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^{13}C-Nuclear Magnetic Resonance and Raman Spectroscopic Studies on Ionization and Mercury Complex of Ergothioneine

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^{13}C-Nuclear magnetic resonance (CMR) and Raman spectra of ergothioneine and its mercury complex have been recorded as function of pH. The ^{13}C-signals of ergothioneine is assigned by comparison with the chemical shifts of histidine, 2-mercaptoimidazole and betaine. Ergothioneine is present in thione form in an aqueous solution or a solid state. In basic solution, ergothioneine exists as thiolate ion upon the deprotonation of the imidazole ring, and the tautomeric form of its imidazole ring is predominantly the 1-H tautomer. The result of ^{13}C-NMR and raman spectra support that ergothioneine coordinates to Hg(II) ion through the thiolate sulfur.

Ergothioneine is an unique biological compound which contains 2-mercaptoimidazole moiety.[2] Although the function of ergothioneine has not been clarified, Heath and Toennis have postulated that ergothioneine may act as a proton donor and acceptor.[3] It has been shown by Stanovnik and Tisler[4] that ergothioneine exists in the form of a zwitter ionic betaine. On the basis of spectral and potentiometric pH measurements, we have already reported[5] that the coordination of ergothioneine with divalent metal ions, such as Co^{2+}, Ni^{2+} and Zn^{2+}, occurrs through the thiolate sulfur donor group. In general, it is suggested that mercaptoimidazoles are present as thione form in uncharged state and as thiolate ion in ionized species.[6] However, the proton ionization and coordination mode of ergothioneine have never been definitely established. In this paper, we have investigated the proton ionization and mercury complex of ergothioneine in detail by ^{13}C-nuclear magnetic resonance (CMR) and Raman spectroscopy.

Experimental

Materials——Ergothioneine was obtained from Sigma Chemical Company. 2-Mercaptoimidazole and 1-methyl-2-mercaptoimidazole were purchased from K and K Laboratories. The solution of mercuric chloride was prepared from reagent grade material. Raman and CMR spectra were measured in distilled water and in heavy water, respectively. In CMR measurements, the pH (pH=pD -0.4)[7] of samples was adjusted by the addition of DCl or NaOD in D_2O. Heavy water, DCl and NaOD were obtained from E. Merck Company.

Apparatus and Procedure——CMR spectra were recorded on a NEVA-NV-21 spectrometer operating in the pulsed-Fourier transform mode. Probe temperature was at $32\pm3°$ under proton-decoupled conditions and sample concentration was 0.2 M in D_2O. Chemical shifts were measured from internal dioxane and converted to a ppm scale in comparison with tetramethylsilane (using a conversion factor of 67.4 ppm). Raman spectra were recorded by using a JEOL-Sl spectrophotometer with the 4880 Å line of an argon ion laser. Sam-

1) Location: *a) Motoyamakita-machi, Higashinada-ku, Kobe; b) Yoshida, shimoadachi-cho, Sakyo-ku, Kyoto.*
2) E.C. Storell, "Organic Sulfur Compounds," Vol. 1, ed. by N. Kharasch, Pergamon Press, New York, N.Y., 1961, pp. 462—490.
3) H. Heath and G. Toennies, *Biochem. J.*, **68**, 204 (1958).
4) B. Stanovnik and M. Tisler, *Anal. Biochem.*, **9**, 68 (1964).
5) N. Motohashi, I. Mori, Y. Sugiura, and H. Tanaka, *Chem. Pharm. Bull.* (Tokyo), **22**, 654 (1974).
6) A. Lawson and H. Morley, *J. Chem. Soc.*, **1956**, 1103.
7) P.K. Glascoe and F.A. Long, *J. Phys. Chem.*, **64**, 188 (1960).

ple concentration for Raman spectra was 0.1 M. The pH measurements were made with a Hitachi-Horiba pH meter, model F-5, equipped with combination pH electrode.

