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LIPOSOMAL HEME AS OXYGEN CARRIER UNDER SEMI-PHYSIOLOGICAL CONDITIONS

ORIENTATION STUDY OF HEME EMBEDDED IN A PHOSPHOLIPID BILAYER BY AN ELECTROOPTICAL METHOD

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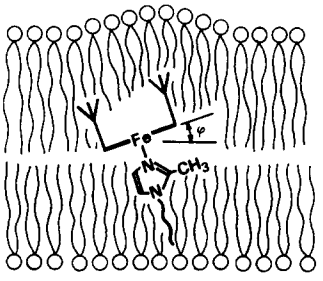
The *meso*-tetra(α,α,α,α(*o*-pivalamidophenyl))porphinato iron-mono(1-lauryl-2-methylimidazole) complex embedded in the bilayer of dimyristoylphosphatidylcholine (liposomal heme) binds molecular oxygen reversibly at pH 7 and 37°C. Orientation of the iron porphyrin complex in the phospholipid bilayer was studied by electric birefringence and dichroism. It was observed that both the phospholipid bibilayer of liposome and the porphyrin plane are oriented nearly in parallel to the electric field. Therefore the angle between the porphyrin plane and the bilayer is considered to be practically small.

Introduction

Much effort has been made to mimic natural oxygen carriers like hemoglobin by using synthetic iron porphyrin complexes. The authors from this laboratory have succeeded in achieving reversible oxygen-binding under semiphysiological conditions (in pH 7.0 aqueous medium at 37°C) by embedding the *meso*-tetra(α,α,α,α-(*o*-pivalamidophenyl))porphinato iron complex of mono-(1-lauryl-2-methylimidazole) in liposomes of phosphatidylcholine (abbreviated as 'liposomal heme') [1]. The oxygen-binding and oxygen-dissociation proceeded very rapidly and the oxygen-binding affinity was similar to that of hemoglobin in blood: the liposomal heme is expected to have high potential for an artificial blood [2,3]. It was recently confirmed that the iron porphyrin complex is embedded in a phospholipid bilayer of a liposome



Scheme I. Liposomal heme.

(Scheme I) and that the hydrophobic environment of the inner region of the liposome protected the oxygen adduct from proton-driven oxidation [4]. The present paper describes the orientation study of heme embedded in phospholipid bilayer by electric dichroism measurements.

Experimental

meso-Tetra(α,α,α,α-(*o*-pivalamidophenyl))-porphinato iron(III) bromide (picket fence iron

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porphyrin [5], abbreviated here as 'heme') was prepared as in the literature [6]. 1-Lauryl-2-methylimidazole was prepared by reacting lauryl bromide and 2-methylimidazole at 200°C. 1,2-Dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) was purchased from Sigma, U.S.A.

The liposomal heme (sample 1) was prepared as follows: 0.1 μmol heme, 5 μmol laurylmethylimidazole, and 20 μmol DMPC were mixed in dichloromethane. By evaporating the solution under reduced pressure, a thin film was prepared on the glass wall of a round flask. 10 ml of pure water were added and the mixture was ultrasonicated for 20 min and homogenized in an ice/water bath. This solution was incubated at 40°C for 2 h.

The liposomal heme (sample 2) was also prepared, with a shorter sonication time (5 min). Its diameter is assumed to be larger than that of sample 1 (detailes are given in the next section).

Sample 3 was prepared as follows. The liposomal heme in 4.5-fold concentration (M/15 (pH 7.0) phosphate buffer) was prepared in a manner similar to sample 1 and was poured into water and diluted 4.5-times. The liposome was expected to be burst by osmotic pressure and to have a lamellar structure.

The DMPC liposome was also prepared in a manner similar to sample 1, without heme.

The transient electric dichroism was measured with the use of a high electric field supplied by an electric field pulse generator (Denken Seiki Co.; 7.5, 40 and 55 kV/cm) which has a square-wave pulse with duration of 1.0 ms [7]. Following the procedures by Dourlent et al. [8], a rotatory polarizer was installed inbetween the monochromator and the cell.

The electric birefringence of the liposome solution was also measured by the same apparatus by setting an analyzer additionally between the cell and the photomultiplier. The polarizer and the analyzer were set at 45° and 135°, respectively, to the electric field.

