Oxygen- and Carbon Monoxide-binding Affinity of the Liposomal Heme under Semiphysiological Conditions

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Oxygen- and carbon monoxide-binding affinity ($P_{1/2}$) of the [$\alpha,\alpha,\alpha,\alpha$-*meso*-tetrakis(*o*-pivalamidophenyl)-porphinato]iron(II)-mono(1-dodecyl-2-methylimidazole) complex incorporated in liposome of phosphatidylcholine (abbreviated as liposomal heme) were measured in pH 7.0 aqueous solution at 15—37 °C. The $P_{1/2}(O_2)$ and $P_{1/2}(CO)$ values at 37 °C were 49 and 0.02 mmHg,† respectively. Enthalpy and entropy changes for the reversible bindings were similar to those of hemoglobin. The relationship between temperature and $P_{1/2}$ for the dimyristoylphosphatidylcholine-liposomal heme had a breaking point at *ca.* 24 °C, which agreed with the phase transition temperature of the liposomal heme.

Much effort has been made to mimic natural oxygen carriers like hemoglobin by using modified synthetic iron-porphyrin derivatives.[1-6] Success was reported in oxygen-binding in aprotic and organic solvents but not in aqueous media.

The authors have recently found that the [$\alpha,\alpha,\alpha,\alpha$-*meso*-tetrakis(*o*-pivalamidophenyl)porphinato]iron(II) (abbreviated as heme) complex of mono(1-dodecyl-2-methylimidazole) incorporated in liposome of phosphatidylcholine (abbreviated as "liposomal heme") binds molecular oxygen reversibly under semiphysiological conditions (in pH 7.0 aqueous media at 37 °C).[7] It was considered that the iron porphyrin complex was embedded in a bilayer of liposome and that the hydrophobic environment of the inner region of liposome protected the oxygen adduct from its proton-driven oxidation.[8] The oxygen-binding and -dissociation proceeded very rapid, and their kinetic constants were comparable to those of hemoglobin (Hb).[9] Oxygen-binding affinity ($P_{1/2}(O_2)$: oxygen pressure at half oxygen-binding) was also preliminarily reported to be similar to that of Hb in blood.[10]

The present paper describes the oxygen- and carbon monoxide-binding affinity ($P_{1/2}(O_2)$ and $P_{1/2}(CO)$) for the liposomal heme composed of dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), and egg yolk lecithin (EYL) in pH 7.0 phosphate buffer solution at 15—37 °C. The binding-affinity was discussed in connection with the phase state of phospholipid, which was estimated by differential scanning calorimetry (DSC) and the fluorescence polarization method.

Experimental

Preparation of Liposomal Hemes. [$\alpha,\alpha,\alpha,\alpha$-*meso*-Tetrakis(*o*-pivalamidophenyl)porphinato]iron(III) bromide was prepared as reported.[11,12] 1-Dodecyl-2-methylimidazole (DMI) was prepared by the reaction of dodecyl bromide with 2-methylimidazole at 200 °C: bp 150 °C/1.5 mmHg; Found: C, 76.1; H, 12.9; N, 11.0%; C/N, 6.91, Calcd for $C_{16}H_{30}N_2$: C, 76.8; H, 12.0; N, 11.2%; C/N, 6.86; NMR ($CDCl_3$) δ=0.95 (3H, dodecyl-CH_3), 1.30—1.85 (20H, dodecyl-$(CH_2)_{10}$-), 2.30 (s, 3H, imidazole 2-CH_3), 3.90 (t, 2H, Im-CH_2-), 6.90 (d, 1H, imidazole 4 or 5-CH), 6.95 (d, 1H, imidazole 4 or 5-CH) ppm.

Dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) were purchased from Sigma Co. (special grade). Egg yolk lecithin (EYL) was obtained and purified by the reported method.[13]

The iron porphyrin (1 μmol), 1-dodecyl-2-methylimidazole (50 μmol) and phosphatidylcholine (200 μmol) were mixed in dichloromethane. By evaporating the solution under reduced pressure, a thin film was prepared on the glass wall of a round flask. Oxygen-free pH 7.0 M/15 phosphate buffer solution (20 cm^3) was added, and the mixture was ultrasonicated and homogenized in an ice-water bath. Then a small excess of L-ascorbic acid (20-fold mol of the iron(III)) was added to the solution under nitrogen atmosphere, and the mixture was incubated at room temperature for 2 h. The red transparent solution showed the ultraviolet (UV) and visible absorption spectrum with maxima at 438, 535, and 562 nm, which agreed with the pentacoordinate deoxy complex of the liposomal heme.[7]

Oxygen-binding Equilibrium Curve. An oxygen adduct (λ_{max}=422 and 546 nm) of the liposomal heme was formed by bubbling oxygen gas through the solution. The spectral change in visible region was measured with a spectrophotometer (Hitachi Co., UV-320) and the oxygen concentration in the solution was monitored with an oxygen probe (Yellow Springs Instrument Co., YSI 5331) at the same time, after equilibrium was reached. Equilibrium between the deoxy heme and the oxygen adduct was shown in Fig. 1 (a); the visible absorption spectrum changes through isosbestic points at 520, 536, 552, and 589 nm by bubbling oxygen and nitrogen gas. The percentage of oxygen-binding was calculated from the differential absorbance at 562 nm.

For reference, the red blood cell suspension was prepared by the method of Imai.[14] The oxygen-binding equilibrium curve of the red blood cell suspension was measured with the above-mentioned apparatus; it was consistent with the curve reported.[14,15] This result supports the validity of the measurements in the present experiment.

Carbon Monoxide-binding Equilibrium Curve. The carbon monoxide adduct (λ_{max}=423 and 540 nm) was formed by bubbling the mixture gas of carbon monoxide and nitrogen; the carbon monoxide concentration was determined by infrared spectroscopy. Equilibrium between the deoxy heme and the carbon monoxide adduct was shown in Fig. 1(b); the UV (Soret band) absorption spectrum was changed through isosbestic points at 403, 431, and 464 nm. The percentage of carbon monoxide-binding was calculated from the differential absorbance at 438 nm after equilibrium was reached.

Differential Scanning Calorimetry (DSC) Measurement. The liposome solution of DMPC(2.5 mmol dm^{-3}) and the DMPC-liposomal heme solution were prepared in the same manner as above. The DSC thermogram of these samples were measured with a differential scanning calorimeter (Seiko Co., SSC-560U).

† 1 mmHg≈133.3 Pa.

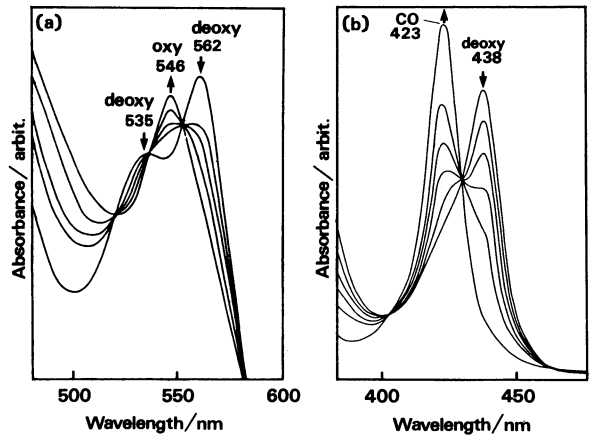


Fig. 1. Visible spectra of the DMPC-liposomal deoxy-heme and its oxygen adduct (a) and ultraviolet spectra of the DMPC-liposomal deoxy-heme and its carbon monoxide adduct (b) in pH 7.0 phosphate buffer solution at 37°C.
[heme]=0.05 mmol dm^{-3}. After bubbling oxygen: $P(O_2)$=0, 25, 50, 100, 760 mmHg (a); after bubbling carbon monoxide: $P(CO)$=0, 0.0034, 0.0071, 0.014, 0.034, 760 mmHg (b).

