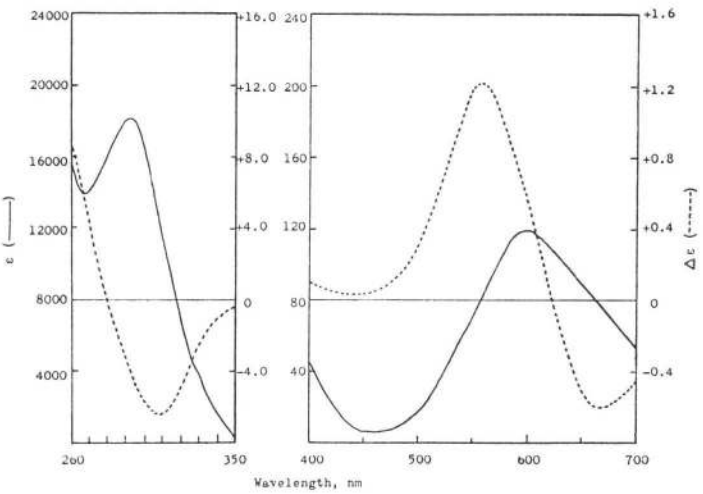
Results and Discussion

Electronic and Circular Dichroism Spectra

Fig. 1 shows the electronic and CD spectra at pH 6.3 for the 1:1 bleomycin-A_2-Cu(II) complex which has an absorption maximum at 595 nm (ϵ 120) and CD extrema at 555 nm ($\Delta\epsilon$ +1.21) and 665 nm (−0.61). The spectrum is typical of a nitrogen-coordinated Cu(II) complex with square-planar (or square-pyramidal and tetragonal) configuration, and the peak at 595 nm is assigned to *d-d* transition of Cu(II). Tetrahedral complexes of Cu(II) are expected to have more intense visible absorption bands, because they lack a center of symmetry and the *d-d* transitions are no longer strictly forbidden[14]. The λ_{max} value is close to that of the 1:1 glycyl-L-histidine-Cu(II) complex [donor set=$N_A N_- N_{Im}$ and $\lambda_{max}=602$ nm (ϵ 60)][15], and shifts to longer and shorter wavelength than those

of the 1:1 Cu(II) complexes of glycylglycyl-L-histidine[$N_A(N_P)_2$-N_{Im} and 525 nm (110)] and glycylglycine[N_AN_PO and 638 nm (90)][16], respectively. In the nitrogen-containing groups, the order of relative effectiveness in the magnitude of the ligand field around the central Cu(II), which is reflected in the λ_{max} values, has been found to be α-amino nitrogen > peptide nitrogen > imidazole nitrogen[15]. The ligand field of pyrimidine ring nitrogen toward Cu(II) is considerably weaker than that of imidazole nitrogen. Thus, shifting of the λ_{max} to lower wavenumber could be brought about by the replacement of a peptide nitrogen with a pyrimidine nitrogen. Furthermore, the 1:1 bleomycin-Cu(II) complex shows a distinct absorption at 292 nm (ϵ 18,000) which is attributed to $\pi \rightarrow \pi^*$ transition of bithiazole residues, because bleomycin-A_2 alone shows also an electronic transition at 287 nm (14,000)[17]. However, the Cu(II) complex has no absorption peaks in the range of 650 ~ 950 nm. The CD spectral sign (+ −) of the bleomycin-Cu(II) complex is similar to those of the glycyl-L-histidine-Cu(II) complex [565 nm ($\Delta\epsilon$ +0.12) and 670 nm (−0.08)][18] and glycylglycyl-L-histidine-Cu(II) complex [490 nm (+0.91) and 580 nm (−0.40)]. The positions of CD extrema are close to the former complex and the magnitudes of the COTTON effects to the latter complex. Signs and magnitude of the CD bands may reflect the chirality and rigidity of the complex structure surrounding the Cu(II) ion.

Fig. 1. Electronic (——) and circular dichroism (------) spectra of bleomycin-A_2-Cu(II) complex at pH 6.3.



