Communications to the Editor

Oxidized Cytochrome P-450. Magnetic Circular Dichroism Evidence for Thiolate Ligation in the Substrate-Bound Form. Implications for the Catalytic Mechanism[1-3]

Sir:

Cytochrome P-450[4] is the only mammalian heme protein normally capable of activating dioxygen for insertion into organic molecules (Scheme I). One of the fundamental questions about the structure and function of P-450 enzymes has been the identity of the axial ligand(s) bound to the iron (II,III) protoporphyrin IX prosthetic group in reaction states **1–5.** Comparative physical properties including absorption spectroscopy,[5,6] of synthetic porphyrin complexes with various axial ligands L[5-12] and enzyme reaction states **1, 2,** and **5** strongly support cysteinate sulfur ligation in the latter. For the substrate-bound high-spin state **2** this conclusion has been drawn from an extensive body of physicochemical data for the complexes Fe^{III}(PPIXDME)L.[5,6] Thus the P-450 cytochromes appear to be the only reasonably substantiated biological examples of cysteinate binding to heme iron.

We have previously demonstrated the utility of magnetic circular dichroism (MCD) spectroscopy as a probe of the P-450 active site structure.[12-14] Here we present additional evidence from MCD spectra[15] that reaction state **2** contains Fe(III)-S-Cys axial ligation. The most suitable enzyme for examining this state is bacterially derived $P\text{-}450_{cam}$[4a] whose MCD spectrum has already been reported.[13,16] Simulated side chain binding by the indicated amino acid residues was studied by MCD spectroscopy (Figures 1 and 2) utilizing the previously described[6,7] Fe^{III} (PPIXDME)L complexes with L = SC_6H_4-*p*-NO_2 (Cys, for x-ray structure: see ref 6), OC_6H_4-*p*-NO_2(Tyr), OEt (Ser, Thr), and OAc (Glu, Asp). A nitrogeneous high-spin synthetic model has not been prepared, but is available (see Figure 2) in acid met-myoglobin (metMb·H_2O) and fluoro metMb (metMb·F) which contain an imidazole (His) axial ligand and are high spin.[18] The most conspicuous and important feature of these spectra is the strong *negative* ellipticity in the Soret region (~400 nm) common to Fe^{III}(PPIXDME)(SC_6H_4-*p*-NO_2) and $P\text{-}450_{cam}$ (**2**) (Figure 1) whereas other species with axial oxygen and nitrogen[17] ligation exhibit strong *positive* ellipticity in this region (Figure 2). The comparison between the thiolate model complex and the enzyme is less exact in the visible region, presumably due to the use of an aromatic rather than an aliphatic thiolate ligand,[19] but overall spectral similarity is evident. Spectra of other species in this region are clearly more dissimilar to that of $P\text{-}450_{cam}$ (**2**) than is the case for Fe^{III}(PPIXDME)(SC_6H_4-*p*-NO_2). The Soret feature of metMb·F[17] (Figure 2), while negative at 405 nm, is the only ca. 20% as intense as the corresponding ones in Figure 1.

