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Examining the Role of Glutamic Acid 183 in Chloroperoxidase Catalysis*

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Site-directed mutagenesis has been used to investigate the role of glutamic acid 183 in chloroperoxidase catalysis. Based on the x-ray crystallographic structure of chloroperoxidase, Glu-183 is postulated to function on distal side of the heme prosthetic group as an acid-base catalyst in facilitating the reaction between the peroxidase and hydrogen peroxide with the formation of Compound I. In contrast, the other members of the heme peroxidase family use a histidine residue in this role. Plasmids have now been constructed in which the codon for Glu-183 is replaced with a histidine codon. The mutant recombinant gene has been expressed in *Aspergillus niger*. An analysis of the produced mutant gene shows that the substitution of Glu-183 with a His residue is detrimental to the chlorination and dismutation activity of chloroperoxidase. The activity is reduced by 85 and 50% of wild type activity, respectively. However, quite unexpectedly, the epoxidation activity of the mutant enzyme is significantly enhanced ~2.5-fold. These results show that Glu-183 is important but not essential for the chlorination activity of chloroperoxidase. It is possible that the increased epoxidation of the mutant enzyme is based on an increase in the hydrophobicity of the active site.

Chloroperoxidase (CPO[1]; EC 1.11.1.10) is a heavily glycocylated heme peroxidase secreted from the filamentous fungus *Caldariomyces fumago* (1–3). Natural product research in the 1940s discovered the biosynthesis of chlorinated products by *C. fumago* (4). Caldariomycin (2,2-dichloro-1,3-cyclopentanediol) is the major chlorinated compound found in *C. fumago* cultures. Much later, enzyme and tracer experiments delineated the role of CPO in the biosynthesis of caldariomycin (5, 6). CPO is undoubtedly the most versatile member of the heme peroxidase family. In addition to catalyzing chlorination, bromination, and iodination reactions (7–9), CPO catalyzes typical one electron oxidations (10) and possesses catalase (11) and monooxygenase activities (12). CPO is especially adept in catalyzing a number of chiral oxidations. The chiral epoxidation of olefins (13–16), sulfides (17–19), and indole (20–22) are carried out in high yield and enantioselectivity. CPO also catalyzes chiral benzylic (23) and propargylic hydroxylations (24–25). The stereoselective abilities of CPO in chiral catalysis suggest that native CPO and/or engineered CPO mutants have the potential to become important industrial catalysts.

The initial reaction of CPO with hydrogen peroxide generates an oxidized intermediate, Compound I. This oxidized intermediate contains one oxygen atom and both of the oxidizing equivalents originally present in hydrogen peroxide. One of the oxidizing equivalents is associated with the conversion of heme ferric iron to an oxyferryl iron species, and the other is generated by the removal of one of the electrons from the heme porphyrin, generating a porphyrin π cation radical (26, 27).

It is assumed that the versatile functions of CPO must be based on a CPO unique active site structure that includes a proximal thiolate ligand to the heme iron and a glutamic acid residue distal to the heme (28). It has been proposed that Glu-183 functions as a general acid-base catalyst in facilitating the formation of Compound I. In contrast, most if not all other peroxidases have histidines functioning in this role. In previous experiments, the proximal thiolate ligand (Cys-29) has been replaced with a histidine residue and the mutant enzyme was expressed in *C. fumago* (29). In this mutant, the k_{cat} for chlorination and peroxidation was reduced ~95%. The catalase and epoxidation activity in the mutant were reduced ~70%. It is obvious that cysteine 29 plays an important role in CPO catalysis. However, unlike the comparable cytochrome P450 Cys to His mutant, the replacement of CPO cysteine 29 with histidine does not totally abolish CPO activities. The distal Glu-183 also plays an important role in the catalytic reactions of CPO. Attempts to express a Glu-183 to His mutant in *C. fumago* were unsuccessful probably because the mutation is a lethal event. Previous attempts to produce CPO knock-outs in *C. fumago* also systematically failed. Because it is highly unlikely that CPO, a secreted enzyme, is an essential gene product in *C. fumago*, there must be an alternate explanation for these failures. The opposite strand DNA to the DNA coding for CPO contains an open reading frame; thus, it is quite possible that CPO mutations could prove to be lethal by producing a mutation in the opposite strand DNA. Recently, Conesa *et al.* (30) have shown that the wild type *CPO* gene can be expressed in *Aspergillus niger* with the recombinant protein retaining the catalytic properties of the native *C. fumago* enzyme. Therefore, this expression system was chosen for the production of the Glu-183 to His mutant. This paper describes the preparation, expression, properties, and catalytic activities of this CPO mutant.