Result and Discussion

The ^{13}C-NMR spectrum of ergothioneine is compared with those of histidine,[8] 2-mercaptoimidazole and betaine[8] in D_2O (see Fig. 1). The C-2 signal of histidine imidazole ring appears at 128.4 ppm, while the C-2 resonance of 2-mercaptoimidazole, substituted by sulfur at the

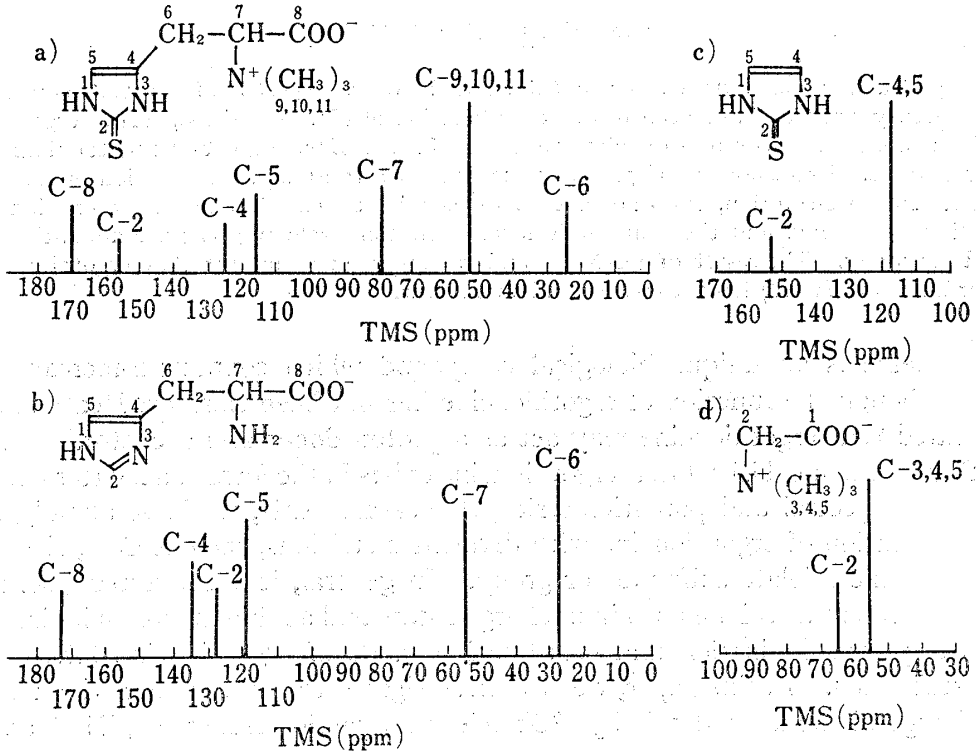

Fig. 1. ^{13}C-NMR Spectra of Ergothioneine, Histidine, 2-Mercaptoimidazole and Betaine

a) ergothioneine, b) histidine, c) 2-mercaptoimidazole, d) betaine

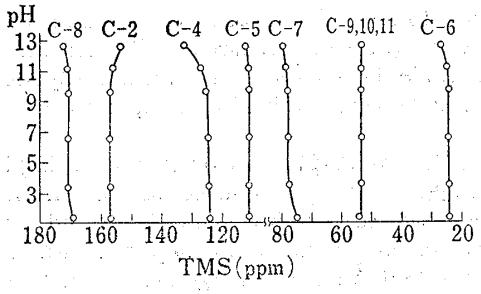

Fig. 2. ^{13}C-Chemical Shift-pH Profiles of Ergothioneine

2-position, is shifted 30 ppm to lower field. In betaine, the N-trimethyl carbons (C-3,4,5) and the α-carbon (C-2) exhibit the signals at 54.8 and 64.6 ppm, respectively. Accordingly, the CMR signals of ergothioneine, which are found at 170.4, 156.6, 124.6, 116.0, 77.9, 53.1 and 23.9 ppm, are reasonably assigned to the C-8, C-2, C-4, C-5, C-7, C-9, 10, 11 and C-6 carbons, respectively. Fig. 2 shows the pH effect on the ^{13}C-resonance of ergothioneine. The major changes are observed at C-2 and C-4 carbons in the pH region from 9 to 13, where the deprotonation occurrs in the imidazole ring. However, the C-5 carbon shows little chemical shift in this pH range. The carbonyl carbon (C-8) of the carboxylic acid shifts 2 ppm to lower field upon deprotonation at pH>2. The lower field shift in pH 1—3 is also found for C-7

8) L.F. Johnson and W.C. Jankowski, "Carbon-13 NMR Spectra," Wiley-Interscience, New York, N.Y., 1972.

