# Topology of the Chloroperoxidase Active Site: Regiospecificity of Heme Modification by Phenylhydrazine and Sodium Azide†

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ABSTRACT: Chloroperoxidase (CLP) from *Caldariomyces fumago* is rapidly and irreversibly inactivated by phenylhydrazine and $H_2O_2$ but not by $H_2O_2$ alone. Inactivation is characterized by a phenylhydrazine-to-CLP partition ratio of ~15, formation of *trans*-azobenzene, and formation of a $\sigma$-bonded phenyl–iron heme complex with a characteristic absorption maximum of 472 nm. Anaerobic extraction of the heme complex from the protein, followed by exposure to dioxygen under acidic conditions, shifts the phenyl group from the iron to the porphyrin nitrogens and yields the four possible *N*-phenylprotoporphyrin IX regioisomers. Oxidation of the iron–phenyl complex within the intact protein by ferricyanide or high peroxide concentrations results in protein-directed phenyl migration to give exclusively the *N*-phenylprotoporphyrin IX regioisomers with the phenyl group on pyrrole rings A and C. CLP also catalyzes the $H_2O_2$-dependent oxidation of azide to the azidyl radical and is inactivated by azide in the presence of $H_2O_2$. Inactivation of CLP by azide and $H_2O_2$ results in loss of heme Soret absorbance and formation of $\delta$-*meso*-azidoheme. These results suggest a topological model for the CLP active site and indicate that the tertiary structure of the enzyme permits substrates to interact with both the $\delta$-meso heme edge and the catalytic ferryl ($Fe^{IV}$=O) species, in agreement with the fact that CLP catalyzes both $H_2O_2$-dependent peroxidation and monooxygenation reactions.

Cytochrome P-450 monooxygenases have a cysteine thiolate as the fifth heme[1] ligand and generally catalyze the two-electron insertion of oxygen from dioxygen or an alternative oxygen donor into their substrates (Ortiz de Montellano, 1986; Poulos et al., 1985; Black & Coon, 1986; Collman & Sorrell, 1975). Bioorganic studies indicate that these two-electron cytochrome P-450 reactions proceed, at least sometimes, by two sequential one-electron transfers (Ortiz de Montellano, 1986). In contrast, horseradish peroxidase and other classical peroxidases with a histidine as the fifth heme ligand catalyze the one-electron oxidation of organic and inorganic substrates (Marnett et al., 1986; Hewson & Hager, 1979; Dunford & Stillman, 1976). Despite the differences in catalytic outcome, similar iron–oxo species appear to be involved in the catalytic chemistry of both classes of enzymes (Ortiz de Montellano, 1986; Marnett et al., 1986). Reactions of the peroxidases with $H_2O_2$ yield compound I structures that are two oxidizing equivalents above the resting ferric state. Compound I of horseradish peroxidase has been identified as a ferryl ($Fe^{IV}$=O) porphyrin radical cation (Penner-Hahn et al., 1986; Hashimoto et al., 1986; Roberts et al., 1981; Schulz et al., 1984; Dolphin et al., 1971) whereas compound I of cytochrome *c* peroxidase consists of a ferryl species and a protein radical (Edwards et al., 1987; Goodin et al., 1987; Yonetani, 1976). These compound I intermediates are reduced by two single-electron transfers, first to compound II by reduction of the porphyrin or protein radical and subsequently to the resting ferric state. The catalytic species of cytochrome P-450 is less well characterized than that of the peroxidases but is thought to be a hypervalent iron–oxo complex related to that of one of the two peroxidase compound I species (Ortiz de Montellano, 1986; Marnett et al., 1986).

The differences in the substrate oxidations catalyzed by cytochrome P450 and the classical peroxidases are frequently attributed to the difference in the heme iron ligand trans to the activated ferryl oxygen (Ortiz de Montellano, 1986; Marnett et al., 1986). However, using various mechanism-based inhibitors as molecular probes, we have obtained evidence that differences in the catalytic outcomes of these two classes of hemoproteins are determined, to a large extent, by differences in their active site topologies (Ortiz de Montellano, 1987). For example, horseradish peroxidase is inactivated when it oxidizes phenylhydrazine (Ator & Ortiz de Montellano, 1987), alkylhydrazines (Ator et al., 1987), or azide (Ortiz de Montellano et al., 1988) by addition of the phenyl, alkyl, or azidyl radical, respectively, to the $\delta$-meso carbon of the prosthetic heme group. Similar results have been obtained with a peroxidase from *Coprinus macrorhizus* using phenylhydrazine or azide as the inactivating agent (DePillis & Ortiz de Montellano, 1989), with lignin peroxidase using azide (DePillis et al., 1990), and with manganese peroxidase using azide or ethylhydrazine (Harris et al., 1991). In contrast, the reaction of aryl- or alkylhydrazines with cytochrome P-450 results in covalent binding of the aryl (Jonen et al., 1982; Delaforge et al., 1986; Swanson et al., 1991; Raag et al., 1990) or alkyl moiety (Ortiz de Montellano et al., 1983) to the iron and/or nitrogens of the prosthetic heme group. Similar results are obtained with hemoglobin, myoglobin (Ringe et al., 1984), and catalase (Ortiz de Montellano & Kerr, 1983), all of which

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[1] Abbreviations: heme, iron protoporphyrin IX regardless of the iron oxidation state; CLP, chloroperoxidase; *N*-phenyl-PPIX, *N*-phenylprotoporphyrin IX; HPLC, high-performance liquid chromatography; EPR, electron paramagnetic resonance spectroscopy; PBN, $\alpha$-phenyl-*N*-*tert*-butylnitrone.

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are known from crystallographic data to have active sites in which the heme group is accessible to substrates. Thus, the active site structures of the peroxidases allow substrate interactions with the heme edge in the vicinity of the δ-meso carbon but physically sequester the iron atom, and presumably the ferryl oxygen, whereas the active site structures of the monooxygenases allow substrates to react directly with the ferryl oxygen.