The extremum at 253 nm in the difference UV spectrum between the metal-free bleomycin-A_2 and Cu(II)-coordinated bleomycin-A_2 appears to be due to the formation of bonding between the pyrimidine ring nitrogen and Cu(II) ,because the spectrum between the deprotonated and protonated pyrimidine chromophores of the model compound, 2-(1-acetamido-2-carbamoylethyl)-6-amino-5-methylpyrimidine-4-carboxamide, shows also the λ_{max} at 257 nm[5]. DABROWIAK *et al.* assigned the absorption near 250 nm to a $d\pi \rightarrow \pi^*$ metal-to-ligand charge transfer transition between the pyrimidine group and the bound Cu(II) ion[9]. The presence of pyrimidine ring nitrogen-Cu(II) bond is well-established in the Cu(II) complexes of nucleic acid constituents[19].

Potentiometric Titration

The acid dissociation constants of bleomycin-A_2 were obtained from the potentiometric titration data by the usual linear least-squares method. These three constants were then refined by a non-linear Fortran IV computer program. The final values, with the standard deviations, are $pKa_1 = 2.912 \pm 0.007$, $pKa_2 = 4.972 \pm 0.007$, and $pKa_3 = 7.716 \pm 0.005$ at I = 0.1 (KNO_3) and 20°C. The pKa values of bleomycin-A_2 are reasonably assigned to the proton loss of the secondary amine group (pKa_1), histidine imidazole group (pKa_2), and amino group of β-aminoalanine portion (pKa_3), respectively. As shown in Fig. 2, the titration curve of 1:1 bleomycin-A_2-Cu(II) system gave clearly

Fig. 2. Titration curves of bleomycin-A_2 with Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}.

The molar ratio of ligand to metal is 1 : 1. The concentration of bleomycin is 2.0 mM.

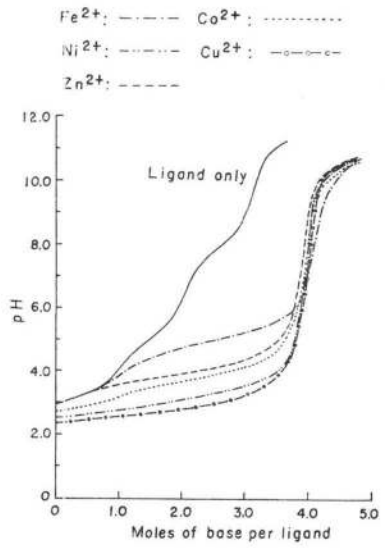


Table 1. Equilibrium constants of divalent metal complexes of bleomycin-A_2.

M^{2+}	Equilibrium constant (log K ± s.d.)	
	$M^{2+}+L^{+} \rightleftharpoons [ML]^{3+}$	$[ML]^{3+} \rightleftharpoons [M(L-H)]^{2+}+H^{+}$
Fe^{2+}	7.428±0.024	−5.478±0.021
Co^{2+}	9.739±0.018	−4.553±0.018
Ni^{2+}	11.324±0.013	−3.846±0.015
Cu^{2+}	12.630±0.011	−3.585±0.013
Zn^{2+}	9.077±0.016	−4.836±0.015

