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Copper(II) Complex of Sulfur-Containing Peptides. Characterization and Similarity of Electron Spin Resonance Spectrum to the Chromophore in Blue Copper Proteins

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Received March 10, 1975

Abstract: A green-colored α-mercaptopropionylglycine-Cu(II) complex which involves thiol, neighboring deprotonated peptide nitrogen, and terminal carboxylate groups as the coordination sites has been obtained. The liberation of peptide proton occurs at the neutral pH region and the infrared spectrum of the complex isolated directly shows the dissociation of a proton from peptide linkage by a disappearance of ν (NH) at 3300 cm^{-1}. The magnetic circular dichroism curve of the complex consists of two negative bands at 450 and 600 nm and the maximum $[\theta]$ was -0.86×10^{-3} (deg cm^2)/dmol at 600 nm under a field of 11.7 kG. The formation of the adduct complex with some heterocyclic bases such as pyridine and imidazole caused a considerable shift of the visible band to a higher frequency. Of interest is the fact that the spin Hamiltonian parameters ($g_\parallel$ = 2.259, $g_\perp$ = 2.040, and $A_\parallel$ = 82 G) and the bonding parameters (α^2 = 0.52 and $^4/_7\alpha'^2 + \kappa$ = 0.51) of the complex are very similar to those of the chromophore in blue copper proteins. The high covalency of the Cu-S bonding is indicative of a decrease in the unpaired electron density on the Cu(II) atom, namely, a small hyperfine coupling constant.

On the basis of optical and ESR properties, copper proteins are grouped into two categories: blue copper proteins and nonblue proteins. The so-called blue copper proteins have an unusually high extinction coefficient in the visible spectrum and an anomalously small copper hyperfine coupling constant ($A_\parallel$ < 100 G) in comparison with that of nonblue proteins ($A_\parallel$ > 140 G).[2a] The unique spectral properties of blue copper proteins have attracted keen interest and various model systems have been investigated.[2b] Most of the model Cu(II) complexes involve coordination to nitrogen ligands where the coordination geometry is distorted tetrahedral. Studies on the copper complexes of thiol ligands have been relatively few, despite the importance of the copper-sulfur interaction in copper enzymes as suggested by Hemmerich[3a] and Beinert.[3b] It is not even known whether the cysteinyl residue involved in a peptide chain can interact with Cu(II) ion through both the thiol and neighboring peptide amide groups.

Evidence for the presence of a thiol group as a ligand for Cu(II) in blue copper proteins has been presented by Graziani et al.[4] From the reactivity with *p*-chloromercuribenzoate and the results of the physicochemical investigations, these authors reported that the sulfur atom could be an invariant ligand for copper in plastocyanin, stellacyanin, and azurin which contain a thiol group of the cysteine residue. The resonance Raman spectra of blue copper proteins between 1700 and 200 cm^{-1} revealed the presence of the Cu-S coordinations (CuN_3S, CuN_4S, CuN_2OS, or CuN_3OS).[5] The proposal that the presence of the sulfur atom provides a logical mechanism for the intensification of the blue copper ligand field bands has been given particular stress. Furthermore, Giordano et al.[6] have implied on the basis of ESR studies that the nonlabile endogeneous axial ligand to the copper in galactose oxidase has π-bonding character and a thiol group may be the ligand involved. The Cu(III) complex of 1,1-dicarboethoxy-2,2-ethylenedithiolate has also been synthesized by Coucouvanis and his colleagues[7] as a model of copper-containing enzymes. Thus, the role of thiol group in copper binding of the proteins is being given much attention.

We have already reported that the red-violet-colored penicillamine-copper complex is isolated from an aqueous solution and has a high extinction coefficient of about 10^3 per copper at 520 nm.[8] The sulfur-containing amino acids and peptides, such as penicillamine and mercaptopropionylglycine (MPG), are useful not only as an oral treatment of Wilson's disease caused by an abnormal metabolism of copper but also as a ligand in the study of the interaction of the Cu(II) ion with the thiol group. This paper deals with ESR similarities of the chromophore between the α-MPG-Cu(II) complex and blue copper proteins. The complex is hereafter abbreviated as green complex.