The phenyl–iron heme complexes formed in the reactions of cytochrome P-450 enzymes with phenylhydrazine or phenyldiazene have recently been shown to rearrange in situ when the intact protein complexes are oxidized with ferricyanide (Swanson et al., 1991). The phenyl group migrates in these reactions from the iron to the nitrogens of the heme group, yielding mixtures of the four possible regioisomers of *N*-phenylprotoporphyrin IX. Separation and unambiguous spectroscopic identification of the pyrrole nitrogen arylated in each of the four regioisomers (Swanson & Ortiz de Montellano, 1991) make this rearrangement a useful topological probe of hemoprotein active sites. Ferricyanide oxidation of the iron–phenyl complexes of several rat isozymes, for example, yields only the two *N*-phenyl-PPIX regioisomers with the phenyl group on the nitrogens of pyrrole rings A and D (Swanson et al., 1991). This finding indicates that the region above pyrrole rings B and C is occluded by the active site amino acid residues.

Chloroperoxidase (CLP), derived from the fungus *Caldariomyces fumago*, is an unusual hemoprotein in that it catalyzes the chloride-dependent chlorination of nucleophilic substrates (Griffin, 1991) and, like cytochrome P-450, has a cysteine thiolate as the fifth heme ligand (Dawson & Sono, 1987; Dawson, 1988). Furthermore, CLP catalyzes $H_2O_2$-dependent substrate oxidations by what appear to be both typical peroxidase and cytochrome P-450 mechanisms. Thus, CLP oxidizes ascorbate, guaiacol, and pyrogallol by simple one-electron abstractions (Thomas et al., 1970a,b), but catalyzes styrene epoxidation and *p*-methoxythioanisole sulfoxidation by oxene-transfer mechanisms (Ortiz de Montellano et al., 1987; Kobayashi et al., 1987). In the present study, we have used phenylhydrazine and azide as probes of the active site architecture of CLP. We report that CLP, like a monooxygenase, allows substrates access to the ferryl oxygen but, like a peroxidase, allows substrates to interact with the δ-meso heme edge. Furthermore, the results of in situ iron–phenyl shift experiments provide a low-resolution topological model of the CLP active site.

## Materials and Methods

*Materials.* Purified CLP (EC 1.11.1.10) from *C. fumago*, myoglobin (horse skeletal muscle), horseradish peroxidase (type VI), bovine liver catalase, guaiacol, diethylenetriaminepentaacetic acid, and sodium ascorbate were purchased from Sigma (St. Louis, MO). Phenylhydrazine hydrochloride, 30% hydrogen peroxide, potassium ferricyanide, 2-butanone (99+%, spectrophotometric grade), butylated hydroxytoluene, sodium ascorbate, hematin, and HPLC-grade ethyl acetate were purchased from Aldrich (Milwaukee, WI). The hydrogen peroxide was quantitated by iodometric titration using $KIO_3$-standardized $S_2O_3^{2-}$, and the phenylhydrazine was recrystallized from ethanol. Stock solutions of phenylhydrazine hydrochloride or sodium azide were prepared fresh in argon-saturated 0.01 N HCl to minimize autoxidation. PBN was purchased from Eastman (Rochester, NY) and methyl phenyldiazenecarboxylate azo ester from Research Organics Inc. (Cleveland, OH). Phenyldiazene was prepared by mixing 2.5 μL of the methyl carboxylate with 200 μL of argon-saturated 1 M NaOH (Kosower & Huang, 1965) to give a 68 mM solution. The concentration could be assayed spectrophotometrically ($\epsilon_{260}$ = 7400 $M^{-1}$ $cm^{-1}$). Deionized, glass-distilled water was used throughout. All CLP incubations were carried out at 25 °C in 0.1 M phosphate buffer (pH 6.0) which had been passed through a column (45 cm × 2.5 cm i.d.) of Chelex 100 resin (Bio-Rad) to remove any contaminating redox-active transition metals.

The commercial CLP preparations (lot nos. 28F0551 and 128F02012) were shown to be >95% pure on the basis of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (data not shown) and exhibited $R_z$ values ($A_{400}/A_{280}$) of 1.46 and 1.40, respectively. The concentration of CLP was determined using $\epsilon_{400}$ = 91 200 $M^{-1}$ $cm^{-1}$ (Morris & Hager, 1966).

Authentic *N*-phenyl-PPIX HPLC standards were prepared using a modification of a previous procedure (Swanson & Ortiz de Montellano, 1991). Myoglobin (20 μM, 0.34 mg/mL) and phenyldiazene (340 μM) were incubatd in 0.1 M Chelexed phosphate buffer (pH 7.0) in a total volume of 20 mL for 15 min at ambient temperature. Hemes were extracted under nominally anaerobic conditions and worked up by reoxygenation under acidic conditions using a scaled-up version of the method described below for CLP. Authentic HPLC standards of δ-*meso*-phenylheme and 8-hydroxyheme were prepared as described by Ator and Ortiz de Montellano (1987) and δ-*meso*-azidoheme as per Ortiz de Montellano et al. (1988).

*Analytical Methods.* Electronic absorption spectra were recorded on a Hewlett-Packard 8450A diode array spectrophotometer. Analytical reverse-phase HPLC was carried out using an Alltech Partisil ODS-3 (5 μm) column (25 cm × 4.6 mm i.d.) and a Hewlett-Packard HP 1090 liquid chromatography system which allowed the acquisition of on-line electronic absorption spectra of eluents. Gas chromatography was carried out with a Hewlett-Packard 5890A gas chromatograph. EPR spectra were acquired with a Varian E-104 instrument custom interfaced with an IBM XT computer. Mass spectra were obtained on a Kratos MS 50 instrument operating in the positive liquid matrix secondary ion mode using 1% trifluoroacetic acid/thioglycerol as the ionization matrix.

*Inactivation of CLP by Phenylhydrazine.* The rate of inactivation of CLP by phenylhydrazine was measured using a reaction mixture which contained 1 μM CLP, 0.2 mM $H_2O_2$, and 0–20 μM phenylhydrazine in a volume of 0.2 mL. At 0, 1, 5, and 10 min after the addition of phenylhydrazine and $H_2O_2$, 5-μL aliquots of the mixture were transferred to quartz cuvettes containing 1.0 mL of an assay solution composed of 0.1 M phosphate (pH 6.0), 5 mM guaiacol, and 0.6 mM $H_2O_2$. Peroxidase activity was measured by the increase of absorbance at 470 nm due to the oxidation of guaiacol to tetraguaiacol ($\epsilon_{470}$ = 26 600 $M^{-1}$ $cm^{-1}$) (Chance & Maehly, 1955).

