# NOVEL HEME: $O_2$ BONDING OF POSSIBLE RELEVANCE TO OXYGEN UTILIZING HEME AND OTHER PROTEINS

William H. Fuchsman[†a], Clyde H. Barlow*, William J. Wallace*, and Winslow S. Caughey*[†b]
Department of Biochemistry*
Colorado State University
Fort Collins, Colorado 80523
and
Department of Physiological Chemistry[†]
Johns Hopkins University School of Medicine
Baltimore, Maryland 21205

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Summary. Solid dipyridine hemes which are unreactive toward oxygen lose both pyridine ligands upon heating under vacuum to give a solid which takes up $O_2$, reversibly, one $O_2$ per heme. Replacement of $^{16}O_2$ by $^{18}O_2$ reduces only infrared bands near 1660 and 1590 $cm^{-1}$, frequencies near the vibrational band for gaseous $O_2$. No Fe-O bands are detected. EPR spectra reveal a free radical and ferric iron; Mössbauer, NMR and infrared spectra support an iron(III) oxidation state. Limited molecular weight data indicate a dimer. Possibly two dioxygen molecules are held sandwich fashion between two porphyrins via donor-acceptor interactions, which are facilitated by electron transfer from iron(II) into the porphyrin forming a $\pi$-anion. Such $O_2$ bonding is not found in oxy Hb and Mb or in oxyhemerythrin but may occur with cytochrome c oxidase and other oxygen utilizing (or producing) heme and other proteins.

Introduction. Vibrational spectra have recently provided important new evidence of the nature of $O_2$ bonding to metal proteins. In the infrared spectra of oxy Hb, an oxy cobalt Hb, and oxy Mb bands due to bound $O_2$ are found near 1100 $cm^{-1}$ consistent with bent end-on binding to metal, $M^{\cdots}O_{\backsim O}$ (1,2,3). Hemerythrin(4) and hemocyanin(5) exhibit bands near 850 $cm^{-1}$ in Raman spectra suggestive of peroxo-type metal bound dioxygen. In contrast, when $O_2$ binds to solid hemes devoid of axial ligands(6,7,8,9), infrared bands near 1600 $cm^{-1}$(8,9) are observed. These oxygenated iron porphyrins must involve oxygen binding quite unlike that in oxy-Hb, -Mb, -hemerythrins or -hemocyanins and we report on their properties and consider their possible significance in hemeproteins.