EXPERIMENTAL PROCEDURES

Reagents

Restriction enzymes were purchased from New England Biolabs (Beverly, MA) and Invitrogen. T4 DNA ligase was purchased from

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[1] The abbreviations used are: CPO, chloroperoxidase; IEF, isoelectric focusing; ALA, δ-aminolevulinic acid.

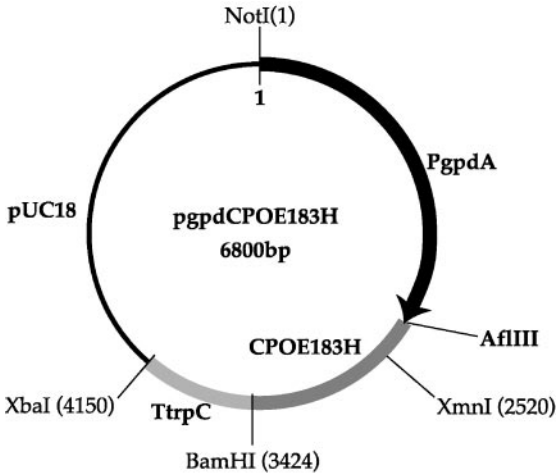

FIG. 1. **The E183H expression vector for co-transformation of *A. niger*.**

Promega (Madison, WI). Purified oligonucleotides were purchased from Keck Biotechnique Center at University of Illinois. Unless otherwise noted, chemicals were obtained from commercial sources and used without further purification.

Site-directed Mutagenesis

Mutations were carried out using the QuikChange site-directed mutagenesis kit of Stratagene (La Jolla, CA). The mutations were introduced via PCR amplification of pTZC using synthetic oligonucleotide primers. The primers contained a silent mutation site that could be used in a convenient mutant-screening assay. Primers for site-directed mutagenesis were designed with the aid of the Wisconsin GCG Package, version 9.1 (Madison, WI). PCR reactions were carried out in a PerkinElmer thermal cycler (PerkinElmer Life Sciences). Standard "hot-start" PCR was performed using high fidelity *pfu* polymerase. The PCR products were extracted and purified from agarose gels using kits purchased from Qiagen (Valencia, CA). The desired mutations were identified by restriction analysis and confirmed by DNA sequencing.

Construction of pgpd/CPOE183H

Expression vector pgpd/CPOE183H was constructed via a three-way ligation. The three DNA sequences used in the construction were: 1) a 230-bp *Afl*III/*Xmn*I fragment derived from pCPO3 (30) that contained the N-terminal CPO coding sequence, 2) a ~900-bp *Xmn*I/*Bam*HI fragment derived from pTZC (31) that contained the remaining CPO coding sequence, and 3) an *A. niger* expression vector, pAN52–5Not (GenBank™ accession number Z32750), that contained an *A. nidulans gpd* promoter and the *A. niger trpC* terminator sequences (32). The *Xmn*I/*Bam*HI fragment contained the E183H mutation. The *A. niger* vector was digested with *Nco*I and *Bam*HI to create an insertion site for the CPO sequences (Fig. 1).

Expression and Selection of Mutant Clones

Plasmid gpd/CPOE183H was co-transformed with pAB4-1 in *A. niger* MGG029 according to previously described procedures (33). Transformants were screened *in situ* by immunodetection as described previously (34).

Culture of Mutant Clones

The mutant clones were grown in roller bottles at 22 °C for 5–7 days. Each bottle contained 300 ml of 5% maltose, 5% yeast extract, 0.5% casein amino acids, 50 μM hemin (Sigma), and 100 μM δ-aminolevulinic acid (Sigma) and the Hutner metal ion supplement (35).