Experimental Section

α-MPG and β-MPG were a gift from Santen Seiyaku Co. and were used after recrystallization from ethyl acetate. Glutathione and glycylcysteine were obtained from Sigma and Tokyo Kasei Co., respectively. The solution of cupric chloride was prepared from reagent grade material and was standardized complexometrically with EDTA. Carbonate-free potassium hydroxide solution was prepared by the procedure described by Armstrong[9] and was standardized by the titration with potassium hydrogen phthalate. Deionized water was used throughout the experiments. All other

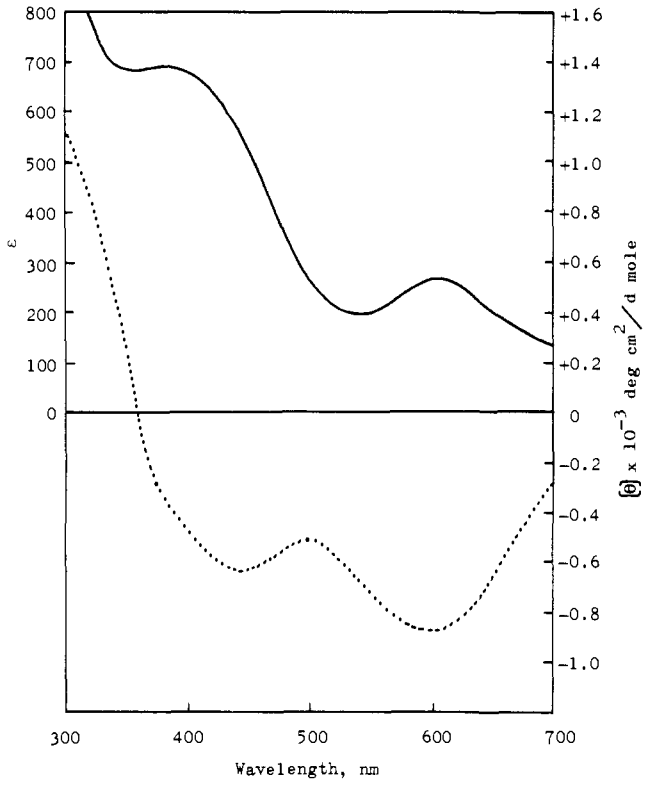

Figure 1. Optical properties of the α-MPG–Cu(II) complex (green). Solid and dotted lines represent absorption and magnetic circular dichroism spectra, respectively.

reagents used were of commercial reagent grade.

Green complex was prepared as described below. An aqueous solution (1 ml) of α-MPG (163 mg) containing NaOH (90 mg) was added to $CuCl_2 \cdot 2H_2O$ (170 mg) dissolved in 2 ml of ethanol. Green-colored precipitates formed in the reaction solution. After standing for 1 hr at 5°, the solid was filtered and washed with 100 ml of ethanol: yield 40%. Anal. Calcd for $Cu(C_5H_8O_4NS)Na \cdot 2H_2O$: C, 19.86; H, 3.89; N, 4.65; Cu, 21.23. Found: C, 19.25; H, 3.57; N, 4.44; Cu, 22.02.

Optical spectra of the complexes were determined in an aqueous solution at 20° using a Shimadzu recording spectrophotometer, Model Double-40R. Circular dichroism (CD) and magnetic circular dichroism (MCD) spectra were recorded on a Jasco J-20 spectropolarimeter. The sample temperature was 20°. MCD is expressed in terms of molecular ellipticity, $[\theta] = 2.303\ (4500/\pi)(\epsilon_L - \epsilon_R)$, with units of (deg cm²)/dmol. MCD measurements using a 11.7-kG magnet were performed with the field direction parallel and then antiparallel to the direction of light propagation. X-Band electron spin resonance (ESR) spectra were obtained on frozen glasses at 77 K with a Joco ME-3X spectrometer equipped with a gauss meter and frequency counter to obtain accurate measurements of the magnetic field and microwave frequency. No attempts were made to apply second-order corrections. Infrared (ir) spectra of α-MPG and its Cu(II) complex were measured in a KBr disk using a Jasco A-2 recording spectrophotometer. The pH titration was carried out with 0.1 *N* carbonate-free potassium hydroxide solution under nitrogen atmosphere. On the titration vessel, 5 ml of 1 *M* potassium nitrate, 5 ml of 37.5 m*M* cupric chloride, and 5 ml of 37.5 m*M* α-MPG were added, and the total volume was adjusted to 50 ml by adding deionized water. Potentiometric measurements were made at 22 ± 0.1°.

