Chemical Models of Hemoglobins and Cytochromes P-450: Influence of the Basicity of the Proximal Ligand on O_2 and CO Binding Kinetics

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Abstract: A number of Fe(II) porphyrins, previously shown to model oxygen carriers when incorporating a nitrogenous base as a proximal ligand, have been investigated for their ability to model the active site of cytochromes P-450 by using a thiolate group as a fifth axial ligand. In spite of difficulties in controlling the chemical preparation of the thiolate pentacoordinated Fe(II) porphyrins, association and dissociation rate parameters have been determined for CO and, for the first time, for O_2 binding, using laser flash photolysis. A large decrease of the affinity ratio $M = K_{CO}/K_{O2}$ is observed when the nitrogenous axial ligand is replaced by a thiolate, as observed between hemoglobins and most cytochromes P-450. The changes in the rate parameters suggested including the ethanolate ligand in this study in order to examine more thoroughly the trans influence exerted by the basicity of the proximal ligand upon O_2 and CO binding over a wide range of pK_a values. The data indicate that the increase of the pK_a of the proximal ligand is accompanied by a decrease of O_2 and CO association rates while dissociation rates increase for CO and decrease for O_2. The opposite trend exhibited by the gaseous ligands is discussed in terms of different relative contributions of σ-bonding and π-back-bonding to the stabilization of the iron(II)−ligand complexes.

Introduction

The combination of chemical modeling and of laser flash photolysis studies has markedly contributed to the understanding of environmental factors governing the reactivity of the active site of myoglobin and hemoglobin. The approach, which was successful in the case of oxygen storage, is faced with much greater difficulties when attempting to mimic other classes of hemoproteins, especially those displaying enzymatic functions such as cytochromes P-450. Chemical models of cytochromes P-450 have been designed over the past 20 years, but their reactivity toward oxygen and carbon monoxide has remained almost unexplored: only three kinetic investigations of carbon monoxide binding are available[1−3] and kinetic data for oxygen binding have not been reported so far. The reasons why the state of knowledge is much less advanced in this case are 2-fold.

First, the crystal structure of the active site of cytochromes P-450 remained unknown up to 1985. As a consequence, most studies of models were focused on elucidating the nature of the coordinated axial ligand(s) in the various Fe^{III} and Fe^{II} functional states of the catalytic cycle of the protein.[4,5] Comparison of optical spectra[1,6−10] and RMN data of CO complexes of models and proteins[2,10−12] provided strong evidence for the coordination of Fe^{II} by a thiolate ligand in the deoxy state. On the other hand, optical,[13−15] RPE,[13−16] MCD,[17] and EXAFS[18] spectroscopic studies of cytochromes P-450 and of some of their complexes compared with appropriate models also suggested a coordination by a thiolate ligand in the resting and in the substrate-bound Fe^{III} states. A definitive proof of the ligation by the S^- group of a cysteine in functional states of the hemoprotein was provided by the crystallographic structures which have accumulated since 1985.[19]

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(1) Chang, C. K.; Dolphin, D. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 3338−3342.

(2) Traylor, T. G.; Mincey, T. C.; Berzinis, A. P. *J. Am. Chem. Soc.* **1981**, *103*, 7084−7089.

(3) El-Kasmi, D.; Tetreau, C.; Lavalette, D.; Momenteau, M. *J. Chem. Soc., Perkin Trans. 2* **1993**, 1799−1803.

(4) Dawson, J. H.; Eble, K. S. *Advances in Organic and Bioinorganic Mechanisms*; Academic Press Inc.: New York, 1986; Vol. 4, pp 1−64.

(5) Momenteau, M.; Reed, C. A. *Chem. Rev.* **1994**, *94*, 659−698.

(6) Stern, J. O.; Peisach, J. *J. Biol. Chem.* **1974**, *249*, 7495−7498.

(7) Collman, J. P.; Sorrell, T. N. *J. Am. Chem. Soc.* **1975**, *97*, 4133−4134.

(8) Chang, C. K.; Dolphin, D. *J. Am. Chem. Soc.* **1975**, *97*, 5948−5950.

(9) Chang, C. K.; Dolphin, D. *J. Am. Chem. Soc.* **1976**, *98*, 1607−1609.

(10) Battersby, A. R.; Howson, W.; Hamilton, A. D. *J. Chem. Soc., Chem. Commun.* **1982**, 1266−1268.

(11) Berzinis, A. P.; Traylor, T. G. *Biochem. Biophys. Res. Commun.* **1979**, *87*, 229−235.

(12) Collman, J. P.; Groh, S. E. *J. Am. Chem. Soc.* **1982**, *104*, 1391−1403.

(13) Collman, J. P.; Sorrell, T. N.; Hoffman, B. M. *J. Am. Chem. Soc.* **1975**, *97*, 913−914.

(14) (a) Ruf, H. H.; Wende, P.; Ullrich, V. *J. Inorg. Biochem.* **1979**, *11*, 189−204. (b) Ruf, H. H.; Wende, P. *J. Am. Chem. Soc.* **1977**, *99*, 5499−5500.

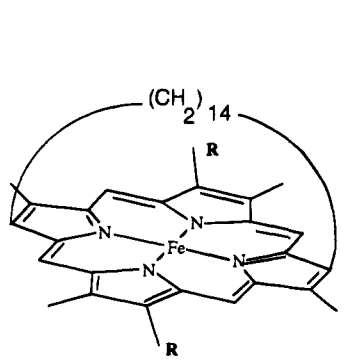
(15) (a) Sakurai, H.; Uchikubo, H.; Yoshimura, T; Maeda, M.; Kawasaki, K. *Biochem. Biophys. Res. Commun.* **1985**, *130*, 1254−1260. (b) Sakurai, H.; Hatayama, E.; Yoshimura, T.; Maeda, M.; Tamura, H.; Kawasaki, K. *Biochem. Biophys. Res. Commun.* **1983**, *115*, 590−597.

(16) (a) Tang, S. C.; Koch, S.; Papaefthymiou, G. C.; Foner, S.; Frankel, R. B.; Ibers, J. A.; Holm, R. H. *J. Am. Chem. Soc.* **1976**, *98*, 2414−2434. (b) Koch, S.; Tang, S. C.; Holm, R. H.; Frankel, R. B.; Ibers, J. A. *J. Am. Chem. Soc.* **1975**, *97*, 916−917.