Table I. Changes in ^{13}C Chemical Shifts of Imidazole Carbons in the pH Region of Imidazole Proton Dissociation

Compound	C-2	C-4	C-5	pK
Ergothioneine	−3.5	+7.2	+0.4	11.5(10.5)[5]
2-Mercaptoimidazole	−2.7	+4.8	+4.8	
1(or 3)-Methyl-2-mercaptoimidazole	−1.8	+9.9(0)	0(+9.9)	
Imidazole[9]	+2.4	+2.7	+2.7	
Histidine[9]	+2.4	+4.8	−0.7	
1-Methylhistidine[9]	+3.5	+6.8	−2.1	
3-Methylhistidine[9]	+3.4	−2.3	+7.1	
N-Acetylhistidine[9]	+2.6	+3.5	+0.9	

Low-field shifts are positive.

in β-position to the carboxyl group. Table I presents the ^{13}C-chemical shifts of imidazole carbons in ergothioneine and 2-mercaptoderivatives upon the ionization of the imidazole ring, together with those of imidazole and histidine derivatives.[9] The C-2 carbons of ergothioneine, 2-mercaptoimidazole and 1(or 3)-methyl-2-mercaptoimidazole shift to high field by 3.5, 2.7 and 1.8 ppm, respectively. Of special note is the fact that upon the ring ionization the C-4 resonance of ergothioneine is shifted to low field by 7.2 ppm while the C-5 resonance is shifted to low field slightly by 0.4 ppm. In 1-methyl-2-mercaptoimidazole substituted by methyl group at the N-1 position, the C-4 carbon shifts to low field by 9.9 ppm, while the C-5 carbon shows no shifts. By contrast, the C-4 and C-5 resonances of 2-mercaptoimidazole equivalently shift 4.8 ppm to lower field upon the ring ionization. Recently, the tautomeric form of histidine was reported by Reynolds, *et al.*[9] and R. Deslauriers, *et al.*[10] As shown in Table I, the changes of imidazole carbons in 1-methylhistidine are identical in sign and similar in magnitude with those in histidine. On the other hand, the changes of chemical shift in 3-methylhistidine are totally inconsistent with those in histidine. It has been shown that the predominant tautomeric form of histidine imidazole ring is the 1-H tautomer in basic solution. On the basis of facts, we are of the opinion that in ergothioneine the 1-H tautomer is also predominant in basic solution. In general, a lowfield shift of the ^{13}C-signals has been observed during deprotonation of SH group in amino-thiol compounds.[11] Of using interesting is that the C-2 carbons of 2-mercaptoimidazole derivatives shift to the higher field upon dissociation of the imidazole proton. In addition, ergothioneine and N-acetylhistidine reveal small positive (low field) shifts of C-5 upon the ring ionization. The proton ionization constant of ergothioneine was calculated from ^{13}C-chemical shift *vs.* pH profiles according to the Henderson-Hasserbach equation.[12,13] The pK vallue of ergothioneine obtained was 11.5. The pH effect on the imidazole carbon signals of ergothioneine-Hg (II) complex is illustrated in Fig. 3, together with that of ergothioneine only. It is known that ergothioneine reacts with Hg(II) ion to form 2:1 complex in neutral and alkaline solutions.[5] As seen in Table II, the C-2 carbon of ergothioneine-Hg(II) complex additionally shifts 14.0 ppm to higher field in basic solution, although the C-2 resonance of ergothioneine-Hg(II) system is already shifted to higher field in acidic solution compared with that of the ligand only. In addition, the C-4 carbon of the Hg(II) complex shifts to lower field in the observed pH range. Ergothioneine can be oxidized to the disulfide form slowly by oxygen and rapidly by hydrogen peroxide in strong acid

