Chem. Pharm. Bull.
24(10)2364—2368(1976)

UDC 547.784.6.04 : 546.562.04

Complexing of Copper Ion by Ergothioneine

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(Received January 12, 1976)

Concurrent redox and complexation reactions between copper(II) and ergothioneine (ESH) have been investigated by means of optical, electron spin resonance (ESR) and nuclear magnetic resonance(NMR) spectra. An equimolar mixture of Cu(II) with ESH gave rise to a green complex. This complex showed an asymmetric peak at 675 nm assigned to *d–d* transition of Cu(II). The ESR spin Hamiltonian parameters ($g_{//}=2.246$, $g_{\perp}=2.068$, and $A_{//}=158G$) of the green complex were typical for the Cu(II) complex of sulfur coordination. In the presence of two molar or excess ESH to Cu(II), an yellow Cu(I) complex was formed. The result of ^{13}C-NMR measurements supports that ESH also coordinates to Cu(I) ion through the thiolate sulfur in the yellow complex.

Ergothioneine (ESH) is an unique biological compound which contains 2-mercapto-imidazole moiety (Fig. 1). On the basis of Raman spectral measurements, we have already reported[2] that ESH is present as thione form in uncharged state and as thiolate ion in ionized species. In general, the interaction of the mercapto group with oxidizing metal ions, such as Cu(II) and Fe(III), is very complicated on account of the redox reaction in addition to complex formation. In the previous paper,[3] it was suggested that the coordination of ESH with cupric ion occurrs through thiolate sulfur. Aurbach and Jakovy[4] have showed that the thiol oxidase from fungi is effective only with thiols possessing the structure $\mathrm{RC}\langle{}^{\mathrm{SH}}_{\mathrm{X}}$, in particular, thiophenol and ESH. This copper-enzyme is the same as the one catalysing diphenol oxidation.[5] Hanlon[6] has also indicated that ESH inhibits a few polyphenol oxidases which maintain the metal moiety of active sites in the cupric form. In addition, ESH prevents the inhibition of sperm motility and fructolysis caused by cupric ion in the boar vesicular secretion[7] and also counteracts the inhibition of purified photoactivated urocanase by cupric ion.[8] However, the interaction between ESH and copper ion has never been quantitatively characterized. Herein, we have investigated about the reaction of ESH with copper ion by means of optical, electron spin resonance (ESR) and nuclear magnetic resonance (NMR) spectra.

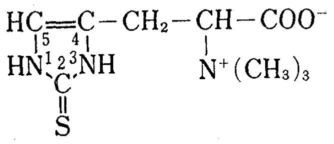

Fig. 1. Numbering in Ergothioneine

Experimental

Materials——ESH was obtained from Sigma Chemical Company. Solutions of Cu(II), Cd(II), and Hg(II) nitrates were prepared from reagent grade materials. Proton magnetic resonance (PMR) and ^{13}C-nuclear

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2) N. Motohashi, I. Mori, and Y. Sugiura, *Chem. Pharm. Bull.* (Tokyo), **24**, 1737 (1976).

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4) G.D. Aurbach and W.B. Jakoby, *J. Biol. Chem.*, **237**, 565 (1962).

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magnetic resonance (CMR) spectra were measured in D_2O. In the measurements of PMR and CMR spectra, the pH(pH=pD−0.4)[9] of samples was adjusted by the addition of DCl or NaOD in D_2O. D_2O, DCl, and NaOD were obtained from E. Merck Company.

Methods——The visible spectra were measured in an aqueous solution (pH 3.4—10.2), with a Shimazu recording spectrophotometer, model Double UV-200. X-Band ESR spectra were obtained at 77 °K and 300 °K with a JEOL ME-3X spectrometer equipped with a gauss meter and frequency counter. PMR spectra were recorded at 60 MHz with a Varian A 60-D spectrometer at about 30°, and DSS was used as the internal standard. CMR spectra were recorded using a NEVA-NV-21 spectrometer operating in the pulsed-Fourier transform mode. Probe temperature was at 32±3° under proton-decoupled conditions. Chemical shifts were measured from internal dioxane and converted to a ppm scale comparison with tetramethylsilane (using a convertion factor of 67.4 ppm). Sample concentrations for PMR and CMR measurements were 0.1M and 0.2M in D_2O, respectively. The pH measurements were made with a Hitachi-Horiba pH meter, model F-5, equipped with combination pH electrode.

Results

Optical Spectra

When ESH was present in two moles or in excess to Cu(II) ion, a pale yellow colored complex was produced and its optical spectrum showed no absorption maximum in the visible region. On the other hand, when Cu(II) ion is present in equimoles to ESH, the solution turns green at pH 9.4. The green colored ES–Cu(II) complex was stable and a broad peak was observed at 675 nm (ε=40) as shown in Fig. 2. Fig. 3 shows the plot of the absorbance at 675 nm against the ratio of Cu(II) ion to ESH, the concentration of ESH being set constant. The intensity at 675 nm rised very sharply until the ratio reached about 1.0 and the absorbance became constant after the ratio reached to 1.0.