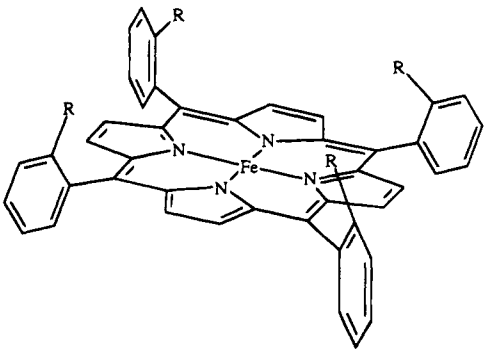
(17) (a) Anderson, K. K.; Hobbs, D. J.; Luo, L.; Stanley, K. D.; Quirke, J. M. E.; Shelnutt, J. A. *J. Am. Chem. Soc.* **1993**, *115*, 12346−12352. (b) Piffat, C.; Melamed, D.; Spiro, T. G. *J. Phys. Chem.* **1993**, *97*, 7441−7450. (c) Sparks, L. D.; Medforth, C. J.; Park, M.-S.; Chamberlain, J. R.; Ondrias, M. R.; Senge, M. O.; Smith, K. M.; Shelnutt, J. A. *J. Am. Chem. Soc.* **1993**, *115*, 581−592. (d) Prendergast, K.; Spiro, T. G. *J. Am. Chem. Soc.* **1992**, *114*, 3793−3801. (e) Shelnutt, J. A.; Medforth, C. J.; Berber, M. D.; Barkigia, K. M.; Smith, K. M. *J. Am. Chem. Soc.* **1991**, *113*, 4077−4087.

(18) (a) Dawson, J. H.; Kau, L.-S.; Penner-Hahn, J. E.; Sono, M.; Eble, K. S.; Bruce, G. S.; Hager, L. P.; Hodgson, K. O. *J. Am. Chem. Soc.* **1986**, *108*, 8114−8116. (b) Hahn, J. E.; Hodgson, K. O.; Anderson, L. A.; Dawson, J. H. *J. Biol. Chem.* **1982**, *257*, 10934−10941. (c) Cramer, S. P.; Dawson, J. H.; Hodgson, K. O.; Hager, L. P. *J. Am. Chem. Soc.* **1978**, *100*, 7282−7290.

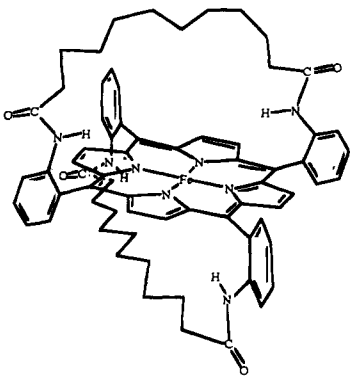
0002-7863/95/1517-6041$09.00/0



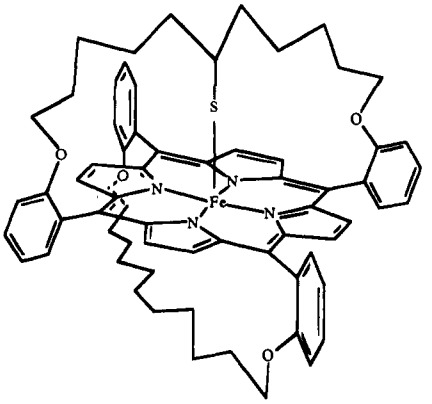
R = CH_2-CH_2-CO_2-CH_3: **"Strapped C14"**



R = H: **TPP**, R = NH-CO-$(CH_3)_3$: **TPivP**



***amide*-BHP(C_{12})(C_{12})**



***ether*-BHP(C_{12})(C_{11}-S^-)**

Figure 1. Structures and formulas for the porphyrins studied in this work.

In the second place, the difficulty of preparing thiolate complexes of isolated Fe(II) porphyrins under controlled conditions certainly accounts for the scarcity of kinetic studies, even using CO as a sixth ligand. In models of oxygen carriers, the porphyrin is five-coordinated by one of a series of nitrogenous bases. These compounds are soluble and stable in an organic solvent like toluene. Except for their binding with the iron porphyrin, they do not present any side reactions. Thiolate derivatives on the contrary are very reactive species which may give rise to numerous oxido-reduction reactions; moreover, they cannot be dissolved in toluene (which is the reference solvent for these studies), unless a crown ether or a cryptand is added. The situation is still worse with the oxygenated complexes: most of them rapidly oxidize at room temperature, and oxygen can react with the thiolate to give a disulfide.[12,20] Genuine oxygenated species have been characterized at room temperature in only two cases.[20-23]

In the present work, we have used several porphyrin structures, which had been previously designed as models of oxygen carriers, to model cytochromes P-450 and to investigate the kinetics of O_2 and CO binding with their thiolate pentacoordinated complexes. For the first time to our knowledge oxygen kinetic rate parameters are reported. The substitution of a thiolate for a nitrogenous base causes large changes in the rate parameters and in the affinity of both gaseous ligands which reproduce the much smaller K_{CO}/K_{O_2} ratio of cytochromes P-450 compared with oxygen carriers. These differences are discussed on the basis of a systematic study of the variations of kinetic and equilibrium parameters over the widest possible range of pK_a of the proximal ligand.

Materials and Methods

Modeling of oxygen-carrying hemoproteins has revealed that distal steric hindrance is one of the important factors which govern the reactivity of the active site. The porphyrin structures used in this work (Figure 1) exhibit a variable amount of steric hindrance and were previously studied as myoglobin models. Whereas TPP (*meso*-5,10,-15,20-tetraphenylporphyrin) itself is not encumbered, BHP ("Basket-Handle" porphyrins) and TPivP ["Picket-Fence", *meso*-tetrakis(α,α,α,α,-*o*-pivalamidophenyl)porphyrin)] on the one hand and "Strapped C14" porphyrin on the other hand are respectively characterized by a small "peripheral" and an intermediate steric hindrance.[3,24,25] The synthesis and characterization of these structures have been previously reported, as well as the kinetic rate parameters for O_2 and CO binding with some of their nitrogenous base complexes [e.g. with pyridine (Py), 1-methylimidazole (1-MeIm), or 1,2-dimethylimidazole (1,2-Me_2Im)].[3,24,25] In the present work butanethiolate and tetrafluorobenzenethiolate were used as proximal ligands to model the coordination of cytochromes P-450 by a thiolate residue. Because of its high basicity (pK_a = 10.7)

(19) (a) Raag, R.; Poulos, T. L. *Biochemistry* **1989**, *28*, 917–922. (b) Poulos, T. L.; Howard, A. J. *Biochemistry* **1987**, *26*, 8165–8174. (c) Poulos, T. L.; Finzel, B. C.; Howard, A. J. *J. Mol. Biol.* **1987**, *195*, 687–701. (d) Poulos, T. L.; Finzel, B. C.; Howard, A. J. *Biochemistry* **1986**, *25*, 5314–5322. (e) Poulos, T. L.; Finzel, B. C.; Gunsalus, I. C.; Wagner, G. C.; Kraut, J. *J. Biol. Chem.* **1985**, *260*, 16122–16130.

