The P-450 Nature of the Carbon Monoxide Complex of Ferrous Chloroperoxidase*

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SUMMARY

The absorption spectrum of the carbon monoxide complex of ferrous chloroperoxidase from Caldariomyces fumago has been shown to be quite similar to the characteristic spectrum of CO complexes of cytochromes of the P-450 type. Comparison of other spectral properties of chloroperoxidase and cytochrome P-450 reveals a striking resemblance between the two proteins. The Soret absorption maxima for native, reduced, cyanide, nitrous oxide, and N-phenylimidazolato complexes are quite similar. N-Phenylimidazolato, a potent inhibitor of the cytochrome P-450(CO)-catalyzed hydroxylation of camphor, is a very effective inhibitor of a chloroperoxidase-catalyzed peroxidation reaction. Like P-450, chloroperoxidase undergoes characteristic spectral changes in the presence of substrates and nitrogenous compounds. Type I and type II spectral changes have been observed. Carefully controlled denaturation of chloroperoxidase resulted in the formation of a species having a spectrum essentially identical with that of cytochrome P-420, the denatured form of P-450. The similar spectral properties described here indicate that both proteins provide quite similar environments for the heme prosthetic group. Both proteins also compare favorably with respect to physical properties such as molecular weight, high content of acidic amino acids, and low isoelectric point.

Cytochrome P-450 is an unusual CO-binding hemoprotein which was first observed in rat liver microsomes (1-3). It has been shown to function as the terminal oxidase (4, 5) in a mixed function oxidase system which is involved in the metabolism of fatty acids, steroids, drugs, carcinogens, and other foreign compounds (6, 7). An unusual characteristic of cytochromes of the P-450 type is the position of the Soret band of the reduced CO complex at an extremely long wave length. The Soret peak for CO complexes of ferrous P-450 hemoprotein occurs in the 450-nm range while the position of the Soret peak for the reduced CO complexes of most hemoproteins is approximately 420 nm (1). Proteins displaying this P-450 anomalous behavior have been shown to be present in various mammalian tissues (5), as well as in yeasts (8), plants (9), and bacteria (10). The extreme catalytic diversity and lack of substrate specificity exhibited by these enzymes is evidenced by their ability to catalyze the hydroxylation of aromatic compounds and alkanes.

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the dealkylation of secondary and tertiary amines, and the oxidation of primary amines (11). These reactions are thought to involve insertion of a hydroxyl group into the substrate and may result in the demethylation of substrates such as nitroanisole, aminopyrine, and N-methyl aniline (12). Cytochrome P-450 from several sources is currently under study in numerous laboratories. The mechanism of the hydroxylation reaction catalyzed by this class of enzymes and the manner in which these enzymes activate oxygen are under intense scrutiny. Most of these studies have been handicapped by the inability to isolate readily soluble protein without resorting to detergent solubilization (13), and by the inherent instability of the enzyme. Cytochrome P-450 readily denatures to a catalytically inactive P-420 form (3, 10-13). Cytochrome P-450(CO) from Pseudomonas putida, which has been isolated by Gunsalus and co-workers (14) and also has been studied by Peterson (15), is the only cytochrome P-450 which can be isolated in a soluble form without resorting to detergents or other solubilizing agents. We report here the striking similarities between chloroperoxidase, a halogenating hemoprotein which has been extensively studied in our laboratory, and cytochrome P-450(CO).

EXPERIMENTAL PROCEDURE

The isolation of chloroperoxidase from Caldariomyces fumago has been reported previously (16). Preparations used for the experiments reported in this paper had specific activities greater than 2000 units per mg and $R_{E}$ values greater than 1.40, indicating that these preparations were at least 85% pure. The visible spectra were recorded on a Cary 15 spectrophotometer using cells with path lengths of 1 cm. Thunberg cuvettes which had been degassed and flushed with nitrogen four times before the anaerobic addition of an excess of sodium dithionite were used for the reduced spectral recordings of chloroperoxidase. For the reduced CO-enzyme complex, the Thunberg cuvette was flushed with CO either before or after the addition of dithionite. The sequence of CO addition had no effect on the final spectrum. The assay for the oxidation of thiourea has been described previously (17) N-Phenylimidazolato was a gift from Dr. I. C. Gunsalus and John Lipscomb. Sodium dithionite was obtained from the J. T. Baker Co., CO from Union Carbide, and all other chemicals were reagent grade available from commercial sources.

