Reversible Oxygen Binding by the Complex of Poly(2-methyl-1-vinylimidazole) and Heme in Cold Aqueous Media †

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Poly(2-methyl-1-vinylimidazole), molecular weight $>10^4$, forms a stable five-co-ordinated complex with heme in aqueous ethylene glycol solution. This complex gave an oxygen adduct at −30 °C, whereas an irreversible oxidation was observed for low-molecular-weight heme complexes with 2-methylimidazoles and for the six-co-ordinated heme complex with poly(1-vinylimidazole). Oxygenation was also observed for heme complexes with copolymers of 2-methyl-1-vinylimidazole but not for those with ionic copolymers. The lifetime of the oxygen adduct decreased with increasing hydrogen-ion concentration and upon adding alcohol or urea. Oxygen-binding and -dissociation rate constants and oxygen-binding equilibrium constants were also measured in cold aqueous media.

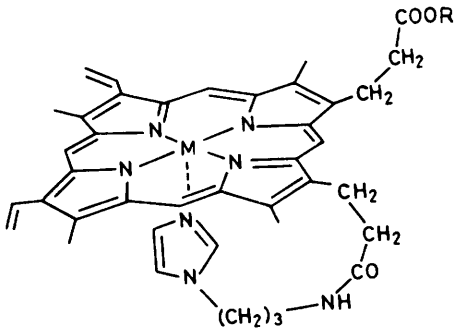
The synthesis of iron–porphyrin complexes which reversibly bind molecular oxygen is of great interest. Two approaches have been successful: one involves the steric modification of porphyrin, *e.g.* picket fence iron–porphyrin,[1] chelated protoheme,[2] doubly bridged iron–porphyrin,[3] *etc.*;[4-6] the other involves linkage of an iron–porphyrin complex to a polymer chain.[7-10] By these means, reversible oxygen binding has been achieved in aprotic solvents or the solid state, but in aqueous media the iron–porphyrin derivatives are irreversibly oxidized.

It is very important for oxygen binding in aqueous media that the iron–porphyrin complex adopts a five-co-ordinated structure in which the sixth co-ordination site is vacant and thus available to bind molecular oxygen, and that the oxygen adduct is surrounded by a hydrophobic environment. We have found, and preliminarily reported,[11] that poly(2-methyl-1-vinylimidazole) (pmvi) predominantly forms a five-co-ordinated complex with iron(II) protoporphyrin IX (heme)‡ and that this polymer–heme complex gives the oxygen adduct even in aqueous solution. This paper describes the co-ordinating properties and the oxygen-binding ability of the pmvi–heme complexes in an aqueous ethylene glycol (1 : 1, v/v) solution at 0 to −30 °C.

Experimental

The polymer pmvi and the copolymers of 2-methyl-1-vinylimidazole were obtained by radical polymerization with 2,2′-azobisisobutyronitrile. The molecular weights and compositions of the polymers were determined by vapour-pressure osmometry and elemental analysis: pmvi, $10^{-4}M$ = 2.1, 3.2, 4.8, and 8.1; poly(2-methyl-1-vinylimidazole–1-vinyl-2-pyrrolidone), content of 2-methyl-1-vinylimidazole residue ($10^{-4}M$) = 74 mol % (5.9), 41 (4.5), and 31 (4.8); poly(2-methyl-1-vinylimidazole–acrylamide), 77 mol % (4.8), 45 (5.2), and 10 (5.6); poly(2-methyl-1-vinylimidazole–methacrylic acid), 44 mol % (4.8); poly(2-methyl-1-vinylimidazole–3-benzyl-2-methyl-1-vinylimidazolium chloride), 60 mol % (7.3). Poly(1-vinylimidazole) ($10^{-4}M$ = 5.3) was also prepared for comparison with pmvi. These polymers were soluble in water, at least to *ca.* 5 wt. %. Imidazole, 1-ethylimidazole, 2-methylimidazole, and 1-isopropyl-2-methylimidazole were used as analogues of low molecular weight.