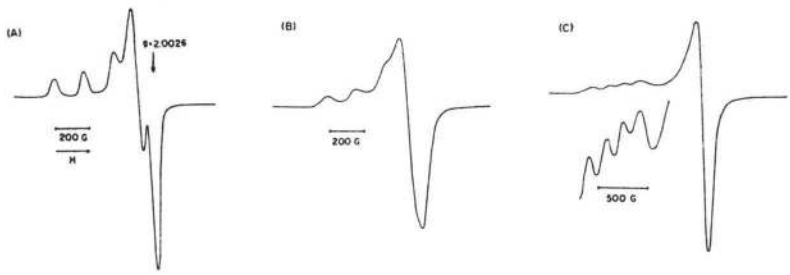
a pH inflection at $a=4.0$ (a=moles of base per ligand), as well as other divalent metal systems. The experimental data suggest strongly that one proton from the peptide linkage dissociates in the course of complex formation and bleomycin behaves as at least a tetradentate ligand. The computer-based analysis of the titration data indicated that the mononuclear species, $[CuL]^{3+}$ and $[Cu(L-H)]^{2+}$, are present predominantly, and the presence of other minor species is negligibly small in this condition. The formation constants of the 1 : 1 bleomycin-A_2-Cu(II) complex determined by the IRVING-ROSSOTTI method[20] were further refined by a non-linear computer program. Table 1 shows the final values of the bleomycin-A_2-Cu(II) complex, together with those of other several metal complexes of bleomycin-A_2. The order of stability, Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II), corresponds well to a so-called "natural order" or "IRVING-WILLIAMS order" for divalent metal complexes. From these constants, a distribution diagram of the complex species was calculated for various pH. Above pH 4, the deprotonated complex species $[Cu(L-H)]^{2+}$ is the predominant species. We obtained pK_c value for the loss of proton from the complex species $[CuL]^{3+}$, and the value ($pK_c=3.585$) is typical of deprotonation from peptide backbone group. In general, the pK_c value (*ca.* 8~9) of a terminal amide group is higher than that (*ca.* 4~5) of an intermediate peptide group[21], and Cu(II)-ion-promoted ionization of peptide hydrogen is well-known in peptide-Cu(II) complexes[22,23]. In the case of the bleomycin-A_2-Cu(II) complex, the secondary amine nitrogen, pyrimidine ring nitrogen, and histidine imidazole nitrogen can form three chelate rings with 5–5–6 ring members if the intervening deprotonated peptide nitrogen of histidine residue is coordinated (see Fig. 4). The planar-configuration is similar to that of the 1 : 1 glycylglycyl-L-histidine-Cu(II) complex, which is coordinated to square-planar with 5–5–6 chelate ring members by amino nitrogen, two peptide nitrogen, and imidazole nitrogen donors, and has been proposed as a reasonable model for the binding of Cu(II) by human serum albumin[24]. The formation constant (log K) of the bleomycin-A_2-Cu(II) complex is considerably larger than that (log $K=9.220$[25] and log $K=7.04$[26]) of the corresponding glycylglycyl-L-histidine-Cu(II) complex. In addition, the deprotonation constant ($pK_c=3.585$) from the peptide group is smaller than those of the 1 : 1 Cu(II) complexes of glycyl-L-histidine ($pK_c=4.00$) and glycylglycyl-L-histidine ($pK_c=4.75$)[27]. The present potentiometric results strongly suggest that bleomycin-A_2 and

Cu(II) form a very stable 1:1 complex, and that Cu(II) is planar coordinated to the secondary amine nitrogen, pyrimidine nitrogen, deprotonated peptide nitrogen, and histidine imidazole nitrogen atoms, and axially to the α-amino nitrogen atom.

Magnetic Susceptibility and ESR Spectra

The magnetic susceptibility of the bleomycin-A_2-Cu(II) complex showed an excellent accordance with the CURIE-WEISS law, with a CURIE constant of 0.38° emu/mol and a WEISS constant of 1.0°K. No important deviation from the straight line was detected on the $1/x-T$ plot throughout the present temperature range (77–293 K). The effective magnetic moment of the Cu(II) complex was 1.75 B.M. The result confirms that the Cu(II) forms a 1:1 complex with the bleomycin ligand and the Cu(II) paramagnetic site is in pure doublet state ($s=1/2$). Fig. 3 shows ESR spectra of the bleomycin-Cu(II) complex at 77 and 293 K. The ESR hyperfine structures remain unchanged between pH 4 and 11.

Fig. 3. X-Band (A, B) and Q-band (C) electron spin resonance spectra of bleomycin-A_2-Cu(II) complex at 77 K (A) and 293 K (B, C).



The splittings due to g-anisotropy, which overlap with the $A_{||}$ hyperfine splitting of Cu(II) in the X-band ESR (see Fig. 3A), can be clearly resolved in the Q-band ESR (see Fig. 3C). All ESR spectra exhibit a typical line shape for nonoriented systems ($S=1/2$, $g_{||}>g_{\perp}$) with near axial symmetry. Furthermore, no ESR signals were detected at half field, $g=4$, resulting from the spin forbidden $\Delta_m=2$-transition in a triplet Cu(II) dimer. Hence, the bleomycin-A_2-Cu(II) complex is considered to be mononuclear in nature, and a dimer structure can be ruled out. The spin Hamiltonian parameters determined were $g_{||}=2.211$, $g_{\perp}=2.055$, and $A_{||}=178\times10^{-4}$ cm^{-1}. Molecular orbital calculations of the Cu(II)-bonding parameters were accomplished by the method of KIVERSON and NEIMAN[28], who wrote the molecular orbital of Cu(II) complexes under the D_{4h} symmetry. The ESR parameters are calculated to be

$$g_{||}=2.0023-(8\lambda/\Delta E_{xy})\alpha^2\beta_1^2-f(\beta_1)$$
$$g_{\perp}=2.0023-(2\lambda/\Delta E_{xz})\alpha^2\beta^2-f(\beta)$$
$$A_{||}=p[-k\alpha^2-(4/7)\alpha^2-2\alpha^2(4\beta_1^2/\Delta E_{xy}+(3/7)\beta^2/\Delta E_{xz})]$$
$$A_{\perp}=p[-k\alpha^2+(2/7)\alpha^2-11\alpha^2\beta^2/7\Delta E_{xz}]$$

