May, 1979] BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN, VOL. 52 (5), 1275—1278 (1979) 1275

Nuclear Magnetic Resonance Studies of the Cu-chlorophyllin Complex with Flavine Mononucleotide

Koe ENMANJI*

Department of Chemistry, Faculty of Science, Kyoto University, Kyoto 606

(Received September 1, 1978)

The NMR relaxation time of the Cu-chlorophyllin (Cu-chln)–flavine mononucleotide (FMN) complex was measured, and its geometrical structure was proposed. The complex has an absorption band at 700 nm which is attributed to complex formation by analogy with the chlorophyll a–FMN complex. Its binding constant is 330 l/mol at 25 °C; this value is larger than those of other Cu-chln complexes. From the analysis of its ESR data, it is clear that the greater part of the electron spin in Cu-chln is distributed on the Cu^{2+} ion. The distance between the nuclear and electron spins was calculated by measuring the spin-lattice relaxation times. From these results, the distance between the porphyrin ring and the isoalloxazine ring in the Cu-chln–FMN complex molecule is found to be 4.2 Å if the two rings are parallel to each other. The chlorophyll a–FMN complex is assumed to have a similar geometrical structure.

There are various kinds of chlorophylls in the lammela structure of the chloroplast. Most of these chlorophylls are called "antana chlorophylls," which harvest solar energy, and part of them exist as P700, which is the reaction center of Photosystem I. It is generally imagined that P700 is formed by interaction between chlorophyll a and an electron acceptor. Tu and Wang[1] have shown that the optical spectrum of the chlorophyll a–flavine mononucleotide (FMN) complex in an acetone–water mixture has a maximum peak at 700 nm and some characteristics of P700. Though the characteristics of this complex are thought to be related to its structure, the structure of the complex was not discussed in their paper. Because the ground state of this complex is not paramagnetic, it is difficult to presume a geometrical arrangement of each constituent molecule in the complex. Cu-chlorophyllin (Cu-chln) is an interesting molecule since the electron magnetism on it will make it easier to study its interaction with itself and with other molecules by means of magnetic resonance methods. Thus, the interaction of chlorophyllin molecules in solution may be studied easily with Cu-chln as a model compound. In the present study, the interaction of Cu-chln with FMN in an aqueous environment was studied in some detail by means of ESR, optical absorption and NMR spectroscopy.

Experimental

Materials. Mg-chlorophyllin was prepared from spinach by the method of Oster and his coworkers.[2] Cu-chln was obtained from Mg-chlorophyllin by the method of Burdick and Carroll.[3]

FMN, reagent grade (Nakarai Chem. Co.), was used without further purification.

Optical Measurement. The optical spectra were measured with a Hitachi PS-3T-type autorecording spectrometer.

NMR Measurement. The proton, phosphorus-31, and carbon-13 NMR spectra were recorded at 100, 40.5, and 25.15 MHz respectively on a JMN PS-100 system equipped with a pulse Fourier transform unit (PFT-100). The sizes of the sample tubes were 5 mm O.D. for ^1H, 8 mm O.D. for ^{31}P. The spin-lattice relaxation time (T_1) was measured by the 180°-τ-90° pulse method. The repetition times were 30 s for both the ^1H and ^{31}P measurements. The spectra were added 16 times for the ^1H and 64 times for the ^{31}P measurements respectively.

Theoretical

For a system containing paramagnetic metal ions and a large excess of free ligands, with magnetic coupling between the metal and a nucleus in the ligand, the following equation holds under appropriate conditions,[4,5]

$$\frac{1}{T_{1P}} = \frac{1}{T_1{}^*} - \frac{1}{T_1{}^\circ} = \frac{f}{T_{1M}+\tau_M}, \tag{1}$$

where T_{1M} is the spin-lattice relaxation time of the ligand in the complex, f is the fractional number of the complexed ligand to the total ligand, $T_1{}^\circ$ is the relaxation time without a paramagnetic substance, $T_1{}^*$ is that with a paramagnetic substance, and T_{1P} is the apparent relaxation time. If we consider a system consisting of two spins that undergo an isotropic Brownian motion, the nuclear relaxation rate due to magnetic dipolar interaction between the nucleus and the electron spins is given by[5] (for $\omega_s\tau_c \gg 1$),

$$\frac{1}{T_{1M}} = \frac{2}{15} S(S+1) \frac{\gamma_I{}^2 g^2 \beta^2}{r^6} \cdot \frac{3\tau_c}{1+\omega_I{}^2\tau_c{}^2}, \tag{2}$$

where ω_I and ω_s are the Larmor angular frequencies of the nucleous and the electron spin respectively. Hence, T_{1M} is proportional to the sixth power of the distance, r, between the unpaired electron and the nuclei. If τ_c is known, T_{1M} can be converted to the electron nuclear distance by means of Eq. 2. The electron-nuclear distance for various neclei in the molecule can give information as to the geometrical arrangement of the intermolecular complex when information about the distribution of the unpaired electron is available.

