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# Chloroperoxidase: P-450 Type Absorption in the Absence of Sulfhydryl Groups†

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ABSTRACT: The oxidation state of the two half-cystine residues in the native ferric form of chloroperoxidase and in the reduced ferrous chloroperoxidase has been examined in order to evaluate the role of sulfhydryl groups as determinants of P-450 type spectra. Mössbauer and optical spectroscopy studies indicate that the ferrous forms of P-450cam and chloroperoxidase have very similar or identical heme environments. Model studies have suggested that sulfhydryl groups may function as axial ligands for developing P-450 character. However, chemical studies involving both sulfhydryl reagents and amperometric titrations show that neither the ferric nor the chemically produced ferrous forms of chloroperoxidase contain a sulfhydryl group. These results rule out the hypothesis that sulfhydryl groups are unique components for P-450 absorption characteristics. The optical and electron paramagnetic resonance (EPR) spectra of the nitric oxide complex of chloroperoxidase have been obtained and compared to those of myoglobin, hemoglobin, and cytochrome *c* and horseradish peroxidase. The EPR spectrum of the NO–ferrous chloroperoxidase complex, which is similar to that of cytochrome P-450cam, does not show the extra nitrogen hyperfine structure which appears to be characteristic of those hemoproteins which have a nitrogen atom as an axial heme ligand.

The absorption spectrum of the CO complex of ferrous chloroperoxidase is quite similar to the characteristic spectrum of the CO complexes of the P-450 type hemoproteins (Hollenberg and Hager, 1973). The unusual characteristic of the CO–ferrous complexes of the P-450 type is the extremely long wavelength Soret band. The Soret peak for the carbon monoxide complexes of ferrous P-450 hemoproteins occurs near 450 nm (Cooper et al., 1965; Lindenmayer and Smith, 1964; Murphy and West, 1970; Appleby, 1969) while the Soret peak for the carbon monoxide complexes of most other reduced hemoproteins is near 420 nm (Klingenberg, 1958).

There has been intense interest in the chemical nature of the heme ligands which give rise to the P-450 type absorption. Recent work has suggested that the ligands in P-450 type cytochromes are thiols. The typical low spin ferric electron paramagnetic resonance (EPR) signals exhibited by P-450 type hemoproteins were first observed by Mason et al. (1965) and Murakami and Mason (1967), who suggested that these signals could arise from the association of the heme iron with thiols. Subsequently, Bayer et al. (1969) prepared a series of Fe(III) hemoglobin and myoglobin thiol complexes and found that these complexes had a pattern of electron paramagnetic resonance EPR *g* values similar to those of cytochrome P-450. Jefcoate and Gaylor (1969) made a similar study of the isopropyl–thiol complex of Fe(III) myoglobin and showed that it too gave the characteristic P-450 *g* values. Blumberg and Peisach (1971a) have shown that the EPR spectra of low spin ferric cytochrome P-450 and cytochrome P-420 were similar to those obtained by the addition of thiols to ferric hemoglobin and myoglobin. Blumberg and Peisach (1971b) have extended their EPR studies to cover a wide variety of heme proteins and they have classified a large number of low spin ferric compounds according to their EPR behavior. From this classification, chloroperoxidase was assigned to the O-type class (Blumberg and Peisach, 1971a). Later information developed from Mössbauer data (Champion et al., 1973) placed chloroperoxidase near the boundary between type-O and type-P (type P = P-450) hemoproteins. In addition, re-

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cent EPR and optical absorption spectroscopy studies on model thiol-hemin complexes (Stern and Peisach, 1974; Collman et al., 1975; Koch et al., 1975a,b) also support the hypothesis that a thiol group serves as an axial ligand in the P-450 hemoproteins. Thus several groups now embrace the theory that a thiol ligand is unique to P-450 hemoproteins and it has been proposed that it is the thiol ligand which confers the unusual Soret absorption on the ferrous-CO complex in the P-450 family. However, deductions from EPR evidence concerning the ligands involved in the P-450 ferrous complexes must be viewed with suspicion because the EPR studies, of necessity, have only inspected ferric heme states and have concentrated on the low-spin ferric states. Presumably all of the low spin ferric states involve two axial ligands attached to heme iron. Since either of these two ferric ligands would be eligible for displacement in the formation of the ferrous-carbon monoxide complex, a thiol ligand present in the ferric state might reasonably be absent in the CO-ferrous state. Thus, it appears quite possible to speculate that a thiol ligand could be responsible for the EPR $g$ values characteristic of the ferric heme in the P-450 family and yet not be the ligand responsible for the P-450 Soret absorption of the ferrous-carbon monoxide complex.

