Rate Parameters for Oxygen and Carbon Monoxide Binding to a Liposome-embedded Heme under Physiological Conditions†

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Rate parameters for oxygen and carbon monoxide binding to a liposome-embedded heme were measured under physiological conditions (pH 7.0 at 37 °C) by stopped-flow spectrophotometry. The rate constants were similar to those of red blood cells in suspension. It is concluded that liposome-embedded heme is a suitable model to study the binding of gaseous molecules to red blood cells.

Hemoproteins, hemoglobin (hb) and myoglobin (mb), which are composed of iron porphyrin complexes and globin proteins, serve to transport and to store molecular oxygen in a living body. Rate parameters for binding of oxygen and CO to the iron porphyrin of hb and mb [equations (1) and (2); L = porphyrinate(2−)] have been well studied,[1-3] as the determination of these parameters is directly relevant to elucidation of the mechanisms of oxygen uptake and release by naturally occurring hemoproteins. In particular, the parameters for red blood cells in suspension[4,5] and overall blood[6,7] yield much information on the oxygen binding in a living body.

$$Fe^{II}L + O_2 \underset{k(O_2)_{off}}{\overset{k(O_2)_{on}}{\rightleftharpoons}} LFe^{II}\text{–}O_2 \qquad K(O_2) = k(O_2)_{on}/k(O_2)_{off} \quad (1)$$

$$Fe^{II}L + CO \underset{k(CO)_{off}}{\overset{k(CO)_{on}}{\rightleftharpoons}} LFe^{II}\text{–}CO \qquad K(CO) = k(CO)_{on}/k(CO)_{off} \quad (2)$$

Recently various iron porphyrin derivatives with oxygen-binding ability have been synthesized in order to mimic hb and mb,[8-10] and the oxygen-binding rate parameters for these hb model compounds have been reported.[11-13] But these parameters were measured using flash photolysis in organic solvents or in aqueous media protected with CO, because the synthetic iron porphyrin derivatives can bind oxygen reversibly only in organic solvents or in the solid state.

We recently found that the [*meso*-5,10,15,20-tetra(*o*-pivalamidophenyl)porphyrinato]iron(II) (heme) complex with 1-dodecyl-2-methylimidazole incorporated into the liposome of phosphatidylcholine (abbreviated as 'liposome-embedded heme') bound molecular oxygen reversibly under physiological conditions (in aqueous media, pH 7.0, at 37 °C).[14,15] The heme complex was considered to be embedded in a bilayer of liposome and the hydrophobic environment of the liposome protected the oxygen adduct from its irreversible oxidation.[15,16] The lifetime of the oxygen adduct for the liposome-embedded heme was *ca.* half a day and oxygen-binding affinity was similar to that of red blood cells.[15] Rapid oxygen binding was measured by using flash-photolysis and stopped-flow methods and the rate parameters for oxygen binding and dissociation for the liposome-embedded heme were briefly reported.[17,18]

In the present study rate parameters for oxygen and CO binding of the liposome-embedded heme were estimated by the stopped-flow method, and the oxygen- and CO-binding reactions of the heme, which is protected by being incorporated into a liposome, are discussed in comparison with those for red blood cells in which hb is encapsulated in a cell membrane.

† *Non-S.I. unit employed:* mmHg ~ 133 Pa.

Experimental

The liposome-embedded heme was prepared as described previously.[14,15] Dimyristoylphosphatidylcholine (dmpc) and dipalmitoylphosphatidylcholine (dppc) were used as phospholipids. The lifetime (half-life period, τ) of the oxygen adduct of the liposome-embedded heme was *ca.* half a day, and the oxygen-binding affinity, $P_{\frac{1}{2}}$ (oxygen pressure at 50% oxygen binding for the heme), was *ca.* 50 mmHg at pH 7.0 and 37 °C.

The burst type of the liposome-embedded heme was prepared as follows. The liposome-embedded heme in 5.0-fold (five times that of measuring sample: [heme] = 1×10^{-5} mol dm^{-3}) concentration [0.067 mol dm^{-3} (pH 7.0) phosphate buffer solution] was prepared in a manner similar to the above sample[14,15] and then diluted in water (1:5). The liposome (vesicle) was expected to burst by osmotic pressure and to have a lamellar structure. The structure of the burst dmpc-liposome-embedded heme was confirmed by the following ^{1}H n.m.r. method. When Eu^{3+} is added to a liposome solution, it interacts only with the choline groups of the outward-facing phospholipid and the n.m.r. signal of the choline methyl group is split.[15] For the burst sample (D_2O solution) the addition of Eu^{3+} caused an upfield shift of the choline methyl signal without splitting. This indicates that this liposome-embedded heme does not possess a vesicle structure but a lamellar structure.