Fluorescence Measurement. Fluorescent compounds, 1,6-diphenyl-1,3,5-hexatriene (DPH) and 8-anilino-1-naphthalenesulfonic acid (ANS), were used as probes for monitoring fluidity in the liposome lipid layers. A thin film composed of heme (0.075 μmol), DMI (3.76 μmol), and DMPC (15 μmol) was prepared according to the above mentioned method. For labelling, DPH (0.05 μmol) or ANS (0.5 μmol) and phosphate buffer solution (5 cm^3) were added and the mixture was ultrasonicated and homogenized in an ice-water bath. In a similar manner, the fluorescent probe-labelled liposome of DMPC was prepared. Fluorescence intensity was measured by fluorescence polarimeter (Japan Spectroscopic Co., JASCO FP-550). The lights at 360 and 366 nm were used for excitation of liposome-incorporated DPH and ANS , respectively. Fluorescence intensities (I) were measured at 433 nm for DPH and 480 nm for ANS, respectively. Fluorescence polarization (P) was calculated by the equation: $P=(I_{\parallel}-I_{\perp})/(I_{\parallel}+I_{\perp})$, where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities polarized parallel and perpendicular to the direction of polarization of the excitation beam, respectively, and simultaneously measured.

Results and Discussion

The oxygen-binding equilibrium curve of the DMPC-liposomal heme was shown in Fig. 2. Deviation of the oxygen-dissociation curve from this oxygen-binding curve was within 5%; the former was obtained by measuring the oxygen-binding % from the oxygen adduct to the deoxy state by bubbling nitrogen gas and the latter from the deoxy state to the oxygen adduct by bubbling oxygen gas. Figure 2 shows that oxygen-binding equilibrium curve is hyperbolic like that of myoglobin (Mb). ($P_{1/2}(O_2)$) of the DMPC liposomal heme is 49 and 22 mmHg at 37 and 25°C, respectively. The ($P_{1/2}(O_2)$) value at 25°C is comparable with the previously reported ($P_{1/2}(O_2)$) value (=38 mmHg at 25°C) of the heme complex with 1,2-dimethylimidazole in toluene.[16,17] Figure 2 also means that the ($P_{1/2}(O_2)$) value of the liposomal heme in aqueous medium is close to $P_{1/2}$ (*ca.* 27 mmHg at 37°C) of Hb in blood[18] but is fairly far from that (0.9 mmHg at 37°C) of Mb.[19] This suggests that the liposomal heme has a potential to act as an oxygen carrier, under physiological conditions, which transports oxygen from the lung ($P(O_2)$=*ca.* 110 mmHg) to Mb in muscle tissue as Hb does.

The carbon monoxide-binding equilibrium curve (Fig. 3) of the DMPC-liposomal heme is also hyperbolic like that of Mb. The $P_{1/2}$ (CO) value is 0.020 and 0.0075 mmHg at 37 and 25°C, respectively. The latter value agrees with the $P_{1/2}$ (0.0089 mmHg at

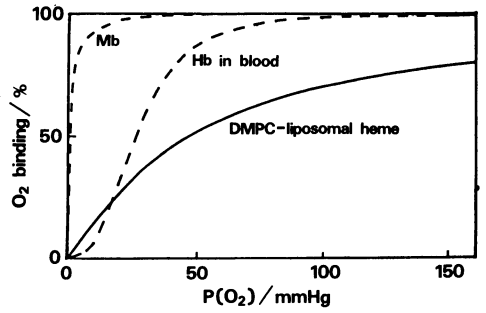


Fig. 2. Oxygen-binding equilibrium curve for the DMPC-liposomal heme in pH 7.0 phosphate buffer solution at 37°C.
[heme]=0.05 mmol dm^{-3}.

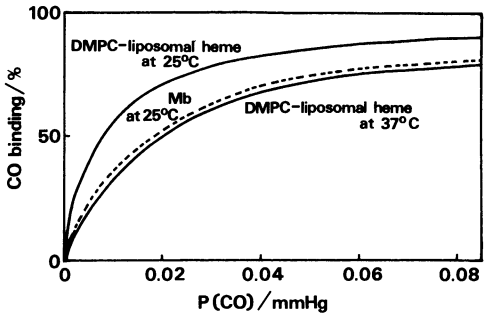


Fig. 3. Carbon monoxide-binding equilibrium curve for the DMPC-liposomal heme in pH 7.0 phosphate buffer solution.
[heme]=0.01 mmol dm^{-3}.

