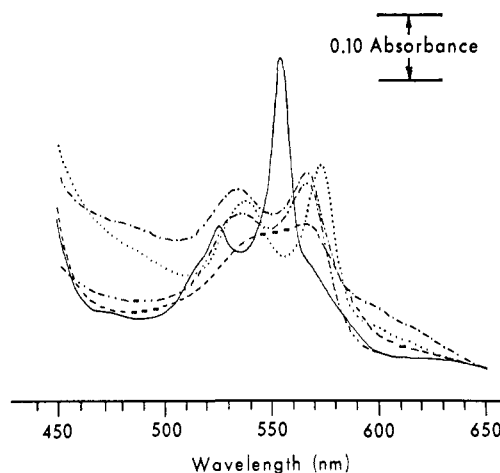Figure 3. Spectral changes of ferrous protoheme IX 2-methyl-imidazole system associated with a reversible oxygenation process. Solution conditions as in Figure 1: A (——) deoxyheme at −80°, B(·····) equilibration of A with O_2 at −80°, C (−·−·−·−) equilibration of B with CO at −80°, D (—·—) solution C at 23°, E (− − − −) equilibration of D with Ar followed by photolysis at 23°.

To support the optical assignment of the oxyheme complex, Mössbauer measurements at 4.2°K of the *tert*-butylamine oxyheme, using ^{57}Fe enriched protoheme IX *ca.* 4 × 10^{-4} *M*, were obtained. The Mössbauer spectrum indicated a sharp doublet, line width *ca.* 0.28 mm/sec, having a quadrupole splitting ΔE_Q of 2.37 ± 0.02 mm/sec and an isomer shift δ of 0.326 ± 0.02 mm/sec, relative to iron metal. These data compare well with ΔE_Q of 2.24 mm/sec and δ of 0.24 mm/sec at 1.2°K, and ΔE_Q of 2.19 mm/sec and δ of 0.26 mm/sec at 77°K for oxyhemoglobin.[14] Allowing this sample to oxidize by warming to ambient temperature produced a paramagnetic Mössbauer spectrum at 4.2°K typical of ferric heme systems, with no indication of the oxyheme doublet.

A number of observations and conclusions can be made from these results. First, the close spectral similarity between the amine oxyheme complex and the native protein is striking when considering the large differences between proximal axial ligands in these heme complexes. Second, the results indicate an imidazolyl-type base is not unique as an axial ligand in its ability to effect the formation of an oxyheme complex. In contrast to imidazole,[15] the *tert*-butylamine is unable to π-bond with the heme and thus indicates that π-bonding between a proximal ligand and oxyheme is not essential. Third, the temperature dependence of the spectra of deoxy and CO complexes in these and other model systems[2] draws awareness to the possible misinterpretation of spectra of heme complexes when observed at below ambient temperatures and to the possible dissimilarities between model complexes and hemeproteins. Thus, deoxymyoglobin does not show

(14) G. Lang and W. Marshall, *Proc. Phys. Soc., London,* **87**, 3 (1966).
(15) C. K. Chang and T. G. Traylor, *J. Amer. Chem. Soc.,* **95**, 8477 (1973).

Figure 4. Spectral changes of ferrous protoheme IX *tert*-butyl-amine system associated with a reversible oxygenation process. Solution conditions as in Figure 2: A (———) deoxyheme at −80°, B (·····) equilibration of A with O_2 at −80°, C (—·—) equilibration of B with CO at −80°, D (−·· −·−) solution C at 22°, E (− − − −) equilibration of D with Ar followed by photolysis at 22°.

absorption spectra changes indicative of either a change in coordination or spin state even at −196°.[4] Finally, the results demonstrate that the natural heme prosthetic group is able to reversibly bind molecular oxygen in solution in the absence of apoprotein or the covalent linkage of any grouping as long as the pertinent chemical and physical conditions are controlled.

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Simple Dioxygen Heme Complexes Formed in *N,N*-Dimethylformamide

Sir:

Two soluble Fe(II) porphyrins recently have been described[1,2] which form reversible oxyhemochromes.[3] Both are iron porphyrin derivatives possessing structural modifications designed to enhance the formation and stability of a complex with molecular oxygen. That