where

$$f(\beta_1)=\alpha\alpha'\beta_1^2+\alpha\alpha'\beta_1(1-\beta_1^2)^{1/2}T(n)/2$$
$$f(\beta)=\alpha\alpha'\beta^2s+\alpha\alpha'\beta(1-\beta^2)^{1/2}T(n)/2$$

with the spin-orbit coupling constant for the Cu(II) ion $\lambda=-828$ cm^{-1}, the free-ion dipole term $p=0.036$ cm^{-1}, and the ligand integral $T(n)=0.333$ for ordinary ligand nitrogen atoms. The overlap integral s for the ligand-to-metal distance ($r=1.9$ Å) has been given the value 0.093 for the Cu-N bond. KIVELSON's approximate formula is also used for estimate of α, based on $A_{||}$, $g_{||}$, and $g_{\perp}$:

$$\alpha^2=-(A_{||}/p)+(g_{||}-2.0023)+3/7(g_{\perp}-2.0023)+0.04$$

Table 2. ESR and bonding parameters for Cu(II) complexes of bleomycin-A_2 and related histidine-containing peptides.

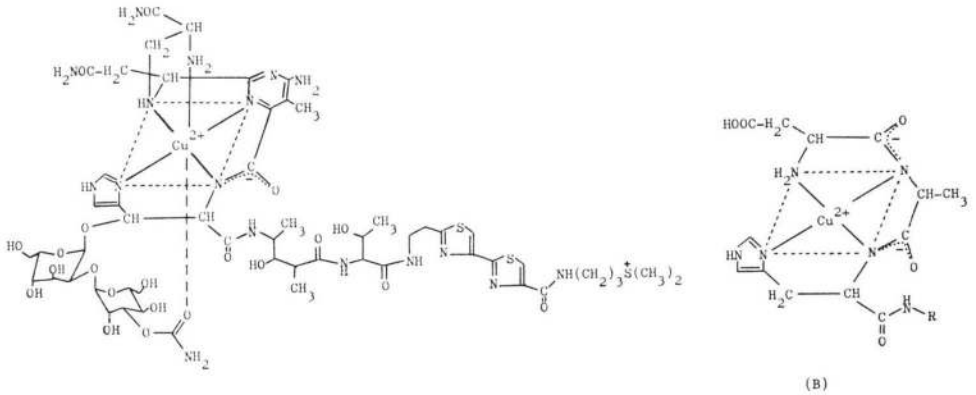
Ligand	$g_{\parallel}$	$g_{\perp}$	$A_{\parallel}$ cm^{-1}, 10^{-4}	α^2	α'^2	β_1^2	β^2
Bleomycin-A_2	2.211	2.055	178	0.77	0.32	0.71	0.85
N-Mercaptoacetyl-DL-histidyl-DL-histidine[16]	2.183	2.060	180	0.75	0.34	0.65	0.96
N-Acetylglycylglycyl-L-histidine[29]	2.179	2.045	204	0.81	0.27	0.64	0.76
N-Mercaptoacetylglycyl-L-histidine[16]	2.206	2.079	195	0.82	0.27	0.67	1.19
Glycylglycyl-L-histidine[16]	2.172	2.065	206	0.81	0.28	0.57	0.94