Results and Discussion

Distribution of the Unpaired Electron in Cu-chln. The ESR spectrum of Cu-chln in an aqueous solution (10 mmol/l) frozen at 77 K shows an almost smeared-out singlet because of the dipolar and the exchange interactions between electron spins on neighboring

---

* Present address: Central Research Laboratory, Mitsubishi Electric Corp., Amagasaki 661.

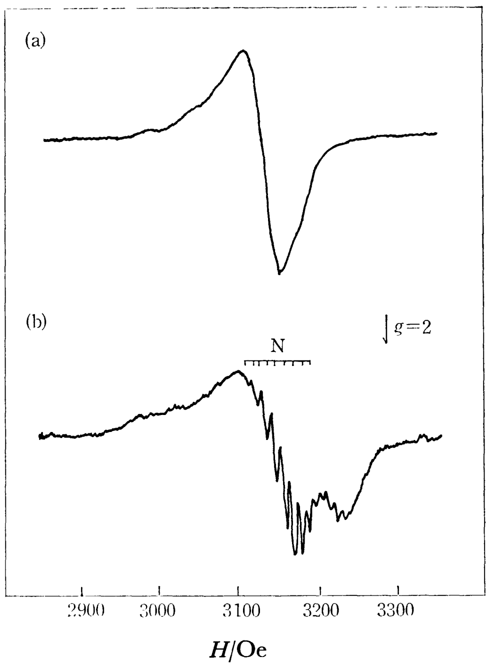

Fig. 1. ESR spectra of Cu-chln at liquid nitrogen temperature a) 0.01 mol/l Cu-chln only, b) 0.01 mol/l Cu-chln plus 0.1 mol/l FMN. Microwave frequency 9.1 GHz, power 2 mW. Modulation width 2.0 G at 100 kHz.

molecules of Cu-chln (Fig. 1(a)), when FMN of 0.1 mol/l is added to this Cu-chln solution and the ESR is measured at 77 K, however a clear superhyperfine structure due to nitrogen atoms is observed, as is shown in Fig. 1(b). The sharpening of the ESR spectrum by the addition of FMN indicates that FMN forms an intermolecular complex with Cu-chln in which the Cu-chln molecules are separated far enough from each other to make the dipolar and exchange interaction between electron spins negligible. The present author previously observed similar effects on the ESR spectrum for Cu-chln with poly(*N*-vinylpyrrolidone) in aqueous solutions and for Cu-chln with Mg-chln in ethanol, both at 77 K.

The hyperfine splitting due to the nitrogen atom is 16.7 G in the Cu-chln–FMN system (Fig. 1(b)). By following the treatment proposed by Koski *et al.*[6] for the ESR spectra of Cu-etioporphyrin and Cu-phthalocyanine, we may conclude that 65% of the unpaired electrons occupy the Cu $d_{x^2-y^2}$ orbital, and while 8.8% occupy each of the four nitrogen atoms, and that the spin density on the pheophytin ring carbons is negligibly small. These findings regarding the spin distribution in Cu-chln and the NMR relaxation measurements to be shown in a later section were used to simulate the structures of the molecular complex between Cu-chln and FMN.

Equilibrium Binding Study between Cu-chln and FMN. The optical difference spectra of the Cu-chln–FMN complex shows a maximum at 700 nm and a minimum at 630 nm. The intensity of the maximum increases with an increase in the concentration of FMN and can be attributed to the complex formation of Cu-chln with FMN. As the change in the concentration of FMN induces no change in the position of the maximum peak, we may assume that the peak height of the difference spectra at 700 nm is proportional to the concentration of the Cu-chln–FMN complex. We further assume this equilibrium:

$$\text{Cu-chln} + n\text{FMN} \rightleftarrows \text{Complex}, \tag{3}$$

for which the equilibrium constant, K, is given by:

$$K = \frac{C_{\text{chlb}}}{C_{\text{chlf}}} \times \frac{1}{C_{\text{F}}{}^{n}}, \tag{4}$$

where C_{chlb}, C_{chlf}, and C_{F} are the concentration of the bund Cu-chln, that of the free Cu-chln, and that of FMN respectively. Equation 4 can be rewritten as:[7]

$$\log \frac{C_{\text{chlb}}}{C_{\text{chlf}}} = \log C_{\text{F}} + \log K, \tag{5}$$