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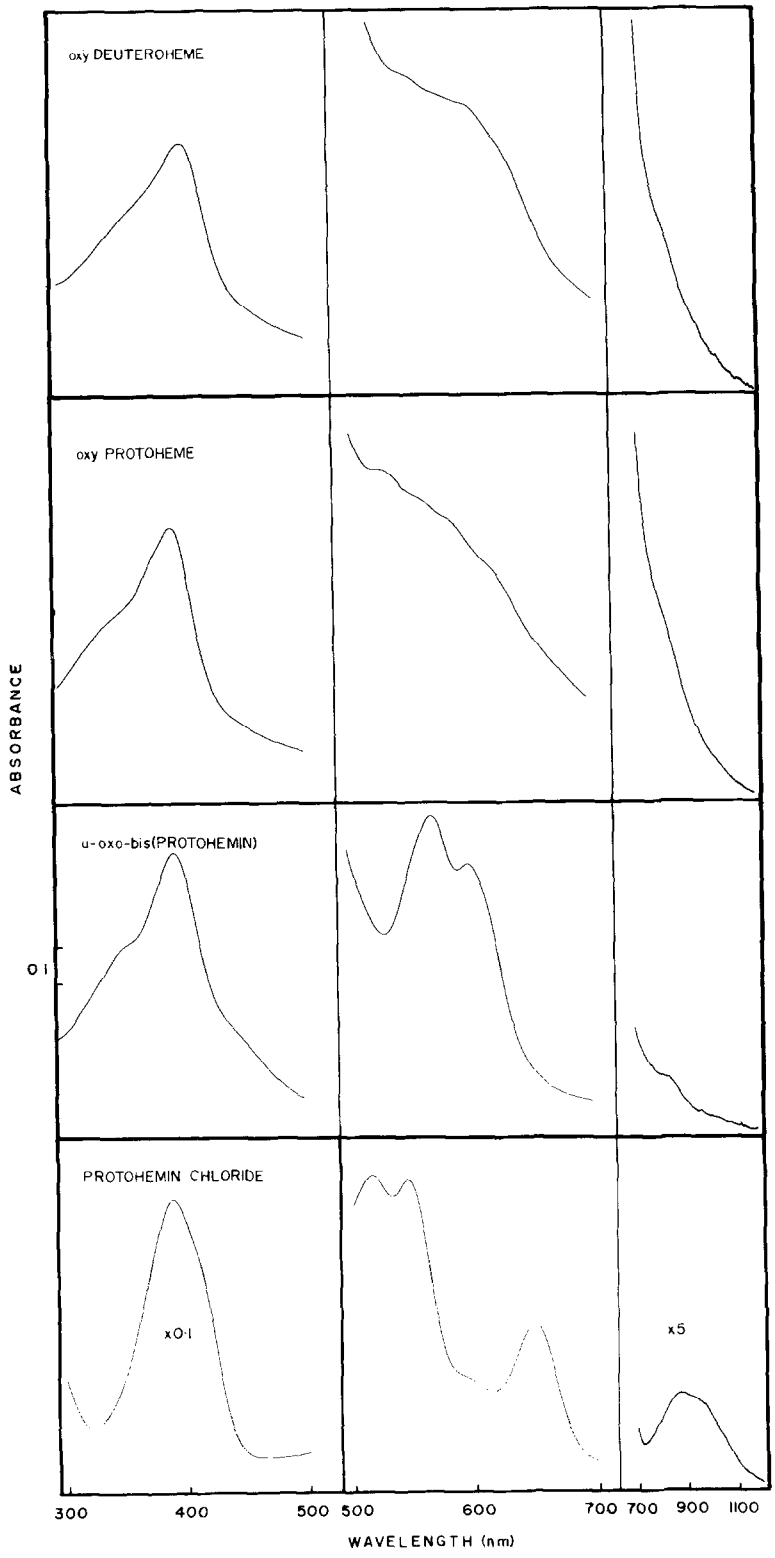
[a]Present address: Department of Chemistry, Oberlin College, Oberlin, Ohio 44074

[b]Address correspondence to this author: Department of Biochemistry, Colorado State University


oxy DEUTEROHEME
oxy PROTOHEME
u-oxo-bis(PROTOHEMIN)
PROTOHEMIN CHLORIDE
x0·1
x5
ABSORBANCE
0·1
300
400
500
500
600
700
700
900
1100
WAVELENGTH (nm)

Methods and Materials. Iron 2,4-substituted deuteroporphyrin IX dimethyl esters were prepared as described earlier(6,10). Infrared spectra of ca. 2 mg of porphyrin in 200 mg of KBr or CsI were determined with a Perkin Elmer model 180 spectrometer. EPR spectra were determined at 77°K with a Jeolco Me-X spectrometer. NMR spectra were determined with a 100 MHz Jeolco spectrometer. Mössbauer spectra were obtained through the kind collaboration of Dr. J. Spijkerman. Electronic spectra were obtained on a Cary 17 spectrophotometer.

Results. When deuteroporphyrin IX dimethyl ester iron(II) dipyridine is heated under vacuum, its vapor pressure becomes measurable ($\sim$ 1 mm) at 80°C and rises to $\sim$ 20 mm at 140°C. Two moles of pyridine/mole of iron are removed by pumping on the sample for 15 to 20 min. at 140°C. Several other dipyridine hemes, including 2,4-diacetyldeutero-, and proto-(6), heme A(7) and etio II-, have also been shown to undergo decomposition via the loss of pyridine under similar conditions. Thus the reaction of equation 1 appears to be a general one

$$\text{py-Fe}^{II}\text{-py} \rightleftharpoons \text{Fe}^{II} + \text{py} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 1$$

where py represents pyridine and $Fe^{II}$ an iron(II) porphyrin without axial ligands.