(20) Schappacher, M.; Ricard, L.; Weiss, R.; Montiel-Montoya, R.; Bill, E.; Gonser, U.; Trautwein, A. *J. Am. Chem. Soc.* **1981**, *103*, 7646–7648.

(21) Ricard, L.; Schappacher, M.; Weiss, R.; Montiel-Montoya, R.; Bill, E.; Gonser, U.; Trautwein, A. *Nouv. J. Chim.* **1983**, *7*, 405–408.

(22) Schappacher, M.; Ricard, L.; Fischer, J.; Weiss, R.; Bill, E.; Montiel-Montoya, R.; Winkler, H.; Trautwein, A. *Eur. J. Biochem.* **1987**, *168*, 419–429.

(23) Budyka, M. F.; Khenkin, A. M.; Shteinman, A. A. *Biochem. Biophys. Res. Commun.* **1981**, *101*, 615–622.

(24) Collman, J. P.; Brauman, J. I.; Iverson, B. L.; Sessler, J. L.; Morris, R. M.; Gibson, Q. H. *J. Am. Chem. Soc.* **1983**, *105*, 3052–3064.

(25) Lavalette, D.; Tetreau, C.; Mispelter, J.; Momenteau, M.; Lhoste, J.-M. *Eur. J. Biochem.* **1984**, *145*, 555–565.

butanethiolate favors a strong coordination in the pentacoordinated state.[2] Tetrafluorobenzenethiolate was included in this study because the structure of the oxygenated complex [TPivP−C_6HF_4S-O_2]$^-$ has been determined by X-ray diffraction.[21,22] In the ether-Basket-Handle porphyrin, the thiolate residue is carried by one handle covalently attached to the porphyrin macrocycle (Figure 1). We also studied ethanolate in order to extend the range of pK_a of the proximal ligand.

The procedures used to obtain the various porphyrin complexes have been previously described[3,26] and are briefly summarized in the Experimental Section. Upon sequential addition of the axial ligand and of the gaseous ligand, the formation of the respective penta- and hexacoordinated species was monitored using UV−visible absorption spectroscopy.

Kinetics. The rate parameters and equilibrium constants were obtained by combining laser flash photolysis experiments and spectrophotometric titrations. Following photodissociation of hexacoordinated complexes B-Fe^{II}-L (L = CO or O_2; B = thiolate or alcoholate) the equilibrium relaxes with a rate k_r given by

$$k_r = k_B^{+L}[L] + k_B^{-L} \quad (1)$$

where k_B^{+L} and k_B^{-L} are the second-order association rate constant and the first-order dissociation rate parameter, respectively (the notations follow the definitions of refs 27 and 28). Equation 1 permits the determination of both rate parameters from the linear plot of k_r *vs* [L] and is valid under strict pseudo-first-order conditions (i.e. [L] ≫ [B−Fe(II)]). However, the dissociation rate k_B^{-L} is measured accurately only if conditions can be found such that k_B^{+L}[L] and k_B^{-L} are of comparable magnitude. If this is not the case, the dissociation rate can be determined by performing the photodissociation experiment at very low concentrations of the gaseous ligand. Equation 1 must then be replaced by the more complete expression

$$k_r = k_B^{+L}([L]_f + [P]_f) + k_B^{-L} \quad (2)$$

where $[L]_f$ and $[P]_f$ are the concentration of ligand and of pentacoordinated porphyrin which are free in the solution after the laser pulse. By choosing [L] ≪ [P], the proportion of hexacoordinated complex remains small and the quantities of L and of pentacoordinated porphyrin which are liberated upon photolysis can be neglected, therefore $[L]_f$ = [L] (i.e. the equilibrium gas concentration at the chosen partial pressure). On the other hand, the equilibrium concentration of free pentacoordinated porphyrin is given by

$$[P]_f = [P]_0/(K_B^L[L] + 1)$$

in which $[P]_0$ is the total porphyrin concentration (5×10^{-5} M in our experiments). Expressing K_B^L as a function of the kinetic rate parameters, one finally obtains for the relaxation rate

$$k = k_B^{+L}\{[P]_0/[(k_B^{+L}[L]/k_B^{-L}) + 1] + [L]\} + k_B^{-L} \quad (3)$$

Equation 3 can be solved for k_B^{-L}, provided that the association rate k_B^{+L} has been previously determined at high ligand concentration. This method was applied in particular to [TPivP−C_6HF_4S-O_2]$^-$, since the method of competitive rebinding[29,30] which has been widely used in oxygen-carrier models turned out to be inappropriate with thiolate ligands. Determining oxygen "off" rates in this way implies that the carboxyhemochrome must be the dominant species at equilibrium and that experimental conditions can be found under which oxygen rebinds faster than CO after flash-off. With heme models of oxygen carriers the conditions can be easily fulfilled since $K_B^{CO} \gg K_B^{O_2}$ and $k_B^{+O_2} > k_B^{+CO}$. With thiolate or alcoholate derivatives the ratio $K_B^{CO}/K_B^{O_2}$ is close to unity or even smaller (see below); therefore protection against oxidation by preferential binding of CO is not effective at the O_2 and CO concentrations required for fast oxygen rebinding. The consequence is that only oxygenated complexes which are intrinsically stable can be studied, using relations 1−3 above. Because of these difficulties the accuracy of the present measurements was also less than usual; the error bars in the present experiments were estimated to be about ±20% and ±40% for association and dissociation rate constants, respectively.