Iron(III) protoporphyrin IX chloride (hemin) and iron(III)



R = Me, M = Fe^{II}: chelated protoheme
R = Me, M = Fe^{III}: chelated protohemin

deuteroporphyrin chloride (deuterohemin) were purified according to Fischer's method.[12] Chelated protohemin was synthesized according to Traylor's method.[2] The aqueous medium was an oxygen-free mixture of a buffer solution (pH 10, 0.2 mol dm^{-3} Na_2CO_3–$NaHCO_3$) with ethylene glycol (1 : 1, v/v). Ethylene glycol was added as a dispersion agent for heme, which aggregates in aqueous solution,[13] and as an anti-freeze agent. The heme complex solution was prepared by adding sodium dithionite to hemin solution ([$Na_2S_2O_4$] : [hemin] = 5 : 1) under a nitrogen atmosphere. The reduction of hemin to heme was also carried out with ascorbic acid, glucose, or reductase. The last was prepared according to the literature.[14]

Flash photolysis and stopped-flow measurements were performed by the use of a pulse flash spectrophotometer (Union Giken RA403) and a stopped-flow spectrophotometer (Union Giken RA401) equipped with a kinetic data processor. Rate constants for the oxygen and carbon monoxide binding and dissociation were determined under pseudo-first-order conditions.[15] The oxygen- and carbon monoxide-binding equilibrium curves were constructed from spectrophotometric measurements taken upon bubbling the gases oxygen and carbon monoxide premixed with nitrogen.

Results and Discussion

Co-ordination Properties of the Poly(2-methyl-1-vinylimidazole)–Heme Complexes.—The visible absorption spectrum of the heme complex with pmvi in aqueous solution was characterized by a single peak at 557 nm (see Figure 1 and Table 1), assigned to a five-co-ordinated complex.[16,17] The apparent formation constant ($K_{app.}$) of the heme complexes with 2-methylimidazole ligands was estimated by spectroscopic

† *Non-S.I. unit employed:* Torr = (101 325/760) Pa.
‡ Protoporphyrin IX = 3,7,12,17-tetramethyl-8,13-divinylporphyrin-2,18-dipropionic acid.

Table 1. Ultraviolet and visible absorption maxima of the heme complexes and of their CO and O_2 adducts (−30 °C)

Ligand	$\lambda_{max.}$/nm deoxy	CO adduct	O_2 adduct
Imidazole, 1-ethylimidazole, poly(1-vinylimidazole)	426, 528, 558	418, 538, 566	(Oxidized) [a]
2-Methylimidazole, 1-isopropyl-2-methylimidazole	430, 557	418, 538, 566	(Oxidized) [a]
pmvi, poly(2-methyl-1-vinylimidazole–1-vinyl-2-pyrrolidone), poly(2-methyl-1-vinylimidazole–acrylamide)	432, 557	418, 538, 566	410, 545, 577
pmvi [b]	424, 545	401, 534, 566	409, 528, 556
Chelated protoheme [c]	422, 530, 558	418, 538, 566	(Oxidized) [a]
Hemoglobin [d]	430, 556	418, 539, 570	414, 542, 578

[heme] = 0.08 mmol dm^{-3}, [imidazoles] = [2-methylimidazoles] = 0.4 mol dm^{-3}, concentration of imidazole or 2-methylimidazole unit of polymers = 40 mmol dm^{-3}; pH 10 buffer solution–ethylene glycol 1 : 1, v/v. [a] Oxidized complex = hemin. [b] Deuteroheme complex. [c] Acid derivative of chelated protoheme: see structure on title page, R = H, M = Fe^{II}. [d] At 25 °C.