Recent Mössbauer spectroscopic studies on reduced chloroperoxidase and cytochrome P-450cam suggest that the chemical environment surrounding the ferrous heme group in both enzymes is very similar if not identical (Champion et al., 1975). Since we have previously reported that chloroperoxidase contains only two half-cystine residues (Morris and Hager, 1966), it became important to carefully examine the oxidation state of these two half-cystine residues both in the ferric and ferrous forms of the enzyme. In this paper we report that neither native chloroperoxidase (ferric form) nor reduced enzyme (ferrous heme) contains a free sulfhydryl group. Hence, we conclude that chloroperoxidase cannot have a thiol heme ligand in either the resting native enzyme or in the P-450 reduced ferrous-CO form. In addition, the EPR spectrum of the nitric oxide complex of ferrous chloroperoxidase suggests that, in contrast to myoglobin and hemoglobin, the ferrous complexes of chloroperoxidase do not contain an imidazole nitrogen as a heme ligand.

## Methods and Materials

*Enzyme Preparation.* Chloroperoxidase was isolated from *Caldariomyces fumago* and purified as reported previously (Morris and Hager, 1966). The preparations used in this paper had a specific activity of at least 2000 units/mg of protein in the standard monochlorodimedone chlorination assay and exhibited Rz values of 1.4.

*Spectral Measurements.* All the optical spectra and spectral changes were recorded on a Cary 15 or Beckman Acta III spectrophotometer.

*Amperometric titrations* were carried out on a Heath Model EUA-19-2 polarograph using a saturated calomel electrode as a reference electrode, platinum metal as the counter electrode, and dropping mercury as the testing electrode. The voltage applied on the testing electrode was −0.6 V with respect to reference electrode. The titration procedure has been described previously by Cecil and Wake (1962). Phenyl mercury acetate was used as a titrant at a concentration of about 2 m$M$. Chloroperoxidase was present at a concentration of approximately 0.015 m$M$

*Reduction of the Disulfide Bond.* The method used for the reduction of the disulfide bond in the denatured enzyme was the sodium borohydride reduction described by Habeeb (1973). The concentration of chloroperoxidase used in these experiments was approximately 0.02 m$M$.

*Anaerobic Titrations.* All anaerobic titration experiments were carried out in an atmosphere of CO which had been purified by bubbling through two towers of acidic vanadium(II) sulfate solution to remove traces of oxygen. The titrations were performed in a Thunberg cuvet similar to that reported by Burleigh et al. (1969). The evacuation and flushing procedure was repeated at least four times. The titrants were added using Hamilton gas tight syringes (volumes ranging from 50 to 100 $\mu$l).

Dithionite solutions were prepared anaerobically in the titrant flask designed by Burleigh et al. (1969). Dithionite solutions were standardized by the anaerobic titration of a solution of FAD. The reduction was monitored by measuring absorbance changes in the visible absorption bands of FAD.

*EPR Measurement.* The EPR spectra of the nitric oxide-chloroperoxidase complex were obtained from a Varian E-9 EPR spectrometer equipped with a low-temperature device. The spectra were measured at −170°. The enzyme concentrations used in the EPR studies were about 0.25 m$M$. The NO complex was formed by placing an enzyme solution (0.25 ml) in a suitable quartz tube which was then flushed with oxygen-free argon for 30 min, and finally sealed with a serum cap. A suitable amount of dithionite solution was added to reduce the enzyme and then oxygen-free nitric oxide was introduced to the EPR tube.