Table 1. Absorption Maxima (nm) of Penta- and Hexacoordinated Complexes of Fe(II) Porphyrins Studied in This Work, in Toluene at 20 °C

[TPP−C_2H_5O]·**K**		417, 445	577	621
[TPP−C_2H_5O−(CO)]·**K**		431		
[TPP−BuS]·**K**		417		
[TPP−BuS−(CO)]·**K**	377	448	545	
[TPivP−BuS]·**K**		417		
[TPivP−BuS−(CO)]·**K**	378	452	552	
[TPivP−BuS−(O_2)]·**K**		427		
[TPivP−C_6HF_4]·**Na**	388 (sh)	418, 441 (sh)		
[TPivP−C_6HF_4−(CO)]·**Na**	382	446		
[TPivP−C_6HF_4−(O_2)]·**K**		427		
[*e*-BHP(C_{12})(C_{11}−S)]·**K**		418		
[*e*-BHP(C_{12})(C_{11}−S)−(CO)]·**K**	379	452		
[*a*-BHP(C_{12})(C_{12})−BuS]·**K**	390	419, 445, 479		
[*a*-BHP(C_{12})(C_{12})−BuS−(CO)]·**K**	390	462		

[a] Measurements of the extinction coefficients were not attempted because of some unavoidable residual oxidation of the hexacoordinated thiolate and ethanolate species.

Results

All hexacoordinated thiolate derivatives displayed the expected typical "hyper" Soret spectra. The main spectral characteristics of the different complexes are summarized in Table 1.

In the presence of oxygen, only TPivP derivatives resisted autoxidation. Oxygenated complexes of all other porphyrins were found to oxidize within minutes, thus preventing any kinetic experiment.

Whereas pentacoordinated B−Fe^{II} complexes (with B denoting either a thiolate or an alcoholate ligand) could not be photodissociated, all hexacoordinated complexes B−Fe^{II}−L were photolabile (L = CO or O_2).

Except for [TPivP−C_6HF_4S−CO]$^-$, clean exponential rebinding kinetics were observed. The relaxation rate was proportional to the gaseous ligand concentration and was independent of the monitoring wavelength. The initial absorption change after photodissociation mirrored the static difference spectrum between the hexa- and pentacoordinated species.

Carbon Monoxide Binding. For most CO derivatives except the Picket-Fence and amide−BHP(C_{12})(C_{12}), the association rates were slow enough to allow an accurate determination of k_B^{-CO} as the intercept of the linear plot of k_r *vs* the ligand concentration. K_B^{CO} was then calculated as the ratio k_B^{+CO}/k_B^{-CO}. The equilibrium constant derived in this way matched the value obtained by spectrophotometric titration within errors. For the Picket-Fence and amide−BHP(C_{12})(C_{12}), K_B^{CO} was obtained by spectrophotometric titration, and k_B^{-CO} was calculated as k_B^{+CO}/K_B^{CO}.

With [TPivP−C_6HF_4S−CO]$^-$, biphasic and wavelength-dependent recombination kinetics were observed; the absorption spectrum revealed the presence of a mixture of both B−Fe^{II}−CO and Fe^{II}−CO species. The reason why the formation of the CO hexacoordinated species is disfavored with $C_6HF_4S^-$ compared with BuS^- is not understood. One would expect a better stabilization of the carbonylated hexacoordinated [TPivP−C_6HF_4S−CO]$^-$ complex in view of the lower basicity of $C_6HF_4S^-$ (see ref 2 and discussion below). The crystallographic structure of [TPivP−C_6HF_4S]$^-$ has revealed a strong interaction of the sodium counterion with one of the carbonyl groups of

(26) Momenteau, M. *Biochim. Biophys. Acta* **1973**, *304*, 814−827.
(27) Rougee, M.; Brault, D. *Biochemistry* **1975**, *14*, 4100−4106.
(28) White, D. K.; Cannon, J. B.; Traylor, T. G. *J. Am. Chem. Soc.* **1979**, *101*, 2443−2454.
(29) Noble, R. W.; Gibson, Q. H.; Brunori, M.; Antonini, E.; Wyman, J. *J. Biol. Chem.* **1969**, *244*, 3905−3908.
(30) Lavalette, D.; Momenteau, M. *J. Chem. Soc., Perkin Trans. 2* **1982**, 385−388.

Table 2. Kinetic Rate Parameters and Equilibrium Constants for O_2 and CO Binding with Various Fe(II) Porphyrin Complexes[a] and Hemoproteins

compound	k_B^{+CO}, M^{-1} s^{-1}	k_B^{-CO}, s^{-1}	K_B^{CO}, M^{-1}	k_B^{+O2}, M^1 s^{-1}	k_B^{-O2}, s^{-1}	K_B^{O2}, M^{-1}	M	ref
[TPP−C_2H_5O]·**K**	7.7×10^2	2	3.5×10^2					this work
[TPP−BuS]·**K**	2.0×10^5	16	1.3×10^4					this work
TPP−Im	4.4×10^6	3.0×10^{-2}	1.5×10^8	5.0×10^7	2.7×10^4	1.7×10^3	8.8×10^4	25, 30
TPP−Py	6.5×10^6			1.0×10^8	1.0×10^5	1.0×10^3		25, 30
[amide−BHP(C_{12})(C_{12})−BuS]·**K**	6.8×10^6	3.4×10^{-1}	2.0×10^7					this work
[ether−BHP(C_{12})(C_{11}−S)]·**K**	8.8×10^4	28	3.1×10^3					this work
["Strapped C14"−BuS]·**K**	2.6×10^3	22	1.2×10^2					3
["Strapped C14"−1-MeIm]	7.4×10^4	1.1×10^{-1}	7.4×10^5					3
[TPivP−BuS]·**K**	2.1×10^7 [b]	10[b]	2.2×10^6 [b]	6.7×10^6	0.2	3.2×10^7	6.9×10^{-2}	this work
[TPivP−C_6HF_4S]·**Na**				2.5×10^8	9.0×10^2	2.8×10^5		this work
TPivP−1-MeIm[c]	3.6×10^7	8×10^{-3}	4.5×10^9	4.3×10^8	2.9×10^3	1.4×10^5	3.2×10^4	24
PF3CUPy[c]	4.8×10^7	3.3×10^{-1}	1.4×10^8	3×10^8	1.9×10^5	1.6×10^3	8.8×10^4	40
PF3CUIm[c]	2.9×10^7	1.4×10^{-2}	2.1×10^9	2.6×10^8	3.9×10^3	6.7×10^4	3.1×10^4	40
[PPIX−BuS]·**K**[d]	1.2×10^5	25	2×10^4					1
P450scc−22*R*-hydroxycholesterol[e]	6.9×10^3	7.6×10^{-1}	9.1×10^3	6.2×10^4	7.7×10^{-1}	8.1×10^4	1.1×10^{-1}	34
sperm whale myoglobin[f]	5.1×10^5	1.9×10^{-2}	2.7×10^7	1.4×10^7	1.2×10^1	1.2×10^6	1.2×10^1	33a