(1) C. K. Chang and T. G. Traylor, *J. Amer. Chem. Soc.,* **95**, 5810, 8477 (1973).
(2) J. P. Collman, R. R. Gagne, T. R. Halbert, J. C. Marchan, and C. A. Reed, *J. Amer. Chem. Soc.,* **95**, 7868 (1973).
(3) In accord with the term "oxyhemoglobin," the term "oxyhemochrome" is proposed to denote complexes composed of a nitrogenous base, an Fe(II) porphyrin, and O_2. The corresponding CO complexes are called carboxyhemoglobin and carboxyhemochrome. Although the term "oxyhemochrome" appears in the older literature in reference to hemochromes in which oxidation of the porphyrin ring has occurred,[4] the term now seems more appropriate for the reversible dioxygen complexes. The stoichiometry of the O_2 complex of I has been established to be one O_2/heme.[5]
(4) R. Lemberg and J. W. Legge, "Hematin Compounds and Bile Pigments," Interscience, New York, N. Y., 1949, p 203.
(5) W. S. Brinigar, C. K. Chang, J. Geibel, and T. G. Traylor, *J. Amer. Chem. Soc.,* **96**, 5597 (1974).
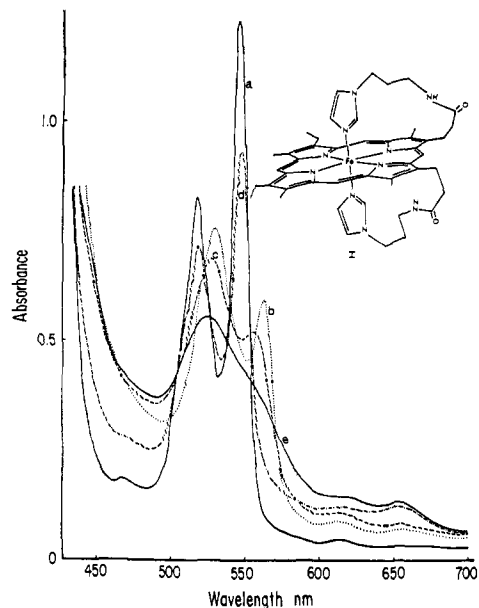
Figure 1. Formation of a reversible oxyhemochrome from ferromesoporphyrinbis[3-(1-imidazoyl)propyl]amide (I) in DMF. Spectra recorded in the sequence: (a) ——, hemochrome at 20°, λ_{max} at 547 and 518 nm; (b) $\cdots$, addition of O_2 (1 atm) at $-45°$, λ_{max} at 562 and 530 nm; (c) ---, replacement of O_2 with CO at $-45°$, λ_{max} at 560 and 528 nm; (d) $-\cdot-$, removal of CO by photolysis; (e) ——, addition of O_2 at 20°; after oxidation was complete.

Figure 2. Formation of a reversible oxyhemochrome from bis-(1-butylimidazole)ferroprotoporphyrin dimethyl ester (IIa) in DMF, [1-butylimidazole]/[protoheme] = 10^2. Spectra follow the same sequence and designation as in Figure 1: (a) λ_{max} at 558 and 528 nm; (b) λ_{max} at 572 and 536 nm; (c) λ_{max} at 565 and 535 nm.
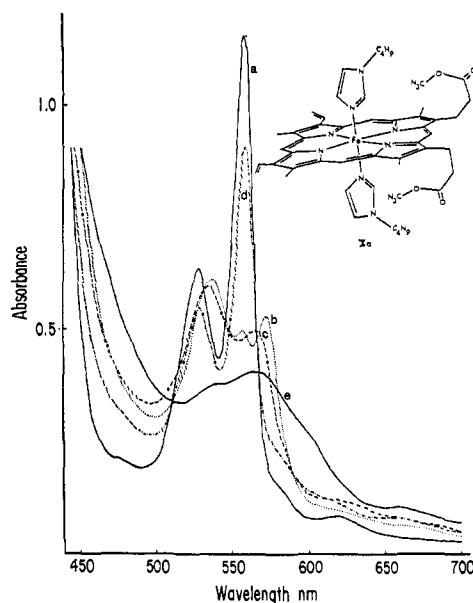
these modifications are not essential to reversible oxygen binding is suggested by our observation that ferromesoporphyrinbis[3-(1-imidazolyl)-propyl]amide (I)[6] in CH_2Cl_2 forms a dioxygen complex stable toward oxidation for hours at $-45°$. This oxyhemochrome is also formed in DMF where it is considerably more stable, Figure 1. These results demonstrate that O_2 is capable of replacing a nitrogenous ligand and therefore is a much stronger ligand in Fe(II) porphyrin complexes than previously suspected.