C_{chlb} can be determined from the ratio of the peak height at 700 nm at a given concentration of FMN to that in the presence of a large excess of FMN at a constant Cu-chln concentration. Thus, the plot of $\log C_{\text{chlb}}/C_{\text{chlf}}$ against $\log C_{\text{F}}$ gives the slope n, *i.e.* the number of FMN molecules bound per Cu-chln; it also gives the equilibrium constant, K. From Fig. 2, we obtain $n=0.91$ and $K=512$ l/mol at 9 °C, $n=0.94$ and $K=330$ l/mol at 25 °C, and $n=1.02$ and $K=101$ l/mol at 84 °C. We may thus conclude that, in this concentration range of FMN (0—7.0×10^{-2} mol/l), Cu-chln forms a one-to-one complex with FMN in this temperature range. Tsuchida *et al.* measured the Cu-chln–pyridine and poly(4-vinylpyridine) complexes with the same method, thus obtaining values of $K=39$ l/mol and $K=1500$ l/mol at pH 10 respectively.[8] The present author previously measured the Cu-chln–adenosine-5′-monophosphate complex and

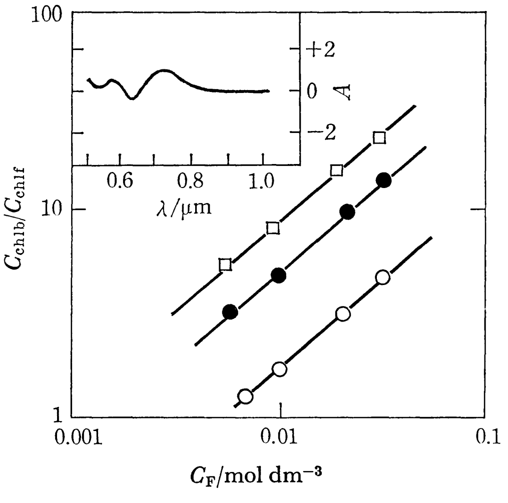

Fig. 2. Optical difference spectrum of Cu-chln–FMN complex and Hill plot for Cu-chln–FMN complex. Reference side: Cell A (Cu-chln 2.99×10^{-4} mol/l, phosphate buffer ,pH 7.0, 0.1 mol/l), Cell B (FMN 7×10^{-2} mol/l, phosphate buffer, pH 7.0, 0.1 mol/l). Sample side: Cell C (Cu-chln 2.99×10^{-4} mol/l, FMN 7×10^{-4} mol/l, phosphate buffer, pH 7.0, 0.1 mol/l), Cell D (phosphate buffer, pH 7.0, 0.1 mol/l) at 25 °C.

obtained $K=19$ l/mol at 30 °C.[9] The view of all these data, it can be said that the Cu-chln–FMN complex has a rather strong binding.

Spin-lattice Relaxation Times of ^1H and ^{31}P Nuclei of FMN. A Probable Structure of the Complex : The detail of the structure may be obtained from studies of FMN and Cu-chln at low concentrations where a one-to-one complex seems to be formed by optical titration. For this purpose, we have measured the spin-lattice relaxation times of nonexchangeable protons, *i.e.*, C(6)–H, C(9)–H, C(7)–CH_3, and C(8)–CH_3 of FMN at 70 mmol/l in the presence (0.9 mmol/l) and the absence of Cu-chln in a neutral D_2O solution (pH 5.8). The results are shown in Fig. 3 (as T_{1P}) as a function of the temperature. The T_1 values vary considerably among different protons, indicating that they are determined by T_{1M} rather than by a common value, τ_M, in Eq. 2. The T_{1P} of each proton shows a minimum at about 30 °C; and according to Eq. 2 we have $\tau_c \approx 1.6\times10^{-9}$ at this temperature. Using this τ_c value and the bound fraction of FMN (f) calculated from the association constant ($K_a=3.3\times10^2$ l/mol) for a one-to-one complex, we obtained the distances from the unpaired electron to the respective protons. The distance from the unpaired electron to the phosphorus atom was similarly calculated using the same τ_c and f value at 30 °C obtained from the T_1 data in Fig. 3. All the results are listed in Table 2.

The long distance obtained for the phosphorus (8.0 Å) indicated that the coordination of the phosphate group to Cu does not occur in the complex formation. The interaction primarily occurs with the known atomic coordinates of FMN and Cu-chln,[11,12] taking the electron-spin distribution of Cu-chln obtained from the ESR study into account.

For this purpose, a computer program developed in our laboratory[13] was applied to this complex. By assuming a parallel arrangement between the two planes of FMN and Cu-chln, we find that the central Cu atom is closer to the methylated ring and that the interplane distance is 4.20±0.05 Å. This interplane distance is a little longer than those of the π-π complex consisting of two aromatic compounds with carbon 2pπ orbitals, but considering the involvement of d-orbitals in the present system, the structure is essentially similar to the stacking arrangement of two aromatic compounds.