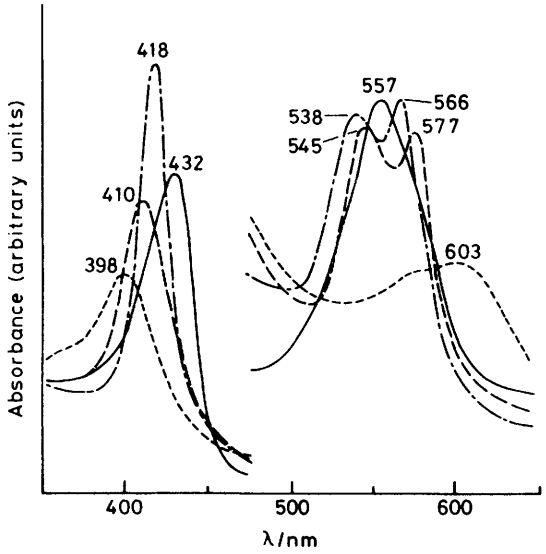


Figure 1. Ultraviolet and visible spectra of the pmvi–heme complex: (———) deoxy complex; (— — —) O_2 adduct; (— · —) CO adduct; (- - -): oxidized complex (hemin). [heme] = 0.08 mmol dm^{-3}, concentration of 2-methylimidazole unit of the polymer = 40 mmol dm^{-3}; pH 10 buffer solution–ethylene glycol (1 : 1, v/v), at − 30 °C

titration.[18] The values at 0 °C for the heme complexes with pmvi ($10^{-4}M$ = 3.2) and poly(2-methyl-1-vinylimidazole–1-vinyl-2-pyrrolidone) (content of 2-methyl-1-vinylimidazole = 41 mol %, $10^{-4}M$ = 4.5) were 2.5 × 10^3 and 6.1 × 10^3 dm^3 mol^{-1} respectively. These values were about 10^2 times those with monomeric ligands, *e.g.* K_{app} for the heme complex with 2-methylimidazole is 41 dm^3 mol^{-1} and with 1-isopropyl-2-methylimidazole is 9.6 dm^3 mol^{-1}. The polymer pmvi predominantly forms a five-co-ordinated complex with heme in aqueous solution, as is also supported by the following result.

It is well known that heme simultaneously binds two nitrogen-containing ligands in aqueous solution. For example, the imidazole–heme complex ([heme] = 0.08 mmol dm^{-3}, [imidazole]/[heme] = 5 × 10^3) shows a visible absorption spectrum with a double peak at 528 and 558 nm assigned to a six-co-ordinated complex. When a small amount of pmvi ([2-methyl-1-vinylimidazole residue]/[heme] = 10^2) was added to this system the spectrum changed to a single peak at 557 nm corresponding to a five-co-ordinated complex. However, when excess of 2-methylimidazole ([2-methylimidazole]/[heme] = 5 × 10^4) was added the spectrum of the six-co-ordinated imidazole–heme complex was not changed. This result indicates that pmvi selectively transforms the stable low-spin imidazole–heme complex into the five-co-ordinated high-spin heme complex. The polymeric pmvi forms the five-co-ordinated heme complex, even in the presence of a large amount of imidazole.

Oxygen Adducts of the Poly(2-methyl-1-vinylimidazole)–Heme Complexes.—The six-co-ordinated heme complexes of imidazole and 1-ethylimidazole are known to be immediately oxidized upon exposure to oxygen. Irreversible oxidation was also observed for the five-co-ordinated heme complexes of 2-methylimidazoles and for the polymeric six-co-ordinated heme complex of poly(1-vinylimidazole) at 30 to −30 °C (Table 1). However, the polymeric five-co-ordinated pmvi–heme complex shows a spectrum (410, 545, and 577 nm, Figure 1) which resembles that of oxyhemoglobin (414, 542, and 578 nm)[19] when its aqueous solution is cooled to −10 to −30 °C and exposed to oxygen. The spectrum of the oxygen adduct changed to that of the heme–CO complex upon bubbling carbon monoxide through the solution and returned to the deoxy-complex after careful introduction of nitrogen gas. This indicates that the oxidation number of the central iron ion (Fe^{II}) was not changed during the exposure to oxygen.

The lack of any contribution of the reducing agent to this reversible oxygenation was confirmed by the following results. (*i*) The heme complex was prepared by reducing the hemin complex with a small excess of sodium dithionite, no trace of which remained before the oxygen exposure. (*ii*) When dithionite was added after the oxygenation, the oxyheme was reduced to the deoxyheme in the same manner as was oxyhemoglobin. (*iii*) The porphyrin ring of heme was degraded and no oxygenation was observed in the presence of a large excess of dithionite. (*iv*) Organic reductants, such as ascorbic acid and glucose, were also effective reducing agents for the formation of the oxygen adduct. (*v*) Enzymatically reduced pmvi–heme complex also gave the oxygen adduct with the same visible absorption spectrum.