Replacement of a nitrogenous ligand by O_2 obviously should also occur when the ligands are not covalently linked to the porphyrin ring. Such is the case. Bis(1-butylimidazole)ferroportoporphyrin dimethyl ester (IIa) yields a stable oxyhemochrome in DMF at $-45°$, Figure 2, and like I oxidizes at 20° with a $t_{1/2}$ of about 2 min. The stability of the oxyhemochromes of both I and IIa appears to be even greater in dimethyl sulfoxide than in DMF. The $t_{1/2}$ in dimethyl sulfoxide is approximately 30 min at 20°.
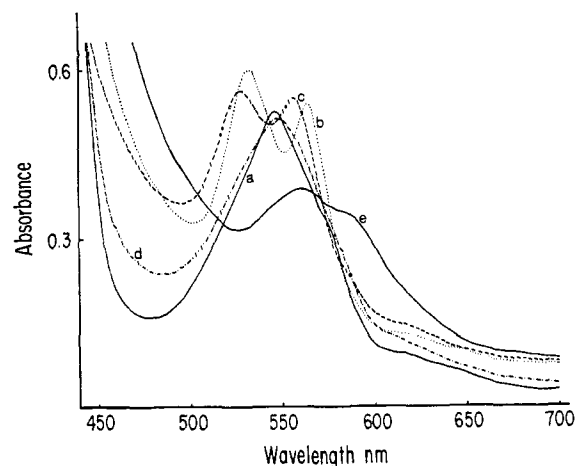
An N-alkyl group on imidazole is not essential for oxygen binding, but its presence does result in increased stability of the dioxygen complex toward oxidation. Bis(imidazole)ferroprotoporphyrin dimethyl ester (IIb) combines with O_2 in DMF at $-45°$, spectral changes being similar to those observed with the N-butyl hemochrome, Figure 2. However, IIb oxidizes immediately at 20°. Imidazole presents a source of both protons and hydrogen atoms for reaction with intermediate reduction products of O_2. The necessity of an aprotic

Figure 3. Formation of a reversible oxyhemochrome from protoheme dimethyl ester in DMF. Spectra follow the same sequence and designation as in Figure 1: (a) λ_{max} at 546 nm; (b) λ_{max} at 563 and 532 nm; (c) λ_{max} at 557 and 528 nm.

environment for stability of oxyheme complexes has been postulated previously.[8]

In the absence of stronger ligands, heme forms a five-coordinate complex in anhydrous DMF.[9] The ligand may be DMF itself or dimethylamine, a likely impurity. This complex also combines reversibly with O_2 at $-45°$, Figure 3, but oxidizes immediately at 20°. The spectra are strikingly similar to the spectra of the corresponding complexes of hemoglobin and myoglobin, although the maxima are shifted by approximately 10 nm toward the violet relative to the heme proteins. Imidazole clearly is not essential for reversible O_2 binding.

Some irreversible oxidation is evident in all four of the systems described above. The CO spectra, obtained

(6) Compound I was synthesized by reaction of mesoporphyrin diacid chloride with 3-(3-imidazole)propylamine followed by iron insertion as previously described[7] for the corresponding pyrroporphyrin derivative. Both the nmr spectrum of the porphyrin in $CDCl_3$ and C and H analysis of the hemin chloride are consistent with structure I.

(7) C. K. Chang and T. G. Traylor, *Proc. Nat. Acad. Sci. U. S.*, 70, 2647 (1973).

(8) J. H. Wang, "Haematin Enzymes," J. E. Falk, R. Lemberg, and R. K. Morton, Ed., Pergamon Press, New York, N. Y., 1961, p 98.

(8) D. Brault and M. Rougee, *Nature (London), New Biol.*, 241, 19 (1973).

after displacing O_2 with CO, are not identical with the corresponding carboxyhemochrome spectra obtained without prior exposure to O_2. Increased absorbance in the region of 660 nm, particularly evident in the oxidation of I and IIb, is indicative of some oxidation at the methine bridge carbons.[10]

All the Fe(II) complexes described herein were obtained by reduction of the corresponding Fe(III) porphyrin with a mixture of CaH_2 and Pd black. A number of Fe(III) porphyrins have been successfully reduced in DMF, CH_3CN, CH_2Cl_2, benzene, and toluene using this technique. The solution must be deoxygenated prior to the addition of CaH_2 and Pd and must contain a small amount of water to generate H_2 for the reduction. Reduction is accomplished by gentle stirring. Removal of the solution from the solid phase gives an anhydrous solution of the Fe(II) porphyrin free of impurities and excess reducing agent. The latter is of particular importance in studies of reversible O_2 binding.