Results

Optical and MCD Spectra. When α-MPG was present in excess to Cu(II) ion, a pale yellow complex is produced and its optical spectrum shows no absorption maximum in the visible region. Generally, if Cu(II) ion is added to a solution of thiol ligand, RSH is oxidized to yield disulfide (RSSR) and Cu(I) ion, and then one more molecule of RSH is necessary to stabilize Cu(I) formed in the form of the RSCu(I) complex as shown in eq 1.[10] On the other hand, when Cu(II) ion is present in equimoles or in a little excess to α-MPG, the solution turns violet at the acid pH region (pH 2.0–4.0) and intensely green at neutral and alkaline pH regions (pH 6.0–10.0). This coloration is attributed to the reaction shown in eq 2. The violet α-MPG–Cu(II) complex (abbreviated as the violet complex hereafter), which has an absorption maximum at near 550 nm, was unstable. The green α-MPG–Cu(II) complex was, however, very stable and broad peaks were observed at 400 and 605 nm as shown in Figure 1.

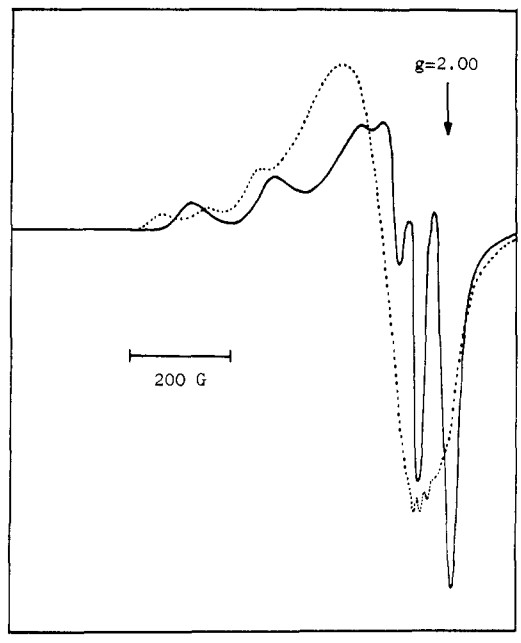

Figure 2. Electron spin resonance spectra of the α-MPG–Cu(II) complex. Solid and dotted lines represent violet and green complexes, respectively.

$$2RS^- + Cu(II) \rightleftharpoons RSCu(I) + 0.5RSSR \quad (1)$$

$$RSCu(I) + 0.5RSSR + Cu(II) \rightleftharpoons 2RSCu(II)$$

or

$$RS^- + Cu(II) \rightleftharpoons RSCu(II) \quad (2)$$

Since CD and MCD are additive, the true MCD has to be determined by subtracting the natural CD contribution. The CD measurement showed, however, that the CD induced from the green complex was negligibly small. The resulting MCD curve shown in Figure 1 consists of two negative bands at 450 and 600 nm. The MCD associated with the major Cu(II) absorption band near 600 nm is largely negative. The maximum $[\theta]$ for the MCD is -0.86×10^{-3} (deg cm²)/dmol at 600 nm under a field of 11,700 G.

Spectral studies on the reaction of Cu(II) ion with similar sulfur-containing peptides, namely β-MPG, glycylcysteine, and glutathione, showed similar features to those of the α-MPG–Cu(II) complex in which the features could be clearly seen.

ESR Spectra. The ESR spectra of the violet and green complexes observed in frozen solution at 77 K are shown in Figure 2. The sample concentration was 0.1 *M* in an aqueous solution (pH 2.7 and 6.3). Both spectra are typical of Cu(II) systems in local environments of C_{2v} and D_{4h} symmetry as normally found for pseudo-square-planar environments except that in the case of the violet complex, anisotropy is detectable in the perpendicular region. In addition,