*Azobenzene Formation.* CLP (20 or 40 μM) was incubated with phenylhydrazine (1 mM) and $H_2O_2$ (2 mM) in 0.25 mL of 0.1 M phosphate buffer (pH 6.0) for 10 min at 25 °C. Controls consisted of reaction mixtures devoid of $H_2O_2$, enzyme, or both. Toluene (2.5 μL of a 0.2 M diethyl ether solution) was added as an internal standard, the incubation was extracted with 0.5 mL of diethyl ether, and the extract was concentrated to ~15 μL. A 3-μL aliquot was analyzed by gas chromatography on a DB-5 capillary column programmed to rise from 30 to 50 °C at 2 °C/min and then from 50 to 250 °C at 20 °C/min. The column was finally held at 250 °C for 5 min before recycling to the initial temperature.

Under these conditions the retention times of benzene, toluene, and *trans*-azobenzene were, respectively, 6.4, 11.4, and 22.3 min. *trans*-Azobenzene formation was also examined by reverse-phase HPLC using the following elution protocol at a solvent flow rate of 1.0 mL/min: 0–10 min, 25% solvent B in solvent A; 10–20 min, linear gradient from 25 to 100% solvent B; 20–30 min, 100% solvent B, where solvent A is 0.1% trifluoroacetic acid in 60:40 methanol/water and solvent B is 0.1% trifluoroacetic acid in methanol. Under these conditions the retention time of *trans*-azobenzene ($\lambda_{max}$ = 318 nm) was 16 min.

*Analysis of Modified Hemes Derived from Phenylhydrazine Systems.* Typical reaction conditions involved incubating CLP (10 $\mu$M) with phenylhydrazine (1 mM) and $H_2O_2$ (2 mM) or with phenylhydrazine alone in 0.1 M phosphate buffer (pH 6.0), in a total volume of 1.0 mL, for 20 min at 25 °C. Each sample was then chromatographed over a column of Sephadex G-25 (9 mL bed volume), and the eluted protein solution (approximately 2 mL) was subsequently made anaerobic by exposure to a vigorous stream of argon for 1 h (final volume ~1.5 mL). The sample was then acidified with 200 $\mu$L of degassed, argon-saturated glacial acetic acid (final pH ~2.5), saturated with NaCl, and extracted with three 1.0-mL aliquots of degassed, argon-saturated 2-butanone containing 0.025% butylated hydroxytoluene as an antioxidant. The butanone extracts were immediately transferred to an aerobic mixture of 2.0 mL of acetonitrile and 400 $\mu$L of 1 M $H_2SO_4$. The mixture was bubbled with $O_2$ for a few minutes and allowed to remain exposed to air for 1 h in the dark. These conditions promote oxidative iron-to-nitrogen phenyl migrations of $\sigma$-bonded phenyl–iron heme complexes resulting in formation of nearly equal amounts of the four *N*-phenyl-PPIX regioisomers (Ringe et al., 1984; Swanson & Ortiz de Montellano, 1991). The acetonitrile and acetic acid were removed in vacuo, 2.0 mL of 1 M $H_2SO_4$ was added, and the samples were extracted with three 3-mL aliquots of ethyl acetate. The solvent was removed in vacuo, and the residue was analyzed by reverse-phase HPLC. The solvent program consisted of 20% solvent B in solvent A from 0 to 35 min followed by a linear gradient to 100% B over 5 min at a flow rate of 1.0 mL/min, where solvent A was 60:40:10 methanol/water/acetic acid and solvent B was 10:1 methanol/acetic acid. Alternatively, a modified gradient program was used consisting of 20% B from 0 to 10 min followed by a linear gradient from 20% to 100% B between 10 and 25 min. The HPLC detector was set at 410 nm.

*Migration of the Phenyl Group within Intact CLP.* Alternatively, oxidative iron-to-nitrogen phenyl migrations were promoted within the CLP active site by reaction of the CLP-bound phenyl–iron heme complexes with ferricyanide (Swanson et al., 1991). The hemes were extracted and worked up using a method which results in degradation of unreacted phenyl–iron complexes, while leaving preformed *N*-phenyl-PPIX intact (Swanson et al., 1991). CLP (20 $\mu$M) was incubated with phenylhydrazine (1 mM) in 0.1 M phosphate buffer (pH 6.0) for 20 min at 25 °C followed by the addition of $K_3Fe(CN)_6$ (3 mM). A concentration of ferricyanide approximately an order of magnitude higher than was used in the cytochrome P-450 studies (Swanson et al., 1991) was required to compensate for the reaction of $Fe(CN)_6^{3-}$ with unreacted phenylhydrazine. The reaction of ferricyanide with excess phenylhydrazine can be monitored spectrophotometrically by measuring the appearance of excess $Fe(CN)_6^{3-}$ ($\lambda_{max}$ = ~420 nm). The hemes were extracted anaerobically as described above and worked up using an alternative method:

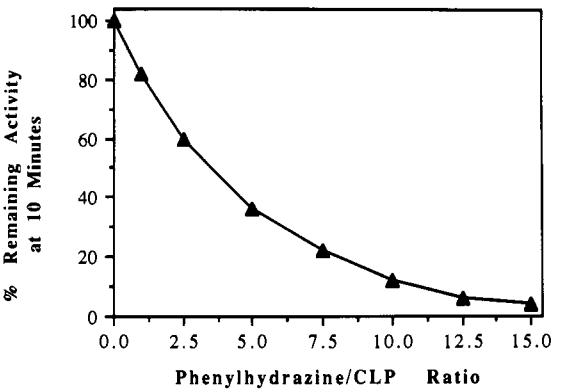


FIGURE 1: Inactivation of CLP as a function of the phenylhydrazine-to-enzyme ratio. CLP (1 $\mu$M) was incubated with 0.2 mM $H_2O_2$ and increasing concentrations of phenylhydrazine. Aliquots were withdrawn after 10 min and the peroxidase activity was measured by the guaiacol oxidation assay as described under Materials and Methods.

the 2-butanone extracts were exposed to air for 30 min in the dark, the solvent was removed in vacuo, and the residue was suspended in 1 M $H_2SO_4$ for 1 h in the dark. The suspension was extracted with ethyl acetate, the solvent was removed in vacuo, and the residue was analyzed by reverse-phase HPLC as described above.