The "bare iron" product of reaction 1, upon exposure to $O_2$ slowly (over about 24 hours) forms an oxygenated complex (oxy Hm). A stoichiometry of 1 mole of $O_2$ per mole of Fe has been observed by both elemental analyses and weight changes. Also, in a gas buret, 40.9 µmole of deuteroheme took up 41.1 µmole $O_2$ and 51.3 µmoles of protoheme took up 52.5 µmole of $O_2$. Heating the oxy Hms under vacuum results in loss of $O_2$ to regenerate $Fe^{II}$. Hence the re-

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Figure 1. Electronic spectra from 1200 to 300 nm showing the near infrared, visible and Soret bands for a) deuteroporphyrin IX dimethyl ester "oxy Hm", 0.065 mM, b) protoporphyrin IX dimethyl ester "oxy Hm" .062 mM, c) µ-oxo-bis[protoporphyrin IX dimethyl ester iron(III)] .063 mM, d) protoporphyrin IX dimethyl ester iron(III) chloride, 0.61 mM. The absorbance in the region from 500 to 700 nm is expanded 10 fold and the 700-1200 nm region 50 fold compared with the 300-500 nm region.

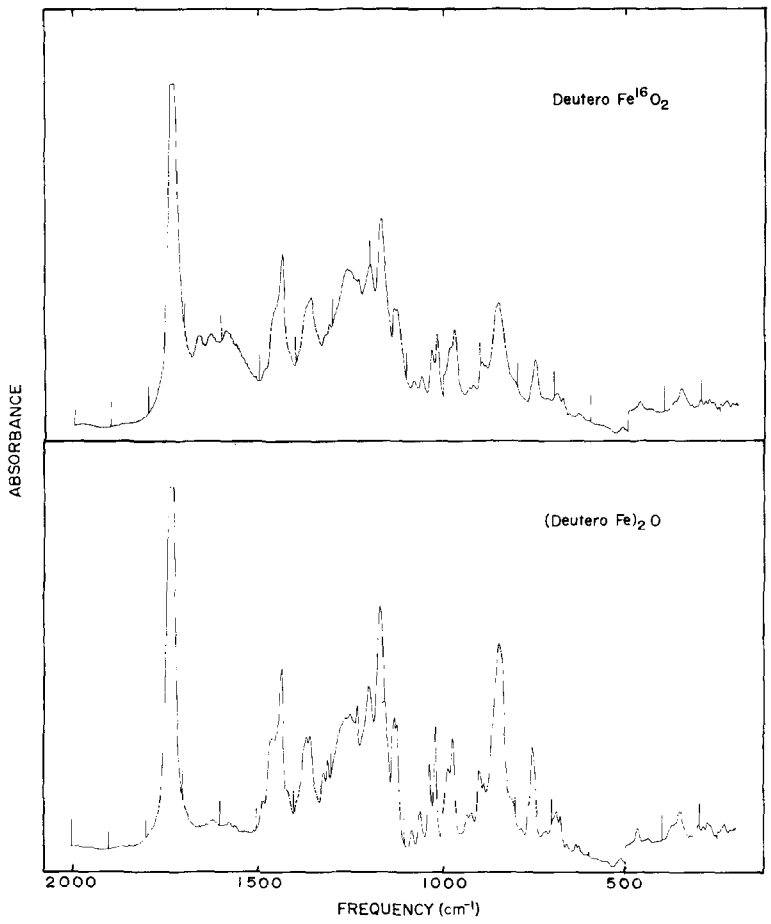



Figure 2. Infrared spectra covering the frequency range from 2000 to 200 $cm^{-1}$ obtained from CsI discs (1 mg/200 mg CsI). a) deuteroporphyrin IX dimethyl ester "oxy Hm" ($^{16}O_2$), b) μ-oxo-bis[deuteroporphyrin IX dimethyl ester iron(III)].