[a] In toluene and 18-crown-6 ether, at 20 °C. [b] Values measured in cryptand [K 222], because of kinetic complications arising in 18-crown-6 ether; a previous study (ref 3) and the close value of $K_B^{CO} = 1.4 \times 10^6$ M^{-1} determined in 18-crown-6 ether suggest only small changes from one co-solvent to another one. [c] Toluene, 25 °C. [d] Values for PPIX (Fe(II) protoporphyrin IX dimethyl ester) measured in toluene, at 23 °C. [e] Values for adrenal mitochondrial cholesterol-side-chain-cleaving cytochrome P450 ($P450_{scc}$) measured in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2, 25 °C. [f] 0.1 M potassium phosphate at 20 °C, pH 7.0.

the picket arms. The presence of the crown ether around the cation near the picket, at a close proximity of BuS^- which likely binds within the cavity,[31,32] might have a stabilizing effect not present in the case of $C_6HF_4S^-$ which does not bind on the picket face of TPivP. Alternatively, the arenethiolate might be destabilized due to the geometry of the aromatic ring which lies almost parallel to the porphyrin macrocycle.[31]

Oxygen Binding. Two oxygenated complexes, [TPivP−C_6HF_4S−O_2]$^-$ and [TPivP−BuS−O_2]$^-$, were found to be sufficiently stable over the duration of a laser flash photolysis experiment. However, the dissociation rate could not be determined accurately from the intercept of k_r *vs* [O_2] since it turned out that $k_B^{+O_2}[O_2] \gg k_B^{-O_2}$.

(a) [TPivP−C_6HF_4S−O_2]$^-$. As previously observed,[32] [TPivP−C_6HF_4S−O_2]$^-$ was stable over hours at room temperature, a fact that may be attributed to the protecting influence of the pickets since the crystallographic structure indicates that oxygen binds within the cavity. However, pentacoordination of [TPivP−C_6HF_4S]$^-$ was only partial, presumably because of the poor basicity of $C_6HF_4S^-$. Therefore the determination of the equilibrium constant using the usual spectrophotometric titration procedure was not possible. We determined instead the dissociation constant by performing laser kinetic experiments at very low oxygen concentration ([O_2] = 5.3×10^{-6} M; [porphyrin] = 5×10^{-5} M) according to eq 3. Using the value of $k_B^{+O_2} = 2.5 \times 10^8$ $M\ s^{-1}$ (determined independently from the slope of k *vs* [O_2] at high oxygen concentration), eq 3 was solved yielding $k_B^{-O_2} = 900 \pm 200$ s^{-1}.

(b) [TPivP−BuS−O_2]$^-$. In contrast to $C_6HF_4S^-$, alkanethiolates have been reported to bind within the cavity of TPivP,[31,32] leaving the unprotected face free for oxygen binding. Surprisingly, the oxygenated complex had a lifetime of the order of an hour. Kinetic measurements were performed, and the partition coefficient $M = K_B^{CO}/K_B^{O_2}$ was determined by spectrophotometric titration of the oxygenated against the carbonylated complex. At the end of the titration, 85% of the carbonylated complex was recovered by bubbling CO, indicating that the amount of oxidation remained within acceptable limits to provide a reasonable estimate of M in this way. $K_B^{O_2}$ was then obtained as K_B^{CO}/M, and $k_B^{-O_2}$ as $k_B^{+O_2}/K_B^{O_2}$.

Discussion

Affinities and kinetic rate parameters are reported in Table 2. Compared with the 1-MeIm complex of the same porphyrin, CO affinities are reduced by at least three orders of magnitude mainly because of considerably higher dissociation rates, indicating a decreased stability of the carbon monoxide complex. A substantial decrease of the association rates can also be noticed. The unusually low association and high dissociation rates for CO binding with a butanethiolate complex (PPIX−BuS^- in Table 2) were noticed a long time ago.[1] These observations are supported by the present results. The stability of O_2 complexes follows the opposite trend. The change from 1-MeIm to BuS^- leads to an increase of $K_B^{O_2}$ by more than two orders of magnitude, also mainly because of the variation of the dissociation rate constant. [TPivP−BuS−O_2]·**K** has the highest affinity constant ever reported for a Fe^{II} porphyrin oxygenated complex. As a consequence, M becomes much smaller than unity with [TPivP−BuS]·**K**, whereas $M = 3 \times 10^4$ for TPivP−1-MeIm. This trend mirrors that observed in proteins, since M is on the average much smaller for cytochromes P-450 than for oxygen carriers (Table 2).[23,33,34]

Trans Effect Due to the Basicity of the Proximal Ligand. The role of the proximal ligand basicity has been examined often. Linear correlations between the pK_a of the axial base and its affinity constant K_B have been reported for numerous metalloporphyrins.[35−39]

Several studies have also addressed the trans influence of the axial ligand on CO and O_2 binding to porphyrins.[5,40,41]

(31) Schappacher, M.; Ricard, L.; Weiss, R.; Montiel-Montoya, R.; Gonser, U.; Bill, E.; Trautwein, A. *Inorg. Chem. Acta* **1983**, *78*, L9−L12.
(32) Schappacher, M.; Ricard, L.; Fischer, J.; Weiss, R.; Montiel-Montoya, R.; Bill, E.; Trautwein, A. *Inorg. Chem.* **1989**, *28*, 4639−4645.