*Azide-Dependent Heme Modification.* CLP (20 $\mu$M) was incubated with $NaN_3$ (2.5 mM) and $H_2O_2$ (5 mM) in 0.1 M phosphate buffer (pH 6.0) in a total volume of 2.0 mL for 20 min at 25 °C before excess $H_2O_2$ was destroyed by adding 15 $\mu$L of 0.28 mg/mL catalase and, 5 min later, 15 $\mu$L of 50 mM sodium ascorbate. The ascorbate returns the enzyme to the ferric state. Glacial acetic acid (0.53 mL) was added, the solution was saturated with NaCl, and the mixture was extracted with three 2.0-mL aliquots of diethyl ether. The ether layers were pooled, the solvent was removed in vacuo, and the residue was analyzed by HPLC. The solvent program consisted of 25% solvent B in solvent A for 10 min followed by a linear gradient from 25% to 100% B over 15 min and 100% B for an additional 5 min, where solvent A was 60:40:0.1 methanol/water/trifluoroacetic acid and solvent B was 100:0.1 methanol/trifluoroacetic acid. The flow rate was 1.0 mL/min, and the detection wavelength was 400 nm.

*Spin Trapping of the Azidyl Radical.* EPR spin-trapping experiments were performed essentially as described by Kalyanaraman et al. (1985). Incubations contained sodium azide (25 mM), $H_2O_2$ (5 mM), diethylenetriaminepentaacetic acid (10 $\mu$M), PBN (70 mM), and CLP (10 $\mu$M) in 0.1 M phosphate (pH 6.0) buffer. CLP was added to the other components to initiate the reaction, and an aliquot of the complete mixture was immediately transferred to a capillary tube that was then dropped into a quartz EPR tube aligned in the sample cavity. All measurements were begun within 15 s of initiating the reaction. Control incubations were run in the absence of CLP, $H_2O_2$, or azide.

## RESULTS

*Inactivation of CLP by Phenylhydrazine.* CLP is inactivated in a concentration-dependent manner by phenylhydrazine and $H_2O_2$, 50% inactivation and nearly complete inactivation being observed at phenylhydrazine-to-CLP ratios of ~3.5 and 15, respectively (Figure 1). Inactivation is very rapid, as evidenced by the essentially identical extents of inactivation (at a given phenylhydrazine concentration) observed 0, 1, 5, or 10 min after the addition of phenylhydrazine and peroxide (data not shown). Phenylhydrazine alone inactivates

Table I: Peroxide Dependence of the Phenylhydrazine Inactivation of CLP[a]

| system | $dA_{470}/dt \times 10^{-5}$ at 10 min ($s^{-1}$)[b] | % remaining activity at 10 min |
|---|---|---|
| control | 274 ± 11 | 100 |
| +$H_2O_2$ (10 μM) | 276 ± 1 | 100 |
| +$PhNHNH_2$ (10 μM) | 128 ± 6 | 47 |
| +$PhNHNH_2$ (10 μM) + $H_2O_2$ (10 μM) | 45 ± 1 | 16 |
| +$PhNHNH_2$ (20 μM) | 42 ± 5 | 15 |
| +$PhNHNH_2$ (20 μM) + $H_2O_2$ (10 μM) | 18 ± 0 | 6 |

[a] Experimental design is described under materials and Methods. [b] Results represent mean ± SD of triplicate measurements.

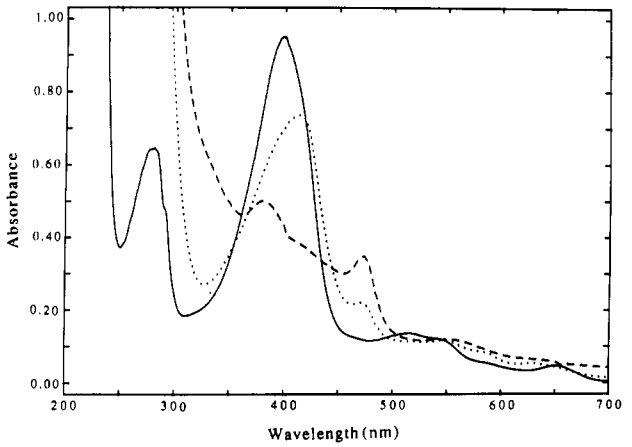


FIGURE 2: Spectroscopic changes accompanying the reaction of CLP with $H_2O_2$ and phenylhydrazine: CLP (10 μM) alone (—); plus phenylhydrazine (1 mM) (···); plus $H_2O_2$ (2 mM) (– – –).

CLP, but the inhibitory effects are substantially enhanced by addition of peroxide (Table I). In contrast, peroxide does not lower the enzymatic activity despite the reported sensitivity of CLP to peroxide-dependent inactivation (Griffin, 1991). These results suggest that, in the absence of exogenously added peroxide, phenylhydrazine autoxidizes in situ to generate the required $H_2O_2$, as proposed earlier for the phenylhydrazine-mediated inactivation of hemoglobin (Augusto et al., 1982), horseradish peroxidase (Ator & Ortiz de Montellano, 1987), and lignin peroxidase (DePillis et al., 1990). CLP activity is not restored by Sephadex G-25 chromatography and is thus irreversibly lost (data not shown).

Catalytic oxidation of phenylhydrazine by CLP produces *trans*-azobenzene, as indicated by the enzyme- and peroxide-dependent formation of a product with the same electronic absorption spectrum ($\lambda_{max}$ = 318 nm) and gas chromatographic (22.2 min) and HPLC (16.1 min) retention times as authentic *trans*-azobenzene. Gas chromatographic analysis shows that benzene is also generated, but its formation is not enzyme- or peroxide-dependent and is therefore due to the autoxidation of phenylhydrazine.

*Spectroscopic Changes Associated with Inactivation by Phenylhydrazine.* Incubation of CLP (10 μM) with phenylhydrazine (1 mM) results in the appearance of an absorption band at 472 nm as well as diminution and shift to longer wavelength (400 → 414 nm) of the Soret absorption (Figure 2). The 472-nm absorption maximum is consistent with formation of a CLP σ-bonded phenyl–iron heme complex (Raag et al., 1990). Subsequent addition of peroxide (2 mM) increases formation of the phenyl–iron intermediate, as evidenced by an increase in the 472-nm absorption, disappearance