Red blood cell suspension was prepared according to a literature method.[19]

The oxygen- and CO-binding reactions were studied using a stopped-flow spectrophotometer equipped with a kinetic data processor (Union Giken RA-401 and RA-601).

Results and Discussion

The oxygen adduct of the liposome-embedded heme was so stable that the stopped-flow study of the oxygen binding could be carried out without the need for CO protection. After rapid mixing of the liposome-embedded heme with the buffer solution saturated with oxygen, the spectrum ($\lambda_{max.}$ 438 nm) of the deoxy-heme changed to one having a maximum at 422 nm assigned to the oxygen adduct, as shown in Figure 1. On bubbling CO gas through the solution the spectrum again changed to that of the CO adduct ($\lambda_{max.}$ 423 nm), which supports the formation of the oxygen adduct.

By changing the monitoring wavelength from 390 to 460 nm, a difference spectrum before and after the rapid mixing could be obtained (Figure 2). This was compatible with the difference between the absorbance of the liposome-embedded deoxy-heme and that of the oxygen adduct. The negative and positive extremes in the difference spectrum, 422 and 438 nm, were

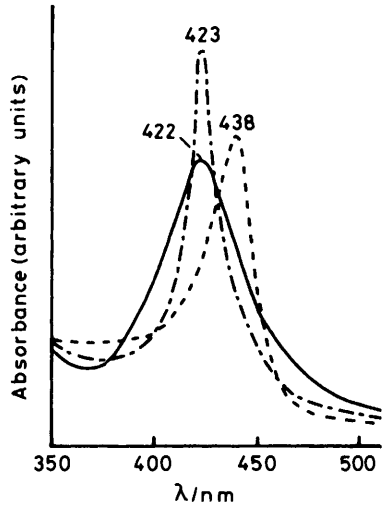

Figure 1. Spectra before (- - - -) and after (——) rapid mixing, and on bubbling carbon monoxide through the mixed solution (- · - · -) at pH 7.0 and 25 °C. [heme] = 1×10^{-5} mol dm ^{3}. Ratio of heme:1-dodecyl-2-methylimidazole:dmpc = 1 : 50:200

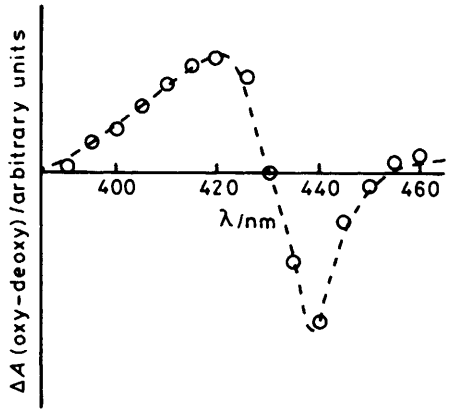

Figure 2. Difference spectrum before and after rapid mixing (○) and that between the liposome-embedded deoxy-heme and its oxygen adduct (- - - -) at pH 7.0 and 25 °C. Conditions as in Figure 1

selected as the monitoring wavelengths thereafter. These wavelengths are in accord with the absorption maxima of the oxygen adduct and deoxy-heme, respectively (Figure 2). The validity of this is supported further by the following two results. (*i*) The absorbance at 430 nm remained constant before and after the rapid mixing [Figure 3(*a*)]; this wavelength is the same as that of the isosbestic point between the spectrum of the deoxy-heme and that of the oxygen adduct. (*ii*) The time courses at 442 and 438 nm were symmetric [Figure 3(*a*)]. For the CO binding, the reactions were followed at 423 and 400 nm. The reproducibility of the measurements was confirmed by using several batches of the sample.

The time curves for the oxygen and CO binding of the liposome-embedded heme were that of a multi-phase system, because the heme complex was heterogeneously dissolved with the liposome in the aqueous medium. However, the curve could be approximated by one of phasic kinetics $f(x) = \ln[1 - (x/a)]$ where a is a constant, within the experimental error [Figure 3(*b*)]. Rate parameters for the oxygen and CO binding and dissociation were determined by first-order kinetics.[2]

Oxygen-binding and -dissociation rate constants $k(O_2)_{on}$ and $k(O_2)_{off}$ are summarized in Table 1. The $k(O_2)_{on}$ values of the