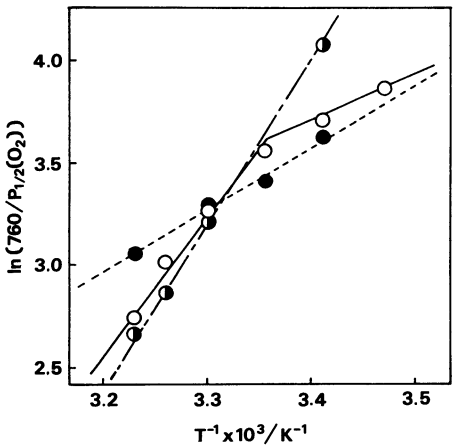


Fig. 4. van't Hoff plots for the oxygen-binding of the liposomal hemes in pH 7.0 phosphate buffer solution.
[heme]=0.05 mmol dm^{-3}. The liposomal heme composed of DMPC; ○, DPPC; ●, EYL; ◑.

TABLE 1. $P_{1/2}$ VALUES AND THERMODYNAMIC PARAMETERS

Iron porphyrin	O_2				CO			
	$P_{1/2}$		ΔH	ΔS	$P_{1/2}$		ΔH	ΔS
	mmHg[a]		kcal mol^{-1}	eu[b]	mmHg		kcal mol^{-1}	eu
	at 37 °C	at 25 °C			at 37 °C	at 25 °C		
Liposomal heme								
DMPC[c] (below T_c)	—	—	- 4.3	- 7.2	—	—	- 8.3	- 4.9
(above T_c)	49	22	-14	-41	0.020	0.0075	-17	-33
DPPC[c]	35	—	- 5.9	-14	—	—	—	—
EYL[c]	51	—	-16	-46	—	—	—	—
Heme-M_2I/Tol[d]	—	38	-14	-41	—	0.0089	—	—
Chelated heme[e]	—	—	-14	-35	—	—	-18	-34
Hemoglobin[f]	27	—	-14—-15	—	0.35	0.10—0.28	-17	—
Myoglobin[g]	0.9	—	-14—-21	—	—	0.012—0.028	—	—

a) 1 mmHg≈133.3 Pa. b) 1 eu=1 cal mol^{-1} K^{-1}. c) The liposomal heme composed of DMPC; dimyristoylphosphatidylcholine, DPPC; dipalmitoylphosphatidylcholine, EYL; egg yolk lecithin. d) [α,α,α,α-*meso*-tetrakis(*o*-pivalamidophenyl)porphinato]iron(II)-mono(1,2-dimethylimidazole) complex in toluene. Taken from Refs. 16, 17, 20. e) Iron(II)protoporphyrin *IX* *N*-[3-(1-imidazolyl)propyl]amide methyl ester in 2% aqueous myristyltrimethylammonium bromide suspension at pH 7.3. Taken from Ref. 23. f) Taken from Refs. 18, 19, 20. g) Taken from Refs. 19, 20.

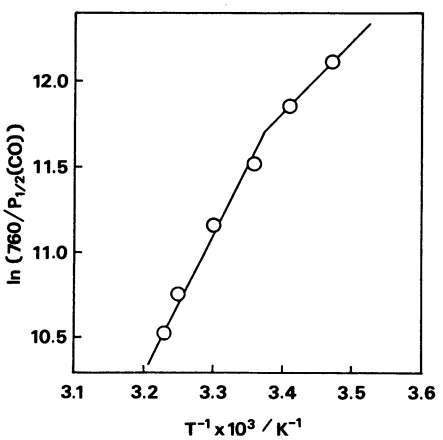


Fig. 5. van't Hoff plots for the carbon monoxide-binding of the DMPC-liposomal heme in pH 7:0 phosphate buffer solution.
[heme]=0.01 mmol dm^{-3}.

25 °C) of the corresponding heme complex in toluene.[20] These gaseous molecule-binding affinities of the liposomal heme suggest that the heme complex behaves as in toluene, although it is solubilized in the aqueous medium.

The oxygen-binding and -dissociation behaviors of the DPPC- and EYL-liposomal hemes were the same as that of the DMPC-liposomal heme except for the $P_{1/2}$ (O_2) of the DPPC-liposomal heme at 37 °C.