RESULTS AND DISCUSSION

Recently, we began an intensive study of the optical, ESR, and Mössbauer properties of chloroperoxidase and its complexes in order to learn more about the environment of the heme and the changes it undergoes upon binding substrates and during catalysis. As shown in Fig. 1, chloroperoxidase readily forms a reduced enzyme-CO complex when the enzyme is reduced by a 2-fold molar excess of sodium dithionite under a carbon monoxide atmosphere. Formation of the reduced CO-chloroperoxidase complex is a fully reversible process, when air is admitted to the cuvette the native oxidized enzyme spectrum is rapidly reformed. The ferrous enzyme-CO complex exhibits a Soret peak at an exceptionally long wave length (approximately 443 nm). This absorption at abnormally long wave lengths is characteristic of
The inhibition of P-450, by N-phenylimidazole is competitive with free complexes with ligands (18), such as CN-, NO, and N-phenylimidazole. Because of this inhibition, the lag period during which there is no oxidation of thiourea is observed. After the lag period the oxidation of thiourea proceeds at a rate which is essentially the same as that in the absence of inhibitor. The lag period during which there is no oxidation of thiourea is observed.

The chloroperoxidase-catalyzed oxidation of thiourea. When N-phenylimidazole is added to the assay mixture, there is an initial inhibition of the oxidation of thiourea as indicated by an initial period of no oxidation. After the lag period the oxidation of thiourea proceeds at a rate which is essentially the same as that in the absence of inhibitor. The lag period during which there is no oxidation of thiourea is observed.

The spectrum of native chloroperoxidase exhibits a Soret maximum at 396 nm with additional peaks at 515 and 650 nm and strongly resembles that of the enzyme-substrate compound of cytochrome P-450cam. The Soret maximum at 391 nm, a peak at 500 with a shoulder at 540 nm and a peak at 645 nm (11).

Further spectral similarities between chloroperoxidase and cytochromes of the P-450 class and has been used to identify these cytochromes as well as serving as a convenient assay and criterion of purity (3). Titrations curves for the formation of the CO complex show spectral intermediates and isosbestic points quite similar to those reported by Peterson et al. for the dithionite titration of cytochrome P-450cam (15).

The addition of a wide variety of substrates and inhibitors to cytochrome P-450 causes characteristic changes in the optical absorption spectrum which reflect changes in the environment and electron density of the heme (19). The types of changes elicited fall into two basic categories (19) which are generally referred to as type I and type II spectral changes. Type I changes involve movement of the Soret peak to shorter wavelengths and are usually detected by the appearance of a peak at 385 to 395 nm in the difference spectrum. Type II changes involve movement of the Soret peak to longer wavelengths and are usually detected by the appearance of a peak at 420 to 430 nm in the difference spectrum.

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As shown previously, chloroperoxidase undergoes distinct spectral changes upon the formation of chloroperoxidase-halide complexes (20). The chloroperoxidase-halide complex shows a typical P-450 type I difference spectrum while the chloroperoxidase-chloride complex has a P-450 type II difference spectrum (20). Table II lists the spectral type of chloroperoxidase complex elicited with several ligands. As with cytochrome P-450, the addition of aniline or pyridine to chloroperoxidase results in typical type II spectral changes (Table II).

Early attempts to solubilize liver microsomal cytochrome P-450 resulted in the conversion of the enzyme to a denatured, P-420 form. The conversion of the P-450 to the P-420 form of chloroperoxidase was accomplished by adding 30 μl of 1 M NaOH to the P-450 cuvette under anaerobic conditions and allowing the solution to stand at room temperature for 30 min.

![Figure 1](image1.png)

**Figure 1.** Absorption spectra of chloroperoxidase. Chloroperoxidase (0.7 mg) was dissolved in 3.0 ml of 0.1 M potassium phosphate buffer, pH 3.0. All spectra were recorded with 0.1 M potassium phosphate, pH 3.0, in the reference cell. The spectral region from 480 to 700 nm was recorded at 10-fold greater sensitivity than the Soret region. Native ferric enzyme (—), reduced chloroperoxidase (——), and the reduced CO complex (· · · ·) were prepared as described in the text. The conversion of the P-450 to the P-420 form of chloroperoxidase was accomplished by adding 30 μl of 1 M NaOH to the P-450 cuvette under anaerobic conditions and allowing the solution to stand at room temperature for 30 min.