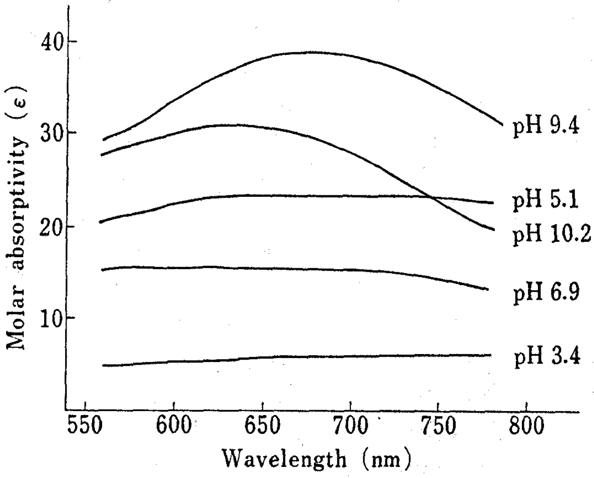

Fig. 2. Visible Spectra of Copper Complexes of Ergothioneine at Different pH Values

ergothioneine (10^{-2} M) to metal ratio=1:1

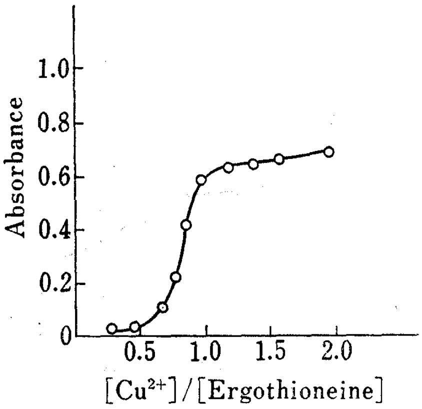

Fig. 3. Change in Absorbance at 675 nm with Ratio of Copper to Ergothioneine

concentration of ergothioneine: 1.0×10^{-2} M, pH 9.5

ESR Spectra

The ESR spectrum of the green complex observed in a frozen solution at 77°K is shown in Fig. 4. In addition, no signals were observed near $g=4$ based on $\Delta M=2$ or spin-forbidden transitions.

NMR Spectra

PMR spectrum of ESH in D_2O (pD 5.2) is presented in Fig. 5. Fig. 6 shows the pH dependence of chemical shift of the H-5 proton for the yellow ES–Cu(I) complex, together

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[9] P.K. Glascoe and F.A. Long, *J. Phys. Chem.*, **64**, 188 (1960).

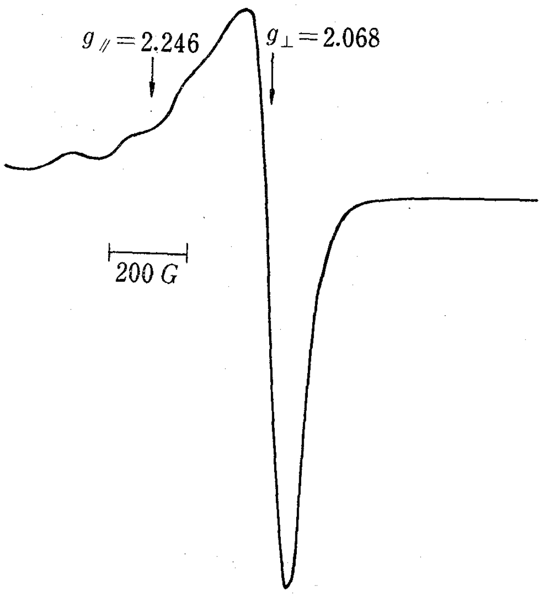

Fig. 4. ESR Spectrum of Green Ergothioneine-Cu(II) Complex at 77 °K

The spectrum was measured in the sample concentration of 5.0 mM. Conditions of ESR spectroscopy: microwave power, 5 mW; modulation amplitude, 6.3 G: scan time, 200G min^{-1}.

