# Cytochrome P-450 and Chloroperoxidase: Thiolate-Ligated Heme Enzymes. Spectroscopic Determination of Their Active Site Structures and Mechanistic Implications of Thiolate Ligation

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## Contents



## I. Introduction

In order to fully understand how a catalytic system operates, it is necessary to establish the structure of the functional unit as well as to determine the changes that the catalytic center undergoes during the reaction cycle. For metalloenzymes, the concept of structure can be broken down into two categories: the structure of the metal binding site(s) and the structure of the protein itself. In this context, cytochrome P-450 and chloroperoxidase are a particularly intriguing pair in light of the extensive evidence that will be presented in this review article that the two enzymes have essentially identical heme iron coordination structures. Surprisingly, however, the two enzymes have rather distinct catalytic properties: P-450 is a monooxygenase that activates dioxygen for incorporation into unactivated C–H bonds, while chloroperoxidase exhibits classic peroxidase and catalase activities and is also able to catalyze the peroxide-dependent halogenation of organic substrates. Clearly, the final catalytic activity of each of these enzymes must be influenced by both the structure of the metal active site as well as the protein environment surrounding the metal. This review will focus on the spectroscopic evidence for the active site structures of these two enzymes and on the interrelationship of metal structure and protein environment in determining the final catalytic activity of each enzyme.

### A. Cytochrome P-450

Mammalian cytochrome P-450 is a ubiquitous membrane-bound monooxygenase that catalyzes the hydroxylation of membrane-entrapped nonpolar substrates including steroids, drugs, and pollutants, yielding partially water soluble products that can be further metabolized.[1–22] Multiple forms (isozymes) of the enzyme, each having slightly different substrate selectivity and reactivity, have been discovered. Ironically, in addition to its very beneficial roles in metabolism, biosynthesis, and membrane detoxification, by producing highly reactive products from certain substrates, P-450 has been strongly implicated as the activator of many chemical carcinogens.[23,24] Thus, it is an enigmatic protein, a sort of Dr. Jekyll and Mr. Hyde, with both beneficial and detrimental properties. An understanding of its unique reactivity, in addition to furthering our knowledge of metalloenzyme catalysis and dioxygen activation, could conceivably lead to the regulation of its role in chemically induced cancer by selective inhibition of the forms of P-450 most reactive in carcinogen activation.

Cytochrome P-450 enzymes have been isolated from numerous mammalian tissues (liver, kidney, lung, intestine, adrenal cortex, etc.), insects, plants, yeasts, and bacteria. Because of its role of dioxygen activation, it has been the subject of a vast number of publications, including numerous review articles and books as well as symposia proceedings. As will be discussed, all P-450 enzymes share a common active site structure and reaction cycle. The most extensively investigated mammalian P-450 has been the major phenobarbital-inducible form from rabbit liver, P-450-LM2, first purified by Coon and co-workers;[20] the bacterial camphor-hydroxylating P-450 from *Pseudomonas putida* known as P-450-CAM is soluble and was the first P-450 to be purified.[4–8] Because of the relative ease of handling P-450-CAM and because it can be obtained in large quantity and studied at high concentration, much of our knowledge about the active site structure and mechanism of action of P-450 in general has been derived from investigations of P-450-CAM.

The P-450 enzymes catalyze the incorporation of one atom of dioxygen into hydrocarbon substrates with

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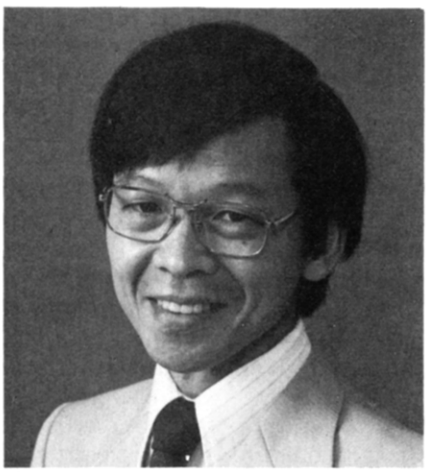


Masanori Sono was born in Nagasaki Prefecture, Japan. He received his B.S. (1970), M.S. (1972), and Ph.D. (1975) degrees from the University of Tokyo, Japan, in pharmaceutical sciences. Following pre- (1972–1975) and post-doctoral work (1975–1977) with Professor Toshio Asakura at the University of Pennsylvania and at the Children's Hospital of Philadelphia on a heme modification study of hemoglobin and myoglobin, he worked under Professor Osamu Hayaishi on oxygenases at the Kyoto University, Japan, as an instructor (1977–1980). In 1980, he moved back to the United States as a research associate with Professor John H. Dawson in the Department of Chemistry at the University of South Carolina. In 1982, he was promoted to Research Assistant Professor and in 1986 to Research Associate Professor. His research interests include spectroscopic and catalytic studies of the active site structures and the mechanisms of actions of several heme-containing oxygenases and peroxidases.

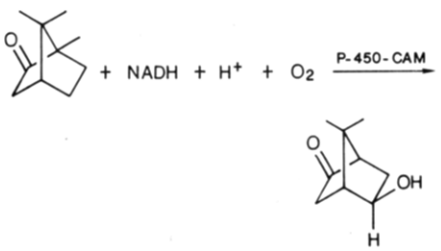
**TABLE I. Major Types of Reactions Catalyzed by Cytochrome P-450[a]**

| reaction type | simplified example | typical substrate |
|---|---|---|
| aliphatic hydroxylation | cyclohexane → cyclohexanol | pentobarbital |
| aromatic hydroxylation | benzene → phenol | phenobarbital |
| alkene epoxidation | cyclohexene → cyclohexene oxide | aldrin |
| N-dealkylation | $CH_3N(H)CH_3 \rightarrow CH_3NH_2 + H_2C{=}O$ | methadone |
| O-dealkylation | $C_6H_5OCH_3 \rightarrow C_6H_5OH + H_2C{=}O$ | codeine |
| oxidative deamination | $(CH_3)_2CHNH_2 \rightarrow (CH_3)_2C{=}O + NH_3$ | amphetamine |
| S-oxidation | $CH_3SCH_3 \rightarrow (CH_3)_2S{=}O$ | chlorpromazine |
| reductive dehalognation | $C_6H_5CH_2Br \rightarrow C_6H_5CH_3$ | halothane |

[a] See ref 1–22 for review articles on cytochrome P-450.

concomitant reduction of the other oxygen atom to water as shown in eq 1. For P-450-CAM (eq 2), this

$$\geq C\text{–}H + O_2 + 2H^+ + 2e^- \rightarrow \geq C\text{–}OH + H_2O \quad (1)$$




reaction involves the stereo- and regiospecific hydroxylation of camphor to the 5-exo alcohol. Several other types of oxygen-transfer reactions are catalyzed by P-450 (Table I), including epoxidation, N-dealkylation, O-dealkylation, and sulfoxidation.[20] One of the most intriguing and least understood oxygen-transfer reactions catalyzed by P-450 involves the oxidative cleavage of carbon–carbon bonds in the biosynthesis of certain steroid hormones as shown in Figure 1.[25] Under anaerobic conditions, P-450 reductively dehalogenates halocarbons[26] as described in Table I.

Both mammalian and bacterial P-450 enzymes share a common reaction cycle with four well-characterized and isolable states (**1**–**4**, see Figure 2). The low-spin six-coordinate ferric resting form **1** is converted to a high-spin five-coordinate state **2** upon substrate binding. Reduction leads to a high-spin five-coordinate derivative **3** capable of binding dioxygen (**4**) or carbon monoxide (**5**). One-electron reduction of the oxygen adduct, the last stable intermediate in the reaction cycle, leads to release of water and the hydroxylated product, likely after transient formation of species such as **6** and **7**. Addition of oxygen atom donors such as alkyl hydroperoxides, peracids, iodosobenzene, amine oxides, $H_2O_2$, $NaIO_4$, or $NaClO_2$ to the ferric substrate complex **2** leads directly to the formation of product, possibly through the intermediacy of species **7**. The spectroscopic evidence for each of the isolable intermediates described in Figure 2 will be presented in this review. Recently, the X-ray crystal structures of P450-CAM states **1** and **2** appeared.[27,28] Remarkably, essentially all of the major structural features, including metal–ligand bond distances, of P-450 states **1**–**5** were independently established through the use of spectroscopic methods, in most cases prior[1] to the publication of the crystal structures. In that regard, the P-450

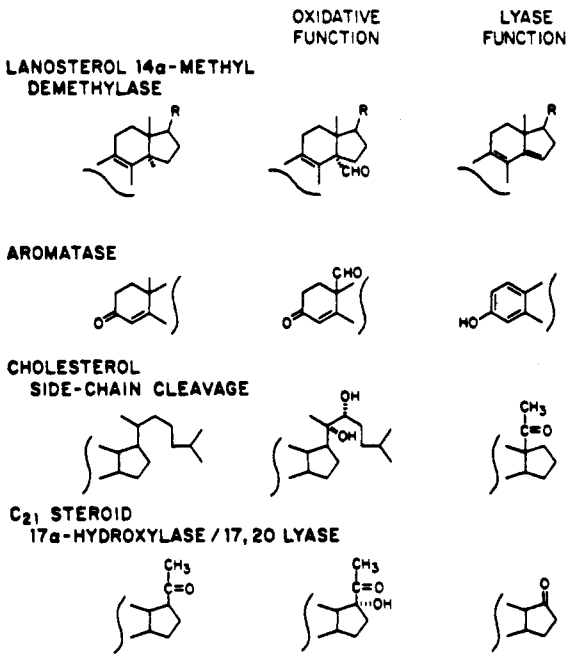



**Figure 1.** Double functional role of cytochrome P-450 dependent enzymes acting on steroid substrates. Reproduced with permission from ref 25. Copyright 1986 Journal of Biological Chemistry.

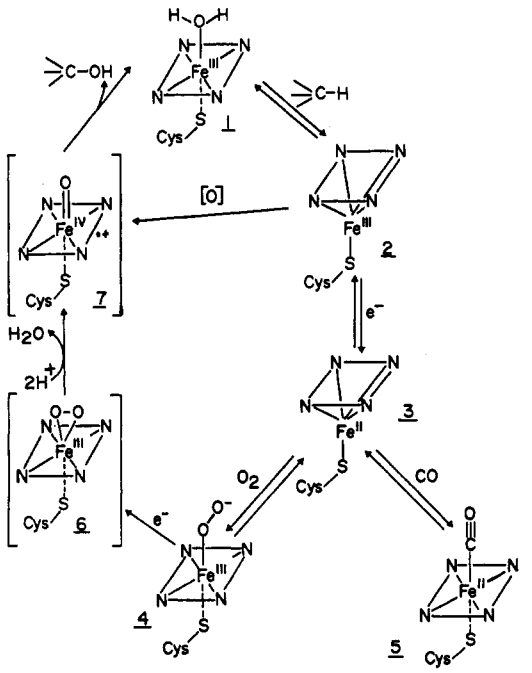



**Figure 2.** Catalytic cycle of cytochrome P-450 and the postulated structures of the intermediates. Oxy-P-450 (4) is shown as a complex of ferric iron and superoxide anion but could also be described as an adduct of neutral dioxygen and ferrous iron. States 6 and 7 are hypothetical intermediates whose structures have not been established. The dianionic porphyrin macrocycle is abbreviated as a parallelogram with nitrogens at the corners. Structures 1, 2, and 7 are neutral, while the overall charge on structures 3, 4, and 5 is minus one and on structure 6 is minus two.

system provides an excellent example of how much information can be derived about the active site of a metalloenzyme through the spectroscopic examination of the enzyme in conjunction with the synthesis and characterization of biomimetic models for its active site.

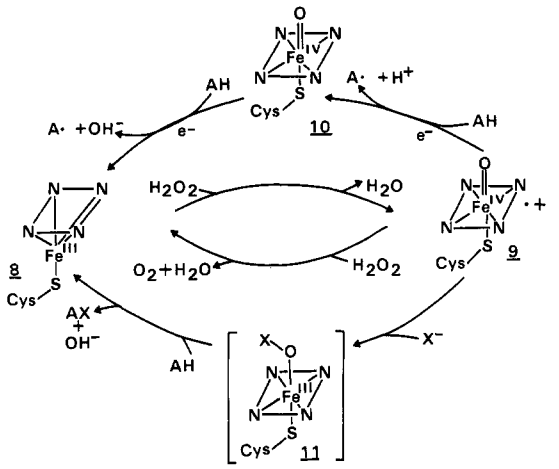



**Figure 3.** Catalytic cycles of chloroperoxidase and the postulated structures of the intermediates. The structures of the intermediates in the peroxidase (8 → 9 → 10 → 8) and catalase (8 → 9 → 8) modes have been partially characterized. The structure of intermediate 11 in the halogenation mode (8 → 9 → 11 → 8) is hypothetical. The dianionic porphyrin macrocycle is abbreviated as a parallelogram with nitrogens at the corners. Structures 8 and 9 are neutral, while the overall charge on structures 10 and 11 is minus one.

## B. Chloroperoxidase

Chloroperoxidase is an enzyme isolated from the marine fungus *Caldariomyces fumago* which has been most extensively characterized in the laboratory of Lowell Hager at the University of Illinois.[29,30] In addition to the usual peroxidase and catalase activities exhibited by other peroxidases,[31] chloroperoxidase is unique in its ability to catalyze the hydrogen peroxide dependent oxidation of $I^-$, $Br^-$, or $Cl^-$ and resultant formation of carbon–halogen bonds with halogen acceptors such as β-diketones. Chlorite can also serve as the halogen source without requiring hydrogen peroxide. Most peroxidases are only able to brominate and iodinate organic compounds by using the halide anion as the source of the halogen. Horseradish peroxidase can chlorinate organic substrates by using chlorite but not chloride as the source of halogen,[32] while myeloperoxidase can use chloride but is inactivated by chlorite.[33] As shown in eq 3, the chloroperoxidase-catalyzed halogenation process occurs with concomitant reduction of hydrogen peroxide to water.

$$AH + X^- + H^+ + H_2O_2 \rightarrow AX + 2H_2O \qquad (3)$$

In contrast to P-450, the catalytic reaction cycle of chloroperoxidase (Figure 3) involves only the ferric and higher oxidation states of iron and therefore is not sensitive to inhibition by carbon monoxide. From the high-spin five-coordinate ferric state 8, chloroperoxidase reacts with hydrogen peroxide to form a high-valent iron–oxo derivative known in the peroxidase literature as compound I (9), which is formally two oxidation equivalents above the ferric resting state. On the basis of extensive data on other peroxidases,[31,34-36] it is likely that chloroperoxidase compound I has an $Fe^{IV}$=O (porphyrin radical cation) structure. This intermediate is the first step in the peroxidase, catalase,[31] as well as halogenation activities of the enzyme. In the catalase mode, compound I interacts with a second molecule of hydrogen peroxide, oxidizing it to dioxygen and returning the heme iron to the ferric resting state. In the

peroxidase mode, compound I serves as a one-electron oxidant, converting organic substrates (AH) into radical products (A·) with concurrent reduction of compound I to compound II (10), which likely has a $Fe^{IV}$=O structure.[31,34,35,37-41] Oxidation of a second substrate molecule regenerates the ferric resting state 8, ready for another round of catalysis. In the chlorination mode, it has been proposed that compound I reacts with chloride to produce a hypochlorite ($OCl^-$) adduct, 11 (also known as compound X), which serves as the active chlorinating agent.[42,43] The exact details of the electronic structure of compound X and how chloroperoxidase chlorinates hydrocarbons are matters of current controversy. Nonetheless, it is clear that a full understanding of the unique modes of reactivity of chloroperoxidase will require a knowledge of its active site structure.