A dramatic difference is observed in the extent of reaction of I and IIa with O_2. This difference may be due to constraints imposed on orientation of the coordinated imidazole(s) in I by its covalent linkage to the porphyrin ring. The effective concentration of N-alkylimidazole in the vicinity of the iron atom is approximately two orders of magnitude higher in I where the imidazoles are covalently linked to the porphyrin ring, than in IIa where the effective concentration of 1-butylimidazole is near the actual concentration, less than 10 mM. However, surprisingly, formation of the dioxygen complex is incomplete in IIa as evidenced by a portion of the parent hemochrome α peak remaining after equilibration with pure O_2 (see Figure 2), whereas it is complete in I (see Figure 1). The P_{O_2} required for conversion of 50% of the hemochrome to oxyhemochrome is less than 1 mm in the case of I and approximately 80 mm for IIa. These results suggest that constraints imposed on the orientation of the coordinated imidazole(s) in I favor formation of the dioxygen complex. Small changes in orientation of the imidazole group would alter the overlap between imidazole π orbitals and Fe t_{2g} orbitals. Since O_2 depends on π bonding for complex formation, the extent of π-bonding interaction between imidazole and Fe will necessarily affect the extent of π back-bonding to the other axial ligand. These results provide experimental evidence for a suggestion originally made by Williams[11] that regulation of oxygen affinity in oligomeric hemoglobins could be due at least in part to slight changes in orientation of the proximal imidazole imposed by protein conformational changes. The transition from the high spin five-coordinate (deoxy) to a low spin six-coordinate (oxy, carboxy) results in displacement of the iron relative to the plane of the porphyrin ring,[12] and probably to changes in puckering of the porphyrin ring system as well. Upon the addition of a sixth ligand, adjustment of the protein in response to changes in the iron porphyrin structure could force the imidazole to alter its orientation. Propagation of the protein conformational change to adjacent subunit(s) would

induce a similar change in orientation of the proximal imidazole in the unliganded subunit(s) increasing the affinity of the heme for π-bonding ligands.

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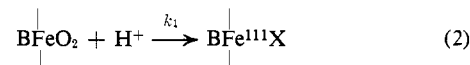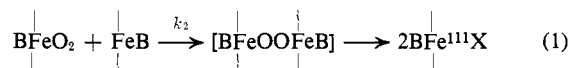*Philadelphia, Pennsylvania 19122*

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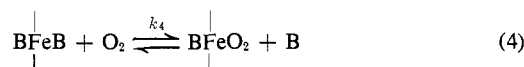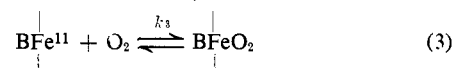*Received February 22, 1974*

Solvent Effects on Reversible Formation and Oxidative Stability of Heme–Oxygen Complexes[1]

Sir:

The stabilities of oxyhemoglobin and oxymyoglobin have been attributed in part to the steric and nonpolar characteristics on the distal side of the heme.[2,3] While this arrangement has been considered to retard both the proposed "binuclear" and "mononuclear" oxidations (eq 1 and 2)[4–8] (B = nitrogen base), it might also affect

$$BFeO_2 + FeB \xrightarrow{k_2} [BFeOOFeB] \longrightarrow 2BFe^{III}X \quad (1)$$

$$BFeO_2 + H^+ \xrightarrow{k_1} BFe^{III}X \quad (2)$$

oxygenation itself.

$$BFe^{II} + O_2 \underset{}{\overset{k_3}{\rightleftharpoons}} BFeO_2 \quad (3)$$

$$BFeB + O_2 \underset{}{\overset{k_4}{\rightleftharpoons}} BFeO_2 + B \quad (4)$$

Such an effect has been demonstrated by Stynes and Ibers[9] for cobalt porphyrins. They found that increased solvent polarity favored oxygenation and interpreted this result as indicating a large contribution of $Co^{III}O_2\cdot^-$ to the complex.

(1) This work was supported by the National Institutes of Health, Grant USPHS HL 13581.

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(3) (a) E. Antonini and M. Brunori, "Hemoglobin and Myoglobin in Their Reactions with Ligands," North Holland Publishing Co., Amsterdam, 1971, p 23; (b) M. F. Perutz, *Proc. Roy. Soc., Ser. B*, **173**, 113 (1969); (c) J. C. Kendrew, *Brookhaven Symp. Biol.*, **15**, 216 (1962).

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(8) J. E. Baldin and J. Huff, *J. Amer. Chem. Soc.*, **95**, 5759 (1973).

(9) H. C. Stynes and J. A. Ibers, *J. Amer. Chem. Soc.*, **94**, 5125 (1972).

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