(33) (a) Carver, T. E.; Olson, J. S.; Smerdon, S. J.; Krzywda, S.; Wilkinson, A. J.; Gibson, Q. H.; Blackmore, R. S.; Dez Ropp, J.; Sligar, S. *Biochemistry* **1991**, *30*, 4697−4705. (b) Martin, K. D.; Parkhurst, L. J. *Biochemistry* **1990**, *29*, 5718−5726. (c) Gibson, Q. H.; Wittenberg, J. B.; Wittenberg, B. A.; Bogusz, D.; Appleby, C. A. *J. Biol. Chem.* **1989**, *264*, 100−107. (d) Wittenberg, J. B.; Wittenberg, B. A.; Gibson, Q. H.; Trinick, M. J.; Appleby, C. A. *J. Biol. Chem.* **1986**, *261*, 13624−13631. (e) Smith, S. E.; Brittain, T.; Wells, R. M. *Biochem. J.* **1988**, *252*, 673−678. (f) Di Iorio, E. E.; Meier, U. T.; Smit, J. D. G.; Winterhelter, K. H. *J. Biol. Chem.* **1985**, *260*, 2160−2164. (g) Parkhurst, L. J.; Sima, P.; Goss, D. J. *Biochemistry* **1980**, *19*, 2688−2692. (h) Giacometti, G. M.; Brunori, M.; Antonini, E; Di Iorio, E. E.; Winterhalter, K. H. *J. Biol. Chem.* **1980**, *255*, 6160−6165. (i) Moffat, K.; Deatherage, J. F.; Seybert, D. W. *Science* **1979**, *206*, 1035−1042.

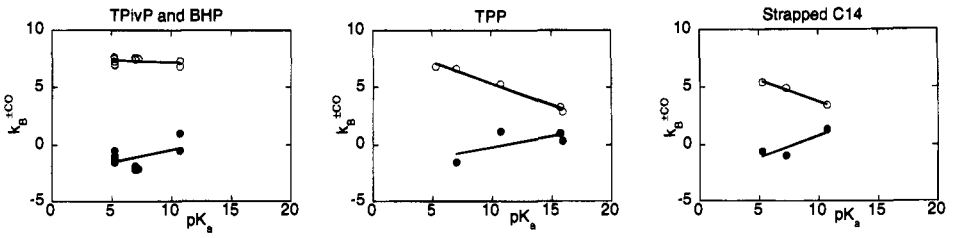


Figure 2. Association (open circles) and dissociation (closed circles) rate parameters as a function of the pK_a of the proximal ligand for CO binding with various Fe(II) porphyrins in toluene at 20−25 °C. The porphyrins are as follows: PF3CUPy,[40] PF3CUIm,[40] TPivP−1-MeIm,[24] TPivP−BuS$^-$, amide−BHPs of ref 25 (TPivP and BHP correlation, left); TPP−Py,[59] TPP−Im,[59] TPP−BuS$^-$, TPP−EtO$^-$ (TPP correlation, middle); strapped-Py−1-MeIm and −BuS$^-$ (strapped correlation, right). The $C_6HF_4S^-$ derivatives were not included in the correlations because the pK_a of this thiolate is unknown. The pK_a values of Py, Im, 1-MeIm, BuS$^-$, and EtO$^-$ were taken from ref 51 as 5.2, 7.0, 7.25, 10.7, and 15.9, respectively. Thermodynamic data[24,60] indicate that the changes in reactivity parameters between 20 and 25 °C do not exceed the size of the symbols. Error bars of the measurements are smaller than the size of the symbols.

Although the overall effect was generally small, correlations were found between the pK_a of various substituted pyridines and $K_B^{O_2}$ for Co(II),[42−44] Mn(II),[45] and Fe(II)[44] porphyrins. Kinetic investigations were also performed, but were limited to the comparison of pyridine and imidazole analogs[40,46] and lead to the conclusion that $K_B^{O_2}$ was reduced by approximately one order of magnitude when pyridine was substituted for imidazole. This effect arises primarily from a destabilization of the final complex, reflected by an increase of the dissociation rate parameter. In spite of the large number of studies, the situation is by no means clear for CO, for which contradictory conclusions were reported. Kinetic[40,46] and equilibrium data[47−49] suggest that the affinity for CO increases with the σ-donor ability of the nitrogenous base upon replacement of pyridine (p$K_a \approx$ 5.3) by imidazole (p$K_a \approx$ 7.0), but that the effect is far less pronounced than for oxygen. A larger increase of the affinity was noticed when an oxygen ligand such as H_2O or methanol (negative pK_a) was replaced by an imidazole ligand.[27] However, deprotonation of the proximal ligand (whether a nitrogenous base, R−OH, or R−SH)[50] or substitution of alkanethiolate for imidazole, which both induce a large increase of basicity, drastically reduce the affinity constant of CO.[1−3]

The proximal ligands considered in the present work (thiolate, nitrogenous bases, alcoholate) cover a considerably wider range

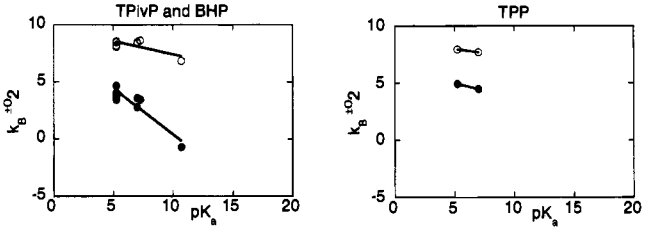


Figure 3. Association (open circles) and dissociation (closed circles) rate parameters as a function of the pK_a of the proximal ligand for O_2 binding with various Fe(II) porphyrins in toluene at 20−25 °C. The porphyrins are as follows: PF3CUPy,[40] PF3CUIm,[40] TPivP−1-MeIm,[24] TPivP−BuS$^-$, amide−BHPs of ref 25 (TPivP and BHP correlation, left); TPP−Py[59] and TPP−Im[59] (plot for TPP, right).

of pK_a[51] extending from 5 up to 16. Although the experiments were performed in toluene, the correlations between K_B and the pK_a mentioned above as well as a previous extensive study of the reactivity of hemochromes[52] have shown that pK_a values remain good indicators of the σ-donor character of ligands in this solvent.

The rate parameters for CO and O_2 binding are plotted versus the pK_a of the proximal base in Figures 2 and 3, respectively. Because steric factors are expected to play an important role in the binding of the distal ligand, we put together only macrocycles which do not differ appreciably in their amount of proximal or distal constraints. The fact that similar trends are observed within each group suggests that the porphyrin nature is of secondary importance. In spite of an important scatter, these trends, expressed by the negative or positive slopes of the linear regression lines, are beyond experimental uncertainties and must be regarded as significant. Whereas $k_B^{+O_2}$ and k_B^{+CO} decrease upon increasing the pK_a of the base, the variations of the dissociation rates (and of the equilibrium constants) are opposite in CO and O_2 complexes.