*Protein Determination.* The concentration of chloroperoxidase was based on the standard chlorination assay (Morris and Hager, 1966) and the heme content of the preparation as measured by the pyridine hemochrome method using a millimolar extinction coefficient of 33 m$M^{-1}$ cm$^{-1}$ at 557 nm. The extinction coefficient of ferric chloroperoxidase at 398 nm was consistent with the value reported by Hollenberg and Hager (1973).

*Materials.* 5,5′-Dithiobis(2-nitrobenzoic acid) ($Nbs_2$)[1] and *p*-chloromercuribenzoate (PCMB) were obtained from Sigma Chemical Co. and used without further purification. Phenyl mercury acetate was obtained from the same company and recrystallized from water prior to use. All other chemicals were reagent grade and were obtained from commercial sources.

NADPH (Type V) and FAD (95% purity) were purchased from Sigma Chemical Co. FAD was used without further purification. Ferredoxin and ferredoxin reductase from spinach leaves were purified by the method of Shin et al. (1963).

## Results

*Sulfhydryl Determination.* $Nbs_2$ and PCMB show unique optical absorption changes when they react with sulfhydryl groups (Ellman, 1959; Boyer, 1954). When native chloroperoxidase was mixed with a $Nbs_2$ or PCMB solution, there was no detectable absorption change. Even after the enzyme had been denatured by exposure to 8 $M$ urea under anaerobic conditions for 2 hr at room temperature, chloroperoxidase failed to give a positive test for sulfhydryl groups.

[1] Abbreviations used are: $Nbs_2$, 5,5′-dithiobis(2-nitrobenzoic acid); PCMB, *p*-chloromercuribenzoate.

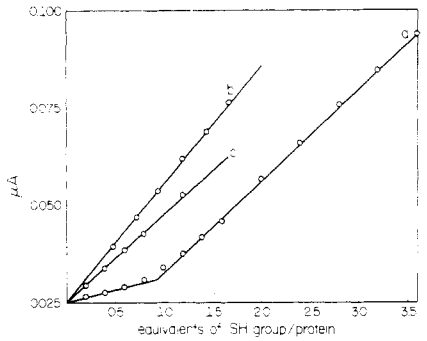


FIGURE 1: The amperometric titration of 5 ml of 0.015 m$M$ chloroperoxidase in 8 $M$ urea with 1.5 m$M$ phenyl mercury acetate at −0.6 V in the presence (a) and absence (b) of a 200-fold molar excess of $Na_2SO_3$. The control experiment, the titration of $Na_2SO_3$ in 8 $M$ urea, is also shown (c).

Table I: Sulfhydryl Group Determination after Sodium Borohydride Reduction.

| Reaction Mixture | $NaBH_4$ | OD at 412 m (after addition of $NbS_2$)[a] | SH ($\mu M$) |
|---|---|---|---|
| + chloroperoxidase (9.4 $\mu M$) | | 0.09 | 0.7[b] |
| − chloroperoxidase | | 0.08 | 0.0[b] |
| + chloroperoxidase (9.4 $\mu M$) | + | 0.325 | 18.2 |

[a] The absorbance at 412 nm was measured against the same reaction mixture without $NbS_2$. [b] Background for control experiment.

Denatured chloroperoxidase in 8 $M$ urea was also titrated amperometrically with phenyl mercury acetate. As shown in Figure 1, the current increases linearly in the amperometric titration indicating that no reaction had taken place between the protein and phenyl mercury acetate. Again, these results indicate that there is no detectable free sulfhydryl group in either the native or denatured form of chloroperoxidase.