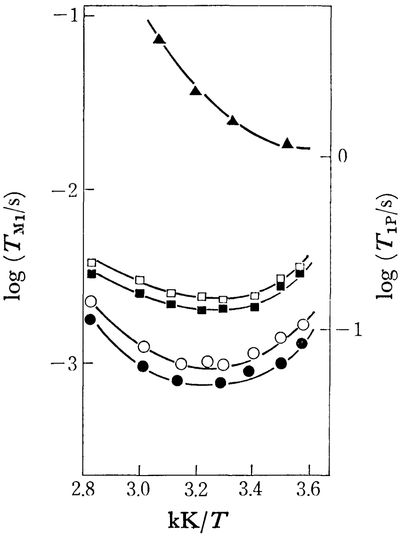

Fig. 3. Temperature dependence of proton and phosphorus relaxation.
FMN (70 mmol/l), Cu-chln (0.9 mmol/l), pH 5.8. C(9)-H: ●, C(6)-H: ○, C(8)-CH_3: ■, C(7)-CH_3: □, phosphorus: ▲.

Table 1. The distances from the electron spin on Cu-chln to the nonexchangeable protons and the phosphorous atom of FMN in the Cu-chln-FMN complex (30 °C, pH 5.8)

	T_{1obsd} [a)] s	R [b)] nm
C(6)-H	8.1×10^{-4}	0.60
C(9)-H	6.1×10^{-4}	0.58
C(7)-CH_3	1.81×10^{-3}	0.69
C(8)-CH_3	1.73×10^{-3}	0.68
P	1.50×10^{-2}	0.80

a) T_{1bosd} is the observed T_{1B}. b) R is the apparent distance between the protons or phosphorus and the electron spin.

Barry *et al.* suggested that the family structures of the Co-mesoporphyrin IX dimethyl ester-2,4,7-trinitro-9-fluorenone complex with 4.00±0.02 Å are closely related to those determined for similar complexes in the crystalline state in that the planes of Co^{2+} MPDE and TNF are parallel.[14] We may, therefore, confirm that the interaction between FMN and Cu-chln in an aqueous environment is probably of hydrophobic origin, with the two planes mutually stacked.[15]

From the close similarity in the optical difference spectra between the Cu-chln–FMN system and the chlorophyll a–FMN system, we can expect that chlorophyll a also forms a similar complex with FMN in an aqueous environment.

The author is deeply indebted to Professor Hiroyuki Hatano for his continual encouragement throughout this work, and also to Dr. Kazuyuki Akasaka for his helpful advice and discussions. He also wishes to thank Mr. Hideaki Kusakawa for his advice on the writing of this paper.

References

1) S. Tu and J. H. Wang, *Biochem. Biophys. Res. Commun.*, **36**, 79 (1969).

2) G. Oster, S. B. Bryode, and J. S. Bellin, *J. Am. Chem. Soc.*, **86**, 1313 (1966).

3) E. M. Burdick and R. G. Caroll, *Ind. Eng. Chem. Soc.*, **46**, 2262 (1954).

4) T. J. Swift and R. E. Connick, *J. Chem. Phys. Soc.*, **37**, 307 (1962).

5) N. Bloembergen, E. M. Purcell, and R. M. Pound, *Phys. Rev.*, **73**, 677 (1948).

6) E. M. Roberts and W. S. Koski, *J. Am. Chem. Soc.*, **96**, 103 (1974).

7) E. M. Baker, M. S. Brookhart, and A. H. Corwin,

J. Phys. Chem., **86**, 4587 (1964).

8) E. Tsuchida, K. Shigehara, and K. Miyamoto, *J. Polym. Sci., Polym. Chem. Ed.*, **14**, 911 (1976).

9) K. Enmanji, *Nippon Kogaku Kaishi*, **1978**, 1317.

10) E. Breitmaier and W. Voeltler, *Eur. J. Biochem.*, **31**, 234 (1972).

11) P. Kierkegaard, R. Norrestar, P. Werner, I. Csoregh, M. Glehn, R. Karlsson, M. Leijonmarck, O. Ronnquist, B. Stensland, O. Tillberg, and L. Torbjornsson, "Flavine and Flavoproteins," John Willy & Sons (1972), pp. 1—22.

12) C. J. Brown, *J. Chem. Soc., A*, **1968**, 2494.

13) Y. Nosaka, K. Akasaka, and H. Hatano, *J. Magn. Reson.*, to be published.

14) C. D. Barry, H. A. O. Hill, P. J. Sadler, and R. J. P. Williams, *J. Am. Chem. Soc.*, **95**, 4533 (1973).

15) G. P. Fulton and G.N. La Mar, *J. Am. Chem. Soc.*, **98**, 2124 (1976).