The scatter in the correlation of dissociation rates may be attributed in part to experimental uncertainties ("off" rates are generally obtained with less accuracy than "on" rates), but structural and electronic factors such as π-additional donor character and hydrogen bonding to the solvent are likely to play a significant role, not reflected by the pK_a. Eventually such factors may even locally dominate when only small variations of the σ-donor ability are considered. Note for instance that a comparison restricted to CO binding with pyridine and imidazole

(34) Tuckey, R. C.; Kamin, H. *J. Biol. Chem.* **1983**, *258*, 4232−4237.
(35) (a) Kadish, K. M.; Shiue, L. R. *Inorg. Chem.* **1982**, *21*, 3623−3630. (b) Kadish, K. M.; Shiue, L. R. *Inorg. Chem.* **1982**, *21*, 1112−1115.
(36) Ellis, P. E.; Linard, J. E.; Szymanski, T.; Jones, R. D.; Budge, J. R.; Basolo, F. *J. Am. Chem. Soc.* **1980**, *102*, 1889−1896.
(37) Linard, J. E.; Ellis, P. E., Jr.; Budge, J. R.; Jones, R. D.; Basolo, F. *J. Am. Chem. Soc.* **1980**, *102*, 1896−1904.
(38) Quinn, R.; Mercer-Smith, J.; Burstyn, J. N.; Valentine, J. S. *J. Am. Chem. Soc.* **1984**, *106*, 4136−4144.
(39) (a) Kirksey, C. H.; Hambright, P. *Inorg. Chem.* **1970**, *9*, 958−960. (b) Kirskey, C. H.; Hambright, P.; Storm, C. *Inorg. Chem.* **1969**, *8*, 2141−2144.
(40) Collman, J. P.; Brauman, J. I.; Docsee, K. M.; Sessler, J. L.; Morris, R. M.; Gibson, Q. H. *Inorg. Chem.* **1983**, *22*, 1427−1432.
(41) Traylor, T. G.; Traylor, P. S. *Annu. Rev. Biophys. Bioeng.* **1982**, *1*, 105−127.
(42) Walker, F. A. *J. Am. Chem. Soc.* **1973**, *95*, 1154−1159.
(43) Stynes, D. V.; Stynes, H. C.; James, B. R.; Ibers, J. A. *J. Am. Chem. Soc.* **1973**, *95*, 1796−1801.
(44) Linard, J. E.; Ellis, P. E.; Budge, J. R.; Jones, R. D.; Basolo, F. *J. Am. Chem. Soc.* **1980**, *102*, 1896−1904.
(45) Jones, R. D.; Summerville, D. A.; Basolo, F. *J. Am. Chem. Soc.* **1978**, *100*, 4416−4424.
(46) Momenteau, M.; Loock, B.; Lavalette, D.; Tetreau, C.; Mispelter, J. *J. Chem. Soc., Chem. Commun.* **1983**, 961−964.
(47) Chang, C. K.; Traylor, T. G. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 1166−1170.
(48) Momenteau, M.; Rougee, M.; Loock, B. *Eur. J. Biochem.* **1976**, *71*, 63−76.
(49) Traylor, T. G.; Campbell, D.; Sharma, V.; Geibel, J. *J. Am. Chem. Soc.* **1979**, *101*, 5376−5383.
(50) Mincey, T.; Traylor, T. G. *J. Am. Chem. Soc.* **1979**, *101*, 765−766.
(51) (a) Vollhardt, K. P. C. *Organic Chemistry*; W. H. Freeman and Company: New York, 1987; p 273. (b) Schoefield, K. S. *Heterocyclic Aromatic Nitrogen Compounds*; Plenum Press: New York, 1967; p 146. (c) Albert, A. *Phys. Methods Heterocycl. Chem.* **1963**, *1*, 1. (d) Arnett, E. M. *Prog. Phys. Org. Chem.* **1963**, *1*, 223−403.
(52) Lavalette, D.; Tetreau, C.; Momenteau, M. *J. Am. Chem. Soc.* **1979**, *101*, 5395−5401.

complexes which differ by only two pK_a units would suggest a stabilization of CO complexes upon increasing the pK_a.

O2−Fe(II) and CO−Fe(II) Bonds. The correlations shown in Figures 2 and 3 indicate that the stabilities of the CO and O_2 complexes are respectively decreased and increased upon increasing the basicity of the proximal ligand, indicating that the σ-donor character of the axial ligand predominantly controls the reactivity toward O_2 and CO.

All quantum mechanical calculations of the mode of binding of the gaseous ligands with hemoglobin agree to conclude that the strength of the Fe−L bond is due to σ-donation from the ligand to iron and to π-back-bonding through $d_\pi \rightarrow p_\pi$ transfer from the iron to the ligand;[53−55] there is also a general agreement to consider that the amount of π-back-bonding is less for CO than for O_2, because the high energy of the CO p_π orbitals makes them less attractive as π acceptors.

Upon increasing the σ-donor character of the proximal base, the electronic density borne by the iron atom increases. The pK_a dependence observed for $k_B{}^{+L}$ is thus likely to result from the repulsion between the electronic density at the ligand L and the increased electronic density at the iron d_{z^2} orbital, leading to a higher energy barrier for bond formation. In the hexacoordinated complex, on the other hand, one may expect the Fe^{II}−L σ-bond strength to decrease because of the competition with the base for σ-donation to the metal. But σ-donation from the base may also indirectly lead to an enhanced π-back-bonding to the ligand, due to the redistribution of electronic density on the various d iron orbitals (including d_π orbitals). Since π-back-bonding is favored in O_2 (compared to CO) this effect may overbalance the relative destabilization of the σ bond resulting in an enhanced oxygen stability in the final complex. The compensation is not sufficient in hexacoordinated CO complexes which show a reduced stability as well as a low frequency of the Fe−C vibration.[56,57]

The presently available data are still too limited to be sure that the variations of $k^{\pm}$ vs pK_a exhibited in these specific cases are indeed general, but the trends are consistent with the assumed modes of binding of CO and O_2 with iron(II) porphyrins.

Conclusion

We have reported the first kinetic investigation of oxygenated models of cytochromes P-450. Further chemical modeling and kinetic investigations are required to reach a state of knowledge comparable to that for oxygen-carrying hemoproteins.