*Studies on the Disulfide Bond.* Previous work has established that there are two half-cystine residues in chloroperoxidase and it had been suggested that these are in the form of a disulfide bond (Champion et al., 1973). Therefore, denatured chloroperoxidase in 8 $M$ urea was reduced with a 200 molar excess of $Na_2SO_3$ and again titrated with phenyl mercury acetate. The reduction of a disulfide bond with $Na_2SO_3$ occurs according to the following equation:

$$R\text{-}S\text{-}S\text{-}R + SO_3^{2-} \rightleftarrows RSSO_3^- + RS^-$$

Thus sulfite reduction of chloroperoxidase should produce one sulfhydryl group and the newly formed sulfhydryl group should react with phenyl mercury acetate. As shown in Figure 1, the amperometric titration of sulfite-reduced enzyme does reveal the presence of a sulfhydryl group. With sulfite-reduced enzyme the rate of reduction of mercury is decreased as revealed by a decrease in the current passing through the solution. In contrast to the titration results with native enzyme, a clear end point was obtained with sulfite reduced chloroperoxidase, at a sulfhydryl group content of 0.93 per chloroperoxidase molecule.

Reduced chloroperoxidase was also reacted with $Nbs_2$ after reduction of the denatured enzyme with sodium borohydride. A molar extinction coefficient of 13,700 $M^{-1}$ $cm^{-1}$ at 412 nm was used to calculate the sulfhydryl content of the reduced enzyme. This coefficient was obtained by reacting $Nbs_2$ with known amounts of glutathione under the same conditions used to reduce the enzyme. The results, shown in Table I, indicate that two sulfhydryl groups are generated by borohydride reductions. These results, taken in conjunction with the other studies, confirm the presence of two half-cystine residues in disulfide linkage in the native protein.

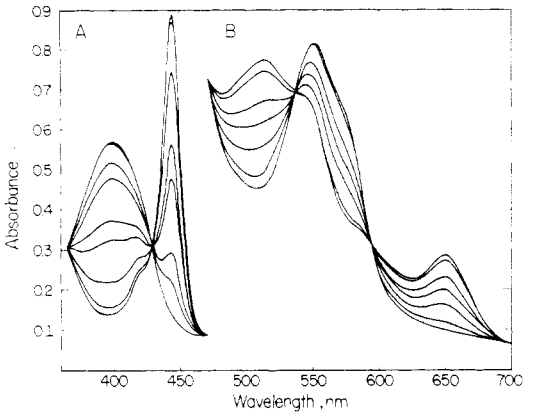


FIGURE 2: Spectral titration of chloroperoxidase with sodium dithionite. (A) Chloroperoxidase (6.13 $\mu M$) in 3 ml of 0.1 $M$ potassium phosphate buffer (pH 6.3) was titrated with 0.25 m$M$ $Na_2S_2O_4$. The curves shown correspond to the addition of 10, 15, 25, 30, 40, 50, and 55 $\mu$l of the dithionite solution. (B) Chloroperoxidase (56 $\mu M$) in 3 ml of 0.1 $M$ potassium phosphate buffer (pH 6.3) was titrated with 0.49 m$M$ $Na_2S_2O_4$. The curves shown correspond to the addition of 50, 100, 130, 160, 210, and 260 $\mu$l of the dithionite solution.

*Anaerobic Reduction of Chloroperoxidase with Sodium Dithionite.* In the presence of excess sodium dithionite, ferrous chloroperoxidase reacts with CO and the resultant complex shows the P-450 type chromophore as reported by Hollenberg and Hager (1973). Since the disulfide bond of native chloroperoxidase could be susceptible to reduction by dithionite, it became important to discover whether or not dithionite was reducing both the heme iron and the disulfide bond of the native enzyme. Accordingly, the stoichiometry of electron uptake by chloroperoxidase in the reductive process was investigated. The sodium dithionite titration was carried out in an atmosphere of CO. Figure 2 illustrates the spectral changes in the Soret, $\alpha$, and $\beta$ bands during the dithionite titration. The absorption maxima at 398, 516, and 650 nm decreased and new maxima at 446 and 550 nm appeared. The extinction coefficient at 446 nm was calculated to be 150 m$M^{-1}$ $cm^{-1}$. During the titration, isosbestic points appeared at 428, 473, 535, and 593 nm indicating a transformation of ferric chloroperoxidase to the reduced chloroperoxidase CO complex. The rate of formation of the CO complex was slow at pH 6.0, usually 30 min were required to achieve full complex formation at low chloroperoxidase concentrations. The absorption at 420 nm ($\delta$ absorption) was also monitored during dithionite reduction. The titration results are plotted in Figure 3. These plots show that the number of electrons taken up by chloroperoxidase during dithionite reduction is approximately 1.1 per chloroperoxidase molecule.