Table I. EPR Parameters for the α-MPG–Copper Complex and Copper Proteins

	$g_{\parallel}$	$g_{\perp}$	$A_{\parallel}$, G	Abs max, cm^{-1}	α^2	$^4/_7\alpha'^2 + \kappa$
Model						
α-MPG–Cu(II) complex (violet)	2.185	2.037	168	18,000	0.66	0.68
α-MPG–Cu(II) complex (green)	2.259	2.040	82	16,400	0.52	0.51
α-MPG–Cu(I) complex (yellow)	No EPR signals					
Type I[a]						
Stellacyanin	2.30	2.04	40	16,550	0.45	0.43
Plastocyanin	2.226	2.053	63	16,750	0.40	0.43
Ceruloplasmin	2.209	2.056	80	16,500	0.47	0.46
Laccase	2.197	2.046	90	16,400	0.48	0.47
Type II[a]						
Erythrocuprein	2.265	2.063	160		0.69	0.75
Denatured ceruloplasmin	2.257	2.056	180		0.77	0.80
Cu(II)–transferrin	2.321	2.053	145		0.74	0.76
Cu(II)–insulin	2.290	2.06	175		0.79	0.82
Tyrosinase	No EPR signals					

[a] See ref 22.

Table II. Selected Bands in the Ir Spectrum of the α-MPG–Cu(II) Complex (green) in Comparison with Those in the α-MPG and α-MPG–Ni(II) Complex

	Observed bands,[a] cm^{-1}				
	ν (H_2O)	ν (NH)	ν (SH)	ν (COOH)	ν (C=O) + ν (CN)
α-MPG		3300 (s)	2530 (m)	1745 (s)	1620 (s), 1545 (s)
α-MPG–Cu(II)	3380 (m, br)				1600 (sh), 1570 (s)
α-MPG–Ni(II)[b]	3380 (m, br)				1585 (s), 1550 (sh)

[a] Intensity designations: s, strong; m, medium; br, broad; sh, shoulder. [b] See ref 12.

no signals were observed near $g = 4$ based on $\Delta M = 2$ or spin-forbidden transitions,[11] and, hence, the α-MPG–Cu(II) complex was believed to be mononuclear in nature, with a dimer structure ruled out. In the green complex, the fine structure observed in the region close to $g = 2$, i.e., around 3400 G, may be due to a superhyperfine interaction with neighboring nuclei, namely one nitrogen atom. The spin Hamiltonian parameters for the copper complexes of α-MPG are given in Table I, together with those of various copper-containing proteins.

Potentiometric Titration. The titration curve of α-MPG consists of two pH buffer zones from which the values of pK_1 (COOH, 3.60) and pK_2 (SH, 8.74) were calculated. The titration curve of 1:1 α-MPG–Cu(II) ion is shown in Figure 3. The curve gave two inflections at $a = 2$ and $a = 3$. This stepwise behavior contrasts with that reported in the case of the 1:1 α-MPG–Ni(II) system, in which the pH inflection occurs in one step at $a = 3$.[12] The following color change was remarkably evident during the titration. The initial pale violet solution shows a greenish tint at $a = 2$.[13] The green color deepened as more base was added and finally the solution became bright green. This color change strongly suggests that a proton from the peptide linkage dissociates in the course of the complex formation and α-MPG behaves as a terdentate ligand in the 1:1 Cu(II) complex. The pertinent equilibrium equations are

$$Cu^{2+} + LH_2 \rightleftharpoons CuL + 2H^+ \quad (3)$$

$$CuL \rightleftharpoons Cu(H_{-1}L)^- + H^+ \quad (4)$$

where LH_2 = α-MPG.

Ir Spectra. Table II shows significant ir absorptions of the green complex, in comparison with those of α-MPG and its Ni(II) complex. The 1:1 α-MPG–Ni(II) complex has a square-planar configuration on which thiol, deprotonated peptide nitrogen, and carboxylate groups take part in the coordination.[12] The disappearances of ν (NH) and ν (SH) at 3300 and 2530 cm^{-1} in the free ligand reveal that the ligand coordinates through the sulfur and peptide nitrogen