Partial Purification of the Mutant CPO

To stabilize the mutant chlorination and epoxidations activities, all of the purification buffer solutions contained 50 μm hemin. Crude culture medium containing the secreted enzyme (1.5 liters) was concentrated 50-fold and applied to a DEAE-Sepharose CL-6B ion-exchange chromatography column (4 × 30 cm) that had been equilibrated with 50 mm Bis/Tris at pH 6.5. The flow rate was adjusted 1 ml/min. Chromatography was carried out at 4 °C. The protein was eluted from the column with a step gradient of 50 mM Bis/Tris buffer at pH 6.5 containing 10–70 mM ammonium sulfate. Each fraction was assayed for chlorination activity to identify the active fractions. The active fractions appeared in the eluent fractions containing 28 mM ammonium sulfate. The enzyme was concentrated by ultrafiltration (Amicon, Newburyport, MA).

SDS-PAGE and Isoelectric Focusing (IEF)

The purity of the mutant enzyme was examined by 12.5% SDS-PAGE gel (Invitrogen) under denaturing condition (36). The enzymes were visualized by staining with Coomassie Brilliant Blue R-250. IEF electrophoresis was performed using the Pharmacia Phastsystem with IEF PhastGels (pH 3–9) and the Pharmacia IEF Mix 2.8–6.5 kit. IEF gels were visualized by silver staining.

Protein Determination

Heme proteins were quantitatively determined using the hemochromogen analysis developed by Deeb and Hager (37). This information provided a basis for comparing the relative enzymatic activities of the mutant and native CPO.

Immuno-dot Blot Assay

Because both apomutant and holomutant enzymes were secreted in the *A. niger* cultures, the total concentration of mutant enzyme was measured in a dot blot assay. Aliquots of the partially purified mutant enzyme or aliquots of the crude culture medium were loaded onto nitrocellulose membranes. The membrane was incubated with anti-CPO rabbit polyclonal IgG antibody for 1 h at room temperature. After incubation, the membrane was washed and then incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G for 1 h. After this second incubation step, the membrane was washed and then reacted with diaminobenzidine and hydrogen peroxide for 10 min for color development. The total concentration of apomutant plus holomutant enzyme was estimated by comparing the dot blot results with the results obtained from serial dilutions of known concentrations of native CPO.

Circular Dichroism

Far-UV-CD spectra were recorded on a JASCO J-720 spectropolarimeter at room temperature. Two spectra were recorded from 250 to 200 nm at 0.1-nm intervals with a spectral bandwidth of 1 nm (the spectra were averaged). The concentration of partially purified samples for both the mutant and native CPO control was identical (50 μg/ml).

Optimum pH for the Chlorination Activity

The relation between activity and pH was examined from pH 2.5 to 7.0 using the potassium phosphate buffers.

Enzyme Activity Assays

All UV-visible absorption measurements were determined in a Shimadzu UV-1201 spectrophotometer using 1-cm path length quartz cuvettes.

1). Chlorination Assay—The chlorination of monochlorodimedone to dichlorodimedone was used for measuring the chlorination activity of the mutant CPO (7). The reaction mixture contained 20 mM potassium phosphate buffer, pH 2.75, 20 mM KCl, 0.5 mM monochlorodimedone, 2 mM H_2O_2, and a suitable aliquot of the enzyme in a total volume of 3 ml. The reaction was initiated by the addition of enzyme, and the decrease in absorbance at 278 nm was monitored at room temperature. One unit of chlorination activity was defined as the formation of 1 μmol of dichlorodimedone per second.

2). Epoxidase Assay—The epoxidation of *p*-nitrostyrene was used to measure epoxidation activity according to the procedure developed by Rai *et al.* (38). The reaction mixture contained 100 mM sodium acetate buffer, pH 4.5, 0.2 mM *p*-nitrostyrene, and 2 mM H_2O_2 plus a suitable aliquot of the enzyme in a total volume of 3 ml. The activity was determined by monitoring the decrease in absorbance at 312 nm as a function of time. One unit of epoxidation activity was defined as the formation of 1 μmol of *p*-nitrostyrene oxide per second.