Oxygen-binding by the liposomal heme was observed as follows. To the solution of the liposomal heme, a small excess of L-ascorbic acid (20-fold mol of iron(III)) was added under nitrogen atmosphere and the mixture was kept at room temperature for 2 h. The red solution thus prepared showed the ultraviolet and visible absorption spectrum with maxima at 438, 535 and 562 nm, which agreed with the pentacoordinate deoxyiron(II) complex of the liposomal heme [1]. By bubbling oxygen through the solution, the oxygen adduct (λ_{max} = 422, 546 nm) of the liposomal heme was obtained.

Results and Discussion

The structure of the liposomal hemes (sample 1–3) was first studied by ^{1}H-NMR measurement. Workers in this laboratory have previously estimated the radius of liposome by NMR spectrum shift with Eu^{3+} addition [9]. When Eu^{3+} is added to a liposome solution, the Eu^{3+} interacts with the choline group of the outward facing phospholipid and shifts the NMR signal of choline methyl group upfield. The same shift was observed for the liposomal heme with addition of Eu^{3+} from (a) to (d) in Fig. 1. This result supports the liposome formation for the liposomal heme. From the intensity ratio of the splitted signals assigned to the outward- and inward-facing choline groups, the radius of liposomal heme (sample 1) was calculated to be about 180 Å. The same measurement of sample 2 gave the radius of 290 Å. On the other hand, for sample 3 the choline methyl absorption at 3.2 ppm was observed as a broad singlet signal. Further-

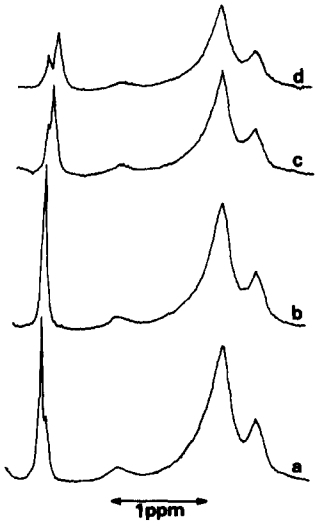

Fig. 1. ^{1}H-NMR spectra of the liposomal heme with added Eu^{3+}. [Eu^{3+}](mM) = a: 0, b: 2.5, c: 6.1, d: 8.7; [DMPC] = 44 mM; [heme] = 0.22 mM; [laurylmethylimidazole] = 11 mM.

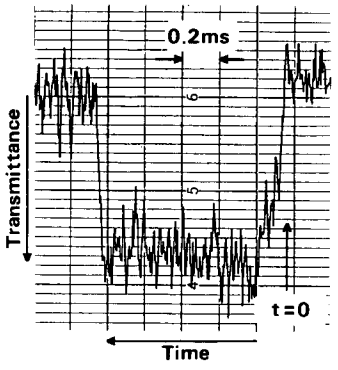

Fig. 2. Birefringence of the DMPC liposome solution at electric discharge. DMPC = 0.2 wt%.

more, the addition of Eu^{3+} to this sample solution caused the upfield shift of the choline methyl signal without splitting. This indicates that the liposomal heme (sample 3) has not a vesicle structure, but rather a lamellar structure.

Next electric birefringence of the liposome solution was measured to study the orientation of liposome under electric field. Fig. 2 shows a rapid increase of transmittance (with 10 μs) of the DMPC liposome solution at electric discharge, followed by a recovery to the original level. This transmittance change (ΔI) is represented by Eqn. 1.

$$I_d + \Delta I = I_0 \sin^2(-\delta/2 + \Delta\delta/2) \quad (1)$$

Here, I_d, δ and $\Delta\delta$ are the initial transmittance, the initial phase delay and change in the phase delay at the electric discharge, respectively. I_0 is transmittance when the polarizer and the analyzer are set in parallel. Substituting the data obtained in Eqn. 1 yielded $\Delta\delta = -0.35$ (rad). $\Delta\delta$ is defined as follows:

$$\Delta\delta = \frac{2\pi l}{\lambda}(n_{\parallel} - n_{\perp}) \quad (2)$$

where $n_{\parallel}$ and $n_{\perp}$ are the refractive indices in parallel and perpendicular to the electric field, respectively; λ and l are the wavelength of the incident light and the cell length (0.6 cm), respectively. Eqn. 2 yielded $n_{\parallel} - n_{\perp} = -3.1 \cdot 10^{-17}$. This means that the liposome solution has a larger refractive index in a perpendicular direction to the electric field than in a parallel direction. The refractive index of the phospholipid is larger than that of water. It is reasonable to assume that the longer axis of liposome is aligned in parallel to the electric field at the electric discharge. For polyelectrolytes such as poly(styrene sulfonate) and albumin, it is known that the macromolecule aligns to orient its longer axis in parallel to electric field [10,11]. It has been also reported by observing a giant liposome with a microscope [12] that the liposome is oriented under an electric field. These results are consistent with the result obtained above on the orientation of liposome in an electric field.

In order to obtain information on the heme embedded in liposome, the effect of electric discharge on the present system was investigated. When the incident light was polarized parallel with the direction of electric field ($\theta = 0°$, Fig. 3a), a rapid decrease in transmittance (within 50 μs) was observed. This was followed by a recovery to the original level. On the other hand, for incident light polarized vertical to the electric field ($\theta = 90°$, Fig. 3b), a rapid increase in transmittance occurred, followed by recovery to the original level. The direction and amplitude of the initial change of absorbance at $\theta = 0°$ and 90° obey the well-known formula for orientational electric dichroism,

$$\Delta A/A = (\rho/6)(1 + 3\cos 2\theta) \quad (3)$$

with $\rho > 0$ [8]. Here ΔA, A and ρ are the transient

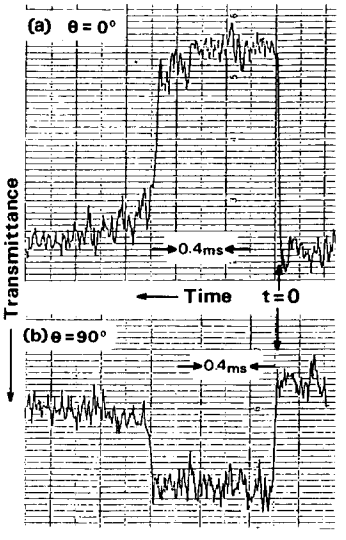

Fig. 3. Electric dichroism signals observed for the liposomal heme (sample 1).

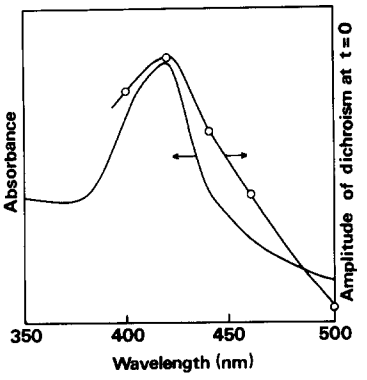

Fig. 4. Amplitude of dichroism in ultraviolet region.

absorbance change, initial absorbance, and reduced linear dichroism, respectively. The reduced linear dichroism is defined by $\rho = (\varepsilon_{\parallel} - \varepsilon_{\perp})/\varepsilon$, with ε the isotropic extinction coefficient in the absence of an electric field, $\varepsilon_{\parallel}$ the extinction coefficient with the light polarized parallel to electric field, and $\varepsilon_{\perp}$ the extinction coefficient for the perpendicular direction, respectively. Obedience to Eqn. 3 means that the electric dichroism observed is caused by orientation of chromophore in the liposomal heme system at electric discharge.

The transient signal at electric discharge was measured at various wavelengths, and the ΔA obtained is plotted in Fig. 4. This agrees with the absorption spectrum of the heme complex and not with that of the DMPC liposome. This result supports that the observed electric dichroism is based on the heme.

Fig. 5 shows the transient signals of samples 2 and 3 at electric discharge. These samples exhibit the same electric dichroism as sample 1. One notices in Fig. 5 that the transmittance change is followed by a slower recovery after electric discharge for samples 2 and 3. The decay rate decreases in the order of sample 1 to 3. This agrees with increasing order of the size of the liposomal heme previously mentioned. From Eqn. 3, ρ values at 7.5, 40 and 55 kV/cm electric fields are calculated for each sample. The extrapolations of the linear ρ vs. $1/E$ plots to $1/E = 0$ gave the ρ values at infinite electric field (ρ_{∞}). ρ values at 7.5 kV/cm electric field ($\rho_{7.5}$) and ρ_{∞} are shown in Table I.