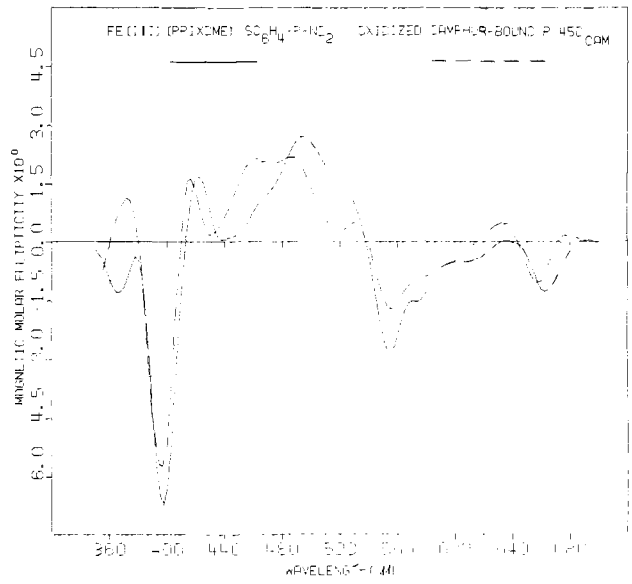
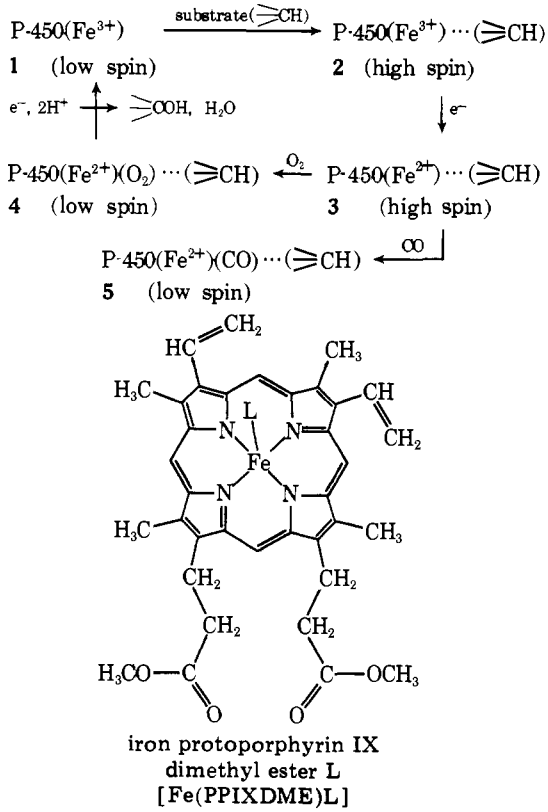


Figure 1. Magnetic circular dichroism spectra of oxidized, camphor-bound $P\text{-}450_{cam}$ at pH 7.0 (replotted from data of Vickery et al.[16]) and a thiolate model heme complex (in toluene).

The present results indicate that axial thiolate ligation in high-spin five-coordination iron(III) protoporphyrin IX species is diagnostically signaled by both the shape and strong negative intensity of their Soret MCD, and provide a further contribution to the collective evidence for cysteinate binding in P-450 (**2**). As shown in the following communication,[20] MCD results now also indicate similar binding in chloroperoxidase despite earlier chemical evidence to the contrary.[21]

Recently, evidence has appeared that P-450 can function as a peroxidase,[22,23] and can achieve hydroxylation anaerobically in the absence of an electron source with alkyl hydroperoxides, H_2O_2, $NaIO_4$, or $NaClO_2$ as the source of oxygen.[24-27] No carbon monoxide inhibition is seen; the enzyme is never reduced. The implication drawn from these results is that the "active oxygen" species in P-450 is the same as the peroxidase "compound I",[24-27] i.e., an oxygen atom bound to

Scheme I



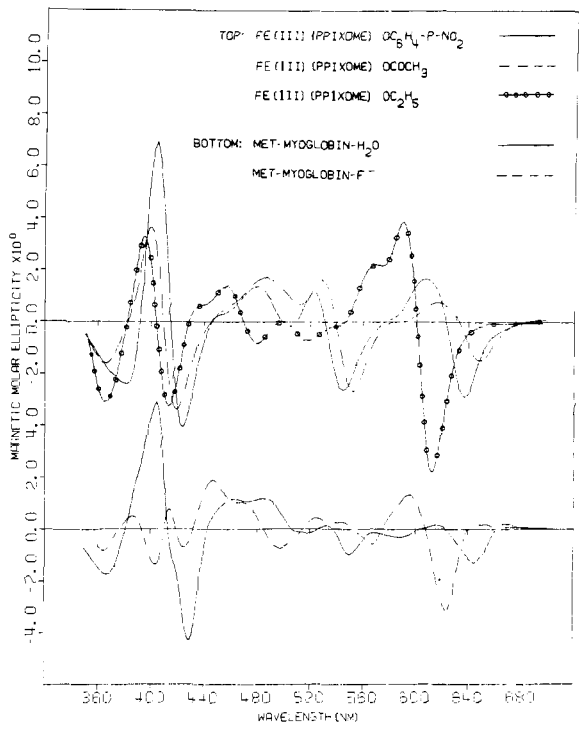
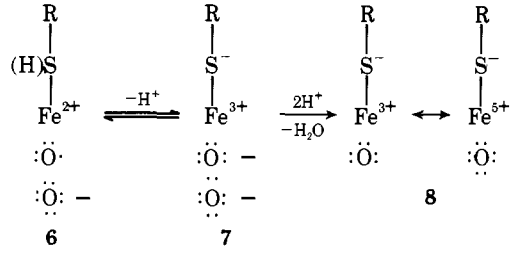