Extensive investigations of chloroperoxidase over the past 15 years have revealed close correspondence between its spectral properties and those of cytochrome P-450 and thiolate-ligated heme iron model complexes.[44-59] Despite these spectral lines of evidence in support of cysteinate ligation to chloroperoxidase, attempts by Chiang et al. to detect a free sulfhydryl group available for ligation to the heme iron were unsuccessful in either the native or denatured enzyme.[60] This was used as evidence that the axial ligand could not be cysteinate. More recently, however, Hager and coworkers sequenced the gene for chloroperoxidase and found an additional cysteine that is very likely the axial ligand.[61] The evidence for each of the structures displayed in Figure 3 will be described in this review together with recent results showing that distinctions exist between P-450 and chloroperoxidase in terms of the nature of the active site heme environment that may account for how two enzymes with such similar metal binding site structures could have such different reactivities.

## II. Low-Spin Ferric States

### A. Identification of the Fifth Ligand to the Heme Iron

On the basis of the observation that chemical modification reagents for thiol groups inactivate P-450, Mason was the first to suggest that a sulfur donor ligand was bound to the heme iron of P-450.[62,63] The initial model system designed to test this hypothesis involved the study of ligand adducts of myoglobin and hemoglobin with thiols.[64-66] The EPR properties of these complexes, now known to be thiolate (as opposed to thiol) adducts,[67] closely matched those of native low-spin P-450, which are unusual among low-spin heme iron proteins because of the narrow spacing between $g_z$ and $g_x$. This suggested that the combination of a sulfur donor ligand and histidine could model the spectral properties of ferric low-spin P-450. Other ligand adducts of myoglobin did not adequately match the EPR properties of P-450. Reexamination of the myoglobin thiolate adducts by Shimizu et al.[68] using MCD spectroscopy added additional support to these conclusions.

More specific evidence for the presence of a thiolate sulfur donor ligand to the heme iron of P-450 came from the elegant model chemistry of Collman and Holm.[69-72] Addition of various biomimetic ligands to well-defined five-coordinate thiolate-ligated heme iron complexes led to several six-coordinate complexes whose EPR properties matched those of low-spin P-450. Only six-coordinate complexes containing a thiolate ligand had EPR properties similar to those of P-450. The identity of the sixth ligand had only a secondary effect on the $g$ values observed, and so the authors did not speculate on the identity of that ligand. Several subsequent studies of models for low-spin ferric P-450 have consistently shown the need for a thiolate sulfur donor to reproduce the EPR properties of the enzyme. Similar results were also obtained by Holm, Ullrich,[73-75] and Sakurai[76,77] using UV–visible absorption spectroscopy to examine thiolate–ferric heme–X model complexes, where X was varied among various biomimetic ligands. Once again, the thiolate ligand was needed to match the spectral properties of low-spin ferric P-450.

In 1977, Ruf and Wende reported that the ligation of two thiolate ligands to ferric heme iron produced a complex with a very unusual UV–visible absorption spectrum with two Soret bands in the 350–500-nm region.[78] The so-called split Soret or hyperporphyrin spectrum had also been seen in the case of ferrous-CO P-450 and was attributed to mixing and splitting of the normal porphyrin $\pi,\pi^*$ transition by a sulfur $p^\dagger \rightarrow$ porphyrin $\pi^*$ transition of the correct energy and symmetry.[79] In the case of ferric low-spin systems, investigations of a large number of ligand combinations revealed only two sets, bis-thiolate[49,73-75,78,80,81] and thiolate/phosphine,[51,73-75,80b,82] that give rise to the split Soret band pattern. Accordingly, the demonstration that addition of acidic thiols or of phosphines to ferric P-450 (vide infra) produces the split Soret spectrum provides additional evidence for the presence of an endogenous thiolate ligand.[49,51]

The most direct spectroscopic evidence for the presence of a sulfur donor ligand to low-spin ferric P-450 has come from EXAFS spectroscopy. Dawson, Hodgson, and their co-workers studied both low-spin ferric P-450-CAM and purified liver microsomal P-450.[54,55,83-86] The EXAFS technique[87-89] is particularly good at distinguishing sulfur donor ligands from nitrogen or oxygen donors and, with good data, is capable of determining metal–ligand bond distances to an accuracy of ±0.02 Å. Although no geometrical information can be directly obtained from the EXAFS data, extensive crystallographic studies of ferric heme iron model complexes revealed that the Fe–N (porphyrin) distance can be used to distinguish six-coordinate from five-coordinate structures.[90] Consequently, determination of the Fe–N (prophyrin) distance from analysis of the EXAFS data can indirectly provide information about the coordination number and, therefore, about the geometry.

Curve fitting of the EXAFS data for low-spin ferric P-450-CAM[91] and microsomal P-450[54] (Table II) clearly demonstrated the presence of a sulfur donor atom as a ligand to the heme iron. In fact, suitable fits could only be obtained by inclusion of a sulfur donor atom. Furthermore, although the EXAFS data do not directly allow the type of sulfur donor (thiolate or thiol) to be determined, the measured Fe–S distances are shorter than those of known Fe(III)–S(thiolate) heme iron systems.[70,92] Since Fe–S(thiol) bond distances are generally about 0.1 Å longer than Fe–S(thiolate) dis-

**TABLE II. Structural Details for Native Ferric Cytochrome P-450, Chloroperoxidase, and Relevant Model Complexes[a]**

| system | Fe–N(porphyrin) method | $R$, Å | $N^b$ | Fe–S(axial) $R$, Å | $N^b$ | ref |
|---|---|---|---|---|---|---|
| | | Low-Spin Ferric | | | | |
| P-450-CAM | EXAFS | 2.00 | 5.0 | 2.22 | 0.6 | 85 |
| P-450-LM2 | EXAFS | 2.00 | 4.8 | 2.19 | 0.8 | 54 |
| P-450-CAM[c] | X-ray | | 4 | 2.20 | 1 | 28, 93 |
| $[Fe(TPP)(SC_6H_5)_2]^-$ | X-ray | 2.008 | 4 | 2.336 | 1 | 92 |
| $Fe(TPP)(HSC_6H_5)(SC_6H_5)$ | X-ray | | 4 | 2.27 ($RS^-$) | 1 | 70 |
| | | | | 2.43 (RSH) | 1 | |
| | | High-Spin Ferric | | | | |
| P-450-CAM | EXAFS | 2.06 | 5.2 | 2.23 | 0.8 | 85 |
| chloroperoxidase | EXAFS | 2.05 | 4.2 | 2.30 | 0.9 | 54 |
| P-450-CAM[d] | X-ray | 2.05 | 4 | 2.20 | 4 | 27, 93 |
| $Fe(PPIXDME)(SC_6H_5NO_2)$ | X-ray | 2.064 | 4 | 2.324 | 1 | 72 |

[a] EXAFS data were obtained by curve fitting. Abbreviations: TPP, tetraphenylporphyrin; PPIXDME, protoporphyrin IX dimethyl ester. [b] Number ($N$) of atoms at the distance indicated. [c] 2.2-Å resolution data. The iron is found to be 0.29 Å out of the plane of the four pyrrole nitrogens toward the cysteinate axial ligand. The sixth ligand was found to be water (or hydroxide). [d] 1.7-Å resolution data. The iron is found to be 0.43 Å out of the plane of the four pyrrole nitrogens toward the cysteinate axial ligand. There is no sixth ligand. The $C_\beta S_\gamma Fe$ bond angle is 105.9°.

tances in comparable systems (Table II), the present data are most consistent with the sulfur donor to ferric P-450 being a thiolate sulfur. The Fe–S bond distance determined from the EXAFS curve-fitting procedure agrees very well with that reported very recently from analysis of the high-resolution crystal structure of the low-spin ferric P-450-CAM.[93a] The accuracy of the metal–ligand bond distance determined by X-ray crystallography for ferric P-450-CAM is about ±0.05 Å.[93b] The Fe–N(porphyrin) bond distance determined by EXAFS is right in the range expected for six-coordinate ferric heme iron systems;[91] unfortunately, the Fe–N bond distance has not been reported for the crystal structure.

As will be discussed in the next section, the concept that the active site structure of chloroperoxidase resembles that of P-450 was first put forth by Hollenberg and Hager[44] on the basis of data for the ferrous–CO states. Since that first report, several studies of ferric chloroperoxidase appeared in which the same conclusion was reached. At ambient temperatures, the ferric resting state of chloroperoxidase is predominately (> 80%) high-spin.[45] Below 200 K, the enzyme is primarily low-spin,[45] presumably as a result of coordination of a sixth ligand. Addition of various exogenous ligands leads to formation of low-spin derivatives of chloroperoxidase, the properties of which, by comparison with those of similar adducts of P-450, can provide information about the endogenous fifth ligand to chloroperoxidase. A low-spin ferric form of chloroperoxidase can also be formed by raising the pH above 7.0.[53] Since the conversion is not reversible, it is not clear whether the ligand that coordinates to the heme iron above pH 7.0 is the same as the one that binds to the native form of the enzyme below 200 K.

Mössbauer studies of the low temperature low-spin form of chloroperoxidase[45,46] revealed close structural similarities between its active site and that of cytochrome P-450. This led Champion et al. to consider the possibility that the two proteins have the same axial ligand. However, the apparent lack of an available sulfhydryl group to coordinate to the iron[60] (see section I) seemingly ruled out that possibility.

Extensive comparisons of the UV–visible absorption and MCD properties of low-spin ligand adducts of ferric

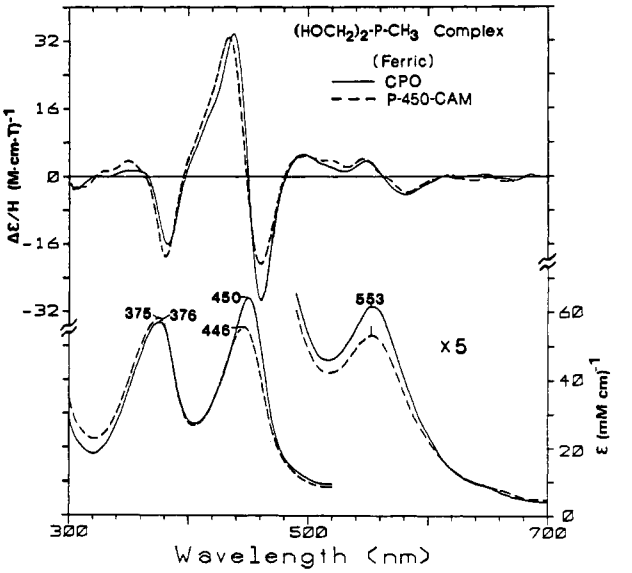



**Figure 4.** UV–visible absorption (bottom) and MCD (top) spectra of the ferric chloroperoxidase (solid line) and P-450-CAM (dashed line) complexes with bis(hydroxymethyl)methylphosphine. Reproduced with permission from ref 51. Copyright 1985 American Chemical Society.

chloroperoxidase with analogous derivatives of ferric P-450 also revealed close similarities.[49–51,53] The most compelling comparison is for the thiolate[49] and phosphine[51] adducts of the two enzymes, which display split Soret spectra that are diagnostic for the presence of an endogenous thiolate ligand. As can be seen in Figure 4, the MCD spectra of the phosphine adducts of ferric chloroperoxidase and P-450 are virtually superimposable. The thiolate adducts also have essentially identical MCD spectra. These data provide strong evidence for the presence of a thiolate ligand in chloroperoxidase as has been established for P-450.

EPR studies of low-spin derivatives of chloroperoxidase also support the proposal that the active site of the enzyme is similar to that of P-450.[58,95] Hollenberg et al.[58] examined a large number of ligand adducts and reached the conclusion that the axial ligand coordinated to chloroperoxidase is similar to that found in P-450. However, sufficient differences were observed between