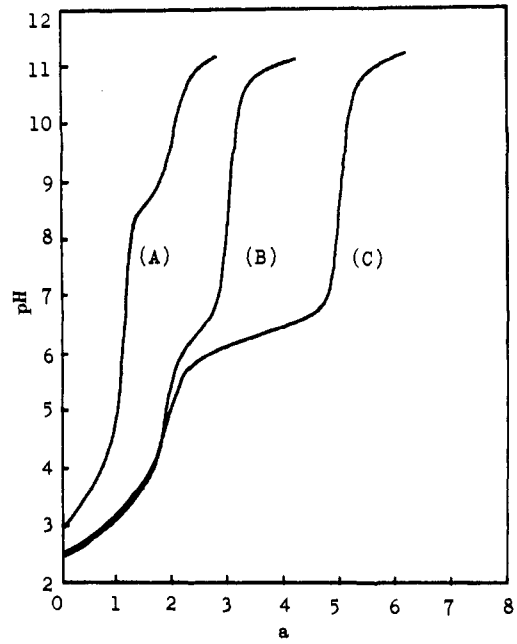

Figure 3. Titration curves of α-MPG with the Cu(II) ion. The molar ratio of ligand to metal is (A) 1:0, (B) 1:1, and (C) 1:2. The concentration of α-MPG is 3.75 mM and "a" represents the moles of base per ligand.

atoms in the green complex. In addition, the shift of carboxylate-stretching frequencies suggests coordination of the COO^- group. A broad absorption centered at 3380 cm^{-1} is due to the presence of water. The results indicate that the green complex has a similar coordination structure to the 1:1 α-MPG–Ni(II) complex. Thus, the structure proposed for the green complex is shown in Figure 4.

Discussion

ESR Characteristics. The values for $g_{\parallel}$, $g_{\perp}$, and $A_{\parallel}$ of the α-MPG–Cu(II) complex are typical for the Cu(II)

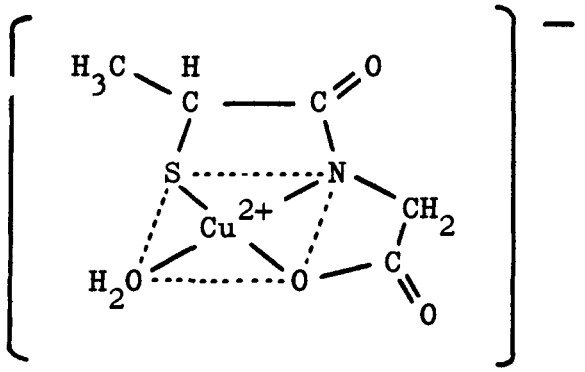

Figure 4. Probable structure of the α-MPG–Cu(II) complex (green).

complex. As to the hyperfine coupling constant in the parallel region, the green complex has a close value to that of the proteins classified as type I, where $A_\parallel < 100 \times 10^{-4}$ cm^{-1}, whereas the $A_\parallel$ of the violet complex is similar to the value of the proteins classified as type II, where $A_\parallel > 140 \times 10^{-4}$ cm^{-1}. From this result, a speculation that the difference in $A_\parallel$ values between two α-MPG–Cu(II) complexes is mainly due to difference in coordinating groups rather than only to difference in the degree of the distortion from square-planar geometry can be persuasively provided.

At 77 K the spectra of the α-MPG–Cu(II) complex showed characteristics similar to those of a mononuclear Cu(II) complex, lacking in $\Delta M = 2$ signals near $g = 4$, which are all attributed to the formation of a dimeric structure by dipolar interaction.[11] On the other hand, the glycylglycine–Cu(II) complex has the ESR parameters for $\Delta M = 2$, $r = 5.0$ Å, $g_\parallel = 2.26$, $g_\perp = 2.06$ and $A_\parallel = 160 \times 10^{-4}$ cm^{-1}, indicating the formation of a dimeric species which involves bridging by the terminal carboxyl group with the amino and peptide nitrogen groups in a planar arrangement around the Cu(II).[14]

The line shape of the green complex is taken to the Gaussian with a half peak-to-peak width, $\Delta H = 160$ G, and a superhyperfine structure in the perpendicular region may be due to ^{14}N ($I = 1$) hyperfine splittings since three lines for one nitrogen atom are expected. The presence of two kinds of isotopes of copper (^{63}Cu and ^{65}Cu) causes the increase of the line width of the spectrum, and therefore an experiment wherein the sample contains only one kind of copper isotope is required for an accurate determination of the interaction with the nitrogen atom.