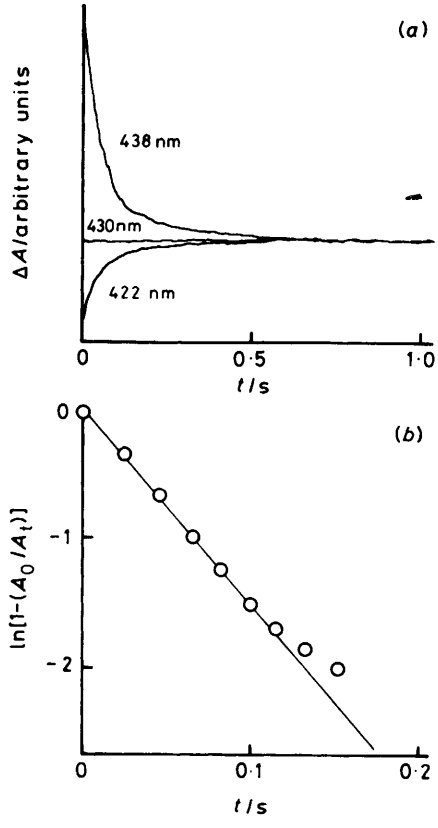

Figure 3. Oxygen binding of the liposome-embedded heme monitored at 422, 430, and 438 nm (*a*), and the approximation to monophasic kinetics (*b*). ΔA = Differential absorbance; A_0 and A_t = differential absorbance at 438 nm at time 0 and t, respectively

dmpc-liposome-embedded heme agreed with that determined by the flash-photolysis method.[17] The $k(O_2)_{off}$ values were compatible with that estimated from the reaction in which the oxygen adduct solution was mixed rapidly with an argon-saturated solution and in which the reverse reaction from the oxygen adduct to the deoxy-heme took place. The $P_{\frac{1}{2}}$ value calculated from the K ($= k_{on}/k_{off}$) value was consistent with that determined from the oxygen-binding equilibrium curve.[15] These results support the validity of this kinetic treatment. The k_{on} values of the liposome-embedded heme are similar to that of a red blood cell suspension. This means that the oxygen-binding reactions for the liposome-embedded heme and the red blood cell suspension show the same characteristics.

The oxygen-binding rate parameters given in the literature for homogeneous systems, [*meso*-5,10,15,20-tetra(*o*-pivalamidophenyl)porphyrinato]iron(II) with 1-dodecyl-2-methylimidazole in toluene,[13] chelated heme in pH 7.3,[20] mb,[21] and stripped hb,[21,22] are also listed in Table 1 and they are about 10^3 times larger than those of the liposome-embedded heme and the red blood cell suspension. It is thought that the oxygen-binding reaction is retarded by the need for oxygen to diffuse in and through the phospholipid membrane.

The oxygen-binding rate was larger for the dmpc- than for the dppc-liposome-embedded heme. This may be explained as follows. The dppc-liposome is below its T_c (gel–liquid crystal phase transition temperature: 41 °C) and the dmpc-liposome is above (23 °C)[23] under these experimental conditions (at 25 °C), so that the membrane fluidity, probably also the oxygen permeability, of the dmpc-liposome is larger than that of dppc. The largest oxygen-binding rate was observed for the burst dmpc-liposome-embedded heme. It is assumed that this liposome has

Table 1. Oxygen-binding rate parameters for liposome-embedded heme at 25 °C

Heme	Solvent	Measurement method[a]	$k(O_2)_{on}$ / dm^3 mol^{-1} s^{-1}	$k(O_2)_{off}$ / s^{-1}	$K(O_2)$ / dm^3 mol^{-1}	$P_{\frac{1}{2}}(O_2)$[b] / mmHg	$P_{\frac{1}{2}}(O_2)^*$[b] / mmHg
Liposome-embedded heme							
dmpc	pH 7.0[c]	s	7.9×10^3	0.32	2.5×10^4	24	22
dmpc	pH 7.0[c]	f	5.4×10^3	1.6			
dppc	pH 7.0[c]	s	2.4×10^3	0.11	2.2×10^4	27	25
Burst dmpc[d]	pH 7.4[d]	s	1.2×10^4	0.29	4.2×10^4	14	
Red blood cell suspension[e]	pH 7.4[e]	s	1.1×10^4	0.16	6.8×10^4	8.8	10
f	Toluene	f	1.1×10^8	46 000	2.3×10^3	262	38
Chelated heme[g]	pH 7.3[g]	f	2.6×10^7	47	5.5×10^5	1.0	
Myoglobin[h]	pH 7.0—7.4	f, s	$(1.0—2.0) \times 10^7$	10—30	$(0.67—1.0) \times 10^6$	0.55—0.82	0.50—1.0
Stripped hemoglobin[i]	pH 7.0—7.4	f, s	3.3×10^7	12—13	$(2.5—2.7) \times 10^6$	0.20—0.22	0.22—0.36

[a] s = Stopped-flow method, f = flash-photolysis method. [b] Calculated from K [$= k(O_2)_{on}/k(O_2)_{off}$] and $P_{\frac{1}{2}}(O_2)^*$ values determined from the oxygen-binding equilibrium curves. [c] Phosphate buffer solution. [d] The liposome-embedded heme in higher concentration was diluted in water (1:5). The liposome is expected to be burst by osmotic pressure and have a lamellar structure. [e] The red blood cell suspension was as in ref. 19. [f] [*meso*-5,10,15,20-tetra(*o*-pivalamidophenyl)porphyrinato]iron(II) complex of 1-dodecyl-2-methylimidazole, data from ref. 13 and J. P. Collman, J. I. Brauman, K. M. Doxsee, T. R. Halbert, and K. S. Suslick, *Proc. Natl. Acad. Sci. USA*, 1978, **75**, 564. [g] From ref. 20, chelated-heme (pH 7.3) = protoheme *N*-(3-imidazol-1-ylpropyl)amide methyl ester solubilized by trimethyl(tetradecyl)ammonium bromide at 20 °C. [h] From ref. 21, at 20 °C. [i] From refs. 21 and 22, at 20 °C.