**TABLE III. EPR Parameters of Ferric Low-Spin Derivatives of Cytochrome P-450-CAM and Chloroperoxidase**

| | P-450-CAM | | | | chloroperoxidase | | | |
|---|---|---|---|---|---|---|---|---|
| derivative | $g_1$ | $g_2$ | $g_3$ | ref | $g_1$ | $g_2$ | $g_3$ | ref |
| native | 2.45 | 2.26 | 1.91 | 98 | 2.62 | 2.26 | 1.83 | 95 |
| | | | | | 2.63 | 2.26 | 1.83 | 58 |
| **Oxygen Donors** | | | | | | | | |
| 1-pentanol | 2.45 | 2.27 | 1.93 | 98 | | no change | | 95 |
| cyclohexanol | 2.46 | 2.27 | 1.91 | 98 | | not examined | | |
| *p*-cresol | 2.43 | 2.27 | 1.93 | 98 | | not examined | | |
| *N*-methylpyrrolidinone | 2.48 | 2.27 | 1.93 | 98 | | not examined | | |
| cyclohexanone | 2.46 | 2.27 | 1.91 | 98 | | not examined | | |
| ethyl acetate | 2.46 | 2.27 | 1.91 | 98 | | not examined | | |
| sodium acetate | 2.46 | 2.25 | 1.91 | 98 | | no change | | 95 |
| potassium propionate | 2.46 | 2.25 | 1.91 | 94 | | not examined | | |
| sodium formate | 2.55 | 2.25 | 1.88 | 94 | 2.59 | 2.27 | 1.84 | 95 |
| **Nitrogen Donors** | | | | | | | | |
| imidazole | 2.56 | 2.27 | 1.87 | *a* | 2.54 | 2.28 | 1.85 | 95 |
| *N*-methylimidazole | 2.54 | 2.26 | 1.87 | 73 | | not examined | | |
| 2-methylimidazole | 2.62 | 2.28 | 1.85 | 98 | | not examined | | |
| *N*-phenylimidazole | 2.47 | 2.26 | 1.90 | *a* | 2.51 | 2.28 | 1.87 | 95 |
| 4-phenylimidazole[b] | 2.50 | 2.25 | 1.89 | *a* | | not examined | | |
| | 2.45 | | | | | | | |
| 2-phenylimidazole | 2.45 | 2.27 | 1.92 | 98 | | not examined | | |
| | 2.41 | 2.25 | 1.91 | *a* | | | | |
| benzimidazole | 2.44 | 2.28 | 1.93 | 98 | | not examined | | |
| 1-octylamine | 2.49 | 2.26 | 1.89 | 73 | | not examined | | |
| indole | 2.41 | 2.26 | 1.93 | 98 | | not examined | | |
| pyridine | 2.48 | 2.27 | 1.92 | 98 | 2.58 | 2.29 | 1.84 | 95 |
| metyrapone | 2.47 | 2.27 | 1.92 | *c* | | not examined | | 95 |
| sodium azide[b] | 2.52 | 2.23 | 1.87 | 94 | 2.56 | 2.22 | 1.85 | 95 |
| | 2.60 | | 1.82 | | 2.73 | 2.24 | 1.77 | |
| | | | | | 2.86 | 2.19 | 1.70 | |
| | | | | | 2.86 | 2.19 | 1.70 | |
| sodium nitrite | | no change | | 94 | 2.63 | 2.24 | 1.82 | 95 |
| potassium thiocyanate | 2.60 | 2.26 | 1.84 | 94 | 2.63 | 2.28 | 1.81 | 95 |
| potassium cyanate | 2.64 | 2.26 | 1.84 | 94 | 2.66 | 2.27 | 1.80 | 95 |
| potassium selenocyanate | 2.51 | 2.28 | 1.86 | 94 | 2.65 | 2.27 | 1.80 | 95 |
| **Sulfur Donors** | | | | | | | | |
| dimethyl disulfide | 2.42 | 2.25 | 1.92 | 98 | | no change | | 95 |
| dimethyl sulfide | 2.50 | 2.27 | 1.89 | 98 | | no change | | 95 |
| methanethiol[b] | 2.50 | 2.27 | 1.89 | 95 | 2.45 | 2.27 | 1.91 | 95 |
| | 2.42 | 2.24 | 1.94 | | | | | |
| | 2.37 | 2.24 | 1.95 | | | | | |
| 1-propanethiol[b] | 2.42 | 2.25 | 1.92 | 67 | | not examined | | |
| | 2.36 | 2.25 | 1.93 | | | | | |
| | 2.33 | 2.25 | 1.95 | | | | | |
| β-mercaptoethanol[b] | 2.42 | 2.27 | 1.92 | 67 | 2.44 | 2.26 | 1.91 | 95 |
| | 2.38 | 2.27 | 1.93 | | 2.44 | 2.25 | 1.81 | 58 |
| ethyl 2-mercaptoacetate[b] | 2.39 | 2.25 | 1.94 | 95 | 2.37 | 2.25 | 1.94 | |
| | 2.37 | 2.25 | 1.95 | | 2.43 | 2.26 | 1.91 | |
| dithiothreitol[b] | 2.42 | 2.24 | 1.93 | 67 | | not examined | | |
| | 2.38 | 2.24 | 1.94 | | | | | |
| | 2.36 | 2.24 | 1.95 | | | | | |
| **Carbon Donors** | | | | | | | | |
| potassium cyanide | 2.45 | 2.28 | 1.83 | *a* | 2.66 | 2.31 | 1.77 | 95 |
| | | | | | 2.72 | 2.30 | 1.75 | 58 |
| *n*-butyl isocyanide | 2.45 | 2.29 | 1.88 | 95 | 2.56 | 2.34 | 1.80 | 95 |
| **Phosphorus Donors** | | | | | | | | |
| dimethylphenylphosphine | 2.51 | 2.28 | 1.86 | 51 | | not examined | | |
| bis(hydroxymethyl)methylphosphine | 2.51 | 2.28 | 1.86 | 51 | 2.59 | 2.28 | 1.82 | 51 |

[a] Lipscomb, J. D. *Biochemistry* **1980**, *19*, 3590. [b] Brackets indicate the presence of multiple sets of signals. [c] Peterson, J. A.; Ullrich, V.; Hildebrandt, A. G. *Arch. Biochem. Biophys.* **1971**, *145*, 531.

the EPR properties of the two enzymes to lead the authors to propose that a less electron-rich sulfur atom than cysteinate, perhaps from an uncommon amino acid containing sulfur, is the fifth ligand to chloroperoxidase. More recently, Sono and Dawson[95] examined a wider range of ligand adducts of chloroperoxidase and compared the data obtained with analogous data for ligand adducts of P-450 (Table III). Fewer differences were observed between data for the two enzymes than in the previous study. This was consistent with the conclusion that the ligand in chloroperoxidase and P-450 is the same.

## B. Identification of the Sixth Ligand to the Heme Iron

Determination of the ligand trans to the endogenous thiolate of low-spin P-450 has been a matter of considerable controversy. Numerous techniques have been used in order to identify the sixth ligand with some results pointing to a histidine ligand and others toward an oxygen donor from water or an alcohol-containing amino acid. As stated above, in the model studies of Collman and Holm, the reported $g$ values for a series of thiolate–ferric heme–X complexes with biomimetic X ligands did not vary sufficiently to allow the identity of the sixth ligand to be ascertained.[69-72] Subsequent reexamination and extension of the data of Collman and Holm by other researchers led to conflicting conclusions, with histidine being proclaimed the ligand in some cases[96,97] and an oxygen donor in others.[73,75-77,98,99] While more studies favored an oxygen donor, the lack of a consensus suggests that it may not be possible to rely solely on EPR data to make comparisons such as this one.

Ruf, Wende, and Ullrich[73] extended the model approach of Holm and Collman by developing methods to generate the samples at temperatures low enough to stabilize the mixed ligand thiolate–ferric heme–X adducts but high enough to still permit examination of their UV–visible absorption spectra in fluid solution. As stated in the previous section, their results showed the need of the thiolate ligand to mimic the spectral properties of low-spin P-450. In addition, careful analysis of the data for several biomimetic X ligands showed that the best fit was achieved when ligand X was an oxygen donor. Sakurai and Yoshimura developed procedures to stabilize the mixed ligand thiolate–ferric heme–X complexes at ambient temperatures and were able to further examine their spectral properties.[99] Once again, the conclusion was reached that the ligand trans to thiolate is an oxygen donor. The same conclusion was also reached recently by Anzenbacher and co-workers who studied a series of thiolate–ferric heme–X complexes by using resonance Raman spectroscopy; comparison of the high-frequency bands revealed a somewhat closer match between the values of oxygen donor adducts and the native protein than for nitrogen donor ligand adducts.[100]

Griffin and Peterson[101] and, more recently, Philson et al.[102] examined the effect of the paramagnetic iron in low-spin ferric P-450-CAM on the relaxation of the bulk water proton NMR signal. Both groups concluded that a proton, located 2.0–2.6 Å from the iron, is in rapid exchange with the solvent. From these data, Griffin and Peterson concluded that the sixth ligand is water; Philson et al. also included the possibility that the sixth ligand could be an amino acid derived alcohol, amine, or amide. With high-spin ferric P-450, both groups reported only outer-sphere relaxation effects consistent with the five-coordinate structure shown in Figure 2. Curiously, with liver microsomal P-450, Grasdalen et al.[103] reported rather different results. They found only outer-sphere relaxation effects in both low-spin and high-spin ferric P-450 states. Such negative evidence does not lend itself to firm structural conclusions. Nonetheless, the data for P-450-CAM is incompatible with histidine as the sixth ligand and is most consistent with an oxygen donor sixth ligand.

ENDOR studies of ferric P-450-CAM by LoBrutto et al.[104] led to essentially the same conclusion as was reached by conventional NMR: the sixth ligand to low-spin ferric P-450-CAM has a dissociable proton located 2.6–2.9 Å from the heme iron, and high-spin ferric P-450-CAM is five-coordinate. Histidine was specifically excluded as a possible sixth ligand to low-spin ferric P-450.

Another approach that has been used in order to identify the sixth ligand to low-spin ferric P-450 has been to compare the properties of ligand adducts of ferric P-450 with the properties of the native protein. The most extensive study of this type has been by Dawson, Andersson, and Sono using UV–visible absorption, MCD, CD, and EPR spectroscopy.[98,105-107] For example, in Figure 5, the UV–visible absorption and MCD spectra of an oxygen donor adduct formed by addition of a neutral nitrogen donor, *N*-methylpyrrolidinone, to ferric P-450-CAM are compared with the spectra of low-spin ferric P-450-CAM. The close similarity between the observed spectral properties of oxygen donor adducts of ferric P-450 and the parallel data on the native low-spin enzyme using four spectral techniques was used to argue that the endogenous sixth ligand is an oxygen donor, either water or an alcohol-containing amino acid. Spectral dissimilarity between ligand adducts and native low-spin P-450 or, in the case of thiol adducts, differences in the effect of pH on the observed spectra were used to rule out all possible nitrogen and sulfur donor sixth ligand candidates, including histidine.[98] White and Coon[108] as well as Yoshida et al.[109] reached a similar conclusion using UV–visible absorption spectroscopy. Competition experiments were used in the Dawson laboratory to demonstrate the coordination of the oxygen donor ligands; White and Coon and Yoshida et al. used P-450 isozymes that are high-spin five-coordinate as isolated so that the conversion to the low-spin six-coordinate form could be easily followed. Shimizu et al.[110] also used this approach to attempt to determine the sixth ligand to ferric P-450. Using MCD spectroscopy, they reached the conclusion that the sixth ligand is a sterically hindered nitrogen donor ligand. Although they considered oxygen donor ligands, in the absence of competition experiments, they were not certain that coordination had occurred. Dawson et al.[98] also considered the sterically hindered nitrogen donor sixth ligand but were able to rule out such a possibility by using EPR data on the complexes.

Perhaps the most puzzling study to address the question of the sixth ligand to low-spin ferric P-450 was the application of electron spin echo (ESE) spectroscopy by Peisach et al.[111] They examined several model compounds of the thiolate–ferric heme–X type and also looked at the native low-spin enzyme. They reported data that seemed to specifically show evidence for a nitrogen donor ligand. With heme systems, the nitrogen of histidine that is observed in the ESE spectrum is not the directly bound nitrogen but the distal nitrogen of the imidazole ring. Quite possibly, the nitrogen that Peisach et al. observed in the ESE of low-spin ferric P-450 was the nitrogen of the Tris buffer in which the sample had been prepared. As has been suggested by Andersson and Dawson,[106] the ferric heme iron of P-450 is capable of binding a wide range of oxygen donor ligands, including buffer components such as Mops and Tris as well as detergents. If Tris were to bind to ferric

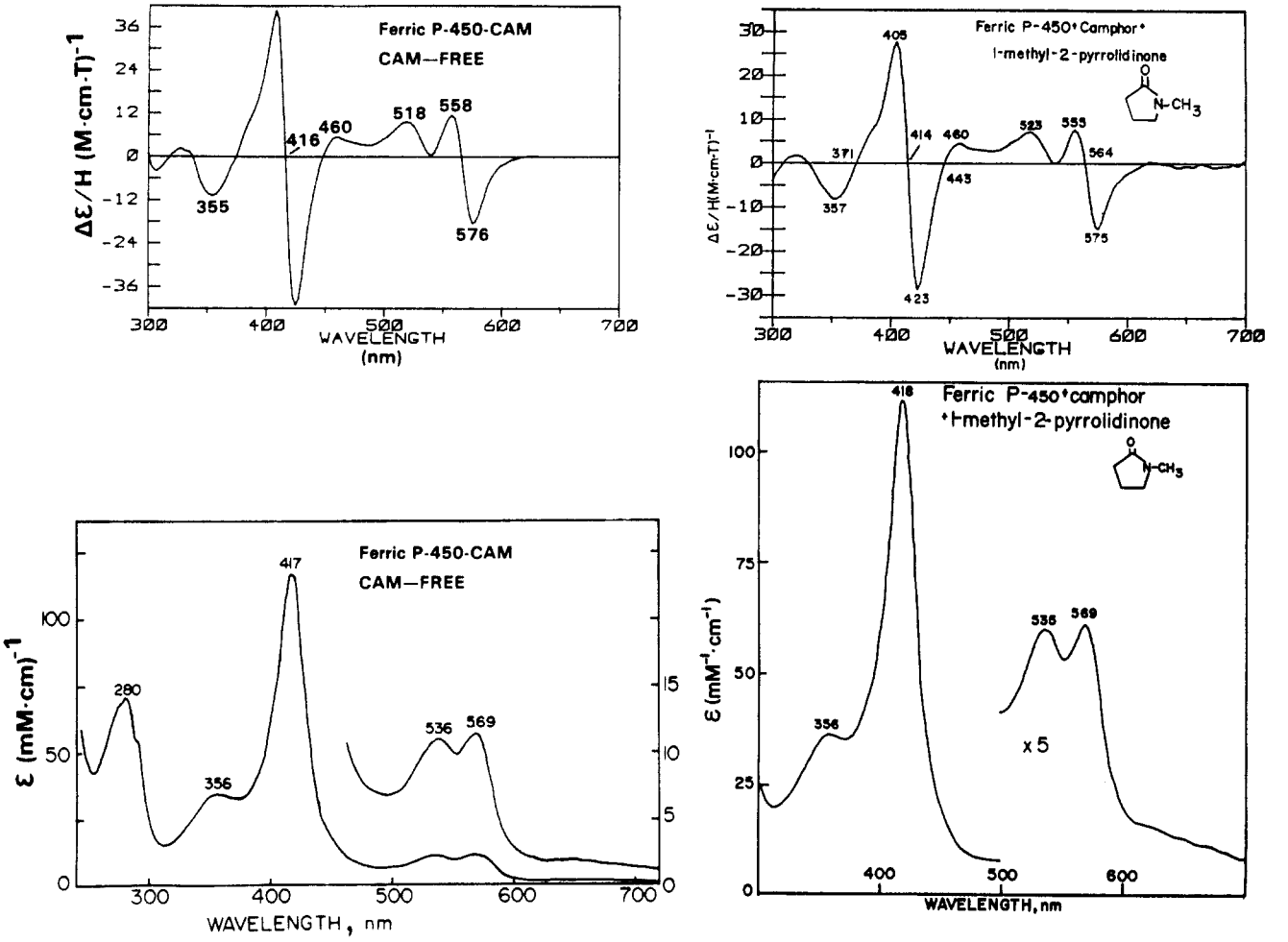



**Figure 5.** UV–visible absorption (bottom) and MCD (top) spectra of resting state ferric P-450-CAM (left) and the neutral oxygen donor adduct formed by addition of *N*-methylpyrrolidinone (right). Adapted from ref 98.

P-450, it would bring a nitrogen atom into close proximity of the iron that might be the origin of the effect seen in the ESE experiment.