For many tetragonal Cu(II) complexes where an unpaired electron is in the $d_{x^2-y^2}$ orbital, the following relations are given approximately.[15]

$$g_\parallel = 2(1 - 4\lambda/\Delta_1) \qquad g_\perp = 2(1 - \lambda/\Delta_2)$$

Here, λ is the spin-orbital coupling constant, and Δ_1 and Δ_2 are the ligand field splitting of $d_{xy} - d_{x^2-y^2}$ and $d_{xz,yz} - d_{x^2-y^2}$, respectively. If Δ_2 is taken as 16,400 cm^{-1} from the visible spectrum of the green complex, $\lambda = 330$ cm^{-1} can be calculated from the experimental g values.[16] The obtained λ value corresponds to about 40% of that of free Cu(II) ion (828 cm^{-1})[17] and reveals a high covalent character in the bonding.

Furthermore, we have calculated the bonding parameters α^2 and $(^4/_7\alpha'^2 + \kappa)$ by the following approximate expressions.[18]

$$\alpha^2 = A_\parallel/P + (g_\parallel - 2.0023) + {}^3/_7(g_\perp - 2.0023) + 0.04 \qquad (P = 0.04 \text{ cm}^{-1})$$

$$A_\parallel = P\,[-{}^4/_7\alpha'^2 - \kappa + (g_\parallel - 2) + {}^3/_7(g_\perp - 2)] \qquad (P = 0.035 \text{ cm}^{-1})$$

For a complex with almost cubic symmetry, α^2 has been correlated with the covalency of the σ bonds, while, for a square-planar complex, covalency in both σ and π bonds in the plane of the ligands reduces the value of α^2 to the same extent. The value of α'^2 has been correlated with the in-plane σ bonding and the term κ comes from the isotropic Fermi electron-nuclear interaction. The fairly good agreement for these parameters between the green complex and blue copper proteins is considered significant (see Table I). Such low α^2 and $^4/_7\alpha'^2 + \kappa$ values reflect a high degree of delocalization of the unpaired hole on Cu(II). Blumberg[19] has proposed that the blue chromophore originates from the Cu(II) ion in a site of distorted rhombic symmetry which deviates from a square-planar toward a tetrahedral geometry. A five-coordinate[20] or flattened tetrahedral geometry[21] has also been proposed. We are of the opinion that the characteristics of the blue copper chromophore can be attributed to the large degree of covalency of the bonding in the complex as well as to the unusual coordination geometry. The high covalency of the Cu–S bonding suggests a decrease in the unpaired electron density on the Cu(II) atom. This is explainable on the basis of a mixing of the s and p orbitals into the 3d orbitals of Cu(II).

Other Spectral Properties. The optical spectrum of the green complex has a weak maximum on a charge-transfer band near 400 nm and one asymmetric peak at 605 nm which is tentatively assigned to d–d transition of Cu(II). The extinction coefficient per mole of copper (ε) is about 300–605 nm. The absorption intensity is smaller than those of the penicillamine–copper complex ($\epsilon = 1000$)[8] and the copper chromophore of typical blue copper proteins ($\epsilon = 1000$–5000).[22] This absorption band is not particularly unusual, therefore, although it is approximately five times as intense as the corresponding bands of $Cu(NH_3)_4^{2+}$ and glycylglycine–Cu(II) complexes, which are 50 and 80 M^{-1} cm^{-1} at 600 and 640 nm, respectively.[23] 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, is found to be α-amino nitrogen > peptide nitrogen > imidazole nitrogen.[24] The lowering λ_{max} value (about 35 nm) of the green complex compared with that of the glycylglycine–Cu(II) complex would reflect the relative effectiveness of thiol and amino groups on the Cu(II) d–d band.

At a room temperature of 20°, the MCD spectrum of the green complex did not resolve the three d–d transitions ($d_{z^2} - d_{x^2-y^2}$, $d_{xy} - d_{x^2-y^2}$ and $d_{xz,yz} - d_{x^2-y^2}$) expected in the visible region any better than in the absorption spectrum. However, the major MCD band at 600 nm corresponds well to the absorption band of the optical spectrum and appears to be induced in the highest energy band, usually assigned to the $d_{xz,yz} - d_{x^2-y^2}$ transition.[25] The MCD curve of the green complex resembles that of bovine superoxide dismutase which has a small negative band at 450 nm and a larger negative band at 600 nm.[26] In addition, the magnitude is somewhat larger than that in the nitrogen coordinated simple Cu(II) complex and is similar to that in the Cu(II) chromophore of bovine superoxide dismutase. Under the condition of a magnetic field of 50,000 G, the maxima [θ] for the MCD of the green complex, tris(hydroxymethyl)aminomethane-Cu(II) complex and bovine superoxide dismutase, are −0.37, −0.05, and -0.29×10^{-4} (deg cm^2)/dmol at near 600 nm, respectively.