The spectroscopic data of the heme complexes in cooled aqueous solution are summarized in Table 1. Oxygenation was also observed with the deuteroheme complex of pmvi. Only the five-co-ordinated heme complex with the polymeric ligand forms an oxygen adduct in the cold aqueous solution. Reversible oxygenation of heme complexes in organic solvents at low temperature has already been reported, but in aqueous media all the heme derivatives were irreversibly oxidized even at low temperature, except in cooled aqueous dimethylformamide [water–dimethylformamide (3 : 7, v/v)].[16,20,21] The pmvi–heme complex contrasts with the low-molecular-weight heme complexes of 2-methylimidazoles in that it is oxygenated whereas the latter ([2-methylimidazole]/

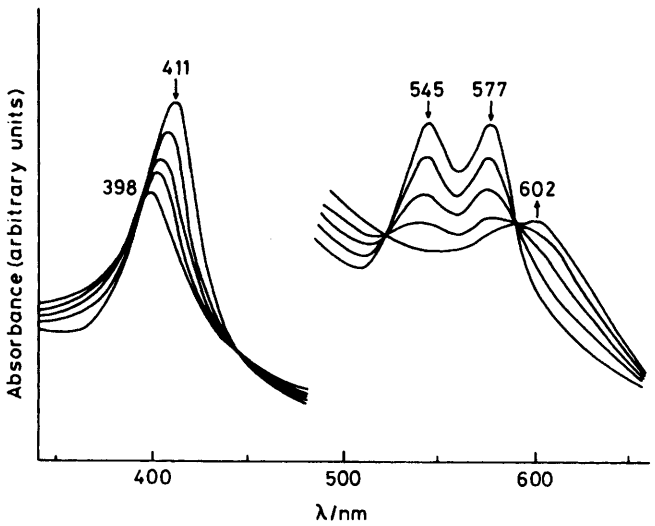


Figure 2. Change in the spectrum of the oxygen adduct after 0 (↓), 15, 30, 60, and 120 min exposure to oxygen. For conditions see Figure 1

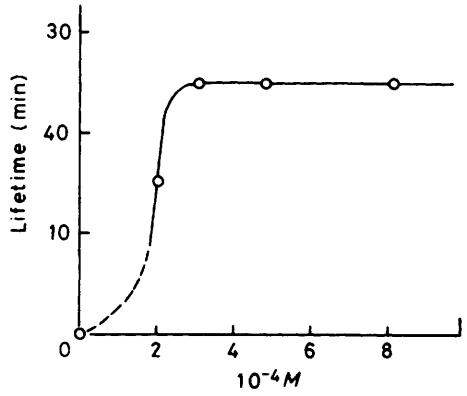


Figure 3. Effect of the molecular weight of pmvi ligand on the lifetime of the oxygen adduct. For conditions see Figure 1

[heme] = 10^3—10^4) which show the same spectra as the deoxyheme and the heme–CO complexes are irreversibly oxidized.

Lifetimes of the Oxygen Adducts.—The oxygen adduct of the pmvi–heme complex was not stable and decayed to the hemin complex, isosbestic points being observed in the visible spectrum (521 and 587 nm, Figure 2). This decay obeyed first-order kinetics from which the lifetime (half-life) of the oxygen adduct was calculated. Figure 3 shows the effect of the molecular weight of the pmvi ligand on the lifetime of the oxygen adduct; the molecular weight has to be high ($>10^4$) to give the oxygen adduct in aqueous solution.