The issue of the sixth ligand has recently been settled in favor of an oxygen donor, specifically water (or hydroxide), with the appearance of the crystal structure of the low-spin ferric state of P-450-CAM by Poulos et al.[28] Given the fact that the spectral properties of low-spin ferric P-450-CAM do not change over the pH range from 5.35 to 9.5,[98] it seems likely that the ligand is either water or hydroxide throughout that pH range. If that is the case, since distal ligands in myoglobin are more acidic than in P-450[67] (see section VIIA), it would seem likely that the sixth ligand is water. Alternatively, one could argue that since the spectral properties of neutral and anionic oxygen donor ligand adducts of ferric P-450 do not differ appreciably,[98] perhaps the techniques that have been used to examine low-spin ferric P-450 are not sensitive to the difference between water and hydroxide. Also puzzling is the fact that attempts in several laboratories to observe the effect of the nuclear spin of $^{17}O$-labeled water on the EPR spectrum of low-spin ferric P-450 have failed.[112]

The sixth ligand to the heme iron of ferric chloroperoxidase that is presumed to be responsible for the conversion of the iron from high-spin to low-spin upon lowering the temperature below 200 K is most likely histidine but could also be a carboxylate-containing amino acid. Surprisingly, this question has not been very actively addressed. The EPR properties of low-spin native chloroperoxidase[58,95] most closely match those of the imidazole adduct of ferric P-450[98] (see Table III). However, native chloroperoxidase and its imidazole adduct exhibit clearly distinguishable EPR parameters, while the EPR properties of the formate-bound ferric chloroperoxidase more closely match those of the native low-spin enzyme (Table III). Hollenberg et al.[58] reported the absorption spectrum of chloroperoxidase at low temperatures where the low-spin form predominates. The spectrum of the sample at pH 3.0 and 77 K has its major absorption peaks at 358, 424, 543, and 582 nm;[58] the imidazole adducts of ferric P-450-CAM[98] and chloroperoxidase[53] at 4 °C have their major peaks at 360, 425, 541, and 582 nm and at 360, 429, 546, and 580 nm, respectively. Corresponding absorption peaks for the formate complex of chloroperoxidase are at 360, 425, 545, and 581 nm.[53] A low-spin form of chloroperoxidase can also be formed by raising the pH above 7.0.[113] Comparison of the MCD spectrum of the high-pH derivative of ferric chloroperoxidase with those of the imidazole adducts of ferric P-450 and low-pH chloroperoxidase (Figure 6) led Sono et al. to conclude that the sixth ligand to high-pH ferric chloroperoxidase is also histidine.[53] As will be discussed in section VIIB, there is evidence for an acidic amino acid with a $pK_a$ of about 5.5 in the vicinity of the heme

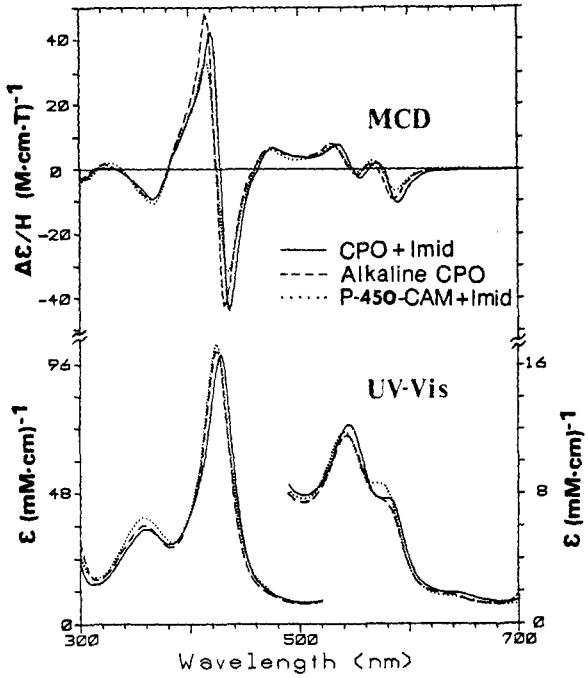



**Figure 6.** UV–visible absorption (bottom) and MCD (top) spectra of alkaline ferric chloroperoxidase (CPO) and the imidazole complexes of native ferric CPO and of ferric P-450-CAM. Reproduced with permission from ref 53. Copyright 1986 American Chemical Society.

iron that effects ligand binding to the ferrous enzyme (ref 53, 114). This ligand could be the same histidine that binds to the heme iron under certain circumstances.

## *III. High-Spin Ferric States*

The EPR spectra of high-spin ferric P-450 (2) and chloroperoxidase (8) are unusual among high-spin ferric heme proteins in the degree of rhombic splitting.[58,115] Whereas most other high-spin ferric heme systems have $g$ values at 6 and 2, high-spin ferric P-450 and chloroperoxidase have $g$ values at 8, 4, and 1.8 and at 7.6, 4.3, and 1.8, respectively. In the midseventies, Holm,[71,72] Collman,[69] Ogoshi,[116] and their co-workers reported that the rhombic splitting observed in high-spin ferric P-450 could be reproduced with five-coordinate ferric porphyrin–thiolate complexes. A nonthiolate high-spin model complex reported by Sato and Kon with rhombic $g$ values could be distinguished from the thiolate complexes by its power saturation behavior.[117,118] Thus, the EPR properties of the thiolate complexes provided strong evidence for endogenous thiolate ligation to the heme iron of both high-spin ferric P-450 and chloroperoxidase.

MCD spectroscopy has also been used to probe the structure of the high-spin ferric states of these two enzymes. Comparison of the MCD spectra of Holm's high-spin ferric porphyrin-thiolate complex with that of camphor-bound ferric P-450-CAM showed great similarity between the two spectra and considerable differences from the spectra of several other high-spin ferric heme systems involving biomimetic ligands.[119] Further, the MCD spectrum of high-spin ferric chloroperoxidase is very similar to that of high-spin ferric P-450-CAM.[48] The three spectra in question are displayed in Figure 7. Again, these data pointed to the

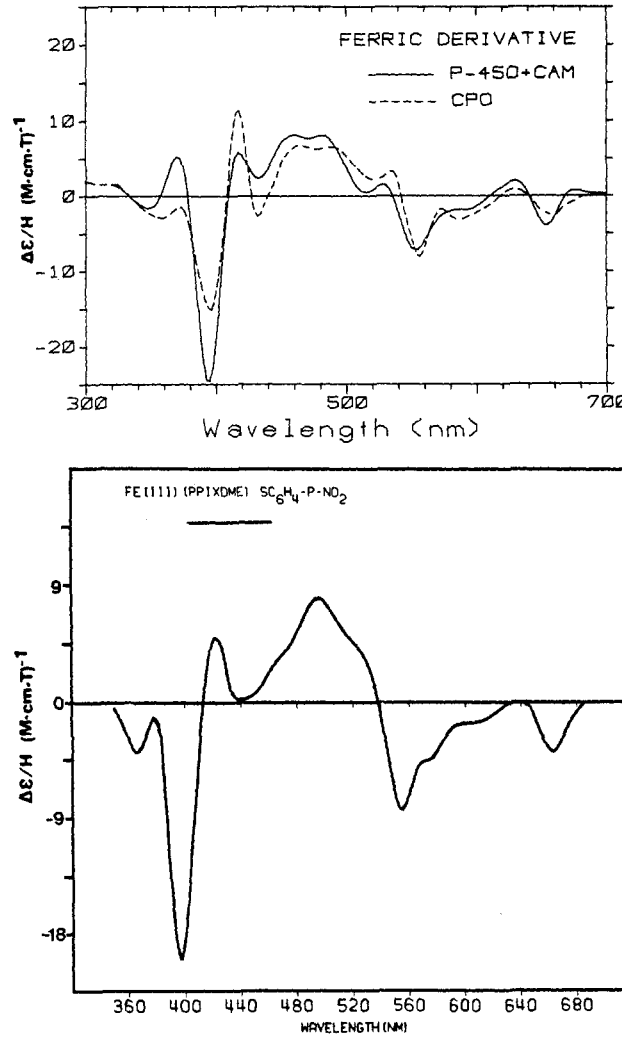



**Figure 7.** MCD spectra of high-spin ferric camphor-bound cytochrome P-450-CAM (top, solid line), high-spin ferric chloroperoxidase (top, dashed line), and a five-coordinate high-spin ferric protoporphyrin–thiolate model complex (bottom, solid line) in toluene. [The P-450 spectrum (Andersson, L. A.; Dawson, J. H., unpublished results) closely matches the published spectrum (Vickery, L.; Salmon, A.; Sauer, K. *Biochim. Biophys. Acta* **1975**, *386*, 87).] The spectra of ferric CPO and the model complex are reproduced with permission from ref 48 and 119, respectively. Copyright 1976 American Chemical Society.

presence of an endogenous thiolate ligand bound to the heme iron of these two enzymes in their high-spin states.

More direct evidence for the presence of a thiolate sulfur has come from the use of EXAFS and resonance Raman spectroscopy. As indicated in Table II, the EXAFS of the high-spin states of the two enzymes reveal the presence of a sulfur donor ligand coordinated to the iron at 2.23 and 2.30 Å for P-450 and chloroperoxidase, respectively.[54,83-85] The Fe–S bond distance for Holm's model complex is 2.324 Å.[72] Again, as in the case of the low-spin state, the fact that the observed Fe–S distance for the protein states is shorter than that of a thiolate model strongly suggests that the protein has a thiolate as opposed to a thiol ligand. The recent publication[27] of the crystal structure of high-spin ferric P-450-CAM clearly showed the presence of a cysteinate ligand as is shown in Figure 8. The Fe–S bond distance from the crystal structure is 2.20[93a] ± 0.05[93b] Å, in close agreement with the value determined by EXAFS (Ta-

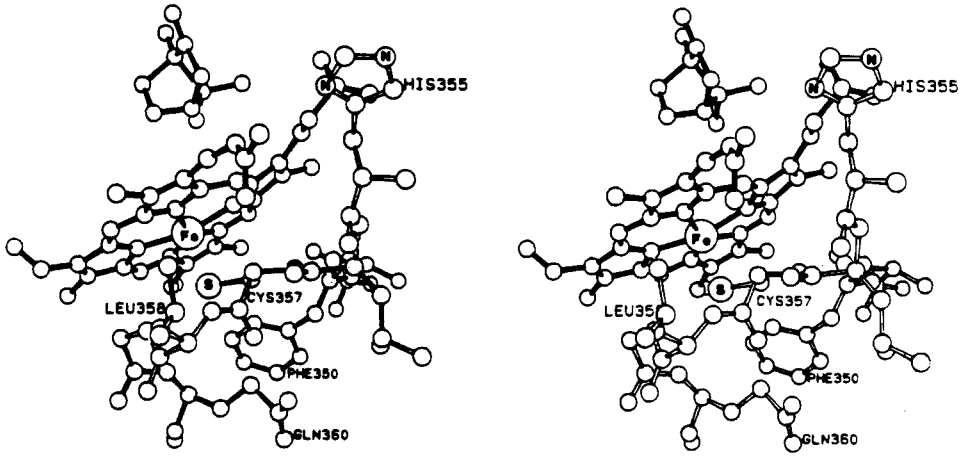



**Figure 8.** Stereoscopic model of cytochrome P-450-CAM in the immediate vicinity of the thiolate ligand Cys-357. Reproduced with permission from ref 27. Copyright 1985 Journal of Biological Chemistry.

ble II). The difference in the Fe–S distance observed by EXAFS for the two enzymes (0.07 Å) is greater than the uncertainty in the data (±0.02 Å) and may help explain some of the subtle differences in physical properties between P-450 and chloroperoxidase. A crystal structure of ferric chloroperoxidase is underway.[120]

Champion and co-workers studied isotopically labeled derivatives of both high-spin ferric P-450-CAM[121] and chloroperoxidase[56] with resonance Raman spectroscopy (Figure 9). Detection of Fe–S vibrations at 351 and 347 $cm^{-1}$, respectively,[122] for the two enzymes, which shifted when the $^{34}S$-labeled enzymes were investigated, provided direct evidence for cysteine ligation in both systems.

## IV. High-Spin Ferrous States

High-spin ferrous P-450 (3) is formed by electron transfer to the high-spin ferric state 2. Although the normal reaction cycle of chloroperoxidase does not involve the intermediacy of a ferrous derivative, such a state can be produced by anaerobic reduction of the high-spin ferric enzyme 8. The spectroscopic properties of these two enzyme states have been extensively explored and have consistently been found to be quite similar to each other as well as to the properties of five-coordinate high-spin ferrous porphyrin–thiolate model complexes.

The first physical study of P-450 state 3 was by Keller et al.[123a] who examined the $^1H$ NMR of the derivative and obtained evidence for the high-spin nature of the heme iron. Champion et al.[123b] confirmed the high-spin assignment by examination of the magnetic susceptibility of camphor-bound ferrous P-450-CAM. Chang and Dolphin[124,125] were the first to prepare a five-coordinate high-spin ferrous tetraarylporphyrin–thiolate complex and show that its UV–visible absorption spectrum closely matched that of ferrous P-450 (and chloroperoxidase) and was quite distinct from that of high-spin deoxymyoglobin.[126] Similar data have since been reported by other laboratories,[127–129] including the crystal structure of a ferrous tetraarylporphyrin–thiolate model complex by Weiss and co-workers.[130] More recently, Parmely and Goff[131] examined the proton NMR properties of a ferrous heme–thiolate model complex

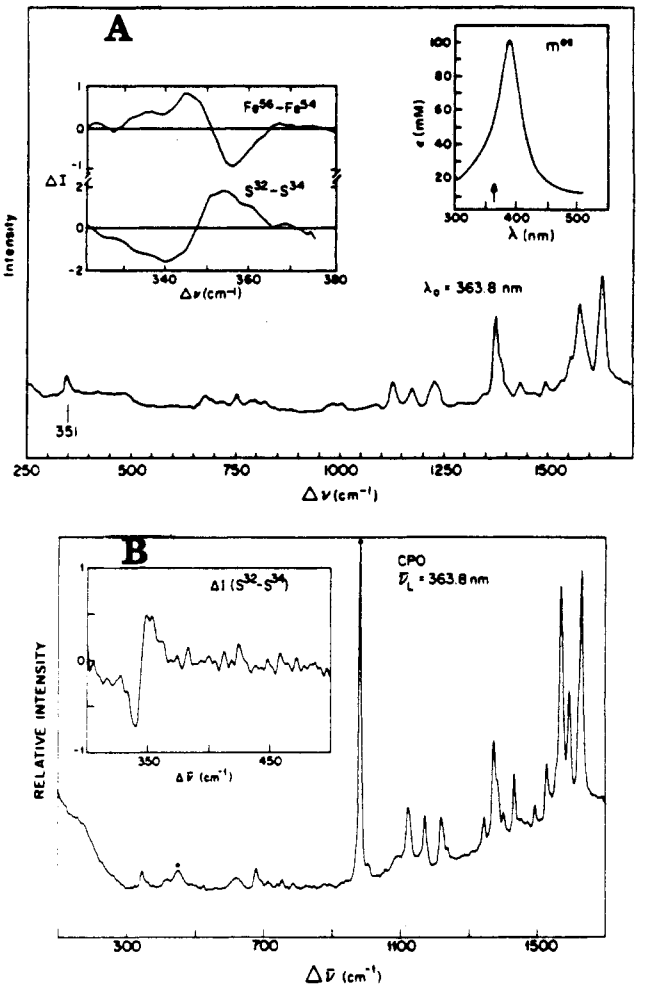



**Figure 9.** (A) Resonance Raman spectrum of high-spin (camphor-bound) ferric cytochrome P-450-CAM (lower curve). The absorption spectrum of ferric P-450-CAM is shown in the upper right-hand corner; the vertical line denotes the position of the resonant laser excitation (363.8 nm). The Raman difference spectra in the region of the 351-$cm^{-1}$ Raman mode are displayed in the upper left-hand corner. The vertical scales for the difference spectra are given in units of 1000 counts $channel^{-1}$. Reproduced with permission from ref 121. Copyright 1982 American Chemical Society. (B) Resonance Raman spectrum of chloroperoxidase (CPO) excited at 363.8 nm. The sulfate vibrations at 450 and 981 $cm^{-1}$ are indicated by asterisks. The insert shows the Raman difference spectrum of a separate experiment using native ($^{32}S$) and $^{34}S$-enriched CPO without sulfate added. Reproduced with permission from ref 56. Copyright 1986 American Chemical Society.