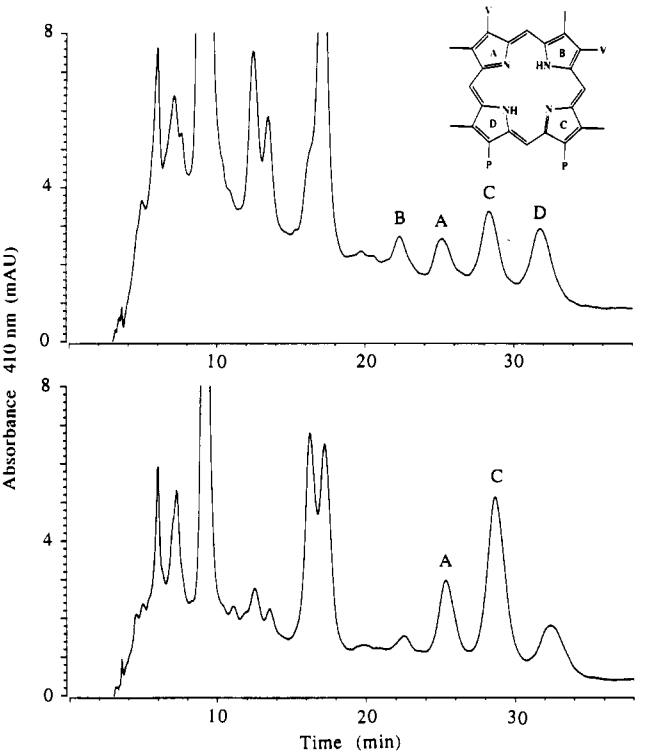


FIGURE 3: HPLC of the *N*-phenyl-PPIX adducts obtained by nominally anaerobic extraction of the phenyl–iron complex followed by exposure to dioxygen and strong acid. CLP (10 μM) was incubated aerobically with phenylhydrazine alone (1 mM) (upper) or with phenylhydrazine (1 mM) and $H_2O_2$ (2 mM) (lower), and the prosthetic groups were extracted and worked up as described under Materials and Methods. The HPLC was run in an isocratic system consisting of 20% solvent B and 80% solvent A. The *N*-phenylprotoporphyrin IX isomers, designated by the letters, correspond to N-arylation of the pyrrole rings shown in the inset (abbreviations: V = vinyl, P = propionic acid). The large peak at 9.15 min is due to underivatized heme.

of the 414-nm band, and emergence of a weak absorption at 382 nm. The final spectrum is very similar to that generated in the reaction of cytochrome P-$450_{cam}$ with phenylhydrazine or phenyldiazene (Raag et al., 1990). The identity of the species that absorbs at 414 nm is not known, but it is not due to coordination of a nitrogenous ligand to the iron because the spectrum of 10 μM CLP is not altered by the addition of 1 mM aniline, benzylamine, 2-phenylethylamine, or azide. Furthermore, if such a coordination complex were formed, it would be expected to have an absorption maximum at higher wavelengths (Schenkman et al., 1967; Dawson & Sono, 1987).

*Prosthetic Heme Modification.* We have previously shown that isolation of prosthetic heme phenyl–iron complexes from myoglobin (Ringe et al., 1984) or cytochrome P-450 (Raag et al., 1990) under anaerobic conditions, followed by exposure to oxygen under acidic conditions, results in an oxidative, iron-to-nitrogen shift of the phenyl group that produces an essentially equimolar mixture of the four *N*-phenylprotoporphyrin IX (*N*-phenyl-PPIX) regioisomers. The same method was therefore employed to unequivocally confirm the formation of a CLP σ-bonded phenyl–iron heme complex. CLP was incubated with phenylhydrazine plus $H_2O_2$ or phenylhydrazine alone, and the excess phenylhydrazine and peroxide were then removed by Sephadex G-25 chromatography. The prosthetic group was extracted as described under Materials and Methods. HPLC analysis of the heme fraction extracted from incubations of CLP with phenylhydrazine alone demonstrated the formation of all four *N*-phenyl-PPIX isomers (Figure 3, upper), each of which coeluted with one of the

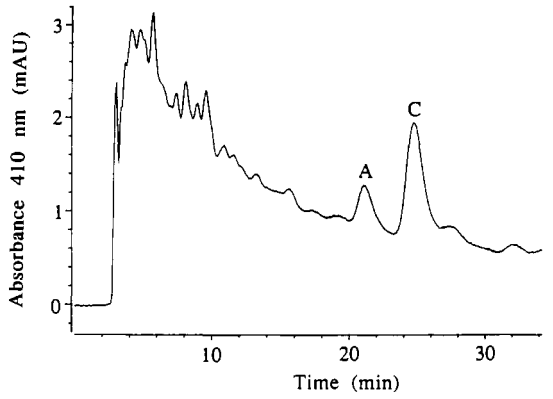


FIGURE 4: HPLC of the *N*-phenylprotoporphyrin IX adducts obtained after oxidation of the intact CLP-bound phenyl–iron complex with ferricyanide. The HPLC conditions are identical to those described in Figure 3, except that a different HPLC column was used.

authentic myoglobin-derived *N*-phenyl-PPIX isomers and had an absorption spectrum with a maximum at 410–416 nm identical to that of the corresponding authentic adduct. The order of elution is isomer B (22.5 min), A (25.4 min), C (28.7 min), and D (32 min) (Swanson & Ortiz de Montellano, 1991). These results unequivocally confirm the generation of a $\sigma$-bonded phenyl–iron heme complex in aerobic incubations of CLP with phenylhydrazine. HPLC analysis of hemes derived from the reaction of CLP with phenylhydrazine and added peroxide also demonstrates formation of the four *N*-phenyl isomers. However, the regioisomers with the phenyl group on pyrrole rings A and C are obtained in a ratio of 1:2.5, and only a small amount of regioisomer D and no more than a trace of regioisomer B are obtained (Figure 3, lower). 8-Hydroxyheme and *δ-meso*-phenylheme, the major heme adducts generated in the horseradish peroxidase system (Ator & Ortiz de Montellano, 1987), are not detected.