The composition of the polymeric ligand affected the oxygenation. The oxygen adduct was observed only for the heme complexes with the non-ionic polymers, poly(2-methyl-1-vinylimidazole–1-vinyl-2-pyrrolidone) and poly(2-methyl-1-vinylimidazole–acrylamide), and not with poly(2-methyl-1-vinylimidazole–methacrylic acid) and poly(2-methyl-1-vinylimidazole–3-benzyl-2-methyl-1-vinylimidazolium chloride) (see Table 1). The ionic residues of the polymers, carboxylate and imidazolium, had undesirable effects on the oxygenation. Figure 4 shows the lifetimes of oxygen adducts formed from

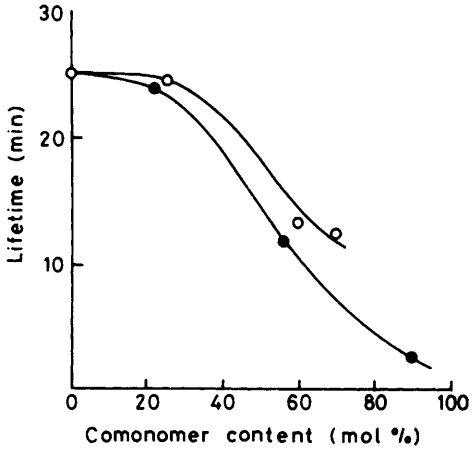


Figure 4. Effect of the composition of the 2-methyl-1-vinylimidazole copolymer on the lifetime of the oxygen adduct. (○) Poly(2-methyl-1-vinylimidazole–1-vinylpyrrolidone); (●) poly(2-methyl-1-vinylimidazole–acrylamide). For conditions see Figure 1

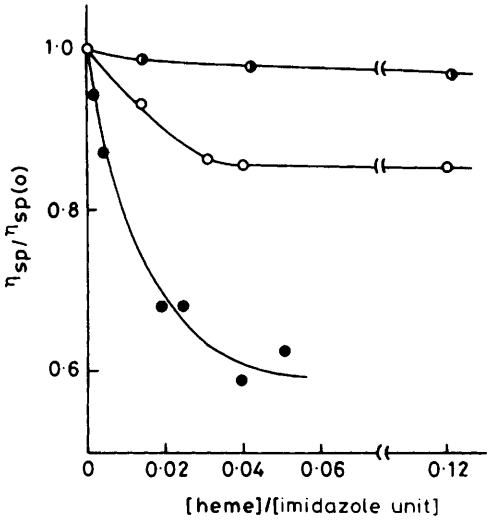


Figure 5. Viscosity of the polymer solutions in the presence of heme: η_{sp} = specific viscosity of the polymer solution, $\eta_{sp(o)} = \eta_{sp}$ in the absence of heme. (○) pmvi with heme; (◑) pmvi with protoporphyrin IX; (●) poly(1-vinylimidazole) with heme. Concentration of 2-methylimidazole or imidazole unit of the polymer = 40 mmol dm^{-3}, pH 10 buffer solution; under a nitrogen atmosphere at 30 °C

the heme complexes with poly(2-methyl-1-vinylimidazole–1-vinyl-2-pyrrolidone) and poly(2-methyl-1-vinylimidazole–acrylamide). The lifetime decreased with increasing content of comonomer, according to the hydrophilicity of the copolymer.

Viscometric measurement of the aqueous solutions of pmvi and of poly(1-vinylimidazole) was carried out to study the shape of the polymer–heme complex (Figure 5). At constant polymer concentration, the addition of heme causes a decrease in viscosity, which reveals that the polymer chain markedly contracts upon complexation of heme. The polymer–heme complex adopts a very compact structure in aqueous solution, heme being occluded within the contracted polymer chain.

The lifetime of the oxygen adduct of pmvi–heme was dependent on the pH of the buffer solution: 29 min at pH 11.5 (0.2 mol dm^{-3} Na_2CO_3–$NaHCO_3$), 24 min at pH 10, and 4 min at pH 8.0 (0.067 mol dm^{-3} Na_2HPO_4–KH_2PO_4). The

oxygen adduct was scarcely detected at pH 7.4. Its lifetime was independent of the heme concentration, ranging from 1 to 0.01 mmol dm^{-3}. These results suggest that the irreversible oxidation (to Fe^{III}) of the oxygenated heme complex with pmvi proceeds mainly *via* a unimolecular process caused by the attack of a proton on the heme-co-ordinated oxygen (proton-driven oxidation) rather than *via* a binuclear μ-oxo-dimer.