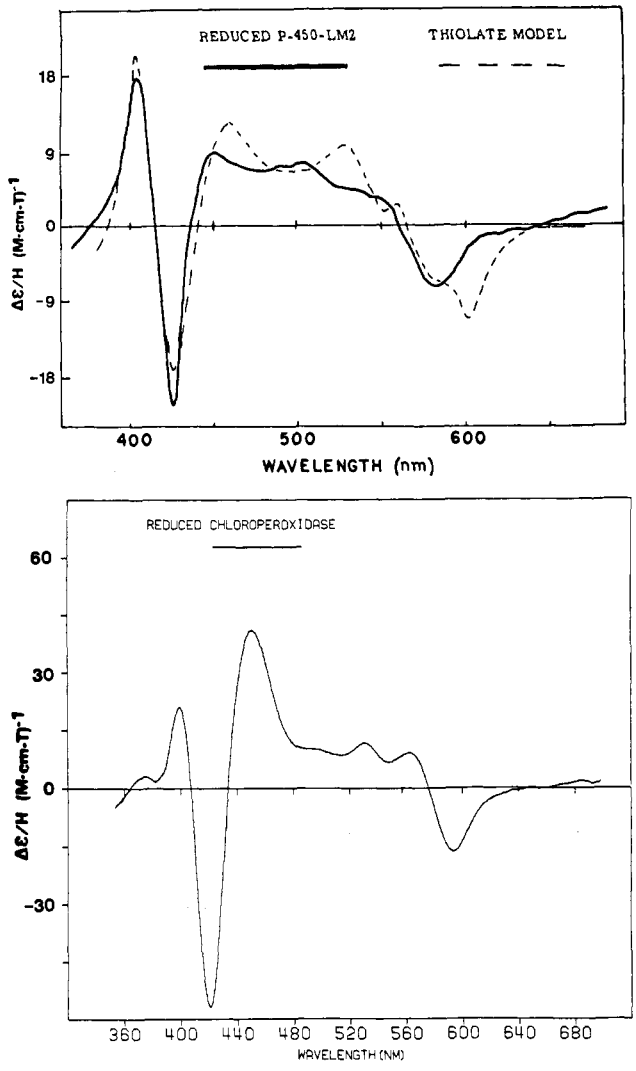



**Figure 10.** MCD spectra of high-spin ferrous cytochrome P-450-LM2 (top, solid line), five-coordinate high-spin ferrous protoporphyrin–thiolate model complex (top, dashed line) in dimethylacetamide, and ferrous chloroperoxidase (bottom, solid line). Data obtained from ref 136, 137, and 48, respectively.

and clearly showed that such a complex is five-coordinate and high-spin. However, the absence of high-field data on the ferrous enzyme and on well-defined heme–thiol complexes prevented the authors from making any firm conclusions about the identity of the fifth ligand to the enzyme.

The ferrous derivatives of P-450 and chloroperoxidase were investigated with Mössbauer spectroscopy and close similarities were observed.[45,46,132-134] Unfortunately, the quadrupole splitting and isomer shift parameters are not that different from those of high-spin ferrous myoglobin, and so the technique has not been that useful in identifying axial ligands. However, the temperature independence of the quadrupole splitting, a property also seen in the ferrous porphyrin–thiolate model complex prepared in Weiss' laboratory,[135] distinguishes P-450 and chloroperoxidase from myoglobin.[46]

The MCD spectra of ferrous P-450[136] and chloroperoxidase[48] are compared with the spectrum of a five-coordinate ferrous protoheme–thiolate model complex[1,137] in Figure 10. As can be seen, the spectra match each other quite closely; the spectra of other high-spin ferrous heme systems such as deoxymyoglobin[138] are rather different. Similar data for ferrous P-450 and the thiolate model were reported by Hatano and co-workers.[128] EXAFS spectroscopy has also been used to examine ferrous P-450[83-85] and a thiolate model complex.[139] The EXAFS data reported in Table IV together with the crystallographic results for the thiolate model[130] clearly show that ferrous P-450 has a sulfur atom in its first coordination shell. The close similarity between the Fe–S bond distances for the enzyme and the thiolate model together with the MCD results strongly suggests that the sulfur donor in the enzyme is a thiolate sulfur. Unfortunately, no EXAFS data have been measured on ferrous chloroperoxidase to date. However, the similarity between the MCD spectra of ferrous P-450 and chloroperoxidase is most consistent with the presence of a thiolate ligand in both cases.

The resonance Raman properties of ferrous P-450[140-143] and chloroperoxidase[47,57,141] and of the Weiss ferrous porphyrin–thiolate model complex[144,145] are also all similar to each other and are distinct from ferrous myoglobin in the position of the "oxidation state marker band" ($\nu_4$). This band is found in the range from 1341 to 1348 $cm^{-1}$ for P-450 enzymes from several sources, chloroperoxidase, and thiolate-ligated models, while the analogous band for ferrous myoglobin, hemoglobin, and horseradish peroxidase[140,141,146,147] occurs in the range from 1355 to 1360 $cm^{-1}$. Conversion of P-450 and chloroperoxidase into enzymatically inactive, partially denatured derivatives known as P-420 and CPO-420, respectively, also causes this band to shift into the range seen for ferrous myoglobin.[57,141-143] The shift of position of this band, which is actually a C–N breathing mode,[148] to lower energies for P-450 and chloroperoxidase has been attributed to the presence of a strongly electron-releasing $\pi$ base such as a thiolate that could be expected to increase the population of the porphyrin $\pi^*$-antibonding orbitals and thereby weaken the porphyrin bond strengths.[140,143] However, as pointed out by Chottard et al.,[144] the energies of the $\nu_4$ band for two imidazole-ligated ferrous tetraarylporphyrin complexes are in the range expected for the P-450 systems and not in the region that would be predicted for myoglobin models (the band for ferrous *protoheme*–imidazole complexes[145] occurs in the myoglobin range). In other words, for tetraaryl porphyrins, the energy of the $\nu_4$ band does not seem to be sensitive to the axial ligand type. Unfortunately, until a ferrous *protoheme*–thiolate complex is examined, it will not be known for sure whether the energy of the $\nu_4$ band is determined by "protein" effects or by the nature of the axial ligand.

## V. Low-Spin Ferrous States

Ligand binding to the five-coordinate high-spin ferrous states of P-450 and chloroperoxidase produces six-coordinate low-spin species such as the oxy- and carbonmonoxy-bound derivatives. As with the ferric forms of these two enzymes, comparison of the resulting spectral properties of such ligand adducts with each other, and with synthetic models whenever possible, provides a further opportunity to deduce the active site structures of the two enzymes.

TABLE IV. Structural Details for Ferrous Cytochrome P-450, Chloroperoxidase, and Relevant Model Complexes[a]

| system | Fe–N(porphyrin) method | R, Å | N[b] | Fe–S(axial) R, Å | N[b] | ref |
|---|---|---|---|---|---|---|
| | | High-Spin Ferrous | | | | |
| P-450-CAM | EXAFS | 2.08 | 3.0 | 2.34[c] | 0.6 | 85 |
| Fe(OEP)(SPr)$^-$ | EXAFS | 2.05 | 3.8 | 2.33 | 0.4[d] | 139 |
| Fe(TPP)(SEt)$^-$ | X-ray | 2.096 | 4 | 2.360 | 1 | 130 |
| | | Low-Spin Ferrous Carbon Monoxide Complex | | | | |
| P-450-CAM | EXAFS | 1.98 | 3.3 | 2.32 | 1.0 | 85 |
| Fe(OEP)(SPr)(CO)$^-$ | EXAFS | 2.00 | 4.4 | 2.33 | 0.2[d] | 139 |
| Fe(TPP)(SEt)(CO)$^-$ | X-ray | 1.993 | 4 | 2.352 | 1 | 130 |
| Fe(OEP)(PrSH)(CO) | EXAFS | 2.01 | 5.1 | 2.41 | 0.8 | 139 |
| Fe(OEP)(THT)(CO) | EXAFS | 2.00 | 5.2 | 2.41 | 0.4[d] | 139 |
| Fe(OEP)(MeSSMe)(CO) | EXAFS | 2.03 | 5.9 | 2.40 | 0.7 | 139 |
| | | Low-Spin Ferrous Dioxygen Complex | | | | |
| P-450-CAM[e] | EXAFS | 2.00 | 7.8[f] | 2.37 | 1.3 | 55 |
| chloroperoxidase[g] | EXAFS | 2.00 | 7.4[f] | 2.37 | 1.4 | 55 |
| Fe(TpPP)($SC_6HF_4$)($O_2$)$^{-h}$ | X-ray | 1.990 | 4 | 2.369 | 1 | 170 |
| Fe(TpPP)(THT)($O_2$)[i] | X-ray | 1.99–2.00 | 4 | 2.49 | 1 | 173 |

[a] EXAFS data were obtained by curve fitting. Abbreviations: OEP, octaethylporphyrin; SPr, *n*-propanethiolate; TPP, tetraphenylporphyrin; SEt, ethanethiolate; PrSH, *n*-propanethiol; THT, tetrathiophene; MeSSMe, dimethyl disulfide; TpPP, *meso*-tetrakis(α,α,α,α-*o*-pivalamidophenyl)porphyrin. [b] Number (*N*) of atoms at the distance indicated. [c] Best fit to filtered EXAFS data. Fe–$S_{ax}$ = 2.38 Å when unfiltered EXAFS data were analyzed. [d] Analysis of solution data using parameters derived from the solid-state EXAFS of structurally defined model complexes may result in low values for N($S_{ax}$) due to Debye-Waller effects. See ref 139. [e] Fe–O(dioxygen) = 1.78 Å; N($O_{ax}$) = 1.1. [f] Analysis of low-temperature data using parameters derived from the study of model complexes at room temperature may result in high N values due to Debye-Waller effects. See ref 55. [g] Fe–O(dioxygen) = 1.77 Å; N($O_{ax}$) = 1.3. [h] Fe–O(dioxygen) = 1.818 Å. [i] "Semiquantitative" structural analysis of X-ray crystal data.

## A. Carbon-Monoxide-Bound Derivatives

The name "cytochrome P-450" is derived from the fact that the ferrous–CO state of the enzyme has its Soret absorption maximum at approximately 450 nm. This led Omura and Sato,[149] in the absence of any information about its specific enzymatic activity, to refer to the heme protein as cytochrome P-450 because it was a pigment that absorbed at 450 nm. This spectral feature is rather unique among CO-binding protoheme proteins; all ferrous–CO protoheme systems other than P-450 and chloroperoxidase absorb maximally at about 420 nm. The similarity in absorption properties of P-450 and chloroperoxidase, most notably in their ferrous–CO states, was first reported by Hollenberg and Hager.[44]

Early attempts to mimic the red-shifted absorption spectrum of ferrous–CO P-450 focused on the possible role of heme–heme interactions.[150] This proposal was eliminated when the soluble and monomeric bacterial P-450-CAM was characterized.[5] Following up on Mason's suggestion that the axial ligand to P-450 might be a sulfur donor, models for the ferrous–CO state were prepared in several laboratories and their spectral properties compared with those of the enzyme. The first success came when Stern and Peisach[151] combined ferrous heme, CO, a thiol, and a strong base and generated a sample with a peak at 450 nm. Shortly thereafter, Collman and Sorrell[152] and then Chang and Dolphin[124,125] reported the preparation of homogenous ferrous–CO adducts in which the trans axial ligand was systematically varied among nearly all possible biomimetic ligands and found that only a thiolate was capable of producing a Soret peak close to 450 nm. The thiolate-ligated ferrous–CO heme models[153] matched two additional unusual features of the ferrous–CO P-450 absorption spectrum,[1] namely coalescence of the normally discrete α and β bands into a single feature at 550 nm and presence of an especially intense δ transition near 370 nm. The crystal structure of a thiolate-ligated ferrous–CO heme model has been published by Caron et al.[130] (Table IV). Single-crystal polarized absorption spectra of ferrous–CO P-450 were reported by Hanson et al.[79a,154] and by Makinen.[155] The extra intense δ band was found to have the same polarization and the same integrated intensity as the Soret band. This fit well with the theoretical description by Hanson, Gouterman, and their co-workers[79a,154] of the red-shifted Soret transition in ferrous–CO P-450 as having resulted from the mixing and splitting of the normal Soret π,π* band by a axial thiolate sulfur $p^\dagger$ to porphyrin π* transition of the correct energy and symmetry.[156]

As with the other derivatives of P-450 and chloroperoxidase described above, comparison of the MCD spectral properties of ferrous–CO protein states and model compounds has been particularly useful in providing compelling evidence for the presence of an endogenous thiolate ligand at the active sites of the two enzymes. In Figure 11, the MCD spectra of ferrous–CO P-450[157] and chloroperoxidase[48] and of a thiolate-ligated ferrous–CO protoheme model[157] are compared. The especially close similarity between the observed spectra coupled with the fact that models with thiol,[157] imidazole,[157] or any other biomimetic ligand[158] trans to CO gave rise to MCD spectra that were significantly different led to the conclusion that the two enzyme states must have an endogenous thiolate ligand.

The most direct evidence for the endogenous thiolate ligand in ferrous–CO P-450 has come from EXAFS spectroscopy.[83–85] As indicated in Table IV, a sulfur donor atom at 2.32 Å provides the best fit for the EXAFS data on ferrous–CO P-450. The Fe–S(thiolate) bond distance for the ferrous–CO model determined crystallographically by Caron et al.[130] was 2.35 Å. Recently, a systematic study of the four possible sulfur donor ligated ferrous–CO model heme complexes was completed.[139] The Fe–S bond distance determined for the thiolate model agreed quite well with that of the

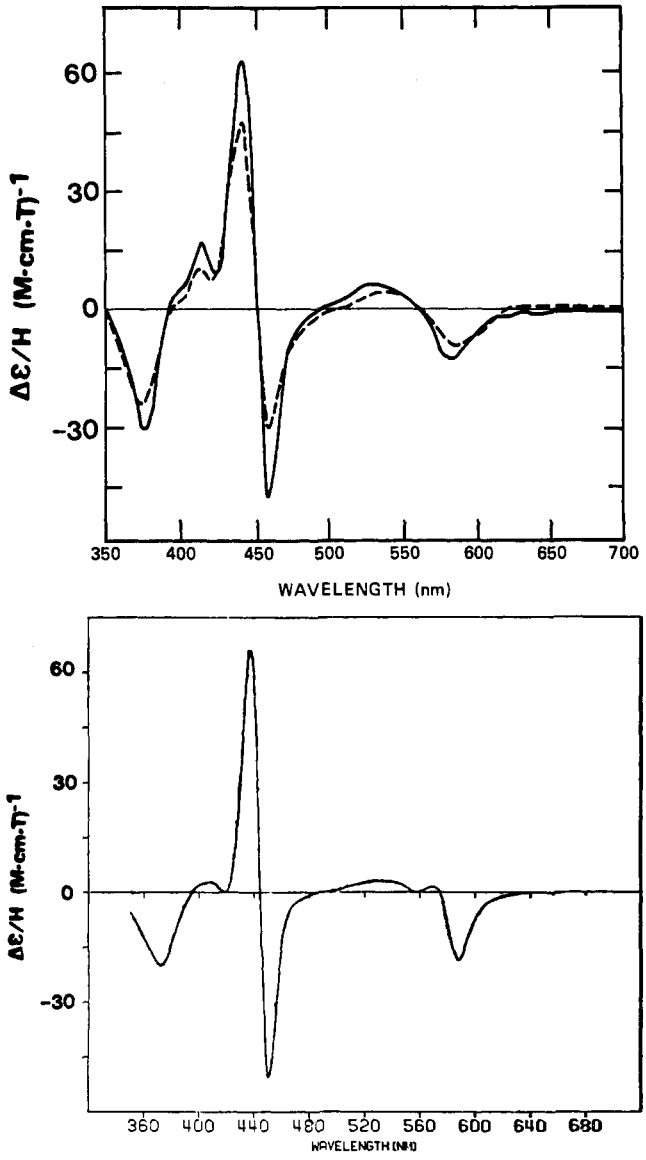



**Figure 11.** MCD spectra of ferrous–CO cytochrome P-450-LM2 (top, solid line), ferrous–CO protoporphyrin–thiolate model complex (top, dashed line) in benzene, and ferrous–CO chloroperoxidase (bottom, solid line). Data obtained from refs. 157, 48, and 157, respectively.

protein, while the distances for the three neutral sulfur donors (thiol, sulfide, and disulfide) were nearly 0.1 Å longer. Thus, the bond distance measured for the ferrous–CO enzyme is most consistent with that of a thiolate sulfur donor ligand. EXAFS measurements on ferrous–CO chloroperoxidase have not yet been reported.