In addition, we have extended the investigation of the trans influence exerted by the proximal ligand basicity on the kinetics of O_2 and CO binding with iron(II) porphyrins. Over a wide range of proximal ligand basicity, the general trend is to stabilize oxygenated complexes and to destabilize carbonylated complexes. Thus, the main differences in the binding of oxygen and CO by the two different classes of hemoproteins considered here (i.e. O_2-carriers and cytochromes P-450) seem to be primarily accounted for by the change of the proximal ligand from histidine to cysteinate.

Experimental Section

Preparation of the Five-Coordinated Mercaptide and Alcoholate−Fe(II) Complexes. Potassium *n*-butylmercaptide and ethanolate were prepared by reacting potassium hydride with *n*-butylmercaptan and ethanol, respectively.[58] KH (1 g; 50% in oil, Aldrich) was washed twice with carefully deoxygenated dry toluene and about 4 mL of butanethiol (Aldrich) was added under argon. Excess thiol and solvent were removed under vacuum (2 h). The resultant white powder was stored in a glovebox (2 ppm O_2). A similar procedure was used to prepare the ethanolate which was used immediately.

To enhance the solubility of the anion in toluene, crown ether complexes were prepared, BuS·**K** and EtO·**K**. A small amount of mercaptide or ethanolate was added to an equivalent amount of 18-crown-6 (Aldrich) in dry deoxygenated toluene; the mixture was stirred for 3 h before use. The solubility of the mercaptide salt in toluene was rather poor, even in the presence of crown ether. Although the exact concentration of a saturated solution is now known, it must be of the order of a few millimolar, since it is higher than that in dibenzo-18-crown-6 where a value of 2 mM has been reported. The solubility of ethanolate is unknown but was not required since the relaxation rates for CO binding were found to be independent of the ethanolate concentration.

A deaerated solution of porphyrin (10^{-4} M in toluene) was stirred for a few minutes with an aqueous solution of sodium dithionite in borate buffer, pH 8 under argon. The organic phase was separated from the mixture and dried by bubbling argon for 2 h. The five-coordinated anion−heme complex was finally prepared by adding an equal volume of a saturated solution of mercaptide (or alcoholate)−crown ether complex. Because of the extreme oxygen sensitivity of the reduced porphyrin and of the anion, all transfers were made under argon.

Due to the possible iron reduction by the thiolate with its concomitant oxidation,[12] the internally chelated thiolate of [ether−BHP(C_{12})(C_{11}−S)]·**K** was protected by an acetyl group before the reduction of the iron by dithionite. Deacetylation and preparation of the five-coordinated anion−heme complex were obtained by adding an equal volume of a saturated solution of potassium hydride−crown ether complex. [TPivP−C_6HF_4S]·**Na** was prepared according to the procedure described in ref 31.

Kinetic Measurements. The experimental setup was as previously described,[52] except for some modifications performed in order to improve the accuracy. The new Q-switched Nd/Yag laser source ("Quantel") had a pulse width of 10 ns, and its energy could be varied between 1 and 450 mJ at 532 nm. The detection system was as before except for digital recording and in-line data processing. Absorbance changes as small as 10^{-3} can be measured with a time constant of 50 ns. The transient absorption changes were recorded on a Lecroy 9450 digital oscilloscope and the data were transferred to an Apple MacIntosh II-CI computer via an IEEE-488 interface for conversion of the recorded signals into transient absorbance changes.

In order to check that the kinetics were not spoiled by base elimination, the base concentration was varied systematically; however, since a large excess of anion was required to ensure pentacoordination of the porphyrins and to avoid autooxidation, the range of variation was only of a factor of 3, obtained by dilution of the saturated solution.

Parallel Reactions. As described by White et al.,[28] the determination of the kinetic rate parameters $k_B{}^{\pm L}$ can be biased by various parallel reactions which may occur in the presence of a free proximal ligand, a porphyrin, and a gaseous ligand. Hemochrome (B−Fe^{II}−B) formation was not a problem with thiolate derivatives, since thiolate ions do not form bis-adducts even with unencumbered Fe^{II} porphyrins.[1−3] At the concentrations used in our experiments, bis-adducts of alcoholate were not observed either, although bis-ligation has been reported under different conditions.[1] Base elimination, which is sometimes observed after photodissociation of the gaseous ligand from nitrogenous bases complexes was not observed, presumably because the greater basicity of the anionic ligand stabilizes the pentacoordinated B−Fe^{II} com-

(53) Case, D. A.; Huynh, B. H.; Karplus, M. *J. Am. Chem. Soc.* **1979**, *101*, 4433−4453.
(54) Eaton, W. A.; Hanson, L. K.; Stephens, J. C.; Sutherland, J. C.; Dunn, J. B. R. *J. Am. Chem. Soc.* **1978**, *100*, 4991−5003.
(55) Dedieu, A.; Rohmer, M.-M.; Bernard, M.; Veillard, A. *J. Am. Chem. Soc.* **1976**, *98*, 3717−3718.
(56) Li, X.-Y.; Spiro, T. G. *J. Am. Chem. Soc.* **1988**, *110*, 6024−6033.
(57) Ray, G. B.; Li, X.-Y.; Ibers, J. A.; Sessler, J. L.; Spiro, T. G. *J. Am. Chem. Soc.* **1994**, *116*, 162−176.
(58) Yu, N.-T.; Kerr, E. A.; Ward, B.; Chang, C. K. *Biochemistry* **1983**, *22*, 4534−4540.
(59) Momenteau, M.; Loock, B.; Bisagni, E.; Rougee, M. *Can. J. Chem.* **1979**, *57*, 1804−1813.
(60) Tetreau, C.; Lavalette, D.; Momenteau, M.; Lhoste, J.-M. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 2267−2271.

plexes.[2,12] Evidence for the absence of base elimination was provided by the fact that relaxation rates were independent of the base concentration over a 3-fold range.

Spectrophotometric Titrations. The partition coefficient $M = K_B^{CO}/K_B^{O_2}$ of TPivP−BuS$^-$ was determined by photometric titration of the oxygenated against the carbonylated complex Carbon monoxide equilibrium constants were obtained by spectrophotometric titration of the carboxyhemochrome against the pentacoordinated species. Calibrated CO/O_2 or CO/argon mixtures were bubbled in the reduced porphyrin solution. The absorption changes were followed in the Soret band.

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