*Anaerobic Reduction of Chloroperoxidase by NADPH.* Ferric chloroperoxidase can also be reduced to the ferrous state by NADPH in the presence of spinach ferredoxin and spinach ferredoxin reductase. When the titration is carried out under an atmosphere of CO the typical P-450 spectrum of the CO complex is formed (see Figure 4). The spectrum

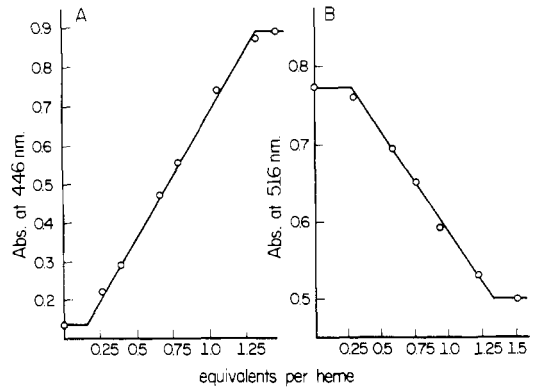


FIGURE 3: End point titration of chloroperoxidase with dithionite. Data points were obtained from Figure 2. The total change in absorbance at 446 nm (a) and 516 nm (b) was determined, corrected for dilution, and plotted as a function of the volume of dithionite added.

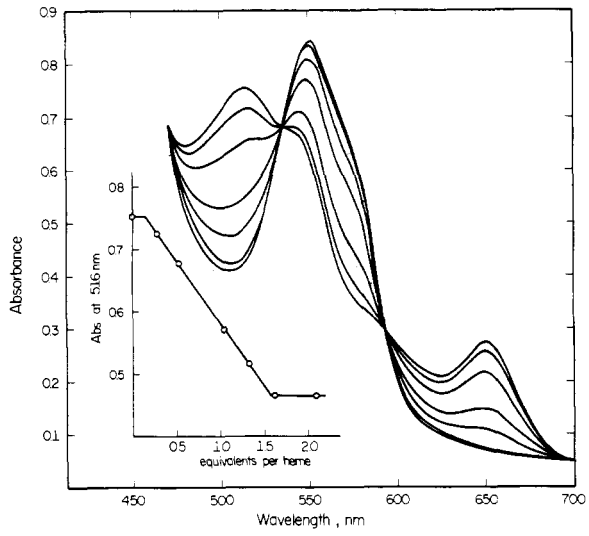


FIGURE 4: Spectral titration of chloroperoxidase with NADPH. Chloroperoxidase (48 $\mu M$) in 3 ml of 0.1 $M$ potassium phosphate buffer (pH 6.3) was titrated with 0.69 m$M$ NADPH in the presence of catalytic amounts of ferredoxin and ferredoxin reductase. The curves shown correspond to the addition of 30, 60, 110, 140, 170, and 220 $\mu$l of the NADPH solution. Spectra are not corrected for dilution. The end point, determined by absorbance at 516 nm, is shown in the insert with the correction of dilution.

of the enzymatically reduced chloroperoxidase CO complex was identical with that formed by sodium dithionite. However, the rate of reduction of chloroperoxidase by the spinach ferredoxin system was very slow. Complete reduction of chloroperoxidase required at least a 1-hr incubation under the conditions shown in Figure 4. Reduction of chloroperoxidase by the ferredoxin system required approximately 0.7 mol of NADPH/mol of heme to achieve the full formation of reduced chloroperoxidase CO complex, as shown in Figure 4.