Temperature (T)-dependence of $P_{1/2}(O_2)$ and $P_{1/2}(CO)$, *i.e. van't Hoff plots*; $ln\ (760/P_{1/2})$ vs. $1/T$, were shown in Figs. 4 and 5. The van't Hoff plots for the oxygen binding of the DPPC- and EYL-liposomal heme give linear relationships. On the other hand, the temperature dependences of oxygen- and carbon monoxide-binding for the DMPC-liposomal heme have breaking points at *ca.* 24 °C. Enthalpy change (ΔH) and entropy change (ΔS) for the oxygen- and carbon monoxide-binding equilibrium were determined and were given in Table 1. $\Delta H(O_2)$ and $\Delta S(O_2)$ for the oxygen-binding of the EYL-liposomal heme and the DMPC-liposomal heme (above 24 °C) were estimated to be *ca.* −15 kcal mol^{-1} and *ca.* −45 eu†, respectively. These values are comparable to those of the corresponding heme complex in toluene[17] and those of Hb[19,21] and Mb.[19,22] They are also comparable to those of iron(II) protoporphyrin *IX* N-[3-(1-imidazoryl)propyl]amide, which was previously evaluated with kinetic methods in aqueous medium.[23] This result indicates that the oxygen- and carbon monoxide-binding by the EYL-liposomal heme and DMPC-liposomal heme (above 24 °C) proceed in the same way as do the bindings by Hb, Mb and iron porphyrin complexes.

On the contrary, both $\Delta H(O_2)$ and $\Delta S(O_2)$ values for the DPPC-liposomal heme and the DMPC-liposomal heme (below 24 °C) are much larger than those of the others (Table 1). The phospholipid environment gives a large effect on the oxygen-binding affinity of the heme through its enthalpy contribution. $\Delta H(CO)$ and $\Delta S(CO)$ for the DMPC-liposomal heme (below 24 °C) are also much larger than those above 24 °C. One can say that the gaseous molecule-binding affinity is relatively decreased for the DPPC-liposomal heme and the DMPC-liposomal heme (below 24 °C) because the enthalpy gain in the binding equilibrium is much reduced (*ca.* 10 kcal mol^{-1}).

The liposome of DPPC has its transition temperature (T_c) between gel and liquid crystal phase of phospholipid at 41 °C, and the liposome of EYL has it at −15—−7 °C.[24] It is reasonable to assume that the DPPC-liposomal heme is situated below its T_c and the EYL-liposomal heme is above its T_c under these experimental conditions. The liposome of DMPC has its T_c at 23 °C[24]; T_c of the DMPC-liposomal heme was measured as described below.

DSC thermograms are shown in Fig. 6. The DMPC-liposome gives T_c at 24 °C, which agrees with the literature datum.[24] For the DMPC-liposomal

† 1 eu=1 cal mol^{-1} K^{-1}.

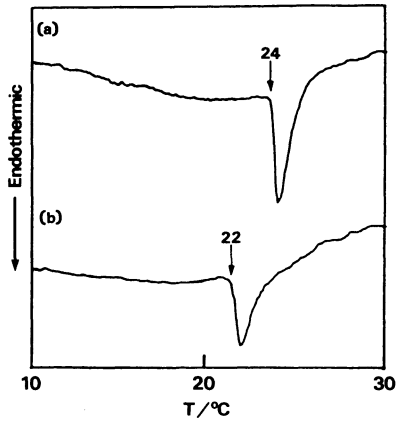


Fig. 6. DSC thermograms of the DMPC-liposome (a) and the DMPC-liposomal heme (b). [DMPC]=2.5 mmol dm^{-3}.

heme, the endothermic peak is brodened and shifted to the lower temperature at 22°C. This means that incorporation of the heme complex into the phospholipid bilayer causes partial disorder in the alkyl chain orientation of phospholipid molecules.

Fluorescence measurement on the liposomal heme was carried out to monitor fluidity in the liposome lipid layers by using DPH and ANS. DPH is known as the probe embedded in the hydrophobic region of the liposome,[25] and ANS is the probe in the hydrophilic region.[26] The reciprocal of the degree of fluorescence polarization ($1/P$) is a parameter to represent the fluidity in the liposome lipid layers; values are given in Fig. 7. Figure 7(a) shows T_c obserbed at 24°C for the DMPC-liposome and the DMPC-liposomal heme, and Fig. 7(b) shows values at 24°C for the former and at 21°C for the latter. From these data, one can say at least that the DMPC-liposomal heme has its T_c at *ca.* 24°C.