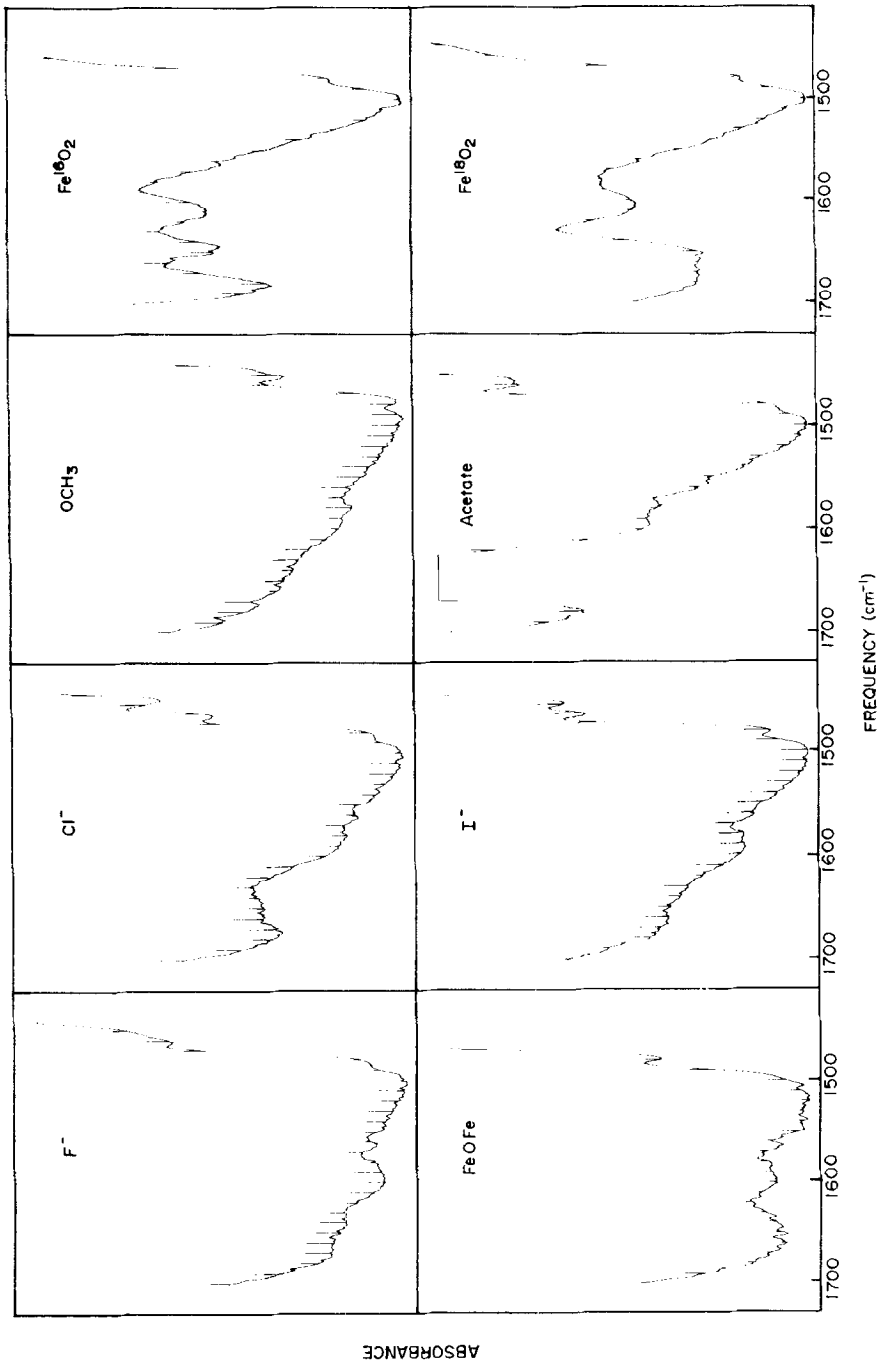
action of equation 2 seems quite general as well. However, alterations

$$Fe^{II} + O_2 \rightleftarrows Fe{:}O_2\ (oxy\ Hm) \quad \ldots\ldots\ldots\ldots\ldots\ldots 2$$

have been observed upon repeated cycling.

The oxy Hms are not only stable as solids but solutions in many solvents e.g., benzene and chloroform, are also stable. The electronic spectra of such solutions are typically quite featureless (Fig. 1), but highly characteristic

Figure 3. Infrared spectra of a series of deuteroporphyrin IX dimethyl ester iron(III) complexes containing different axial ligands. The frequency range 1700-1500 $cm^{-1}$ shows the unique character of the oxygen isotope-sensitive set of bands around 1600 $cm^{-1}$ in the oxy Hm complexes.