*The Oxidative Titration of Reduced Chloroperoxidase CO Complex with Potassium Ferricyanide.* The reduced chloroperoxidase CO complex was prepared by the addition of 0.6 mol of sodium dithionite/mol of chloroperoxidase solution under an atmosphere of CO. The reduced enzyme-CO complex was then back titrated with potassium ferricyanide to regenerate the oxidized enzyme species. Upon the addition of ferricyanide, the absorption at 446 nm decreased at a slow rate. Complete oxidation of the reduced chloroperoxidase CO complex by ferricyanide was obtained by the addition of 0.92 mol of ferricyanide/mol of chloroperoxidase (based on heme content) as shown in Figure 5. The spectrum of reoxidized chloroperoxidase was identical with the spectrum of native ferric chloroperoxidase. This result demonstrates that the redox process used to generate the ferrous-CO complex is completely reversible and further shows that dithionite reduction only involves the heme iron of the enzyme.

*Optical and EPR Studies on Reduced NO-Chloroperoxidase Complexes.* Some ferrous hemoproteins, when treated with nitric oxide, will form an iron bonded nitric oxide derivative. These NO complexes have characteristic absorptions in the Soret, $\alpha$, and $\beta$ regions of the optical spectrum. Since the EPR spectrum of nitric oxide derivatives of ferrous hemoproteins can reflect the character of the axial ligand coordinated on the opposite side of the heme group, the NO complexes of ferrous chloroperoxidase were prepared and examined. Yonetani et al. (1972) concluded that the EPR hyperfine structures of the NO complexes of hemoglobin, myoglobin, horseradish peroxidase, lactoperoxidase, cytochrome $c$ peroxidase, and cytochrome oxidase reflect the interaction of the NO radical with an axial nitrogen ligand on the opposite side of the iron. Kon (1975) has interpreted recent studies with heme model compounds as indicating that the presence of a nitrogen atom as an axial ligand produces a nine-line EPR hyperfine structure whereas a

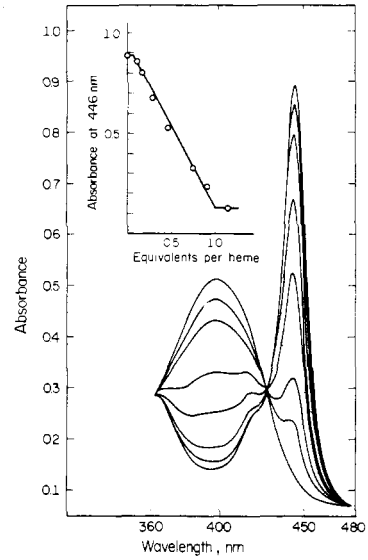


FIGURE 5: Spectral titration of reduced chloroperoxidase CO complex with potassium ferricyanide. Chloroperoxidase (6.1 $\mu M$) in 3.55 ml of 0.1 $M$ potassium phosphate buffer (pH 6.3) was titrated with 0.1 m$M$ $K_3Fe(CN)_6$. The curves shown correspond to the addition of 20, 30, 50, 80, 130, 160, and 210 $\mu$l of the ferricyanide solution. The end point, determined by absorbance at 446 nm, is shown in the insert.

five-coordinated heme iron NO derivative yields a three-line hyperfine structure. Reduced chloroperoxidase reacts with nitric oxide to form an iron bonded derivative having the characteristic spectrum shown in Figure 6. EPR measurements of the nitric oxide complex were also carried out as shown in Figure 7. The EPR spectrum of the NO complex of chloroperoxidase with $g$ values of 2.082, 2.004, and 1.975 indicates rhombic symmetry for the paramagnetic center of this complex. The $g$ values for the chloroperoxidase-NO complex are very similar to the $g$ values recorded for the NO complex of cytochrome P-450cam (2.076,

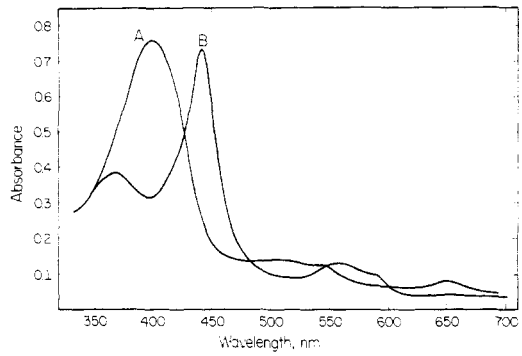


FIGURE 6: Spectra of chloroperoxidase and the NO derivative of chloroperoxidase. In curve A, the cuvet contained 9.2 $\mu M$ chloroperoxidase in 0.1 $M$ potassium phosphate buffer (pH 3.0). In curve B chloroperoxidase (9.2 $\mu M$) was reduced with an eightfold excess of dithionite and reacted with NO (bubbled through the cuvet).