Thus T_c of the DMPC-liposomal heme agrees with the breaking point of the temperature dependence of oxygen- and carbon monoxide-binding by the DMPC-liposomal heme. The oxygen- and carbon

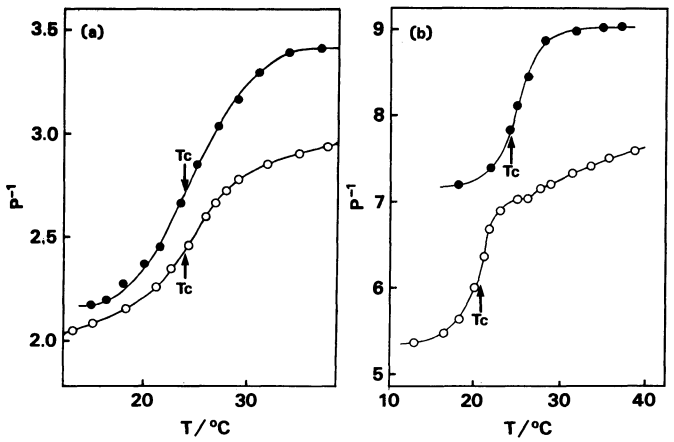
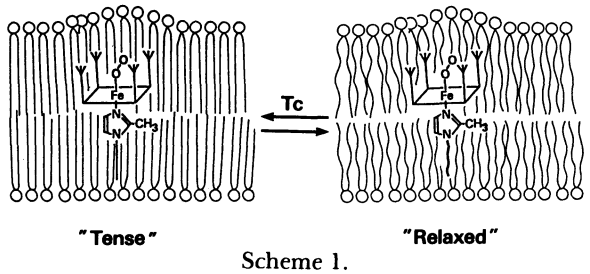


Fig. 7. Membrane fluidity of the DMPC-liposome and the DMPC-liposomal heme by using DPH (a) and ANS (b) in pH 7.0 phosphate buffer solution. [heme]=0.0015 mmol dm^{-3}. [DPH]=0.0015 mmol dm^{-3}. DPH; *Ex.* 366 nm, *Em.* 433 nm. [ANS]= 0.0010 mmol dm^{-3}. ANS; *Ex.* 360 nm, *Em.* 480 nm.



Scheme 1.

monoxide-binding affinity of the liposomal heme is dependent on the phase state of the phospholipid.

The solubility of oxygen in a DPPC-liposome has been previously reported;[27] it was slightly affected by the phase state of the phospholipid. Thus the dependence of gaseous molecule-binding affinity on T_c can not be explained only by the solubility of oxygen in the liposome phospholipid layer.

The gaseous molecule-binding affinity of the liposomal heme may be explained by using Scheme 1 as follows. Above T_c, phospholipid molecules are in the liquid crystal state, which provides an environment just like organic solvents such as toluene around heme; the heme complex is in a "*relaxed*" state, and the gaseous molecule-binding affinity and the ΔH values are comparable with those of the corresponding heme complex in toluene and with those of Hb and Mb. On the other hand, below T_c, phospholipid molecules are in the crystal state which causes an orientation of the DMI ligand; this is supported by the existence of the phase transition for the liposomal heme as well as the liposome without the heme complex (see the results by the DSC and fluorescence measurements). The orientation of the DMI ligand probably causes a structural distortion of the heme complex, because the bulky heme molecule is embedded in the phospholipid-DMI bilayer in the crystal state. The heme complex is in a "*tense*" state, and this may be one of the reasons for the low oxygen-binding affinity of the DMPC-liposomal heme (below T_c) and the DPPC-liposomal heme. The "*tense*" and "*relaxed*" states of the heme complex for the liposomal heme are not the same ones as the tense(T)- and relaxed(R)-states for Hb; The latter are caused by globin protein. But the gaseous molecule-binding phenomenon of the liposomal heme resembles that of Hb.

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