The orbital excitation energies are estimated to be $\Delta E_{xy} = 15038\ cm^{-1}$ and $\Delta E_{xz} = 18018\ cm^{-1}$ from CD extrema previously described. For the proposed planar Cu-N_4 structure of the bleomycin paramagnetic site (see Fig. 4A), the bonding parameters of the bleomycin-A_2-Cu(II) complex are thus calculated and the results are compared with those for a histidine-containing peptide in Table 2. Under the square-planar geometry, values of bonding parameters of numerous peptide complex coincide within well-defined ranges; in-plane σ-bonding: $0.81 \leqslant \alpha^2 \leqslant 0.82$, $0.27 \leqslant \alpha'^2 \leqslant 0.29$, in-plane π-bonding: $0.64 \leqslant \beta_1^2 \leqslant 0.74$, out-of-plane, $0.65 \leqslant \beta^2 \leqslant 0.76$[25]. In fact, the α^2, α'^2, and β_1^2 values of N-acetylglycylglycyl-L-histidine, N-mercaptoacetylglycyl-L-histidine, and glycylglycyl-L-histidine indeed satisfy each range limit. Large values of β^2 mean that out-of-plane π-bondings are rather weak in these complexes. Of importance is a remarkable decrease in α^2-value seen for the bleomycin complex. The similar decreases in α^2-values have been reported for Cu(II)-porphirin complexes ($0.74 \leqslant \alpha^2 \leqslant 0.79$), but the structure of the bleomycin-Cu(II) complex should not be referred to the rigid square of Cu(II)-porphirins, because both in-plane and out-of-plane π-bonding characters are too different ($\beta_1^2 \simeq 1$, $\beta^2 \simeq 1$) from each other[30]. Recently, ESR investigations have been accomplished for a square-pyramidal Cu(II)-peptide complex, N-mercaptoacetyl-DL-histidyl-DL-histidine, and the fact that σ-covalency of the Cu(II) site is effectively enhanced by the apical coordination of the extra imidazole residue, has been demonstrated[16]. A striking similarity of the ESR parameters, as well as the bonding parameters can be seen between the Cu(II) complexes of N-mercaptoacetyl-DL-histidyl-DL-histidine and bleomycin. This suggests that a square-pyramidal structure may be proposed for the chromophore of the bleomycin-Cu(II) complex. In the case of bleomycin-Cu(II) complex, however, the absence of nitrogen superhyperfine splittings is likely to reflect a distortion from the square-planar configuration, since a nitrogen-coordinated Cu(II) site with high square-planar geometry shows well-defined nitrogen splittings. In addition, a lower field shift of the $g_{\parallel}$-value noted in the bleomycin-Cu(II) complex can be interpreted in terms of some tetragonal distortion occurring in the square-planar arrangement[31]. It was previously proposed that an apical coordination of the carbamoyl group prevents the isomerization of bleomycin (see the following section). The ESR spectra of the isobleomycin-Cu(II) complex ($g_{\parallel} = 2.209$, $g_{\perp} = 2.057$, $A_{\parallel} = 179 \times 10^{-4}\ cm^{-1}$) were recorded under the same conditions, but no important changes were detected in the hyperfine structure.

Specific Cu(II)-Binding Site of Bleomycin-A_2

On the basis of the present spectroscopic and potentiometric investigations, it is proposed that the natural bleomycin-Cu(II) complex has substantially a square-pyramidal configuration. This corresponds well to the previous proposal for the bleomycin-Cu(II) complex[5] and the recent X-ray

Fig. 4. Probable structure for Cu(II) complexes of bleomycin-A_2(A) and human serum albumin(B).



(A) (B)

crystallographic result of the P-3A-Cu(II) complex[10]. As shown in Fig. 4, the secondary amine nitrogen, pyrimidine(N-1) ring nitrogen, deprotonated peptide nitrogen of histidine residue, and histidine imidazole(N-1) nitrogen coordinate to Cu(II) as square-planar ligand donors, and the α-amino nitrogen as axial donor. The weak coordination from the carbamoyl group, probably carbonyl oxygen, is presumed on the basis of the chemical reaction[32,33]. Substantially, a tetragonal or square-pyramidal Cu(II) complex has electronic and magnetic properties similar to the corresponding square-planar Cu(II) complxes[16].

Of special interest is the fact that the planar Cu(II)-binding sites between bleomycin and human serum albumin are similar, and the bleomycin-Cu(II) complex is more stable than the albumin-Cu(II) complex. The bleomycin-Cu(II) complex has four chelate rings with 5–5–5–6 ring members, and the albumin-Cu(II) complex possesses three chelate rings with 5–5–6 ring members. It is well-known that human serum albumin has a specific Cu(II) transport site with histidine as the third amino acid residue, Asp-Ala-His, and its Cu(II) complex is a stable complex with donor set of $N_A(N_P)_2N_{Im}$ (see Fig. 4)[25,26,34]. The presence of histidine as the third amino acid residue in tripeptide complexes of Cu(II) drastically decreases their susceptibility to nucleophilic attack[35]. In addition, the strong ability of bleomycin to chelate of Cu(II) ion may result in a marked reduction of the tissue stores of copper in WILSON's disease, which is a copper-dependent disease of a peculiar metabolic defect characterized by excessive amounts of copper in the brain, liver, cornea, and urine.

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