$Fe^{18}O_2$
$OCH_3$
$Fe^{18}O_2$
$Cl^-$
Acetate
$I^-$
$F^-$
Fe O Fe
1700
1600
1500
FREQUENCY ($cm^{-1}$)
ABSORBANCE

and different from spectra for μ-oxo-bis-hemins (oxidation products obtained when dipyridine hemes are dissolved in such solvents open to air(10)), for typical high spin hemins and for oxy Hb or oxy Mb(11). Porphyrin dimers frequently have less well-resolved spectra than monomers and dimers are also suggested in limited molecular weight determinations by vapor phase osmometry (e.g., oxy protoheme in benzene gave a found mol. wt. of 1600 vs. a calculated value of 1354). The solutions failed to show multiple components when subjected to chromatography in several systems.

Infrared spectra of oxy Hms typically reveal characteristic bands sensitive to isotope exchange ($^{18}O_2$ for $^{16}O_2$) only near 1600 $cm^{-1}$ (Fig. 2,3). No O-O bands appeared in the 1100 $cm^{-1}$ (bent end-on) or the 850 $cm^{-1}$ (peroxo) regions (Fig. 2). And, although with NO and CO hemes Fe-N (at 480 $cm^{-1}$) and Fe-C (at 354 $cm^{-1}$) bands are seen, the corresponding Fe-O bands were not found for oxy Hms.

Mössbauer spectra suggest an iron(III) oxidation state (9)[c] as do EPR spectra where absorption is found in g=6 and 4 regions (Fig. 4). A striking feature of the EPR spectrum is a rather broad g=2 (radical) signal. The paramagnetism in solution is shown by PMR spectra with strong broadening and paramagnetic shifts. In the diacetyldeutero derivative, the acetyl C=O stretch, an infrared band shown to be sensitive to oxidation state(8,9,12) is consistent with iron(III).

Discussion. When solid hemes from which the axial ligands have been removed are treated with gaseous dioxygen dimeric complexes containing two hemes and two oxygen molecules are formed. The bound dioxygen is infrared active and absorbs around 1600 $cm^{-1}$. This must mean that the character of the oxygen-oxygen bond is little changed in consequence of its binding within the complex, but the bonding environment is asymmetric. Although the complexes

[c]For example, the oxyHm from 2,4-diacetyldeuteroporphyrin IX dimethyl ester exhibited Mössbauer spectra at 298K with a quadrupole splitting of 0.70 mm/sec and a chemical shift of 0.55 mm/sec with respect to sodium nitroprusside.

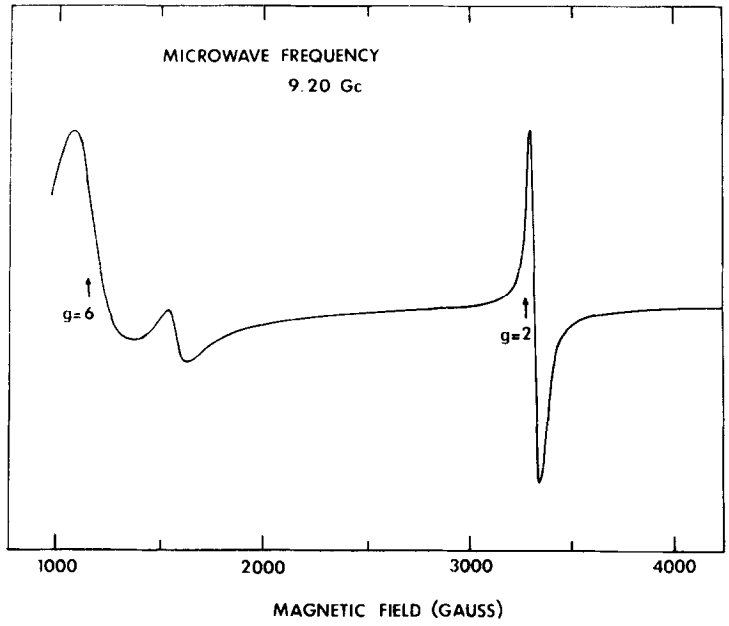



Figure 4. EPR spectrum of solid oxy diacetyl Hm at 77°K.