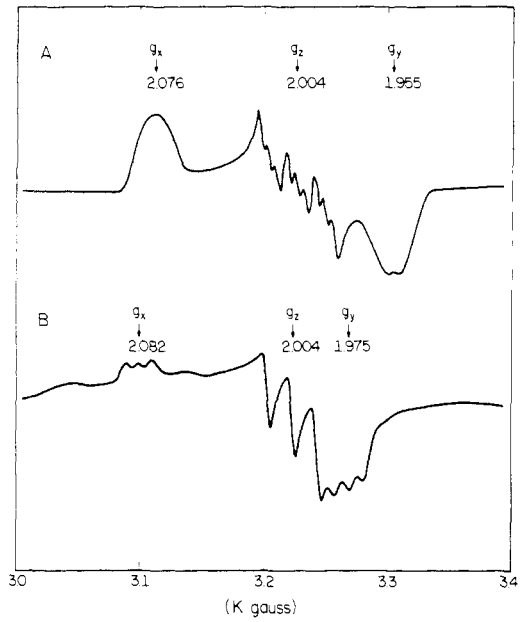


FIGURE 7: X-Band EPR spectrum of $^{14}NO$-ferrous horseradish peroxidase (A) and chloroperoxidase (B) at −170°.

2.004, and 1.966).[2] The EPR spectra of both cytochrome P-450cam and chloroperoxidase exhibit well-resolved triplets centered at $g$ = 2.004 and having a hyperfine splitting of 20 G. The chloroperoxidase-NO complex also shows resolved triplets at the other two $g$ values (Figure 7). Tsibris[2] indicates that the cytochrome P-450cam-NO complex shows only a partially resolved triplet at $g$ = 1.966. The $^{15}NO$ complexes of both chloroperoxidase and cytochrome P-450cam[2] yield well-resolved doublet resonances at the three $g$ values indicated above. At $g$ = 2.004, both of the $^{15}NO$ complexes show the same hyperfine splitting of 28 G.

The hyperfine structures of the ferrous-NO complex of chloroperoxidase are not at all similar to those of hemoglobin and horseradish peroxidase. Thus it would appear that ferrous cytochrome P-450 and chloroperoxidase NO complexes do not have a nitrogen atom as a proximal ligand to the iron. A detailed interpretation of the optical and EPR results is now being carried out in this laboratory.

[2] J. Tsibris, personal communication; also see Schlosnagle et al (1974).

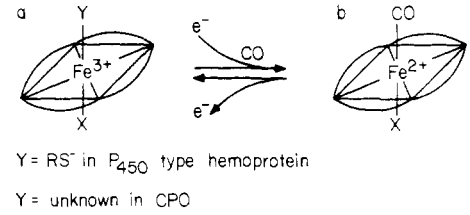


FIGURE 8: Proposed heme axial ligands of chloroperoxidase and cytochrome P-450 in ferric (a) and CO-ferrous (b) form.