Additional support for the presence of a thiolate axial ligand in ferrous–CO chloroperoxidase was recently presented by Goff and co-workers[59] based on the location of the $^{13}C$ NMR signal for the $^{13}CO$-bound enzyme in comparison with data for other heme proteins[59,159] and model complexes[160] (Table V). Imidazole-ligated systems were found to have the resonance in a range from approximately 205 to 209 ppm, while ferrous–CO chloroperoxidase[59] as well as P-450[159] had their peaks between 200 and 201 ppm. Traylor and co-workers previously showed that the peak occurs at about 197 ppm for a thiolate-ligated ferrous–CO heme model and is shifted to above 204 ppm in the case of a thiol-bound

**TABLE V. Carbon-13 Chemical Shifts for the $^{13}C$ Signal in Ferrous–CO Heme Proteins and Model Compounds**

| ferrous heme–CO system | chemical shift, ppm | ref |
|---|---|---|
| Nitrogen Donor Ligand Trans to Carbon Monoxide | | |
| myoglobin | 207.9 | 59 |
| horseradish peroxidase $A_1$ | 208.9 | 59 |
| horseradish peroxidase B/C | 209.1 | 59 |
| lactoperoxidase | 208.3 | 59 |
| cytochrome *c* hemopeptide | 205.8 | 59 |
| Fe(protoheme)(imidazole)(CO) | 206.8 | 59 |
| Fe(protoheme)(imidazolate)(CO) | 204.8 | 59 |
| Sulfur Donor Ligand Trans to Carbon Monoxide | | |
| cytochrome P-450-CAM | 200.3 | 159 |
| chloroperoxidase | 200.8 | 59 |
| Fe(protoheme)(thiolate)(CO) | 197.0 | 160 |
| Fe(protoheme)(thiol)(CO) | 204.7 | 160 |

model.[160] These data, taken together, support the conclusion that the axial ligand to ferrous–CO P-450 and chloroperoxidase is a thiolate.

Of the other physical methods to have been used to investigate ferrous–CO P-450 and chloroperoxidase, none has contributed strongly to the identification of the endogenous ligand. In the resonance Raman spectrum, there is again a shift to lower energies of the $\nu_4$ band for ferrous–CO P-450[141-143] (1365–1368 $cm^{-1}$) relative to the analogous data for myoglobin or hemoglobin[147] (1370–1372 $cm^{-1}$). However, the shift is very small (about 2 $cm^{-1}$), and essentially no difference in band energy is seen between thiolate[144] and nitrogen donor ligated ferrous–CO tetraarylporphyrins.[146,161] Furthermore, the band for ferrous–CO chloroperoxidase[56] (1370 $cm^{-1}$) is in the range expected for ferrous–CO myoglobin. The use of infrared absorption spectroscopy to study the ferrous–CO state of P-450 has not proven useful in assigning the ligand trans to CO, because the energy of the C–O stretch is more sensitive to geometrical factors than to the trans ligand.[162] Likewise, the Mössbauer parameters for CO-bound P-450, chloroperoxidase, and myoglobin are not very sensitive to the identity of the trans ligand.[45,134]

## B. Dioxygen-Bound Derivatives

A dioxygen-bound derivative of ferrous cytochrome P-450 was first reported by Peterson and co-workers in 1971.[163] Initial reports of the UV–visible absorption spectral properties of the oxygenated enzyme revealed that in contrast to the red-shifted Soret peak of ferrous–CO P-450 relative to CO-bound myoglobin, the Soret maximums of oxy-P-450[163,164] and oxymyoglobin[126] were at essentially the same wavelength. This led to the proposal that both proteins have a histidine imidazole ligand trans to dioxygen.[96] Further support for this idea came from data reported by Chang and Dolphin[165] on a purported thiolate-ligated ferrous–$O_2$ model complex that *did* have a substantially red-shifted Soret peak, suggesting that the oxyenzyme *did not* have a thiolate ligand. However, as can be seen in Figure 12, the MCD spectrum of oxy-P-450, initially reported by Dawson and Cramer,[166] is considerably different from that of oxymyoglobin. This rules out the possibility that both have the same ligand. In addition, the above-mentioned model complex for oxy-P-450 turned out to be a bis(thiolate)–ferric porphyrin adduct,[78,166,167] eliminating that part of the argument against a thiolate

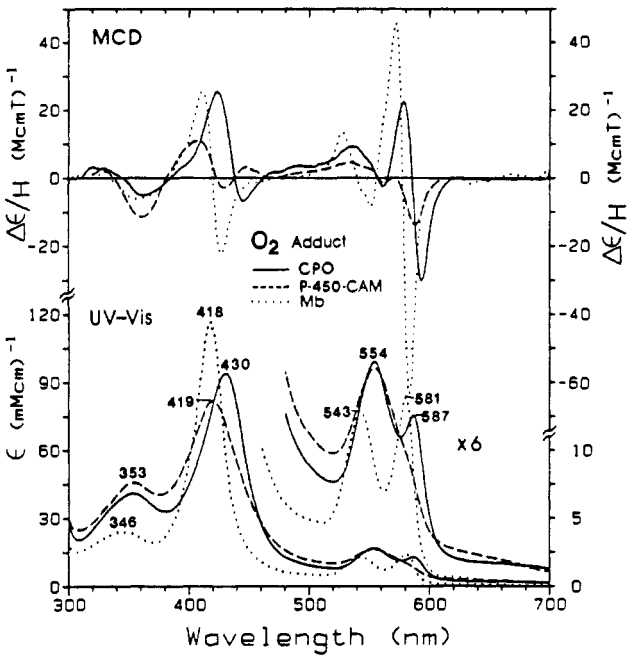



**Figure 12.** UV–visible absorption (bottom) and MCD (top) spectra of oxychloroperoxidase (CPO) and P-450-CAM measured at −30 °C in 65% (v/v) ethylene glycol, 0.035 M potassium phosphate buffer (pH 6.0 and 7.4, respectively) and myoglobin (Mb) measured at −10 °C in 50% (v/v) ethylene glycol, 0.5 M potassium phosphate buffer (pH 7.0). Reproduced with permission from ref 52. Copyright 1985 Journal of Biological Chemistry.

axial ligand in the oxygen-bound state. In 1980, Dolphin et al.[168] demonstrated that no proton uptake occurs during interconversion of P-450 states 3–5. Given the strong evidence available at that time for thiolate ligation in state 5, this was used to argue that the thiolate ligand was retained in the oxygenated enzyme.

Especially convincing evidence for the structure of the oxygenated form of P-450 began to appear in the early 1980s with the report of a crystalline thiolate–ferrous heme–$O_2$ model by Weiss and co-workers.[169,170] The complex displayed an O–O stretch in the infrared at 1139 $cm^{-1}$ that shifted to 1076 $cm^{-1}$ upon use of $^{18}O_2$ (Table VI, see below) and was interconvertible with the CO-bound complex.[169] A crystal structure of the complex revealed a Fe–S(thiolate) bond distance of 2.369 Å (Table V).[170] Unfortunately, in order to increase the stability of the complex, Weiss used the "picket fence" porphyrin first developed by Collman and co-workers[171] in designing models for oxyhemoglobin. Since this is a tetraarylporphyrin derivative, its optical properties are not as useful for comparison with data on the oxyenzyme. Budyka et al.[127] and Okubo et al.[128] reported the UV–visible absorption and MCD spectra of partially characterized thiolate–ferrous protoheme–$O_2$ models. In the former case, the MCD spectrum bears sufficient resemblance to that of oxy-P-450 to suggest that the thiolate ligand is likely present in the protein state.

Further studies of the crystalline oxy-P-450 model focused on the use of Mössbauer spectrocopy. Curiously, two different crystalline forms of the model, varying in the choice of crown ether or cryptand used to solubilize the thiolate but both clearly having a thiolate axial ligand, were found to have very different temperature dependence for the quadrupole-splitting parameter.[172] In one case, the complex displayed a very temperature-dependent splitting, while in the other system, the measured value was temperature independent. The temperature independence of the quadrupole splitting observed for oxy-P-450,[132,134] which contrasts with the case for oxyhemoglobin,[132] had been thought to be a property imparted on the system by the thiolate ligand. Clearly, the recent model complex data show that the temperature independence of the quadrupole splitting is not a function of the trans axial ligand. Instead, Montiel-Montoya et al. proposed that the property results from dynamic jump behavior of the bound dioxygen between the various sites in the crystal lattice.[172]

**TABLE VI. Vibrational Frequencies for the O–O Stretch in Ferrous–$O_2$ Heme Proteins and Model Complexes**

| ferrous–$O_2$ heme system | $^{16}O$–$^{16}O$ stretch $cm^{-1}$ | method | ref |
|---|---|---|---|
| hemoglobin | 1159 | infrared | 175 |
| Fe(TpivPP)(imidazole)($O_2$)[a,b] | 1163 | infrared | 176 |
| cytochrome P-450-CAM | 1140 | resonance Raman | 174 |
| Fe(TpivPP)(thiolate)($O_2$)[a,c] | 1139 | infrared | 169 |

[a] Abbreviation: TpivPP, *meso*-tetrakis(α,α,α,α-*o*-pivalamidophenyl)porphyrin. [b] The imidazole employed was *N*-methylimidazole. [c] The thiolate employed was $C_6HF_4S^-$.

As in the case of P-450 states 3 and 5, the most direct evidence for the presence of an axial thiolate sulfur donor ligand in oxy-P-450 has come from EXAFS spectroscopy[55] (Table IV). Once again, the best fit to the EXAFS data was achieved by inclusion of a sulfur atom as a ligand with a Fe–S bond distance of 2.37 Å. This is essentially identical with the Fe–S distance in Weiss' thiolate/$O_2$ model.[170] Unfortunately, only one other sulfur donor ligated oxygen complex, one involving a thioether ligand, has been reported. The Fe–S(thioether) bond distance, based on "semiquantitative" crystallographic data,[173] was 2.49 Å. These data suggest that a neutral thiol ligand would likely have a bond distance much longer than the anionic thiolate in Weiss' model and therefore that the sulfur donor in oxy-P-450 is a thiolate.[55]

Resonance Raman spectroscopy has recently been employed to detect the O–O stretch in oxy-P-450-CAM.[174] As listed in Table VI, the frequency found is in remarkable agreement with the value previously determined by IR spectroscopy for the thiolate model complex.[169] In addition, the shift observed upon use of $^{18}O_2$ was almost identical with that seen in the model. Furthermore, the frequencies observed for the thiolate model, and now for the oxyenzyme, are shifted by about 20 $cm^{-1}$ from the values reported for oxyhemoglobin[175] and an imidazole-ligated model[176] (Table IV). This decreased frequency of the dioxygen stretch indicates the presence of a larger charge on the iron.[174]

Given the extensive parallels between cytochrome P-450 and chloroperoxidase, it is surprising that 14 years went by between the first report of a oxygenated derivative of P-450 and preparation of a similar complex for chloroperoxidase. Nonetheless, in 1985, three research groups independently reported the preparation of a ferrous–dioxygen adduct of chloroperoxidase.[52,177,178] Nakajima et al.[177] prepared the derivative from compound II by addition of excess hydrogen peroxide in a fashion similar to the preparation of compound III (the oxy form) of horseradish peroxidase.[179] These experi-

menters noted that the UV–visible absorption spectrum of oxychloroperoxidase was more like that of oxylactoperoxidase than that of oxy-P-450. Lambeir and Dunford[178] generated the oxygenated derivative directly from the reduced state of the enzyme by oxygen addition and measured the oxygen binding rate [$(5.5 \sim 1.0) \times 10^4$ $M^{-1}$ $s^{-1}$] over the pH range from 3.5 to 6.0. Sono et al.[52] used cryogenic techniques to stabilize the dioxygen adduct of ferrous chloroperoxidase and were able to investigate its properties by using homogeneous samples. They reported its MCD and UV–visible absorption spectra (Figure 12) in comparison with parallel data on oxy-P-450 and oxymyoglobin, showed that the oxyderivative did not display an EPR spectrum, showed that the adduct was formed reversibly and could be converted into the CO-bound enzyme, and measured its rate of autoxidation to the ferric state. The rates of autoxidation of the oxygenated derivatives of P-450-CAM and chloroperoxidase (7.0 and $5.8 \times 10^{-4}$ $s^{-1}$, respectively) were essentially identical at −10 °C and pH 6.0.[52]

Surprisingly, despite the extensive similarities between the spectral properties of parallel derivatives of chloroperoxidase and P-450 discussed above, the UV–visible absorption and MCD spectra of oxychloroperoxidase and oxy-P-450-CAM are distinct from each other.[52] In particular, the Soret peak of the UV–visible absorption spectrum of oxychloroperoxidase is over 10 nm red-shifted from that of oxy-P-450-CAM, and the former has a sharp $\alpha$ peak at 587 nm, where only a slight shoulder is seen in that region of the spectrum of oxy-P-450-CAM. As for the red-shift of the Soret peak of oxychloroperoxidase, the oxy-P-450 model system of Budyka et al.[127] displayed large solvent effects on the wavelength of the Soret peak with the peak position shifting up to 10 nm to longer wavelength with increasing solvent polarity. Other than the two major differences just noted between the absorption spectra of the two oxygenated enzymes, the overall absorption spectral properties of oxychloroperoxidase are more like those of oxy-P-450-CAM than oxymyoglobin.[52] Note for example, the red-shifted $\beta$ band positions and the relative weakening of Soret band intensities and increased intensities of the $\delta$ transitions. Very recently, Dawson et al. reported the investigation of oxychloroperoxidase using EXAFS spectroscopy (Table IV) and obtained evidence for a sulfur atom, likely a thiolate, with a Fe–S bond distance of 2.37 Å, the same as the Fe–S distance found for oxy-P-450-CAM.[55] Clearly, considerable structural similarity exists between the two oxygenated heme enzymes despite some spectral dissimilarity.