Figure 2. Magnetic circular dichroism spectra of oxygen and nitrogen derived model heme complexes. The latter are replotted from data of Vickery et al.[17] The solvent for Fe^{III}(PPIXDME)OC_6H_4-P-NO_2 is toluene; for Fe^{III}(PPIXDME)$OCOCH_3$ is 95% CH_2Cl_2, 5% CH_3CO_2H; for Fe^{III}(PPIXDME)OC_2H_5 is 90% CH_2Cl_2, 10% C_2H_5OH. The myoglobin derivatives were examined at pH 6.8.[17]

ferric iron.[28] This intermediate clearly has a very electrophilic oxygen, most likely possessing oxygenase capability. The real question, then, is how the protein goes from a dioxygen bound species to an oxygen atom bound species—*a process not achieved by other oxygen binding heme proteins such as hemoglobin.* Two properties of P-450—the unusual thiolate ligand[29] and the acceptance of two electrons by the protein—may make this possible.

The cysteine anion ligand appears capable of transferring electron density to the iron. Collman et al.[12] have shown, for example, that the ferrous carbonyl adduct of P-450 is electron rich. Furthermore, the inability to prepare an iron(III) protoporphyrin IX *alkyl* thiolate complex for this work has been due *in part* to the ease of electron transfer from thiolate to iron to give an iron(II) porphyrin and disulfide.[19]

Reduction and oxygen binding by P-450 leads to the catalytically active intermediate **4** (Scheme I). If sulfur binding is retained, the equilibrium mixture **6**–**7** (shown as charge-limiting forms) is a possibility.[29,30] We conjecture that the highly charge-separated form **7** can relieve itself of two charge equivalents by loss of water, achieving the crucial dioxygen bond cleavage and affording "compound I",[28] **8.** This process could be facilitated by electron "push" of the relatively polarizable thiolate, with the same effect operating to stabilize at least transiently, the highly electrophilic species **8,** which is presumably the actual hydroxylating agent.



In conclusion, we have presented spectral evidence using MCD that P-450 has a thiolate ligand in its oxidized, high-spin form. This unusual ligand, by transferring electron density to iron, may facilitate dioxygen bond cleavage leading to a peroxidase "compound I" intermediate capable of oxygenation.[31]

Acknowledgments. We wish to thank Ruth Records for running the MCD spectra, Dr. Larry Vickery (University of California, Berkeley) for permission to replot data,[16,17] and the National Institutes of Health (Grant No. GM 20276-02) and the National Science Foundation (Grants GP-40089X and MPS 75-09806) for financial support.

