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-phenylimidazolyl complexes are quite similar. N-Phenylimidazole, a potent inhibitor of the cytochrome P-450-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 spectral similarities 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 hemeproteins is approximately 420 nm (1). Proteins displaying this P-450 anomalous behavior have since 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, 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 hemeproteins is approximately 420 nm (1). Proteins displaying this P-450 anomalous behavior have since been shown to be present in various mammalian tissues (5), as well as in yeasts (8), plants (9), and bacteria (10).

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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). Titration 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-450 (15).

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 complex of cytochrome P-450. CO complex, in the presence of camphor, has a Soret maximum at 391 nm, a peak at 500 with a shoulder at 540 and a peak at 645 nm (11).

Further spectral similarities between chloroperoxidase and P-450 become apparent upon comparison of the spectra of various complexes of the two proteins. As can be seen in Table I, the Soret positions and extinction coefficients of the two proteins display a good correspondence for both oxidation states and complexes with ligands (18), such as CN−, NO, and N-phenylimidazole.

In addition to forming characteristic spectral complexes with P-450 and chloroperoxidase, N-phenylimidazole is an extremely effective inhibitor of P-450-catalyzed camphor hydroxylation and of chloroperoxidase-catalyzed peroxidations. The inhibition of P-450 by N-phenylimidazole is competitive with the lag period the oxidation of thiourea proceeds at a rate which is essentially the same as that in the absence of inhibitor. 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 results depicted in Fig. 2 indicate that the length of the lag period is roughly proportional to the amount of N-phenylimidazole which was added initially to the reaction mixture. These results 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.

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 correspond to a shift of the Soret to longer wavelengths and are usually detected by the appearance of a peak at 420 to 430 nm in the difference spectrum. As shown previously, chloroperoxidase undergoes distinct spectral changes upon the formation of chloroperoxidase-halide complexes (20). The chloroperoxidase-iodide 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 by various 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,

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**Fig. 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 380 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.

**Table I**

<table>
<thead>
<tr>
<th>Enzyme species</th>
<th>P-450 Soret peak</th>
<th>CPO Soret peak</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Max.</td>
<td>Min.</td>
</tr>
<tr>
<td>Oxidized</td>
<td>406</td>
<td>408</td>
</tr>
<tr>
<td>Reduced</td>
<td>406</td>
<td>408</td>
</tr>
<tr>
<td>CO complex</td>
<td>443</td>
<td>446</td>
</tr>
<tr>
<td>+CN−</td>
<td>436</td>
<td>436</td>
</tr>
<tr>
<td>+NO</td>
<td>430</td>
<td>435</td>
</tr>
<tr>
<td>+N-Phenylimidazole</td>
<td>424</td>
<td>424</td>
</tr>
</tbody>
</table>

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1 I. C. Gunsalus, Personal communication.
TABLE II

<table>
<thead>
<tr>
<th>Ligand added</th>
<th>Final concentration</th>
<th>Peak</th>
<th>Trough</th>
<th>Spectral type</th>
</tr>
</thead>
<tbody>
<tr>
<td>F^-</td>
<td>0.0 M</td>
<td>420</td>
<td>380</td>
<td>II</td>
</tr>
<tr>
<td>Cl^-</td>
<td>0.0 M</td>
<td>430</td>
<td>390</td>
<td>II</td>
</tr>
<tr>
<td>Br^-</td>
<td>0.0 M</td>
<td>393</td>
<td>430</td>
<td>II</td>
</tr>
<tr>
<td>I^-</td>
<td>0.0 M</td>
<td>266</td>
<td>420</td>
<td>I</td>
</tr>
<tr>
<td>Pyridine</td>
<td>100 mM</td>
<td>420</td>
<td>375</td>
<td>II</td>
</tr>
<tr>
<td>Aniline</td>
<td>50 mM</td>
<td>418</td>
<td>382</td>
<td>II</td>
</tr>
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</table>

CPO was diluted in 0.1 M potassium phosphate buffer (pH 3.0) to a final concentration of 0.4 mg per ml and the solution was divided equally into two cuvettes. After establishing a baseline of equal light absorption, solutions of the various ligands were added to give the final concentration indicated. An equal volume of 0.1 M potassium phosphate was added to the reference cell and difference spectra were recorded. CPO, chloroperoxidase.

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 analogous, 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 ferric-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 Wittenberg 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.

One reaction mechanism suggested for cytochrome P-450-catalyzed hydroxylation involves the generation of an enyme-bound OH· cation from a hydroperoxide intermediate which could then serve as the active hydroxylating species (29). Studies in our laboratory with 18O-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 those 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.

REFERENCES

*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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