The oxygen binding in organic solvents (toluene and benzene) was so fast that the rate parameters, $k(O_2)_{on}$ and $k(O_2)_{off}$ could not be measured with this stopped-flow apparatus.

Table 2. Carbon monoxide-binding rate parameters for liposome-embedded heme at 25 °C

Heme	Solvent	Measurement method	$k(CO)_{on}$ / dm^3 mol^{-1} s^{-1}	$P_{\frac{1}{2}}(CO)^*$[a] / mmHg
Liposome-embedded heme				
dmpc	pH 7.0[b]	s	2.8×10^5	0.0075
dmpc	pH 7.0[b]	f	6.9×10^6	
Burst dmpc	pH 7.4[c]	s	6.3×10^5	
Red blood cell suspension[c]	pH 7.4[c]	s	1.4×10^4	
d	Toluene	f	1.4×10^6	0.0089
Chelated heme[c]	pH 7.3[c]	f	3.6×10^6	
Myoglobin[e]	pH 7.0—7.4	f, s	$(3.0—5.0) \times 10^5$	0.012—0.028
Stripped hemoglobin[f]	pH 7.0—7.4	f, s	4.6×10^6	0.0010—0.0040

[a] Determined from the carbon monoxide-binding equilibrium curves. [b] Phosphate buffer solution. [c] See Table 1. [d] See footnote *f* in Table 1 and J. P. Collman, J. I. Brauman, and K. M. Doxsee, *Proc. Natl. Acad. Sci. USA*, 1979, **76**, 6035. [e] From ref. 21, at 20 °C. [f] From refs. 21 and 24, at 20 °C.

a lamellar structure or a non-closed (non-vesicle) bilayer structure, and this is advantageous for the supply of oxygen to the heme.

The CO-binding and -dissociation rate constants $k(CO)_{on}$ and $k(CO)_{off}$ are summarized in Table 2. The $k(CO)_{on}$ value of the dmpc-liposome-embedded hemes is close to that of the red blood cell suspension and distant from those given in the literature for homogeneous systems. It seems that the CO-binding reaction is also retarded by the diffusion of CO in and through the phospholipid membrane. The CO-binding rate was larger for the burst dmpc-liposome-embedded heme. This also supports the assumption that the CO-binding rate is influenced by the gas diffusion. However, in Table 2 the $k(CO)_{on}$ value of the dmpc-liposome-embedded heme differs from that determined by the flash-photolysis method[17] while the latter coincides with those for the homogeneous systems. The same kinetic behaviour has been reported for red blood cells,[24,25] *i.e.* the $k(CO)_{on}$ value determined by flash photolysis was much larger than that by the stopped-flow method, whereas there was no difference in the $k(O_2)_{on}$ values determined by these methods. Though the further discussion on this result is not allowed here, one can say that the gaseous molecule-binding property of the liposome-embedded heme is governed by the same feature as for red blood cells.

The oxygen-binding profile of red blood cells has been studied in detail by Roughton[4] taking the diffusion of oxygen into account. The overall oxygen-binding rate constant for red blood cells (k_h) was expressed as in equation (3), where k is the

$$k_h = (kDc_0)^{\frac{1}{2}}/a \quad (3)$$

true oxygen-binding rate constant of stripped hb, D is the diffusion constant, c_0 is the concentration of hb in the red blood cell, and a is the average half-thickness of the red blood cell. For the liposome-embedded heme the k_h value could be estimated from previous data: $a = 400$ Å,[15] $k = (1.0—2.0) \times 10^7$ dm^3 mol^{-1} s^{-1},[21] $D = (1.8—1.0) \times 10^{-5}$ cm^2 s^{-1},[26,27] and $c_0 = 1 \times 10^{-5}$ mol dm^{-3}. Substituting these values into equation (3) yielded $k_h = 8.8 \times 10^3$ dm^3 mol^{-1} s^{-1} for the liposome-embedded heme. This value is similar to $k(O_2)_{on}$ determined in this experiment (Table 1). The liposome-embedded heme is clearly a suitable model with which to study the mechanism of oxygen uptake and release by red blood cells.

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