References and Notes

(1) Magnetic Circular Dichroism Studies, Part 43; for Part 42 cf. R. E. Linder, G. Barth, E. Bunnenberg, C. Djerassi, L. H. Seamans, and A. Moscowitz, *Chem. Phys. Lett.*, in press.
(2) Presented in part at the 59th Annual Meeting, Federation of American Societies for Experimental Biology, Atlantic City, N.J., April 1975.
(3) This work will be presented by one of us (J.H.D.) as partial fulfillment of the requirements for the Ph.D. degree in chemistry at Stanford University.
(4) For reviews cf. (a) I. C. Gunsalus, J. R. Meeks, J. D. Lipscomb, P. Debrunner, and E. Münck in "Molecular Mechanisms of Oxygen Activation", O. Hayaishi, Ed., Academic Press, New York, N.Y., 1974, Chapter 14; (b) J. E. Tomazewski, D. M. Jerina, and J. W. Daly, *Ann. Rep. Med. Chem.*, **9,** 290 (1974); (c) H. A. O. Hill, A. Röder, and R. J. P. Williams, *Struct. Bonding (Berlin),* **8,** 123 (1970).
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(15) MCD measurements on the model complexes were made on a JASCO (Japan Spectroscopy Company) J-40 circular dichroism instrument using a 15 KG electromagnet. The protein MCD spectra have been corrected for natural circular dichroism - (MCD_{obsd} = MCD + CD). All data have been normalized and are expressed in the units of molar magnetic ellipticity, $[\theta]_M$, deg cm^2 dmol^{-1} G^{-1}. Measurements were made at ambient temperatures. The MCD spectrum of oxidized $P\text{-}450_{cam}$ (Figure 1) is a replot of data presented by Vickery et al.[16] and the MCD spectra of metMb·H_2O and metMb·F (Figure 2) are replots of data presented by Vickery et al.[17]
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(29) Sulfur ligation is assumed here when oxygen is bound by extension of its apparent presence in reaction states **1, 2,** and **5.**
(30) Recent experiments on $[Ru(NH_3)_5(H_2S)^{2+}$, (pK_{a_1} 4)] provides an indication that Fe(II) thiol complexes, as yet uncharacterized, may be moderately weak protonic acids: C. G. Kuehn and H. Tabue, *J. Am. Chem. Soc.*, **98,** 689 (1976).
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that it, too, has a negative MCD effect in the Soret region. We thank Professor Ogoshi for communicating synthetic details and R. W. Lane, Jr., for helpful discussions. We also note the recent communication by Chang and Dolphin involving thiolate ligation of a porphyrin-oxygen complex: C. K. Chang and D. Dolphin, *J. Am. Chem. Soc.*, **98**, 1607 (1976).

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Chloroperoxidase. Evidence for P-450 Type Heme Environment from Magnetic Circular Dichroism Spectroscopy[1,2]

Sir:

Chloroperoxidase (CPO) and cytochrome P-450, two heme proteins with fundamentally different native activities,[3,4] have a surprising number of similar physical properties as judged by electronic absorption, electron paramagnetic resonance (EPR), and Mossbauer spectroscopy.[5] Here we present additional evidence, obtained with magnetic circular dichroism (MCD) spectroscopy, for the equivalence of their oxidized high spin, and reduced + CO heme environments. More importantly, a comparison of the MCD spectra of CPO with model heme compounds[6,7] indicates that the similarity between CPO and the P-450 cytochromes is due to thiolate ligation of the heme iron. This is contrary to the conclusion of Chiang et al.[8] based on chemical evidence that the axial ligand is *not* sulfur derived in either the native or urea-denatured protein.

Similarities between CPO and P-450 were first observed by Hollenberg and Hager[5a] who studied the absorption spectra of CPO. In addition to similarities in their oxidized and reduced states, they found that CPO, like P-450, forms a reduced + CO complex absorbing at an abnormally long wavelength (443 nm). An explanation for the unusual spectral characteristics of P-450 has been reached as a result of model heme experiments[6,7,9] which strongly implicate an axial thiolate ligand as the causal structural feature.

Figures 1-3 compare the MCD spectra[10] of CPO[11] and P-450[12-14,17-19] in their oxidized high-spin, reduced, and reduced + CO states. Similarity between the spectra of the two

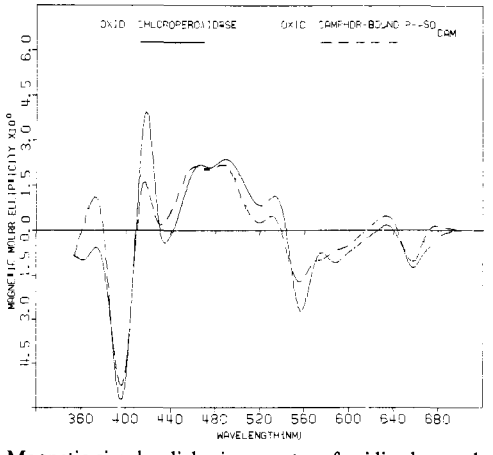

Figure 1. Magnetic circular dichroism spectra of oxidized, camphor-bound P-450$_{cam}$ at pH 7.0 (replotted from data of Vickery et al.[13b]) and oxidized chloroperoxidase at pH 3.8.