## Discussion

In this paper we have shown that the two half-cystine residues in native chloroperoxidase are present in a disulfide linkage. Thus there can be no sulfhydryl group available for coordination to the heme iron of the native enzyme. Furthermore, it has been shown that chloroperoxidase can be reduced by dithionite or by the spinach ferredoxin reductase system under conditions which only allow the iron atom of the heme group to become reduced. The titration experiments clearly show that only a one-electron reduction step is required to produce the ferrous form of chloroperoxidase which can combine with carbon monoxide and yield a P-450 spectrum. If a sulfhydryl ligand were required for the expression of P-450 character, a total of three electrons per chloroperoxidase molecule would be required in order to produce the P-450 spectrum. A one-electron reduction step would be required to produce the ferrous heme protein and two electrons would be required to cleave the cystine disulfide linkage to produce two cysteine residues. The titration of the native chloroperoxidase with dithionite is open to criticism because sulfite ion is a product of the reaction between dithionite and ferric heme. Thus it could be argued that sulfite ion, produced as an intermediate product in the reaction of the native enzyme with dithionite ion, could serve as a reducing agent for the disulfide bond. However, these concerns are not valid in the experiments where NADPH served as the reductant. Again, the results of the NADPH titration with ferredoxin and ferredoxin reductase support a single electron reduction process. In addition, the results from the back titration of the reduced chloroperoxidase CO complex to native ferric chloroperoxidase by ferricyanide also confirm the one-electron reduction process for the generation of the reduced chloroperoxidase CO complex. Thus all of the facts are consistent with a one-electron reduction model for the formation of the ferrous-CO P-450 complex of chloroperoxidase and the results indicate that the ferric heme of chloroperoxidase is reduced to the ferrous state with the single disulfide bond in the protein remaining intact.

Recent studies on heme model complexes and heme proteins using absorption and EPR spectroscopy have been interpreted to suggest that P-450 type cytochromes have a sulfhydryl ligand attached to the heme iron. The results presented in this paper indicate that although sulfhydryl ligands may be sufficient to generate P-450 character, sulfhydryl groups are not mandatory requirements for the expression of P-450 behavior.

In order to reconcile the results presented in the paper with the observations on P-450 models and the EPR studies on ferric P-450 forms, we propose the system of heme ligands shown in Figure 8. The Y ligand of the ferric form of P-450 hemoproteins may well be a sulfhydryl group. We suggest that the Y ligands of the ferric forms of chloroperoxidase and P-450cam are probably different since both op-

tical and EPR spectroscopy reveal differences between the low-spin ferric forms of these two proteins. However, we propose that the X ligands in the chloroperoxidase and the P-450 hemoproteins are identical. This proposal is based on the observation that both proteins upon reduction yield high spin ferrous forms having almost identical Mössbauer spectra (Champion et al., 1975). In contrast, the high spin ferrous forms of hemoglobin and horseradish peroxidase show quite different Mössbauer spectra (Champion et al., 1975). Furthermore, if one assumes that in the low spin ferrous carbon monoxide complexes, the ferric Y ligands are replaced by CO, then the ferrous CO complexes of chloroperoxidase and the P-450 proteins should also be identical. This model can therefore explain some of the physical and chemical data currently available on the comparative aspects of these two heme protein systems.

The present demonstration that P-450 absorption can occur in chloroperoxidase in the absence of sulfhydryl ligands suggests that the P-450 model studies should be extended to include a variety of iron porphyrins and ligands other than sulfur anions. The chloroperoxidase studies indicate sulfhydryl groups are not mandatory components for P-450 optical absorption. Furthermore, Dolphin[3] has indicated that the P-450 sulfhydryl model system originally studied by Stern and Peisach is quite sensitive to substituents on the porphyrin ring.

The hypothesis concerning X and Y ligands in Figure 8 raises a new question concerning the chemical nature of the X ligand. Since the EPR spectrum of the reduced chloroperoxidase–NO complex does not show the extra nitrogen hyperfine structure that comparable EPR data on the NO derivatives from myoglobin, hemoglobin, cytochrome *c* peroxidase, and horseradish peroxidase show (Yonetani et al., 1972), it can be argued that chloroperoxidase (and hence the P-450 proteins) do not have a nitrogen axial heme ligand in the ferrous complexes. An imidazole nitrogen has been firmly established as the axial ligand in both myoglobin and hemoglobin by X-ray crystallography. At various times, sulfur ligands from methionine residues or oxygen ligands from tyrosine residues or carboxyl groups have been considered as potential heme iron ligands. However, with the limited information available at this time, it is not possible to reach any decisions concerning ligands for chloroperoxidase complexes.

[3] D. Dolphin, personal communication.

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