In Table 2 the effect of additives is shown. The oxygen adduct was formed in aqueous ethylene glycol solution regardless of its ethylene glycol content, and in aqueous poly(ethylene oxide) (*M ca.* 400) solution at −30 °C. It was destroyed by adding alcohol or urea, as was oxyhemoglobin.

From these results, it is considered that the proton-driven irreversible oxidation has to be suppressed to enable the oxygenation in aqueous media and that by combining the five-co-ordinated heme complex with a water-soluble but hydrophobic polymer the oxygen adduct can be observed in the cold aqueous medium due to the hydrophobic environment.

Kinetic and Equilibrium Parameters for Oxygen Binding.—The oxygen-binding affinity, $P_{\frac{1}{2}}(O_2)$, *i.e.* the oxygen pressure at 50% oxygen binding for the heme, was calculated from the midpoint of the oxygen-binding equilibrium curve for the pmvi–heme complex in aqueous solution at −30 °C (Figure 6).

Table 2. Effect of additives on the lifetime of the pmvi–heme–O_2 complex in aqueous solution (pH 10)

Additive	θ_c/°C	Lifetime/min
70 vol. % Ethylene glycol	−30	15
50 vol. % Ethylene glycol	−30	24
40 vol. % Ethylene glycol	−20	4
10 vol. % Ethylene glycol	−5	<0.5
20 vol. % Oligo(ethylene oxide)	−30	22
20 vol. % Ethanol *	−30	(Oxidized)
20 vol. % Urea *	−30	(Oxidized)

* 50 vol. % Ethylene glycol solution. For conditions see Figure 1.

The value is shown in Table 3 together with reference data for the chelated protoheme estimated by the CO protection method and for hemoglobin. Similarly, $P_{\frac{1}{2}}(CO)$ (carbon monoxide pressure at 50% CO binding) was determined from the CO-binding equilibrium curve (Figure 6) and is given in Table 3. Both oxygen- and carbon monoxide-binding affinities for pmvi–heme were decreased relative to protoheme, in particular the carbon monoxide-binding affinity was very small, and $P_{\frac{1}{2}}(O_2)/P_{\frac{1}{2}}(CO)$ was reduced to 200, close to that of hemoglobin in the tense (T) state.[22-24] These low gaseous molecule-binding affinities may be explained by the following two reasons: (*i*) pmvi forms the very stable five-co-ordinated heme complex, as mentioned above, which reduces the binding

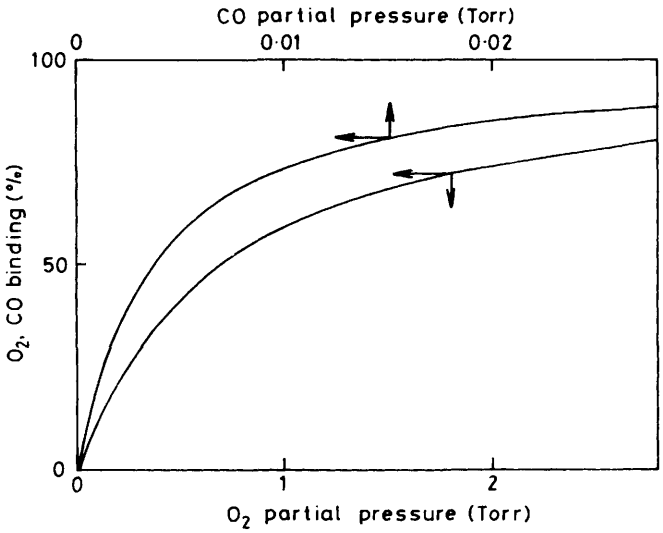


Figure 6. Oxygen- and carbon monoxide-binding equilibrium curves. For conditions see Figure 1