The relation between electric field, E, and ρ is given as Eqn. 4 by supposing that the transition

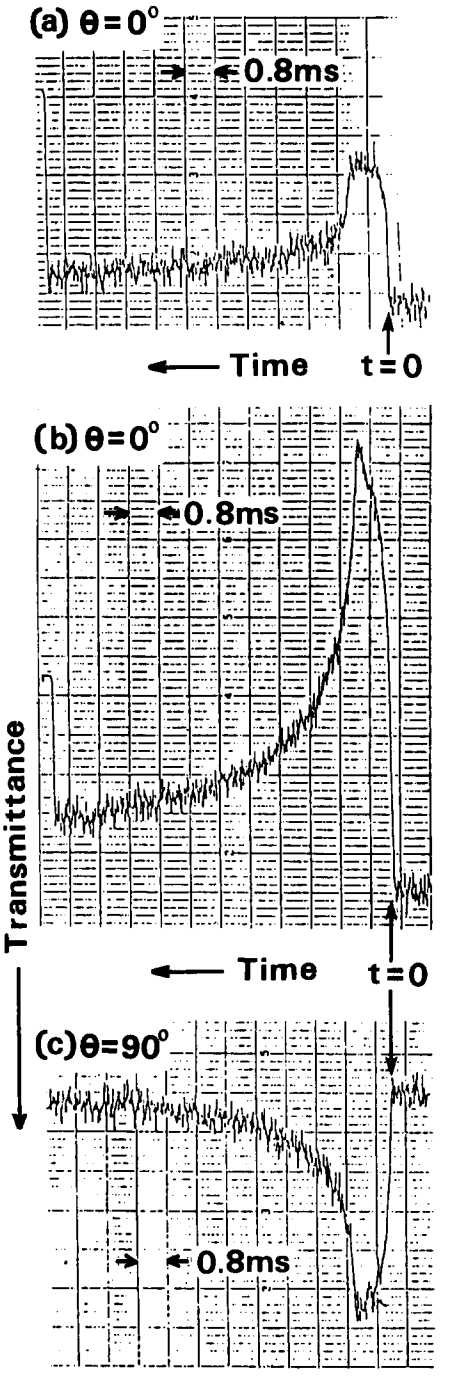

Fig. 5. Electric dichroism signals observed for the liposomal heme (sample 2 (a); sample 3 (b), (c)).

moment of heme at Soret band lies symmetrically in the heme plane.

$$\rho = (3/8)(3\cos 2\phi - 1)\cdot\psi(E) \qquad (4)$$

TABLE I

REDUCED LINEAR DICHROSIM (ρ) AND ORIENTING ANGLE OF HEME PLANE TO ELECTRIC FIELD (ϕ)

Sample:	1	2	3
$\rho_{7.5}$	+0.15	+0.15	+0.5
ρ_{∞}	0.32	0.25	> 0.6
$\psi(E)$	0.47	0.60	> 0.83
$\phi(°)$	26	28	< 15

where ϕ is orienting angle of the heme plane to electric field. $\psi(E)$ is the orientation fuction to represent the degree of orientation: The $\psi(E)$ values at 7.5 kV/cm electric field were calculated from $\rho_{7.5}/\rho_{\infty}$ and shown in Table I. ϕ is estimated from the ρ values and given in Table I. At least for sample 3, the heme plane is oriented parallel to electric field. The phospholipid bilayer of liposome being oriented parallel to electric field as mentioned above, it is reasonable to consider that the angle between the heme plane and the phospholipid bilayer (φ in Scheme I) is close to zero. It is assumed that the steric structure of the heme (four substituent groups build up on the heme plane) and lauryl group of the imidazole ligand set the heme plane in parallel to the phospholipid bilayer.

The liposomal deoxy-hemes were prepared from samples 1, 2 and 3. Immediately after bubbling oxygen through the solution, each liposomal deoxy-heme formed the oxygen adduct with a lifetime (half-life period) of approx. 3 h at 37°C. On the other hand, the corresponding deoxy-heme complex solubilized in water with cetylpyridinium bromide resulted in rapid irreversible oxidation upon exposure to oxygen gas. Reversible oxygen-binding in aqueous medium is efficient when the heme complex is situated in the phospholipid bibilayer with the orientation of the molecules.

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