3). Catalase Assay—The oxidation of reduced methylene blue was used to measure catalase activity (38). The reaction mixture contained 100 mM sodium acetate buffer, pH 4.5, 0.2 mM dithionite reduced methylene blue, 2 mM H_2O_2, and a suitable aliquot of enzyme in a total volume of 3 ml. One unit of catalase activity was defined as the formation of 1 μmol of the oxidized form of methylene blue per second.

RESULTS

Isolation of the E183H Mutant—*A. niger* produces the E183H mutant enzyme at levels comparable to the expression

of wild type CPO in *A. niger*. However, a majority of the mutant CPO expressed in *A. niger* is in the form of apoenzyme having no heme incorporated. By comparing the relative levels of apomutant and holomutant enzymes estimated from a hemochromogen assay and a dot blot immunoassay, the ratio of apoenzyme to holoprotein was ~1.5 to 1. A similar observation has been made in the expression of recombinant wild type CPO in *A. niger* (30). Most disturbingly, the E183H presented serious problems in early attempts to purify and characterize the mutant enzyme. In early studies, the Glu-183 mutant lost most of its chlorination and epoxidation activity in simple dialysis and ultrafiltration steps. Acetone precipitation, a regular purification step used with native CPO, could not be used because the mutant lost almost all activity when exposed to acetone at −20 °C. Subsequently, it was discovered that the loss of the chlorination and epoxidation activity during dialysis and ultrafiltration could be prevented by the addition of iron protoporyphyrin IX to the mutant enzyme preparations. The presence of heme greatly stabilized the E183H extracts, and in addition, 90% of the mutant protein now appeared in the form of holoenzyme. In contrast, holoenzyme represented only one-third of the mutant enzyme population in untreated extracts.

Conesa *et al.* (30) found that secreted wild type CPO in *A. niger* was subject to partial degradation by secreted protease activity. In our initial problems concerning the instability of the E183H mutant enzyme, we considered protease degradation as a potential cause of the instability. This turned out not to be true. Ten different protease inhibitors either singly or in kit form were tested and were found to have no effect. The inhibitors tested were antipain-dihydrochloride, bestatin, chymotatin, E-64, leupeptin, pepstatin, phosphoramidon, pefablioc SC, aprotinin, and phenylmethylsulfonyl fluoride. Other reagents such as bovine serum albumin, glycerol, and sorbitol had no effect on the stability of the E183H enzyme.

Increasing the Yield of the E183H by the Addition of δ-Aminolevulinic Acid (ALA)—In regular culture broth, the secretion level of the mutant protein (apoenzyme plus holoenzyme) was quite low (~2 mg/liter). The addition of ALA to the culture medium increased the secretion level ~4-fold. ALA synthase is thought to be a crucial enzyme to regulate heme synthesis. There are a few reports regarding an increase in heme protein production by ALA inducement. For example, Herbaud *et al.* (39) found that the highest cytochrome c_3 production was obtained when *Escherichia coli* was grown in LB medium supplemented with δ-aminolevulinic acid under aerobic condition (39). Further studies showed that the regulative effects of ALA and hemin on ALA synthase were done through transcriptional regulation (40). In these studies on the production of mutant CPO by *A. niger*, the addition of δ-aminolevulinic acid significantly enhanced the production of the E183H mutant enzyme.

Partial Purification of E183H Mutant—The instability of the mutant enzyme precluded the normal initial acetone precipitation routinely used to purify native CPO. Even the presence of heme did not protect Glu-183 from denaturation by acetone. DEAE ion exchange chromatography was used exclusively for purification of the mutant enzyme. As described under "Experimental Procedures," all purification buffers contained 50 μm of hemin to stabilize the mutant enzyme. The specific activity of the partially purified E183H increased by 145-fold, and the enzyme showed a major band in SDS-PAGE (Fig. 2) and had a *RZ* value of 1.2 (Fig. 3).