9) W.F. Reynolds, I.R. Peat, M.H. Freedman, and J.R. Lyerla, Jr., *J. Am. Chem. Soc.*, **95**, 328 (1973).
10) R. Deslauriers, W.H. McGregor, D. Sarantakis, and E.C.P. Smith, *Biochemistry*, **13**, 3443 (1974).
11) L. Flohè, E. Breitmaier, W.A. Günzler, W. Voelter, and G. Jung, *Hoppe-Seylers Z. Physiol. Chem.*, **353**, 1159 (1972).
12) E.G. Finer, "Macromolecules and Solids in R.K. Harris, Nuclear Magnetic Resonance," Vol. 1, Chemical Society, London, 1972, p. 281.
13) J.S. Cohen, R.I. Shragen, M. McNeel, and A.N. Schechter, *Nature*, **228**, 642 (1970).

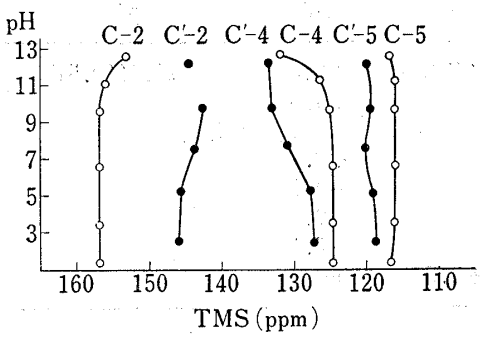

Fig. 3. ^{13}C Chemical Shift-pH Profiles of Imidazole Ring Carbons of Ergothioneine and its Mercury Complex

○: ergothioneine
●: ergothioneine-mercury complex

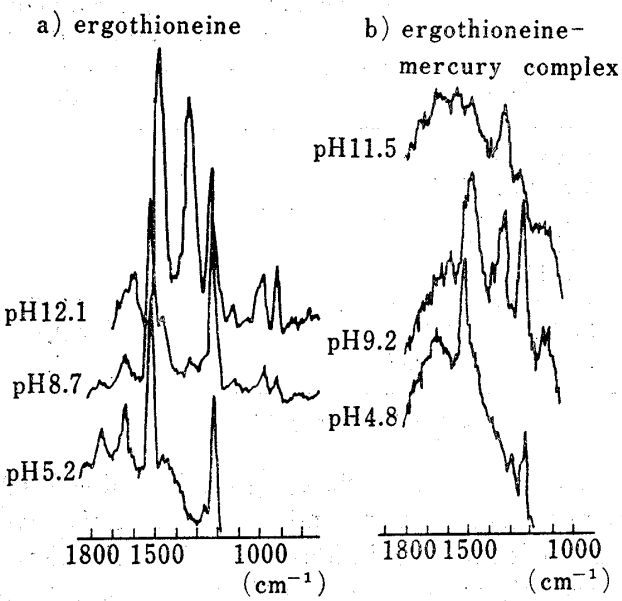

Fig. 4. Raman Spectra of Ergothioneine and its Mercury Complex at Different pH Values

TABLE II. Changes in ^{13}C Chemical Shifts of Imidazole Carbons in Ergothioneine-Mercury Complexes and Ergothioneine Disulfides

Compound	C-2[a]	C-4[a]	C-5[a]
Ergothioneine-mercury complex (pH 5)	−11.0	+3.2	+2.7
Ergothioneine-mercury complex (pH 9.5)	−14.0	+8.3	+3.3
Ergothioneine disulfide (5N-DCl)	−8.2	+0.4	+2.5
Ergothioneine disulfide (5N-DCl+H_2O_2)	−14.3	+3.5	+5.2

Low-field shifts are positive. *a*) relative to pH 6 of ergothioneine

solution.[3] The C-2 resonance of ergothioneine disulfide is markedly shifted to higher field and similar to the C-2 resonance of ergothioneine-Hg(II) complex. These results indicate that ergothioneine coordinates with Hg(II) ion through its sulfur atom to form 2: 1 complex in basic solution. In addition, it suggests that ergothioneine has already interacted with Hg(II) ion through its sulfur atom in acidic solution. It seems reasonable to consider that the remarkable change in the C-2 chemical shift of ergothioneine-Hg(II) complex is caused by the π-back donation from Hg(II) to sulfur of ergothioneine. The decrease of the C-2 chemical shift in ergothioneine-Hg(II) complex at pH>11, may be due to its decomposition.