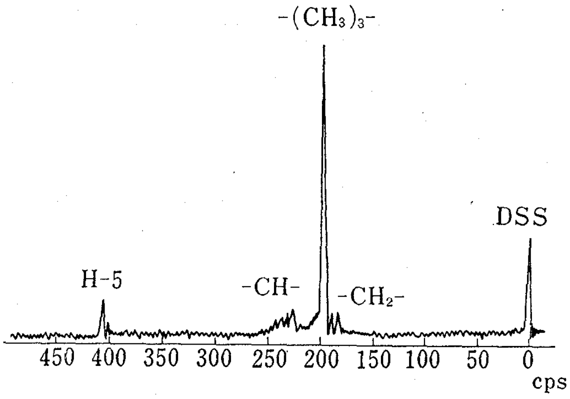

Fig. 5. Proton Magnetic Resonance Spectrum of Ergothioneine at pD 5.2

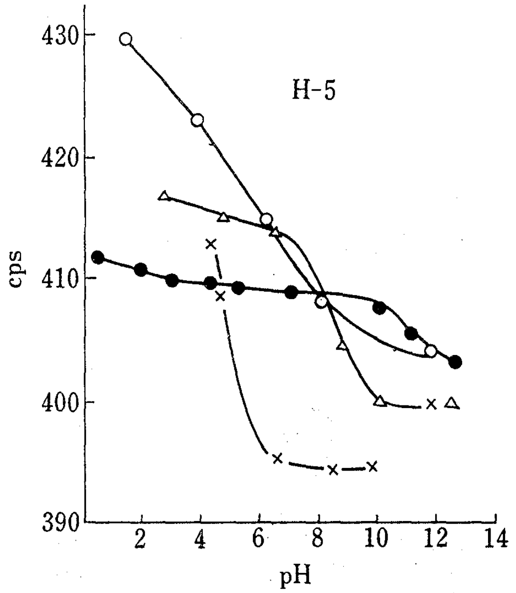

Fig. 6. ^{1}H Chemical Shift-pH Profiles of Ergothioneine and Its Metal Complexes

ergothioneine (0.2 M) to metal ratio=2:1
●: ergothioneine, ○: ergothioneine-mercury complex, ×: ergothioneine-copper complex, △: ergothioneine-cadmium complex

with those of Hg(II) and Cd(II) complexes of ESH and the ligand only. The chemical shifts of the H-5 proton of ESH only is slightly influenced by pH in the range from pH 10 to 13, where the mercaptoimidazole ring of ESH exists as thiolate ion.[2] On the other hand, the H-5 proton of yellow Cu(I) complex shifts to higher field in pH 4—8. Fig. 7 shows the pH effect of the imidazole ring carbon signals of ESH, the yellow Cu(I) complex and two other metal complexes. ^{13}C-Signals of ESH have been already assigned in the previous paper.[2] The C-2 and C-4 carbon signals of ESH-metal complexes are markedly shifted to higher and lower field, respectively, in comparison with that of the ligand only. In particular, the C-2 and C-4 carbons of the yellow Cu(I) complex show the remarkable inflections between pH 6 and 8. The decrease of the C-2 chemical shift in the Hg(II), Cu(I) and Cd(II) complexes at pH 11—12, may be due to the decomposition of these complexes.

Discussion

If Cu(II) ion is added to a solution of ESH, one molecule of ESH is oxidized to yield disulfide (ESSE) and Cu(I) ion, and then one more molecule of ESH is necessary to stabilize Cu(I) formed in the form of the ES–Cu(I) complex (yellow complex) as shown in Eq. 1. This complex showed no ESR signals.

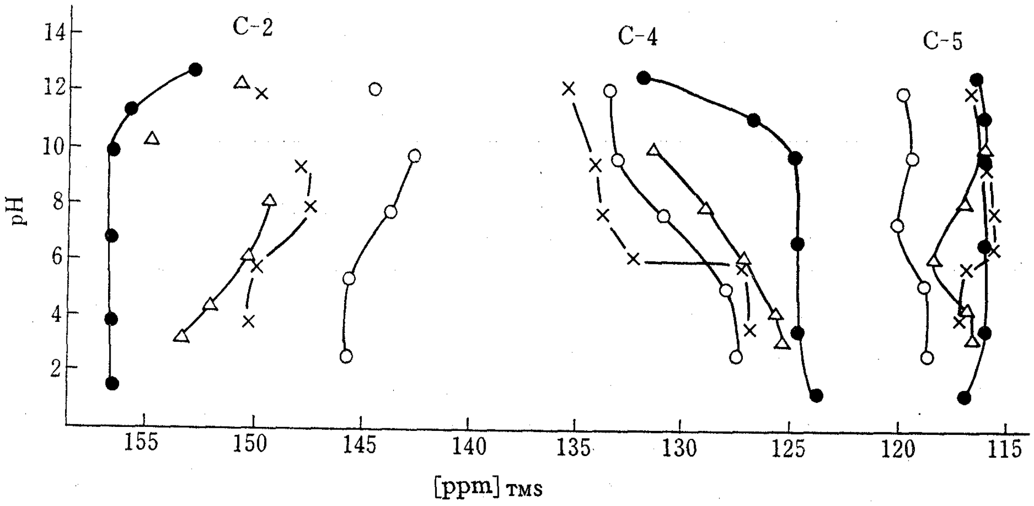

Fig. 7. ^{13}C Chemical Shift-pH Profieles of Imidazole Ring Carbons of Ergothioneine and Its Metal Complexes