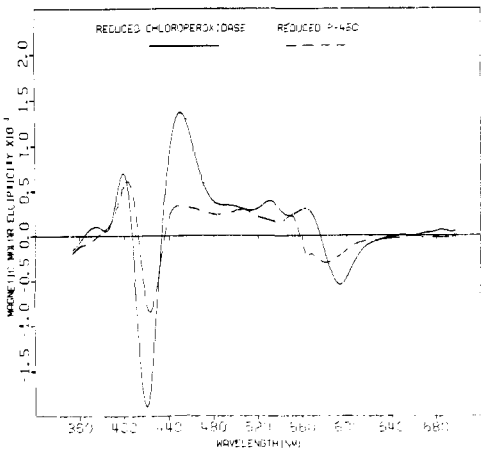

Figure 2. Magnetic circular dichroism spectra of purified, reduced P-450$_{LM2}$ at pH 7.4 and purified, reduced chloroperoxidase at pH 3.8.

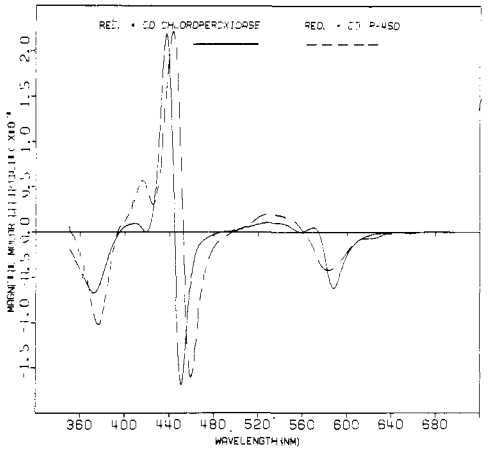

Figure 3. Magnetic circular dichroism spectra of purified, reduced + CO P-450$_{LM2}$ at pH 7.4 and purified, reduced + CO chloroperoxidase at pH 3.8.

proteins is at once apparent, particularly in the oxidized high-spin and reduced + CO states. The MCD spectrum of oxidized CPO (Figure 1) reproduces virtually all the features of oxidized high-spin P-450$_{cam}$.[12,13] Particularly noteworthy are the negative bands of nearly equal intensity in the 395-nm Soret and 660-nm charge transfer regions. While the spectra of the reduced proteins[14] (Figure 2) show gross overall resemblance, too many differences are present to conclude that their heme environments are alike. The *low temperature* Mossbauer results of Champion et al.,[5b] however, indicate that reduced CPO and P-450 do have equivalent heme environments. Whether or not it is possible to extrapolate the low temperature Mossbauer data to ambient temperatures is questionable. The inconsistencies seen in the MCD spectra may be due to differences in the spin states of the two proteins at ambient temperatures. The reduced + CO spectra are displayed in Figure 3. As discussed by Collman et al.,[7] the locations of the Soret MCD crossover points (~450 nm) for the two proteins, which correspond to the positions of their absorption maxima, may be shifted when the local polarity of their heme environment changes. The small shape differences in the 520-620-nm region are reflected in the corresponding absorption spectra.[5a,16] Aside from these minor variations, the spectra are quite similar, exhibiting equally intense MCD effects in the 450-nm Soret region, shoulders at about 420 nm, and "extra" negative bands at 370 nm. The pair of features at 450 and at 370 nm are also observed in the *hyper* spectra[20] exhibited by a number of metallo porphyrins.[21]

Our previous work comparing the MCD spectra of P-450 and models for its oxidized high-spin[6] and reduced + CO[7]