In the MCD spectra (Figure 12), fewer similarities are seen between the three oxygenated proteins.[52] The spectral features of oxychloroperoxidase are consistently red-shifted from those of the other two proteins. In the Soret region, asymmetric MCD curves are seen for oxychloroperoxidase and oxy-P-450-CAM, while oxymyoglobin has symmetrical line shapes. In the 500–620 nm region, the spectrum of oxychloroperoxidase more closely resembles that of oxymyoglobin, although it is substantially less intense and red-shifted. Of the two MCD studies of thiolate–ferrous protoheme–$O_2$ models for oxy-P-450 that have been published, curiously, the MCD spectrum of the model reported by Okubo et al.[128] more closely resembles that of oxychloroperoxidase, while the MCD spectrum reported by Budyka et al.[127] is somewhat similar to that of oxy-P-450-CAM. In summary, although the spectral properties of oxychloroperoxidase and oxy-P-450-CAM are clearly distinguishable from those of histidine-ligated heme proteins, the observed spectral dissimilarities between the dioxygen adducts of chloroperoxidase and P-450-CAM are somewhat surprising given the extensive similarities observed between analogous derivatives as discussed elsewhere in this review. Significant differences between chloroperoxidase and P-450-CAM were recently reported (see section VIII) in the manner in which they bind ligands, which likely reflects differences in the environment surrounding the metal binding site.[53] Given the reported sensitivity of the spectral properties of a thiolate–ferrous heme–$O_2$ model to "solvent effects",[127] it may be that the differences observed between oxychloroperoxidase and oxy-P-450-CAM also reflect these environmental factors. Additional studies of oxychloroperoxidase with Mössbauer and resonance Raman spectroscopy should provide further insight into its properties vis-a-vis oxy-P-450.

### C. Other Ligand-Bound Derivatives

The binding of additional ligands other than CO and $O_2$ to the ferrous states of P-450 and chloroperoxidase will be discussed in three contexts: (a) the investigation of the nitric oxide adduct as a further probe of the identity of the endogenous axial ligand, (b) the comparison of the spectral properties of parallel ligand-bound forms of the two enzymes as a way of comparing active site structures, and (c) the use of ligand binding to probe the origin of the unusual red-shifted nature of all low-spin ferrous derivatives of the two enzymes other than the dioxygen adducts.

The investigation of the nitric oxide adducts of ferrous heme proteins with EPR spectroscopy can provide information about the ligand trans to NO. Positive evidence for a nitrogenous ligand can be derived from observation of the appropriate ligand superhyperfine-splitting pattern. This approach has been elegantly illustrated in the recent work on cytochrome *c* oxidase by Chan and co-workers.[180] In the case of P-450, the absence of superhyperfine splitting of the EPR signal beyond that expected for the nitrogen in NO is consistent with the presence of a non-nitrogenous ligand such as a thiolate.[181-183]

The Soret peak in the UV–visible absorption spectrum of ferrous–NO P-450,[181] like that of the ferrous–CO derivative, is appreciably red-shifted relative to the analogous form of myoglobin. In 1976, Stern and Peisach[184] reported the preparation of a thiolate–ferrous heme–NO model complex with an absorption spectrum that closely matched that of ferrous–NO P-450 and thereby provided additional support to the growing evidence for an endogenous thiolate ligand in the latter.

Comparison of the UV–visible absorption and MCD spectral properties of analogous ligand adducts of ferric P-450 and chloroperoxidase, as discussed in section II, has provided considerable support for the conclusion that both enzymes have structurally similar active sites. Extension of this comparison to the low-spin ferrous ligand adducts with CO and $O_2$ has already been discussed. Additional comparisons have been reported between the adducts with NO,[50,52] *n*-butyl isocyanide

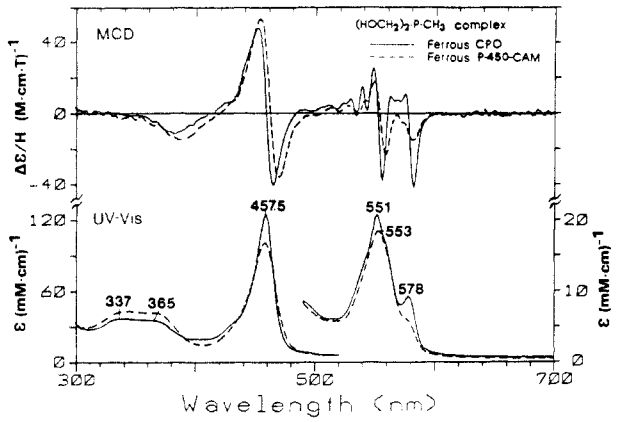



**Figure 13.** UV-visible absorption (bottom) and MCD (top) spectra of the ferrous chloroperoxidase (solid line) and P-450-CAM (dashed line) complexes with bis(hydroxymethyl)methylphosphine. Reproduced with permission from ref 51. Copyright 1985 American Chemical Society.

(ref 50, 52), and bis(hydroxymethyl)methylphosphine (ref 50, 51). In all three cases, the UV–visible absorption and MCD spectra of the parallel derivatives of the two enzymes are quite similar as can be seen for the phosphine adducts in Figure 13. This adds additional weight to the body of evidence for the close structural similarity between the active sites of the two enzymes.

The UV–visible absorption, CD, and MCD properties of a series of ligand adducts of ferrous P-450-CAM were investigated by Dawson, Andersson, and Sono[156] in order to probe the origin of the unusual red-shift observed for all such ferrous species other than oxy-P-450. In the case of ferrous–CO P-450, theoretical investigations in conjunction with single-crystal polarized absorption spectroscopic studies by Hanson et al.[79a,154] led to the conclusion that the unusual red-shifted Soret maximum was the result of a mixing and splitting mechanism. This explanation proposed that a sulfur $p^{\dagger}$ to porphyrin $\pi^*$ transition of the correct energy and symmetry mixed with and split the normal porphyrin $\pi,\pi^*$ band resulting in two transitions, one at longer wavelengths from the "normal" position for the Soret transition and one at shorter wavelengths. Clearly, this explanation required the red-shift of the Soret band as well as the appearance of a reasonably intense band in the near-UV region. Related theoretical work by Jung and Ristau,[185] also involving mixing of porphyrin transitions and sulfur donor ligand based charge-transfer bands, differed from the above treatment in the extent to which the ligand trans to thiolate might participate in the process. Dawson et al.[156] found that the ferrous P-450 ligand adducts fell primarily into two categories: (i) those with intense near-UV transitions in the UV–visible absorption spectrum and with relatively simple MCD spectra, and (ii) those with appreciably less intense near-UV bands and much more complicated MCD spectra, especially in the 500–600 nm region. They concluded that the mixing and splitting mechanism was probably not operative in the latter category, which lacked the intense near-UV band. In such cases, the red-shifted Soret peaks were proposed to result from the particular combination of a strongly electron-donating cysteinate axial ligand trans to a good $\sigma$ donor ligand. Gouterman[186] and Valentine[187] and their co-workers showed that electron-releasing ligands can induce red-shifts in the spectral properties of porphyrin systems.

## VI. High-Valent States: Chloroperoxidase

As can be seen in Figures 2 and 3, the current proposals for the mechanisms of action of P-450 and chloroperoxidase both include the involvement of high-valent iron–oxo intermediates. For P-450, only circumstantial evidence is available for the existence of either of the species beyond oxy-P-450 shown in Figure 2. With functional heme iron model systems, more convincing data has appeared to show that a compound I type iron–oxo intermediate plays a crucial role in hydroxylating alkanes and epoxidizing alkenes.[10,34,35] By inference, it is often assumed that the same intermediate is the active hydroxylation catalyst in the reaction cycle of the enzyme as well. The fact that P-450 can be turned over by using single oxygen atom donors such as peracids, hydroperoxides, iodoxyaromatics, amine oxides, or inorganic oxygen donors (Figure 2, **2** → **7**)[1,5,11–13] *suggests* the involvement of a common iron–oxo intermediate such as **7**. With mammalian liver microsomal P-450, Coon and co-workers used stopped flow techniques and observed two structurally undefined intermediates beyond oxy-P-450.[188] Single-turnover studies of oxy-P-450-CAM have not revealed the buildup of any intermediates between the oxygenated enzyme and the resting ferric state.[189] A number of experiments hinting at the formation of a compound I-type intermediate in the P-450 system have been reported,[190–192] but none have provided really hard evidence for such an species. Mechanistic evidence against a compound I-type intermediate has also appeared.[193] Very recently, Kobayashi et al.[194] examined the interaction of 2,4-diacetyldeuterioheme-substituted[167,195] oxy-P-450-CAM with hydrated electrons and suggested that an intermediate having a composition more like chloroperoxidase compound II[177,196,197] was involved. However, they were not able to directly demonstrate product formation from the same sample, and the amount of spectral change observed for the new intermediate by difference spectroscopy was very small. Clearly, despite frequent statements to the contrary in the literature, no compelling evidence has yet appeared to show that the intermediates beyond oxy-P-450 depicted in Figure 2 actually participate in the reaction cycle of P-450.

In spite of considerable effort to determine the mechanism of halogenation of organic substrates by chloroperoxidase, only a limited consensus has been reached concerning the details of the process. There is agreement that a compound I type [$Fe^{IV}$=O, porphyrin $\pi$ cation radical] intermediate (**9**) forms first and that, in its peroxidase reactivity mode (Figure 3), the enzyme follows the classic peroxidase path producing compound II (**9** → **10**) and then regenerating the ferric resting state **8**.[30,198,199] Disagreement exists as to the fate of compound I during the halogenation process. Hager and co-workers[32,42,200] proposed that the interaction of compound I with chloride ion leads to production of a ferric hypochlorite (**11**, [$Fe^{III}$OCl]) adduct they called compound X. Recent mechanistic investigations by Dunford et al.[43] provided support for this hypothesis. They carefully analyzed the steady-state kinetics of the

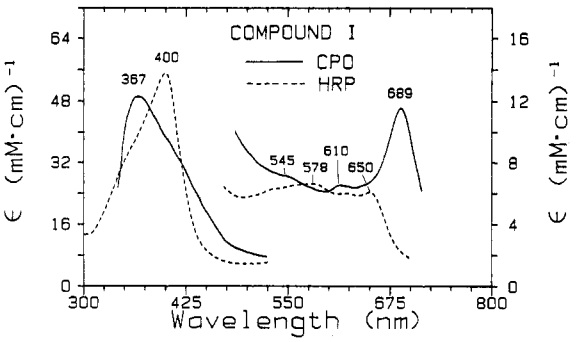



**Figure 14.** UV–visible absorption spectra of chloroperoxidase compound I[203] (solid line) and of horseradish peroxidase compound I[204] (dashed line). Reproduced with permission from ref 203. Copyright 1980 Academic.

chlorination of monochlorodimedone by chloroperoxidase and obtained evidence for chloride ion addition to compound I to produce compound X, which then halogenated the substrate as shown in Figure 3.

The kinetic analysis of Dunford et al.[43] also shows that compound X intermediate in chloroperoxidase-catalyzed chlorinations has only a very transient existence. Therefore, essentially no direct evidence is available for its structure. However, Hager and co-workers[201] also described a ferric hypochlorite (compound X) intermediate in the low-pH chlorite ($NaClO_2$) dependent chlorination reaction catalyzed by horseradish peroxidase. This intermediate can be generated in stable homogeneous form above pH 10 and has therefore been the object of spectroscopic examination. Most recently, Terner and co-workers[202] used resonance Raman spectroscopy to show that the intermediate has a Fe–O stretching frequency that is very similar to that in the compound II state of the same enzyme. Earlier studies of horseradish peroxidase compound II with both resonance Raman[37,39] and EXAFS[34,35] spectroscopy showed it to consist of a $Fe^{IV}$=O unit. Of course, the active form of horseradish peroxidase compound X exists at low pH, while the spectroscopic investigation was carried out above pH 8,[201] and so it is quite possible that lowering the pH causes a structural change to take place, converting the $Fe^{IV}$=O unit into a [$Fe^{III}$OCl] center. In any event, more work is clearly necessary to establish the structure of the active chlorinating catalyst in either horseradish peroxidase or chloroperoxidase.

As for the structures of chloroperoxidase compounds I and II (Figure 3, **9** and **10**), although each intermediate has been detected and partially characterized, the properties of the two species are much less well understood than the analogous forms of horseradish peroxidase. Palcic et al.[203] used rapid-scanning stopped flow techniques to measure the UV–visible absorption spectrum of chloroperoxidase compound I, which is compared with the spectrum of horseradish peroxidase compound I[204] in Figure 14. The unique features of the spectrum of the chloroperoxidase derivative are the blue-shifted Soret peak and the appearance of a distinct peak at 689 nm. In contrast, horseradish peroxidase compound I has its Soret peak at 400 nm with a broad envelope of absorption between 500 and 650 and only weak intensity at 690 nm. In fact, the absorption spectrum of chloroperoxidase compound I more closely resembles that of catalase compound I,[205] which has an anionic tyrosinate axial ligand[206] trans to the oxo group, a much broader Soret peak than that of horseradish

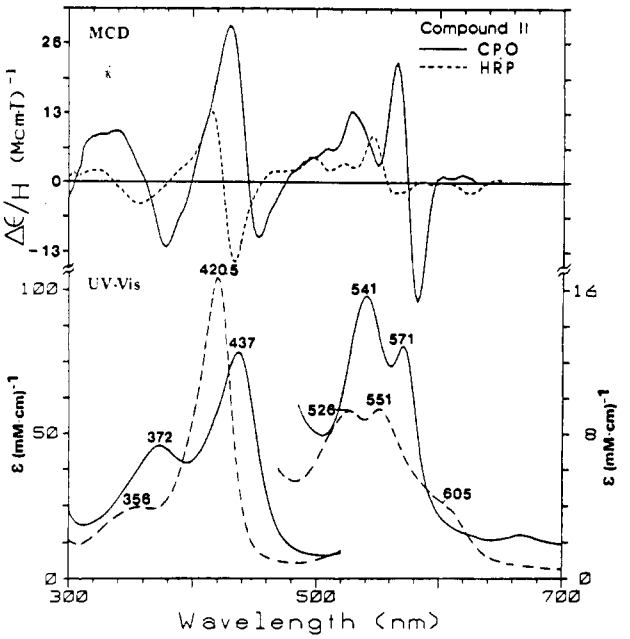



**Figure 15.** UV–visible absorption (bottom) and MCD (top) spectra of compound II derivatives of chloroperoxidase (CPO) (solid line) and horseradish peroxidase (HRP) (dashed line). The measurements of CPO (1.5–2.0 $\mu$M) were made in 0.1 M potassium phosphate buffer, pH 6.0, with peroxides (~0.15 mM) and ascorbic acid (~3 mM) and those of HRP (10–15 $\mu$M) in 0.005 M sodium carbonate buffer, pH 10.5, with equivalent amounts of EtOOH and ascorbic acid, at ~4 °C. The MCD spectrum of CPO compound II is the average of three measurements. Reproduced with permission from ref 196. Copyright 1985 Instituto Superior Tecnico, Avda.

peroxidase compound I, and a prominent peak at about 660 nm. Dolphin[205] used the spectral differences between horseradish peroxidase and catalase compounds I to argue that the two porphyrin $\pi$ cation radicals have different ground states, $^2A_{2u}$ and $^2A_{1u}$, respectively. On the basis of the NMR properties of ruthenium-substituted horseradish peroxidase compound I, Morishima[207] proposed a more complex ground state for iron horseradish peroxidase compound I consisting of an admixture of $^2A_{2u}$ and $^2A_{1u}$ states. The Mössbauer and EPR properties of chloroperoxidase compound I[208] have been interpreted in terms of an exchange-coupled spin $S = 1$ Fe(IV) iron and a spin $S' = {}^1/_2$ porphyrin radical. In any event, the spectral similarity between chloroperoxidase and catalase compounds I suggests that chloroperoxidase compound I has a ground-state electronic structure that is similar to that of catalase compound I. An additional type of compound I ground state consisting of a Fe(IV, $S = 1$) oxygen radical was proposed for P-450 compound I by Loew et al.[112] However, the Mössbauer and EPR data[208] on chloroperoxidase compound I are inconsistent with that formulation.