Chlorination and Dismutation Activities of the E183H Mutant—To evaluate the effect of the distal substitution on the various activities of CPO, the activities of the E183H mutant were measured in three different assays. The chlorination activity of the mutant enzyme decreased to a level of ~15% of the wild type activity, and the catalase activity decreased to 50% of the native CPO catalytic activity (Fig. 4).

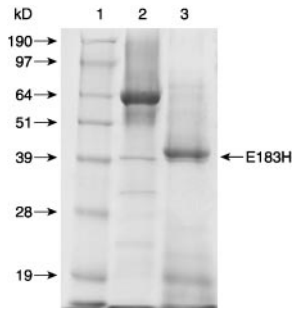

FIG. 2. **SDS-PAGE analysis of E183H after DEAE-Sepharose ion-exchange chromatography purification.** Aliquots of concentrated crude extract and the purified samples were subjected to 12.5% SDS-PAGE followed by Coomassie Brilliant Blue staining. *Lane 1*, prestained molecular weight standard; *lane 2*, concentrated crude culture media; *lane 3*, DEAE column-purified E183H.

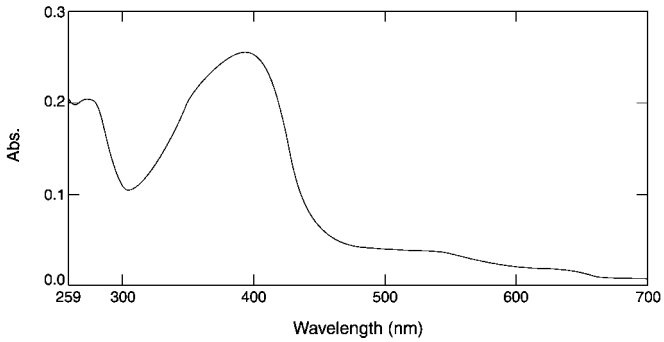

FIG. 3. **Absorption spectrum of the partially purified E183H.** The *RZ* value, ratio of A_{400} to A_{280}, is 1.2.

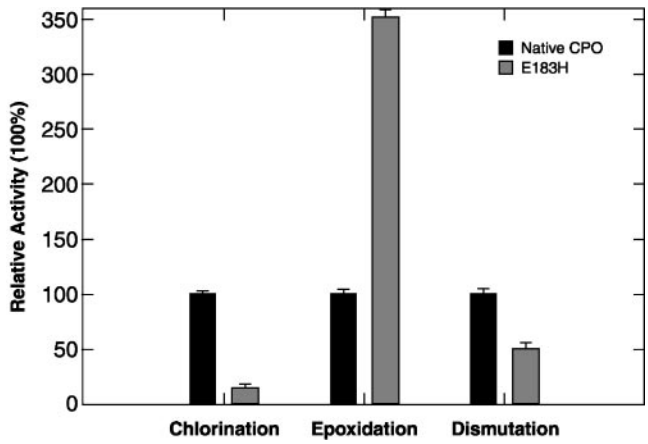

FIG. 4. **Relative activities of mutant enzyme and native CPO in the chlorination, dismutation, and epoxidation assays.**

Epoxidation Activity in the E183H Mutant—As outlined under "Experimental Procedures," the epoxidation of *p*-nitrostyrene was used to assay the epoxidation activity of the E183H mutant according to the procedure developed by Rai *et al.* (38). In contrast to the results obtained for the chlorination and dismutation mutant activities, epoxidation activity unexpectedly increased 2.5-fold (Fig. 4).