ergothioneine (0.2 M) to metal ratio=2:1
●: ergothioneine, ○: ergothioneine-mercury complex, ×: ergothioneine-copper complex,
△: ergothioneine-cadmium complex

$$\begin{array}{l} \mathrm{ES^- + Cu(II) \rightleftharpoons 0.5ESSE + Cu(I)} \\ \mathrm{Cu(I) + ES^- \rightleftharpoons ES\text{-}Cu(I)} \\ \hline \mathrm{2ES^- + Cu(II) \rightleftharpoons ES\text{-}Cu(I) + 0.5ESSE} \end{array} \qquad (1)$$

Whereas, if Cu(II) ion is present in equimoles to ESH, the ES–Cu(II) complex (green complex) is formed according to reaction shown in Eq. 2.

$$\begin{array}{l} \mathrm{ES^- + Cu(II) \rightleftharpoons ES\text{-}Cu(II)} \\ \text{or} \\ \mathrm{ES\text{-}Cu(I) + 0.5ESSE + Cu(II) \rightleftharpoons 2ES\text{-}Cu(II)} \end{array} \qquad (2)$$

The optical spectrum of the green complex has one asymmetric peak at 675 nm which is assigned to d–d transition, $d_{xz,\,yz}$–$d_{x^2-y^2}$transition, of Cu(II).

The values for $g_{//}$, $g_{\perp}$ and $A_{//}$ of the green complex are typical for the Cu(II) complex. As to the copper hyperfine coupling constant ($A_{//}$), the green complex has a smaller value than those of histidine and imidazole-Cu(II) complexes, suggesting the coordination through sulfur donor (see Table I).[10]

Table I. ESR Parameters for Cu(II) Complexes of Ergothioneine, Histidine and Imidazole

Ligand	Donor	$g_{\perp}$	$g_{//}$	$A_{//}(G)$	$\lambda_{\max}(\mathrm{cm}^{-1})^{a)}$
Ergothioneine	1S	2.068	2.246	158	14800
Histidine	4N	2.063	2.230	180	15600
Imidazole	4N	2.063	2.267	180	16800

a) $\lambda_{\max}$ are the ligand field splitting of $d_{xz,yz}-d_{x^2-y^2}$ and were obtained from the visible spectra.

At 77°K the spectrum of the green 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]

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10) G. Malmström and T. Vänngard, *J. Mol. Biol.*, **2**, 118 (1960); Y. Sugiura, Y. Hirayama, H. Tanaka, and K. Ishizu, *J. Am. Chem. Soc.*, **97**, 5577 (1975).
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TABLE II. Changes in ^{13}C Chemical Shifts of Imidazole Carbons in Copper, Cadmium and Mercury Complexes of Ergothioneine at pH 9

Compound	C-2[a]	C-4[a]	C-5[a]
Copper complex	−9.3	+9.0	0
Cadmium complex	−7.4	+6.8	+0.2
Mercury complex	−14.0	+8.1	+3.2

Low field shifts are positive.
a) relative to pH 9 of ergothioneine

Table II shows the changes of ^{13}C-chemical shifts for the imidazole ring carbons. The C-2 resonance of the yellow ES–Cu(I) complex is shifted 9.3 ppm to higher field at pH 9 and similar to the C-2 resonance of Hg(II) and Cd(II) complexes of ESH. ESH coordinates with divalent metal ions, such as Hg^{2+} and Cd^{2+}, through its sulfur atom to form 2:1 complex.[3] The remarkable change in the C-2 chemical shift of the ES–Cu(I) complex is attributed to the π-back donation from d^{10}-Cu(I) ion to sulfur of ESH. In addition, as shown in Fig. 7, the C-2 resonance of the ES–Cu(I) complex is shifted to higher field even in acidic solution, compared with that of the ligand only. The fact suggests that ESH already interacts with copper through its sulfur atom in acidic solution. In Hg(II) and Cd(II) complexes of ESH, furthermore, the C-4 resonances are markedly shifted to lower field and the C-5 resonances are slightly shifted. The changes of the C-4 and C-5 carbons in the ES–Cu(I) complex are identical in sign and similar in magnitude with those of the Hg(II) and Cd(II) complexes. These results indicate that ESH coordinates with Cu(I) ion through its sulfur atom and dissociates the N-3 proton of its imidazole ring in basic solution. In addition, the degree of the change in the chemical shifts of the C-4 carbon of the Cu(I), Hg(II) and Cd(II) complexes of ESH decrease in order of Cu(I)>Hg(II)>Cd(II). This order is in good agreement with that of the formation constants of these complexes calculated by means of potentiometric titration.[3]