![Figure 2](image2.png)

**Figure 2.** Effect of N-phenylimidazole on the oxidation of thiourea by chloroperoxidase. The reaction mixture contained 300 μmoles of potassium phosphate buffer (pH 4.0), 40 amoles of potassium chloride, 6 amoles of hydrogen peroxide, 0.4 μmole of thiourea, and 0.25 μg of chloroperoxidase in 2 ml. The reactions were initiated by the addition of peroxidase. The amount of N-phenylimidazole added was: 0 (Curve 1); 0.1 μmole (Curve 2); 0.3 μmole (Curve 3); and 0.45 μmole (Curve 4).

suggest that N-phenylimidazole may serve as an oxidizable substrate and that the release of inhibition may be due to oxidation of N-phenylimidazole to a compound which cannot exhibit an inhibitory effect on thiourea oxidation.

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1 I. C. Gunsalus, Personal communication.
trum of the reduced CO complexes of these two proteins and other have the same axial ligands on the heme iron and almost identical interaction with 5. Peisach and W. E. Blumberg. ESR studies show the g values for P-450,,, to be 7.81, for chloroperoxidase and 4.55 for P-450,,,) (16, 21).

45,000), exhibit a predominance of acidic amino acids over basic residues, and have fairly low isoelectric points (approximately 4.45). The results presented here suggest that chloroperoxidase may be a valuable adjunct to aid in the understanding of the functional importance of these structural properties which are unique to cytochrome P-450. These studies therefore should lead to a better understanding of the mechanisms of oxygen activation and hydroxylation. The functional similarities and possible structural correlates between chloroperoxidase and P-450 are currently under study in our laboratory. This work will be facilitated by the ready availability of gram quantities of crystalline chloroperoxidase.

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P-450 cytochromes suggest that these proteins differ from other heme proteins in having unique structures for one or both axial ligands or alternatively perhaps the P-450 types differ from other heme proteins in more subtle heme-protein interactions. Whatever the source of this abnormal behavior, it is extremely interesting and is currently under study from a variety of points of view.

In addition to structural similarities as evidenced by spectral analogies, peroxidases and cytochrome P-450-type enzymes share common functional relations. Peroxidases are able to catalyze a number of oxidations using molecular oxygen as the electron acceptor (23). Since several of these reactions are inhibited by CO, and a ferroperoxidase-CO complex has been observed during the reaction (24), the over-all reaction is thought to involve a ferri-ferrous valency change similar to that involved in P 450 hydroxylations (25).

Further support for a structural and functional relationship between cytochrome P-450 and peroxidases comes from the work of Hrycay and O'Brien (26, 27) who have reported that cytochrome P-450 is responsible for most of the peroxidase activity in liver microsomes.

The results of oxygen binding studies with horseradish peroxidase led Wittgenberg et al. (28) to propose that horseradish peroxidase might serve as a prototype for a terminal oxidase. However, the reduced CO complex of horseradish peroxidase, as well as lactoperoxidase and catalase, displays the Soret band at the usual 420 nm.3

One reaction mechanism suggested for cytochrome P-450-catalyzed hydroxylation involves the generation of an enzyme-bound OH+ cation from a hydroperoxide intermediate which could then serve as the active hydroxylating species (29). Studies in our laboratory with 10-labeled peroxy acids, hydrogen peroxide, and water have led us to postulate that chloroperoxidase-compound I contains a single substrate oxygen atom which can behave like an OH+ species (30).

The results presented here suggest that chloroperoxidase may be a valuable adjunct to aid in the understanding of the functional importance of these structural properties which are unique to cytochrome P-450. These studies therefore should lead to a better understanding of the mechanisms of oxygen activation and hydroxylation. The functional similarities and possible structural correlates between chloroperoxidase and P-450 are currently under study in our laboratory. This work will be facilitated by the ready availability of gram quantities of crystalline chloroperoxidase.

4 The presence of manganese in the sample makes EPR analysis difficult in the vicinity of g = 2. The EPR studies were done in collaboration with J. Peisach and W. E. Blumberg.
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