Optimum pH and IEF—To further elucidate the role of Glu-183 as a general acid-base catalysis, the pH optimum of the E183H mutant was examined. The enzyme activity was measured at various pH values ranging from pH 2.5 to 7.0. The concentration of H_2O_2 used in these studies was optimized for each pH value. The mutant was most active at pH 3.0, whereas

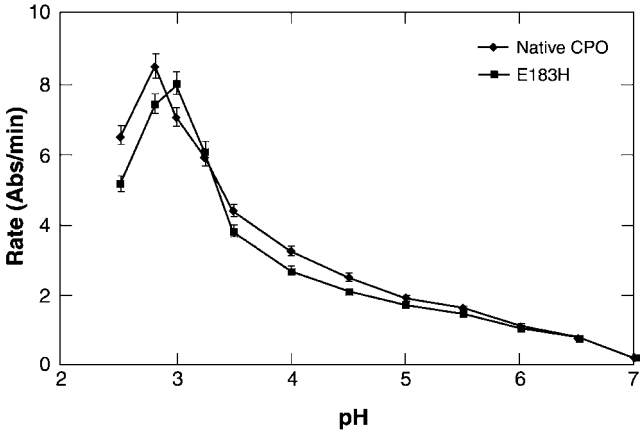

FIG. 5. **pH profile for chlorination activity in the E183H mutant enzyme and native CPO.** The optimum pH for the E183H mutant is 3.0, and the optimum pH for native CPO is 2.75. All analyses were carried out at 22 °C with an appropriate concentration of enzyme in the standard monochlorodimedone assay.

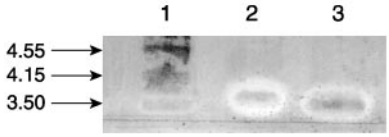

FIG. 6. **pI of the E183H mutant.** This figure illustrates IEF using a PhastGel (pH 3–9) for the E183H mutant and native CPO, which shows that the pI of the mutant is slightly higher than that of native CPO. *Lane 1*, markers; *lane 2*, mutant CPO; *lane 3*, native CPO.

its native counterpart was at pH 2.75 (Fig. 5). The E183H mutant showed a slightly higher pI value based on their isoelectric focusing gel properties (Fig. 6), but this was expected for the replacement of a negatively charged by a positively charged one.

Circular Dichroism—CD spectrum was used to exclude the possibility that activities of mutant enzyme were decreased by large structure changes. The CD data showed that there were some small differences in intensity in the peptide bond region between the mutant and native CPO; however, the overall shapes of the two spectra were the same (Fig. 7). The CD spectra of the mutant proteins (80.5% α-helix, 6.2% β-turn, and 15.7% unordered) were very similar to that of wild type (79.6% α-helix, 0.6% β-sheet, 6.4% β-turn, and 16.2% unordered). The result indicated that the amino acid substitutions had no significant effect on the overall folding of the mutant enzyme.

DISCUSSION

The discovery that hemin protects the mutant enzyme during purification and the observation that the apoform of the mutant can be converted to the holoenzyme in the presence of hemin were extremely important observations because hemin allowed the purification and characterization of this mutant enzyme. This discovery may also offer a new approach to the production of mutant CPOs. Wild type apoCPO can be produced in *E. coli* in large quantities; however, under the very best conditions, only small amounts of holoenzyme could be obtained by reconstitution procedures (41). Because the E183H apomutant readily reconstitutes to holoenzyme and because the E183H mutant is more active than wild type CPO in chiral epoxidation catalysis, it appears logical to assume that further mutation of E183H in *E. coli* is an obvious choice for examining the active site requirements for chiral catalysis.

Histidine plays a key role as a general acid-base catalyst in the formation of Compound I in the plant peroxidases. Poulos and Kraut (42) proposes that the distal histidine assists the