Even less is known about chloroperoxidase compound II than about compound I. A UV–visible absorption spectrum of compound II in the Soret region measured during the steady state of reaction was reported by Thomas et al.[197] in 1970 and was thought to consist of a mixture of compound II in the presence of some compound I or the ferric enzyme. The absorption and MCD spectra of a nearly homogeneous preparation of compound II in its steady state were reported by Dawson et al.[196] and are displayed in Figure 15 in compar-

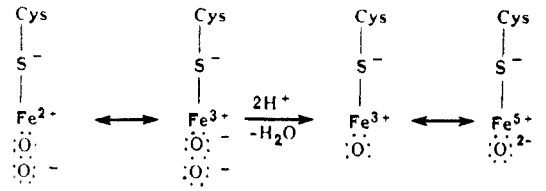



**Figure 16.** Proposed role of the thiolate ligand in the catalytic mechanism of cytochrome P-450. Adapted from ref 119.

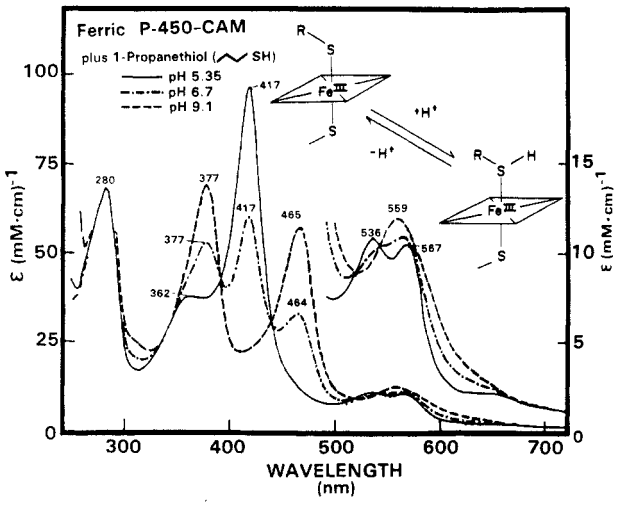



**Figure 17.** UV-visible absorption spectral changes of the 1-propanethiol complex of ferric cytochrome P-450-CAM as a function of pH. Adapted from ref 67.

ison with analogous data on horseradish peroxidase compound II. As can be readily seen, the spectra of the parallel derivatives of the two peroxidases are not all that similar. As with the compound I state, the spectral properties of chloroperoxidase compound II are more closely related to those of catalase compound II.[209] A similar absorption spectrum of chloroperoxidase compound II was reported by Nakajima et al.,[177] working with partially purified enzyme. Unfortunately, chloroperoxidase compound II has not yet been investigated by EXAFS, resonance Raman, or Mössbauer spectroscopy.

## VII. Mechanistic Implications of Thiolate Ligation

Given the extensive evidence that cytochrome P-450 and chloroperoxidase both have an endogenous cysteinate axial ligand, at least two questions remain to be answered: (a) what roles do the thiolate ligands play in the reactivity of the two enzymes, and (b) is it possible to account for such disparate reactivities for the two enzymes given such similar active sites? While the complete answers to these questions are not yet at hand, some evidence has recently appeared that can help begin to provide the answers.

### A. Cytochrome P-450

In 1976, Dawson et al.[119] speculated on the role that the proposed thiolate ligand to the heme iron of P-450 might play in the activation of dioxygen for incorporation in organic substrates. As shown in Figure 16, it was suggested that the relatively polarizable thiolate ligand might push electron density into the trans position of intermediate **6** (Figure 2), thereby weakening the O–O bond and facilitating its cleavage to give an iron–oxo compound I type product (**7**). It was also proposed that the electron-releasing thiolate ligand might help to stabilize the highly electrophilic compound I product resulting from cleavage of the oxygen–oxygen bond.

**TABLE VII. Acidities of 1-Propanethiol Bound to Ferric Heme Proteins**[a]

| thiol states | $pK_a$ | deprotonation reaction |
|---|---|---|
| free | 10.7 | $RSH \rightleftharpoons RS^- + H^+$ |
| bound to ferric P-450-CAM | 6.7 | $R(H)S-Fe^{3+}-Cys \rightleftharpoons RS^--Fe^{3+}-Cys + H^+$ |
| bound to ferric myoglobin | <4.0 | $R(H)S-Fe^{3+}-Imid \rightleftharpoons RS^--Fe^{3+}-Imid + H^+$ |

[a] Taken from data in ref 67.

In 1982, experimental evidence for the electron-releasing nature of the thiolate ligand in P-450 relative to the histidine imidazole of myoglobin was presented by Sono, Andersson, and Dawson.[67] In a thorough study of the binding of sulfur donor ligands to the two proteins, it was observed that while thiol (RSH) addition to ferric myoglobin always led to thiolate ($RS^-$) ligation to the heme iron, in the case of ferric P-450-CAM either thiol or thiolate ligation was possible, depending on the thiol $pK_a$ and the solution pH. In the myoglobin case, even at acidic pH and even with very weakly acidic thiols, the resulting complexes had spectral (UV–visible absorption, EPR, and MCD) properties that closely resembled imidazole adducts of P-450, strongly suggesting that both systems consisted of a thiolate–ferric heme–imidazole coordination structure. In other words, upon interaction with the myoglobin heme iron, the thiol would deprotonate. Since the lowest pH accessible was pH 4, this required that the $pK_a$ of the bound thiol was less than 4 in all cases. For P-450, acidic thiols also deprotonated upon ligation to form a bis(thiolate) complex. Ruf and Wende[78] had shown previously that bis(thiolate)–ferric heme iron complexes had characteristic split Soret or hyperporphyrin absorption spectra (vide supra). This made the deprotonation of the added thiol particularly easy to monitor. With a very weakly acidic thiol, 1-propanethiol, it was observed that upon lowering the pH of the ligand-bound enzyme, the spectrum changed to that of a normal low-spin ferric heme iron system as would be expected if the bound thiolate were to become protonated. The pH-dependent spectral change is shown in Figure 17, and the derived $pK_a$ values for 1-propanethiol bound to the two proteins are listed in Table VII.

These data on thiol/thiolate ligation to P-450 and myoglobin show that a ligand will be more acidic when bound to myoglobin than when bound to P-450. Conversely, a ligand is more basic or more electron rich bound to P-450 than to myoglobin. Since the major difference at the active sites of the two proteins is the endogenous thiolate of P-450 compared with the histidine imidazole of myoglobin, the data show that the thiolate releases electron density through the iron into the trans ligand as proposed[119] in Figure 16. Further evidence in support of this hypothesis was also reported by Sono and Dawson[94] on the basis of significant dif-

ferences observed in the affinities of anionic ligands to ferric P-450 and myoglobin. In addition, the reduction potentials for P-450[104] (ferric/ferrous couple) are appreciably lower than that of myoglobin[210] as would be expected for a more electron-rich metal center. The fact that functional model systems are able to transfer an oxygen atom from the heme iron to the substrate without needing a thiolate ligand[10,211] suggests that the main function of the thiolate ligand in P-450 is to facilitate cleavage of the O–O bond in order to produce the key iron–oxo intermediate.

## B. Chloroperoxidase

As part of an effort to obtain new information about the active site heme environment of chloroperoxidase, Sono et al.[53] recently investigated the binding of a large number of exogenous ligands over a wide pH range. This work demonstrated that the active site environment of chloroperoxidase contains polar amino acid residues as will be described below. In contrast, the recent X-ray crystal structure analysis of substrate-bound P-450-CAM[27] confirmed earlier conclusions reached from ligand binding studies of P-450 that the substrate binding site and the active site environment in general are very nonpolar. Essentially all the amino acids that contact the bound camphor except for tyrosine-96, which forms a hydrogen bond to the camphor ketone oxygen, are hydrophobic amino acids. There are no acidic or basic amino acids anywhere near the dioxygen binding site to participate in oxygen activation.

The study by Sono et al.[53] of ligand binding to ferric and ferrous chloroperoxidases revealed the presence of acid/base sensitive amino acids in the active site heme environment that affect ligand binding and could therefore also play a role in catalysis. For example, weakly acidic anionic ligands such as cyanide, azide, nitrite, formate, and acetate bind to the ferric enzyme with the apparent $K_d$ values shown in Figure 18A. Except for cyanide, all these ligands have p$K_a$ values in the pH range 2–7 over which the study was performed. Recalculation of the $K_d$ value, based on the actual concentration of protonated (neutral) ligand form present at the various pH values of the study, reveals that in all cases the intrinsic $K_d$ values are pH independent (Figure 18B). Similar data were also reported for fluoride binding. These results suggest that the protonated (neutral) forms of the weakly acidic ligands bind to the enzyme. The spectral similarities observed between the analogous ligand adducts of ferric P-450 and chloroperoxidase suggest that when bound to the heme iron, the ligands have the same protonation state, likely deprotonated. This requires that anionic ligands bind to chloroperoxidase in their protonated state and then deprotonate upon binding to the iron with the proton being picked up by a nearby basic amino acid such as histidine imidazole. Very similar pH effects were previously reported by Chance[212,213] for catalase and by Dunford, Erman, and their co-workers[214–217] for horseradish and cytochrome *c* peroxidases. In other words, such pH effects seem to be indicative of a peroxidase-type active site heme environment.

Further support for the presence of an acidic amino acid in the close proximity of the active site came from examination of the pH profile of binding of a neutral

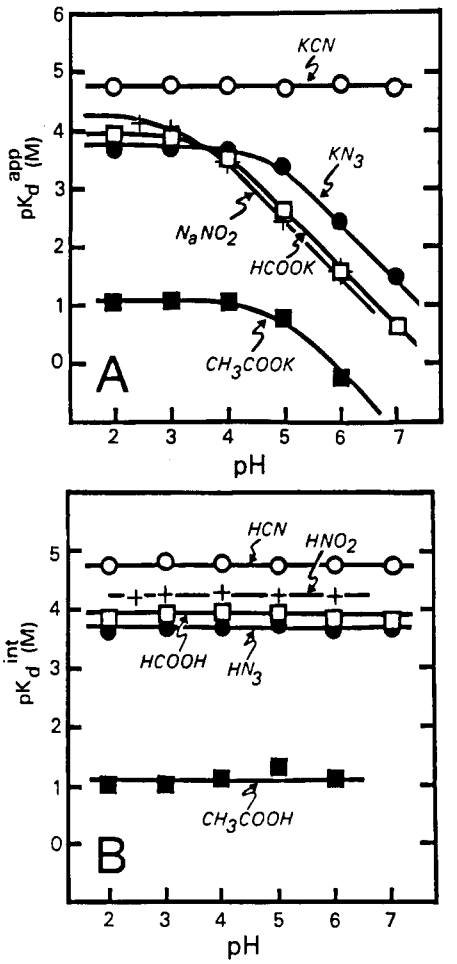



**Figure 18.** Effects of pH on the affinities of ferric chloroperoxidase for weakly acidic anionic ligands: (A) apparent equilibrium dissociation constants ($K_d^{app}$) and (B) intrinsic $K_d$($K_d^{int}$) values as a function of pH. The $k_d^{int}$ values were calculated on the basis of protonated (neutral) ligand concentrations. Reproduced with permission from ref 53. Copyright 1986 American Chemical Society.

alkyl isocyanide to ferrous chloroperoxidase.[53] An ionizable group with a p$K_a$ of 5.5 was detected. Campbell et al.[114] previously presented evidence for the presence of an amino acid with the same p$K_a$ in the environment of the active site that effected CO binding to ferrous chloroperoxidase. The possible role of an active site histidine in the catalytic mechanism of cytochrome *c* peroxidase was suggested by Poulos and Kraut[218] based on the crystal structure of the enzyme.

Taken together, these data reveal that while the spectroscopic properties of chloroperoxidase closely resemble those of P-450, the active site environment is that of a hydroperoxidase. In other words, chloroperoxidase has a P-450-type metal coordination structure located in a peroxidase active site heme environment.[53] Chloroperoxidase and P-450 have only a limited set of enzymatic activities in common,[219] such as the N-dealkylation of certain organic substrates,[220] the epoxidation and hydroxylation of cyclohexene,[221,222] and the oxygenation of sulfides and sulfoxides.[223,224] Evidently, the combination of a P-450 metal binding structure in a peroxidase active site environment produces a unique mode of reactivity, namely the chlorination of organic substrates.

## VIII. Summary

The active site structures of cytochrome P-450 and chloroperoxidase have been carefully and extensively investigated with a wide range of spectroscopic methods. In conjunction with equally thorough studies of synthetic models for the metal binding sites, these efforts have established that both enzymes have an axial cysteinate sulfur donor ligand trans to the dioxygen or peroxide binding site. This assignment has recently been verified for ferric P-450 by using X-ray crystallography. The most direct spectroscopic evidence for this structure has come from EXAFS and resonance Raman studies with MCD and EPR spectral comparisons between protein and model systems providing strong supporting data. Studies of high-valent chloroperoxidase intermediates (Figure 3), while not yet as complete as might be desired, are beginning to provide information about similar proposed intermediates in the P-450 reaction cycle (Figure 2). Evidence supporting the role of the thiolate ligand in providing an electron "push" to facilitate cleavage of the O–O bond in the P-450 reaction mechanism has been obtained through comparative studies of the ligand binding properties of P-450 and myoglobin. The active site structural similarities between P-450 and chloroperoxidase present an enigma, given the rather different reactions catalyzed by the two enzymes. Recent studies have shown that while the two enzymes have similar metal binding sites, their heme environments are quite different in polarity. The investigations of P-450 and chloroperoxidase described herein provide an excellent example of the extent to which the active site structure of a metalloenzyme can be deduced through spectroscopic means.

## IX. Acknowledgements

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**Registry No.** Chloroperoxidase, 9055-20-3; cytochrome P-450, 9035-51-2.

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