When the effect of additional binding of pyridine and imidazole ligands on the visible absorption spectrum of the green complex was investigated, it was found that the d–d band near 600 nm shifts linearly to a shorter wavelength with the order of the basicity in pyridine and imidazole li-

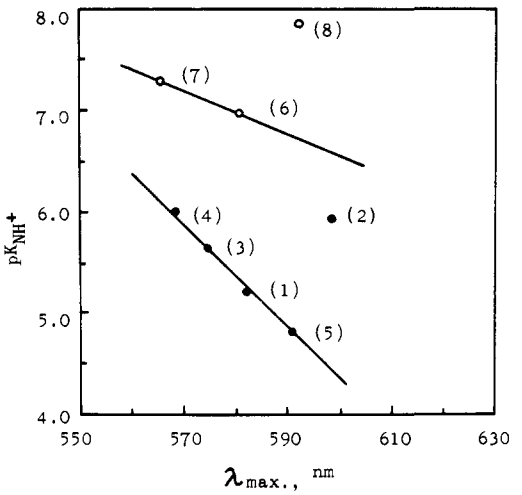

Figure 5. Correlation between shift of the main absorption band and basicity of pyridine and imidazole ligands. The numbers indicate ligands: (1) pyridine; (2) 2-methylpyridine; (3) 3-methylpyridine; (4) 4-methylpyridine; (5) quinoline; (6) imidazole; (7) 1-methylimidazole; (8) 2-methylimidazole.

gands added, except for 2-methylpyridine and 2-methylimidazole (see Figure 5). The pK values of the protonated bases used are as follows: Py, 5.17; 2-CH_3-Py, 5.97; 3-CH_3-Py, 5.68; 4-CH_3-Py, 6.02; Qu, 4.80; Im, 6.95; 1-CH_3-Im, 7.25; 2-CH_3-Im, 7.86.[27] Replacement of H_2O by pyridine or imidazole leads to a high ligand field and causes the shift of the visible band to a higher frequency. The larger shift of 4- (or 3-) methylpyridine than in that of pyridine is attributed to the inductive effect of the 4- (or 3-) methyl group. The smaller shift in 2-methyl derivatives is attributed to the steric interference. The addition of 4-chloro- and 4-cyanopyridines which involve electron-withdrawing groups, however, gave no changes on the original absorption spectrum. From the result of the spectrophotometric titrations, it was clearly observed that 3 mol of pyridine and 1 mol of imidazole coordinate to the green complex, respectively. The pyridine adduct of the green complex is considered most likely to be a mononuclear Cu(II) species with tetragonal symmetry. In the imidazole adduct of the green complex, the existence of dimeric or polymeric as well as monomeric species can also be assumed at the present stage. Characterization of the molecular and electronic structures is now under way. The dimeric and polymeric structures involving imidazole as the bridging group have already been proposed in alanylhistidine-Cu(II),[28] glycylhistidine-Cu(II),[29] and glycylhistidine-Ni(II)[30] systems.

In conclusion, the present investigation on the interaction of the sulfur-peptide and Cu(II) ion clarified that the thiol group can bind to Cu(II) together with the neighboring deprotonated peptide nitrogen atom, and that the high covalency of the Cu-S bonding has a significant effect on the small copper hyperfine coupling constant. ESR similarities between the green complex and chromophore in blue copper proteins are suggestive of the presence of the sulfur atom as one of the coordinates in the copper proteins.

Acknowledgments. Gratitude is due to Dr. H. Ohya of Kyoto University for ESR measurements and for pertinent discussion, Mr. W. Mori of Osaka University for MCD measurement, Dr. N. Toshioka of Santen Seiyaku Co., Ltd., for providing MPG, and Mrs. M. Ohara for assistance with the manuscript.

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