Fig. 4 shows Raman spectra of ergothioneine and its mercury complex at different pH values. The Raman spectra of ergothioneine in an aqueous solution and in the solid state show no bands between 2500 and 2600 cm^{-1}, which are assigned to SH stretching vibration. On the basis of infrared and PMR spectra, Hayden and Maienthal[14] have reported that 2-mercaptoimidazole and its derivatives exist as the thione form in an aqueous solution. It is also presumed that ergothioneine exists as the thione form in an aqueous solution and in the solid state. In addition, ergothioneine exhibits bands at about 1200 and 1500 cm ^{1}. The latter band is attributable to the thioamide group. At pH 12.1, a sharp band is newly observed at near 1300 cm^{-1} together with the previous bands. This alteration of the Raman spectrum of ergothioneine is probably attributed to the ionization of its imidazole group. The Raman spectra of ergothioneine-Hg(II) complex show a few strong bands in the 1500—1200 cm^{-1}

14) A.L. Hayden and M. Maienthal, *J. Assoc. Offic. Agr.*, **48**, 596 (1965).

region. Of interesting is that the Raman spectrum of ergothioneine-Hg(II) complex in the pH region formed 2: 1 complex is similar to that of ergothioneine of which imidazole proton dissociates. In view of a marked resemblance between the Raman spectra of ergothioneine at pH 12.1 and its Hg(II) complex at pH 9.2, it is indicated that ergothioneine and its Hg(II) complex exist as the same anionic form in basic solution. In the Raman spectrum of ergothioneine-Hg(II) complex at pH 11.5, the broad bands near 1500 cm^{-1} may be due to its decomposition.

On the basis of these data, the ionization of ergothioneine is summarized in Fig. 5. Ergothioneine exists as the thione form in an aqueous solution and in the solid state, and the

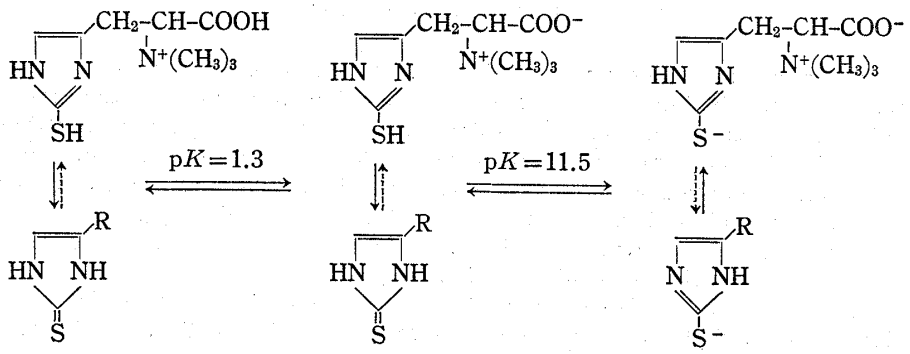

Fig. 5. Ionizations of Ergothioneine

ionization step of its imidazole ring involves the transition from the neutral form to the anionic form.[4,15] Two tautomeric forms, 1-H and 3-H tautomers, are considered for the anionic imidazole ring. On the other hand, the anionic imidazole ring is predominantly the 1-H tautomer. In addition, the Raman spectral data support strongly that the mercaptoimidazole ring of ergothioneine exists as the thiolate ion in basic solution.

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15) J. Carlsson, M.P.J. Kierstan, and K. Brocklehurst, *Biochem. J.*, 139, 221 (1974).