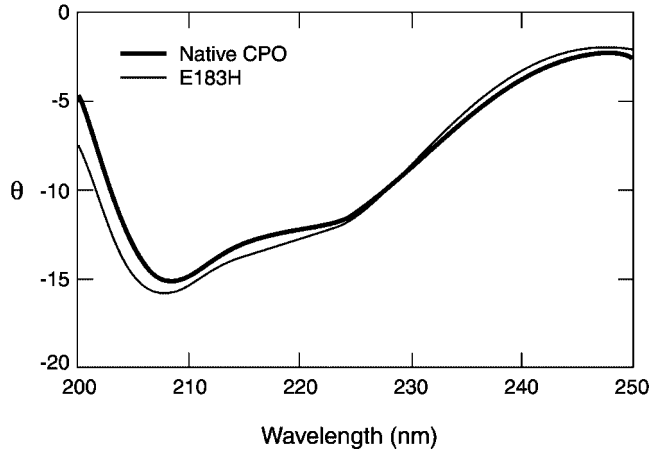

FIG. 7. **CD spectra of CPO and the E183H.** The CD spectra of E183H mutant (80.5% α-helix; 6.2% β-turn, and 15.7% unordered) were very similar to that of native CPO (79.6% α-helix, 0.6% β-sheet, 6.4 β-turn, and 16.2% unordered). The experiments were carried out at room temperature. The length of cuvette was 0.1 cm. Two spectra were recorded from 250 to 200 nm at 0.1-nm data intervals with a spectral bandwidth of 1 nm (the spectra were averaged). Concentrations of partially purified samples for the mutant and native CPO control (*RZ* value was 1.2 for each one) were 50 μg/ml.

peroxide anion to bind the heme iron by acting as a general base catalyst while accepting a proton from the neutral hydrogen peroxide when it binds to the iron and by acting as a general acid catalyst by donating a proton to the departing hydroxide anion (42). Several mutant peroxidases have revealed histidine as an important active site residue. In wild type CPO, a glutamic acid residue replaces the plant peroxidase histidine distal residue and is postulated to function as the acid-base catalyst, facilitating the formation of Compound I. By analogy to the experiments with horseradish peroxidase where replacement of the distal histidine leads to large losses in activity (43), it would be expected that replacement of the distal glutamic acid in CPO also should be deleterious. This is certainly true in terms of the halogenation activity of CPO. The rate of enzymatic chlorination in the Glu-183 to His mutant falls by ~85%. This indicates that the distal Glu residue in native CPO is important but not essential for the halogenation activity of the enzyme. The van der Waals volume of histidine (98.8 cm^3 mol^{-1}) is larger than that of histidine (85.9 cm^3 mol^{-1}), so some distortion of the mutant active site should be expected. The fact that the mutant heme prosthetic group is more loosely held supports the conclusion that there has been a change in the geometry of the active site. The x-ray structure of native CPO identifies a proton shuttle among Glu-183, His-105, and Asp-106 (44). When Glu-183 is replaced with a histidine residue, the normal orientation of this triad could be partially destroyed. Such a change could obviously impair the chlorination activity of the mutant. In studies with horseradish peroxidase mutants, it has been shown that the loss of a hydrogen bond between the distal His-42 and Asp-70 induces severe functional defects in peroxidase activity (45). In addition, previous experiments have shown that the modification of a single histidine residue with diethyl pyrocarbonate totally inactivates CPO with respect to chlorination activity (46).

A quite different picture emerges from a comparison of the catalytic activity of the mutant and native CPOs in the epoxidation reaction. In this case, the mutant is ~2.5-fold more active than the native enzyme. In focusing on the origin of this increase in activity, it is highly unlikely that the increase in activity could be associated with the formation of Compound I, the first step in all CPO oxidations. The rate constant for the formation of Compound I in the reaction of native enzyme with

hydrogen peroxide is 2.4×10^6 $M^{-1}sec^{-1}$ (47), whereas the rate constants for both the chlorination and epoxidation activity of native CPO are magnitudes lower. Thus, the overall rate for both chlorination and epoxidation reactions must reside in the second step involving the reaction between Compound I and the oxidizable substrate. It seems probable that the distortion of the active site that inhibits the chlorination activity must actually facilitate the reaction between the olefinic substrate and the oxygen atom in the oxyferryl Compound I species, perhaps by allowing a closer approach of the olefinic substrate to the oxygen atom of the oxyferryl group in Compound I. Clearly, the olefinic substrate must approach the Compound I intermediate from the distal side of the heme. In the mechanism-based suicide reaction between CPO Compound I and *cis*-β-methyl styrene, an oxygen-carbon bond is formed in the inactive intermediate (48). In a similar vein, the proton shuttle among Glu-183, His-105, and Asp-106 that is important for chlorination activity appears unimportant for epoxidation activity. The diethyl pyrocarbonate treatment that kills chlorination activity does not impair epoxidation activity.

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