are quite stable, the bonding forces that lead to the stability are evidently largely non specific dipolar interactions. This is in marked contrast to the highly specific formal bonding involved in oxy hemoglobins, myoglobins and hemerythrin which is characterized by a change in $\nu_{O-O}$(1,2,3,4) and by a $\nu_{M-O}$(13). The oxy Hms described in this work do not exhibit a metal-oxygen stretch or a $\nu_{O-O}$ greatly different from that of the free gas (1555 $cm^{-1}$).

The argument regarding the nature of the bound dioxygen clearly rules out the possibility of electron transfer from iron to oxygen. Nevertheless, the evidence, particularly the ESR and Mössbauer spectra, clearly indicates that iron is oxidized and is present in the complex in the +3 state. The electron released in this oxidation process must be trapped in the $\pi$ system of the porphyrin to produce a porphyrin $\pi$ anion radical. The g=2 ESR signal is a logical consequence of the formation of such a radical. The cytochrome c peroxidase ES complex shows a rather similar g=2 signal(14).

A model that is consistent with the bonding requirements of these oxy Hms is obtained by placing the dioxygen molecules between and lying parallel to

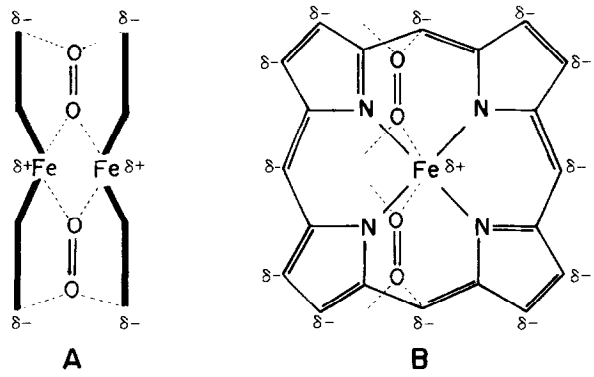



Figure 5. A schematic representation of a possible structure for the oxy Hm complexes in which each dioxygen ($O_2$) is held in the dimeric complex by weak donor-acceptor bonding through the interaction of an induced dipole on dioxygen with the positive charge centers at both iron atoms and with the negative charge resident in the $\pi$ system of each porphyrin. If this interaction reduces the electron density in the $\pi^*$ orbital system of the dioxygen then the fractional increase in order for the O-O bond observed in the infrared could be explained. By spanning across and interacting with charge centers as different as iron ($\delta$+) and ring carbon ($\delta$-) electronic assymmetry can be induced in dioxygen allowing it to become infrared active. A. A view parallel to the porphyrin plane illustrating the placement of the dioxygen molecules between the two hemes. B. A view perpendicular to the porphyrin plane (top heme removed) illustrating the placement of the dioxygen molecules with respect to the porphyrin ring system.

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the $\pi$ systems of two adjacent hemes in such an orientation that one oxygen atom of each dioxygen could interact weakly with the iron atoms while the other extends out between the planes of the porphyrin ring systems. The presence of two isotope sensitive bands in the infrared (e.g., at 1663 $cm^{-1}$ and 1597 $cm^{-1}$ for oxy deutero Hm) may be a result of the asymmetry in the porphyrin system. In this respect it is worthy of note that the oxy etio Hm complex in which the porphyrin is more nearly symmetric shows only a single band at 1595 $cm^{-1}$. A model which embodies this interpretation of the data is shown in Fig. 5.

Thus in some hemeproteins the porphyrin ring may be directly involved in oxygen binding with strong interaction between Fe and $O_2$ not required. Possibly $O_2$ - porphyrin ring interactions can also occur in a protein with only a single heme especially if an amino acid residue or a cofactor were to serve the role of the second porphyrin in the oxy Hms. Indeed the reactions of

flavo and other non-heme proteins with $O_2$ may involve analogous binding of $O_2$.

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