Table 3. Oxygen- and carbon monoxide-binding affinity

	θ_c/°C	$P_{\frac{1}{2}}(O_2)$/Torr	$P_{\frac{1}{2}}(CO)$/Torr	$P_{\frac{1}{2}}(O_2)/P_{\frac{1}{2}}(CO)$
pmvi–heme [a]	−30	0.7	3.5×10^{-3}	200
Chelated protoheme [b]	20	1.0	1.8×10^{-3}	560
	−30	7.0×10^{-3} [c]	3.7×10^{-6} [c]	1 900
Hemoglobin (T) [d]	20	35 (α)		50
		115 (β)	0.7	164
	−30	0.24 [c] (α)		160
		0.81 [c] (β)	1.5×10^{-3} [c]	540

[a] [heme] = 0.08 mmol dm^{-3}, concentration of 2-methylimidazole unit of polymer = 40 mmol dm^{-3}; pH 10 buffer solution–ethylene glycol (1 : 1, v/v). [b] Suspended in 2% trimethyltetradecylammonium bromide–phosphate buffer at pH 7.3 (T. G. Traylor and A. P. Berzinis, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 3171). [c] Estimated by using ΔH and ΔS for hemoglobin and chelated protoheme. [d] Refs. 22—24.

Table 4. Oxygen- and carbon monoxide-binding rate constants for heme complexes

	$k_{on}(O_2)$/dm^3 mol^{-1} s^{-1}	$k_{off}(O_2)$/s^{-1}	$K(O_2)$/dm^3 mol^{-1}	$k_{on}(CO)$/dm^3 mol^{-1} s^{-1}	$k_{off}(CO)$/s^{-1}	$K(CO)$/dm^3 mol^{-1}
pmvi–heme [a]	1.0×10^5	170	6.2×10^2	2.5×10^4	—	—
b,c	—	—	—	1.6×10^4	0.04	4.0×10^5
Chelated protoheme [b,d]	—	—	—	2.4×10^6	0.2	1.2×10^7
Chelated protoheme [b,e]	2.6×10^7	47	5.5×10^5	3.6×10^6	8.9×10^{-3}	4.0×10^8
Hemoglobin (T) (α) [b,f]	2.9×10^6	180	1.6×10^4	1.0×10^5	0.09	1.0×10^6
(β) [b,f]	1.2×10^7	2 500	4.8×10^3			

[a] [heme] = 0.08 mmol dm^{-3}, concentration of 2-methylimidazole unit of polymer = 40 mmol dm^{-3}; pH 10 buffer solution–ethylene glycol (1 : 1, v/v), 25 °C. Stopped-flow method. [b] Flash-photolysis method. [c] In pH 10 buffer at 20 °C. [d] Suspended in hexadecylpyridinium chloride–pH 10 buffer at 20 °C. [e] Suspended in trimethyltetradecylammonium bromide–pH 7.3 buffer at 20 °C (T. G. Traylor and A. P. Berzinis, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 3171). [f] Refs. 22—24.

affinity of gaseous molecules at the sixth co-ordination site of heme; (*ii*) heme is complexed with and occluded in the contracted pmvi polymer chain, which sterically prevents the co-ordination of gaseous molecules.

Oxygen-binding kinetic parameters for the pmvi–heme complex were estimated by a stopped-flow method and are listed in Table 4 together with reference data. Because the oxygen adduct of the pmvi–heme complex had a short lifetime even at room temperature, the oxygen-binding and -dissociation rate constants could be estimated from the spectral change within *ca.* 1 s. Carbon monoxide-binding and -dissociation rate contants were measured with both flash-photolysis and stopped-flow methods. The small oxygen-binding rate constant, $k_{on}(O_2)$, and carbon monoxide-binding rate constant, $k_{on}(CO)$, corresponding to the large $P_{\frac{1}{2}}(O_2)$ and $P_{\frac{1}{2}}(CO)$ values, may also be explained by the reasons mentioned above.

In conclusion, the pmvi–heme complex forms a semi-stable oxygen adduct in cold aqueous media where other synthetic hemes were rapidly oxidized. The oxygen- and carbon monoxide-binding affinities of pmvi–heme were relatively low and comparable with those of hemoglobin due to the small rate constants for binding of gaseous molecules.

Acknowledgements

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