*Migration of the Phenyl Group within the CLP Active Site.* Earlier work demonstrated that ferricyanide oxidizes the intact phenyl–iron heme complexes of several mammalian cytochrome P-450 enzymes, resulting in protein-directed iron-to-nitrogen migration of the phenyl group. Addition of ferricyanide to the phenyl–iron complexes of cytochromes P-450IA1, P-450IIB1, P-450IIB2, and P-450IIE1 preferentially yields the *N*-phenyl-PPIX A and D regioisomers (Swanson et al., 1991). This suggests that the active site topologies of the four cytochrome P-450 isozymes are characterized by exposed and sterically unhindered access to the nitrogens of pyrrole rings A and D. The same method has been used to investigate the active site topology of CLP. CLP (20 $\mu$M) was incubated with phenylhydrazine (1 mM) followed by the addition of $K_3Fe(CN)_6$ (3 mM). The prosthetic group was extracted anaerobically after the ferricyanide reaction and was then exposed to oxygen in the absence of acid, a method that promotes degradation of unreacted $\sigma$-bonded phenyl–iron heme complexes to lower wavelength absorbing materials but does not alter the *N*-phenyl-PPIX isomers. This method prevents distortion of the protein-directed *N*-phenyl-PPIX isomer ratio by nonregioselective conversion of residual iron–phenyl complexes to *N*-phenyl-PPIX isomers outside of the active site. HPLC analysis of the *N*-phenyl-PPIX isomers formed by ferricyanide-promoted rearrangement of the intact CLP phenyl–iron complex shows that isomers A and C are formed in a ratio of 1:2.5, whereas the isomers substituted on pyrrole rings B and D are formed, if at all, in much smaller amounts (Figure 4). Interestingly, the ratio of the A and C isomers generated by oxidation of the protein-bound complex with ferricyanide is the same as that obtained when CLP, phenylhydrazine, and excess peroxide are incubated and the prosthetic group is directly extracted under aerobic, acidic conditions (Figure 3, lower). High peroxide concentrations thus appear to promote the oxidative phenyl migration within the CLP active site. Formation of the D regioisomer in the peroxide- but not ferricyanide-mediated reaction indicates that the ferricyanide-mediated reaction is slightly more regioselective.

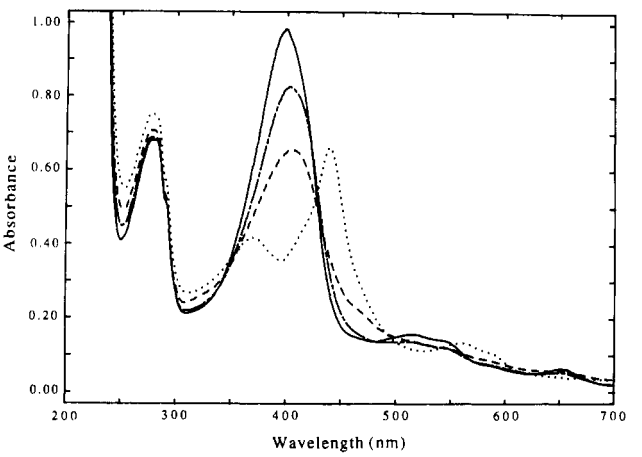


FIGURE 5: Spectroscopic changes accompanying the reaction of CLP with $H_2O_2$ and sodium azide: CLP (10 $\mu$M) alone (—); plus $H_2O_2$ (1.25 mM) and azide (2.5 mM), $t$ = 0 (···); $t$ = 30 s (– – –); $t$ = 20 min (–·–).

*Reaction of CLP with $H_2O_2$ and Azide.* Azide was chosen as the molecular probe to determine if the meso heme edge of CLP is exposed because it gives meso heme adducts with the peroxidases even when aryl- or alkylhydrazines do not (DePillis & Ortiz de Montellano, 1989; DePillis et al., 1990; Harris et al., 1991). Incubation of CLP (10 $\mu$M) with azide (1.25 mM) does not alter the CLP spectrum, suggesting that azide, at this concentration, does not form a coordination complex with the heme iron. Addition of $H_2O_2$ (2.5 mM), however, results in immediate loss of the native Soret peak at 400 nm and the emergence of absorption bands at 372 and 440 nm attributable to the Soret absorbances of compounds I and II, respectively (Figure 5) (Thomas et al., 1970a,b). These bands are rapidly replaced by a Soret absorbance at 402 nm that reaches a maximum absorbance corresponding to 87% of that of the native Soret band (Figure 5). These results suggest that reaction of azide with the higher oxidation states of CLP results in partial heme degradation.

The above conditions (i.e., $N_3^-$:CLP ratio = 125; $H_2O_2$:CLP ratio = 250) result in ~22% inactivation of CLP whereas azide alone or peroxide alone does not decrease enzymatic activity. The relatively low extent of inactivation at such a high azide-to-CLP ratio indicates that azide is a much poorer inactivator of CLP than phenylhydrazine. The oxidation of azide by CLP has been confirmed by EPR spin-trapping experiments. Incubation of CLP with azide and peroxide in the presence of the spin trap PBN results, as previously reported (Kalyanaraman et al., 1985), in the appearance of an EPR signal characteristic of the PBN–$N_3$ nitroxide adduct (not shown). Reaction mixtures devoid of CLP, peroxide, or azide failed to yield any detectable signal.

Reverse-phase HPLC analysis of the prosthetic group extracted from incubations of CLP (20 $\mu$M), azide (2.5 mM), and $H_2O_2$ (5 mM) demonstrates the formation of *δ-meso*-azidoheme (Figure 6). The modified heme eluting at 10.2 min cochromatographs with an authentic standard of *δ-meso*-azidoheme, exhibits an identical electronic absorption spectrum with $\lambda_{max}$ = 399 nm, and is not detected in incu-

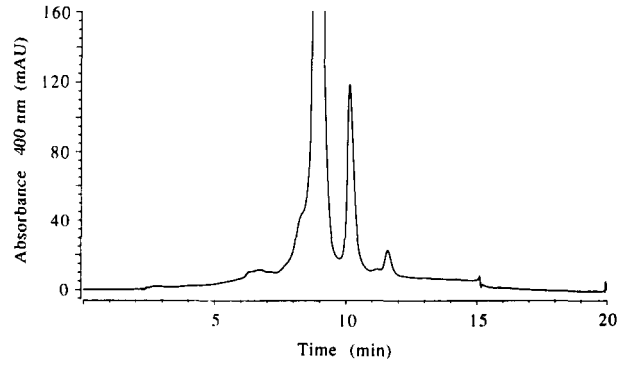


FIGURE 6: HPLC analysis of the prosthetic group extracted from CLP after partial inactivation by sodium azide and $H_2O_2$. Reaction and HPLC conditions are described under Materials and Methods.

bations of CLP alone, CLP plus azide in the absence of $H_2O_2$, or CLP plus peroxide in the absence of azide. The only other significant 400-nm-absorbing material was unreacted heme ($t_R$ = 8.9 min, Figure 6), which represented ~88% of the material detected in the chromatogram. These results suggest that the protein structure of CLP allows substrates access to the heme δ-edge.

## Discussion

CLP is irreversibly inactivated during the peroxide-dependent catalytic turnover of phenylhydrazine (Table I) with the concomitant formation of *trans*-azobenzene. The partition ratio for the reaction indicates that approximately 15 molecules of phenylhydrazine are oxidized by each enzyme molecule before it is inactivated (Figure 1). The *trans*-azobenzene metabolite presumably arises by combination of catalytically generated phenyldiazenyl and phenyl radicals. The phenyl radical, however, also adds to the iron of the prosthetic group because enzyme inactivation is caused by the formation of a σ-bonded phenyl–iron heme complex. The formation of this complex, suggested by the appearance of a long-wavelength absorption band at 472 nm in the inactivated enzyme (Figure 2), is confirmed by the fact that anaerobic extraction of the complex followed by reoxygenation under acidic conditions yields a mixture of the four *N*-phenyl-PPIX regioisomers (Figure 3, upper). Oxidative iron-to-nitrogen shift of the phenyl group under these conditions has been demonstrated with the phenyl–iron heme complexes extracted from a number of other hemoproteins (Swanson et al., 1991; Raag et al., 1990; Ortiz de Montellano & Kerr, 1983; Swanson & Ortiz de Montellano, 1991). The four regioisomers are obtained in equal amounts when the rearrangement occurs after the complex is removed from the protein under fully anaerobic conditions (Raag et al., 1990; Swanson & Ortiz de Montellano, 1991). Formation of isomers C and D in slightly higher amounts in the present instance (Figure 3, upper) suggests that the solution was not completely ananaerobic when the heme was extracted, so that the iron–nitrogen shift occurred to some extent before the phenyl–iron complex was completely free of the protein. Similar control of the phenyl shift by the denaturing protein is observed when the myoglobin complex is added to an acid solution under aerobic conditions (Swanson & Ortiz de Montellano, 1991). Formation of a phenyl iron complex with phenylhydrazine, in and of itself, distinguishes CLP from the majority of classical peroxidases, which do not form such complexes (Ortiz de Montellano, 1987; Ator & Ortiz de Montellano, 1987; DePillis & Ortiz de Montellano, 1989; DePillis et al., 1990; Harris et al., 1991). The 472-nm absorption maximum of the complex is similar to that observed for the complexes of cytochromes P-450$_{cam}$ and P-450IA1, but is at shorter wavelengths than the 480-nm absorbance of the corresponding complexes of cytochromes P-450IIB1, -IIB2, and -IIE1 (Swanson et al., 1991; Raag et al., 1990). The crystal structure of the cytochrome P-450$_{cam}$ complex shows that the phenyl group is tilted 10° away from a vertical to the heme by steric interactions with the active site residues (Raag et al., 1990). This suggests the possiblity that the phenyl group is tilted in complexes with a 472-nm maximum but not in those with a 480-nm maximum. The 472-nm maximum observed with CLP thus is consistent with the formation of phenyl–iron complex in which the ligand trans to the iron is a thiolate and in which the phenyl group may be sterically compressed and therefore tilted.

Oxidation of the phenyl–iron complex within the protein with ferricyanide or by exposure to high peroxide concentrations results in protein-directed phenyl migration to give predominantly at 1:2.5 ratio of the regioisomers of *N*-phenyl-PPIX with the phenyl group on pyrrole rings A and C (Figures 3, lower, and 4). The peroxide oxidation also yields a small amount of the *N*-phenyl adduct substituted on pyrrole ring D, but the ferricyanide-mediated shift gives little of this adduct. Ferricyanide thus appears to mediate a cleaner in situ shift than does peroxide, but with the same pattern of dominant products. This is the first example of a peroxide-mediated phenyl–iron shift. Formation of a phenyl–iron complex indicates that the active site of CLP is more open than that of horseradish and other classical peroxidases. This suggests that substrates have access to the heme iron and the ferryl oxygen, in agreement with the fact that CLP catalyzes ferryl oxygen transfer to olefins to give epoxides and sulfides to give sulfoxides (Ortiz de Montellano et al., 1987; Kobayashi et al., 1987). Predominant formation of the pyrrole ring A and C *N*-phenyl-PPIX regioisomers when the iron–phenyl shift occurs within the intact active site indicates that the space over these two pyrrole rings is relatively open whereas that over pyrrole ring B is occluded by protein residues. The space over pyrrole ring D appears to also be occupied by protein residues, although the difference in the yield of the D regioisomer between the peroxide and ferricyanide reactions suggests that conformational changes may occur readily in this region of the protein. The CLP active site topology, as defined by this technique, is significantly different from those of the active sites of four rat liver microsomal cytochromes P-450, all of which are suggested by the *N*-phenyl-PPIX patterns to be open above pyrrole rings A and D but closed above pyrrole rings B and C (Swanson et al., 1991). However, the *N*-phenyl-PPIX patterns obtained in similar experiments with other cytochrome P-450 enzymes vary, in some cases even selectively yielding the A and C *N*-phenyl-PPIX regioisomers (unpublished results), so the topological difference between CLP and the rat enzymes does not differentiate peroxidase from monooxygenase active sites. It is of substantial interest, however, that the nitrogen of pyrrole ring B is almost completely unavailable in CLP because this same nitrogen is consistently unavailable in the cytochrome P-450 enzymes (Swanson et al., 1991; Raag et al., 1990; unpublished data). The evidence suggests that pyrrole ring B is masked in the cytochrome P-450 enzymes by a helix that corresponds to the I-helix of cytochrome P-450$_{cam}$. The present results suggest the intriguing possibility that the pincer action of a helix in the region of pyrrole ring B helps to bind the heme in CLP, as it does in the cytochrome P-450 enzymes. Blanke and Hager have shown that residues 30–38 of chloroperoxidase have high sequence identity with residues 45–53 of cytochrome *c* peroxidase. The cytochrome *c* peroxidase residues are part of a helix containing the catalytic

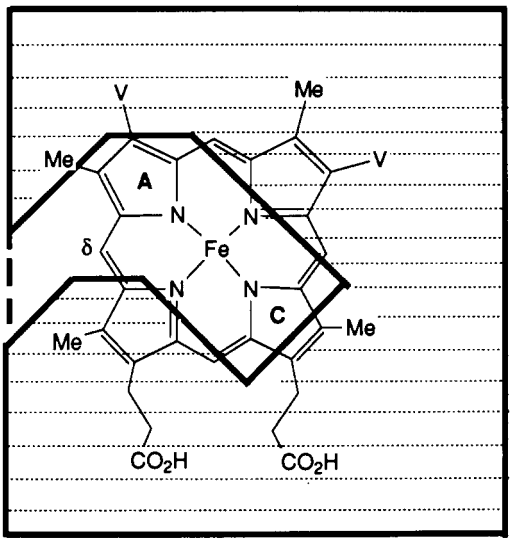


FIGURE 7: Model of the CLP active site based on the regiochemistry of in situ *N*-phenyl and *meso*-azido adduct formation (abbreviations: V = vinyl, P = propionic acid).

histidine that provides one side of the heme binding site. It is therefore possible that the helix defined by residues 30–38 serves a similar function in chloroperoxidase.

The CLP-catalyzed oxidation of azide to the azidyl radical results in partial inactivation of the enzyme and partial conversion of its prosthetic heme group to the *δ-meso*-azido derivative (Figures 5 and 6), although the relatively low yield of adduct and the decrease in the Soret band suggest that some heme degradation also occurs. Exclusive formation of the *δ-meso*-azido adduct indicates that the structure of CLP permits substrates to interact with the heme in the vicinity of the δ-meso carbon. Substrate access to the δ-edge, as evidenced by formation of *δ-meso*-aryl, -alkyl, or -azido adducts, has been observed with horseradish peroxidase, ligninase, manganese peroxidase, and *C. macrorhizus* peroxidase and thus appears to be a general property of peroxidase enzymes (Ator & Ortiz de Montellano, 1987; Ator et al., 1987; Ortiz de Montellano et al., 1988; DePillis & Ortiz de Montellano, 1989; DePillis et al., 1990; Harris et al., 1991). The δ-meso edge is not so readily accessible that it permits the detectable formation with phenylhydrazine of the *δ-meso*-phenyl adduct, although this may not be due so much to inaccessibility of the δ-edge as to preferential reaction of the phenyl radical with the heme iron atom. Access to the δ-heme edge and regioselective formation of the pyrrole ring A and C isomers of *N*-phenyl-PPIX suggest the active site topology shown in Figure 7. The active site is portrayed as being relatively exposed to the medium because indirect evidence suggests a moderately polar active site (Ortiz de Montellano et al., 1987; Dawson & Sono, 1987).

In summary, CLP is a unique hemoprotein with the cysteine fifth iron ligand characteristic of a cytochrome P-450 enzyme, an open active site much like that of the cytochrome P-450 enzymes, and the ability to catalyze cytochrome P-450-like oxygen-transfer reactions. However, it also permits substrates to interact with the δ-meso heme edge, as do all other peroxidases so far examined, has a relatively polar active site, as do the other peroxidases, and catalyzes the one-electron oxidation of classical peroxidase substrates. The topology of the CLP active site is thus fully consistent with the dual catalytic activity of the enzyme in that it exhibits properties characteristic of both peroxidase and monooxygenase active sites. The hermaphroditic nature of the active site may be related to the unique ability of the enzyme to oxidize chloride ion to a chlorinating species, a reaction thought to require direct reaction of chloride ion with the ferryl oxygen to give hypochlorous acid or a related iron-bound chlorinating species (Griffin, 1991; Dawson & Sono, 1987).

**Registry No.** CLP, 9055-20-3; $H_2O_2$, 7722-84-1; $PhNHNH_2$, 100-63-0; $N_2^-$, 14343-69-2; heme, 14875-96-8.

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# NMR Studies of the Active Site of Isopenicillin N Synthase, a Non-Heme Iron(II) Enzyme†

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ABSTRACT: The active site structure of isopenicillin N synthase (IPNS) has been previously studied by the use of Mössbauer, EPR, electronic absorption, and NMR spectroscopies [Chen, V. J., Frolik, C. A., Orville, A. M., Harpel, M. R., Lipscomb, J. D., Surerus, K. K., & Münck, E. (1989) *J. Biol. Chem. 264*, 21677-21681; Ming, L.-J., Que, L., Jr., Kriauciunas, A., Frolik, C. A., & Chen, V. J. (1990) *Inorg. Chem. 26*, 1111-1112]. These studies have revealed three coordinated His residues along with three sites for substrate [δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine, ACV], NO, and water binding on the active Fe(II) of IPNS. We report here NMR studies of Fe(II)IPNS and its Co(II)-substituted derivative [Co(II)IPNS]. By the use of NOE techniques on the Co(II)IPNS-ACV complex, we have recognized a $-CH_2-CH<$ spin system at 14.6, 24.3, and 38.6 ppm that is assigned to the α and β protons of a coordinated Asp residue. Corresponding solvent nonexchangeable features are found near 40 ppm in Fe(II)IPNS and the Fe(II)IPNS-ACV complex, but the peaks are too broad for NOE effects to be observed. The binding of NO to the Fe(II) center results in a significant change in the configuration of the metal site: (a) The $C_\beta H_2$ resonances due to the coordinated Asp residue disappear. The loss of the signal may indicate a change of the carboxylate configuration from syn-like to anti-like or, less likely, its displacement by NO. (b) The imidazole NH resonance for one of the coordinated His residues in the Fe(II)IPNS-ACV complex also disappears, suggesting that this His residue is strongly perturbed and may be detached from the metal site. These results allow us to propose a scheme showing the effects of exogenous ligand binding on the active site of IPNS. To date, this is the first successful NMR study of the endogenous ligands of the Fe(II)-NO center in a non-heme Fe(II) protein.

The key steps in the biosynthesis of penicillin- and cephalosporin-related antibiotics in some microorganisms are the oxidative ring closure reactions of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) forming the β-lactam ring and the thiazolidine ring of isopenicillin N, the precursor of other penicillins and cephalosporins (Baldwin & Bradley, 1990; Baldwin, 1989; Baldwin & Abraham, 1988; Robinson 1988).

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The enzyme isopenicillin N synthase (IPNS) that aerobically catalyzes the four-electron oxidative process contains a single high-spin non-heme iron(II) center in its active site (Chen et al., 1989a,b). However, unlike in the oxidative cleavage reactions catalyzed by non-heme iron-containing dioxygenases in which oxygen atoms are incorporated into the substrates (Que, 1989), dioxygen is completely reduced to 2 equiv of $H_2O$ in the oxidative ring closure reactions of ACV in the IPNS catalysis (Baldwin & Bradley, 1990; Baldwin, 1989; Baldwin & Abraham, 1988; Robinson, 1988).

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