Directed Evolution of Chloroperoxidase for Improved Epoxidation and Chlorination Catalysis

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Abstract: Chloroperoxidase (CPO) from the fungus *Caldariomyces fumago* is undoubtedly the most versatile member of the heme protein family. In addition to functioning as a halogenating enzyme and a classical peroxidase, CPO catalyzes the dismutation of peroxides in a catalase-type reaction and carries out cytochrome P450 oxygen insertion reactions. From the viewpoint of biocatalysis the most important CPO reactions are chiral epoxidations, hydroxylations, and sulfoxidations. CPO catalyzes a variety of chiral epoxidation reactions with high yields and high enantioselectivities. However, the industrial use of native CPO for the synthesis of chiral epoxides is limited because of its relatively low epoxidation rates in comparison to its high catalase activity, which robs the epoxidation reaction of oxidant. The use of CPO is also restricted by its poor reactivities in organic solvents. Directed evolution technology has been used to address these problems. After three rounds of PCR-based random mutagenesis, we have isolated mutants of chloroperoxidase having greatly enhanced epoxidation activity compared to the wild-type enzyme. In addition, in the screening of a first generation library of random mutation transformants, we have isolated three CPO mutant clones having improved chlorination activity and enhanced stability in a ternary solvent microemulsion comprised of toluene, isopropanol and water. Surprisingly, all three recombinant variants carry a single mutation in the cysteine residue that functions as the proximal heme ligand in the native enzyme. Two of these mutant clones are identical, having the proximal cysteine heme-ligand replaced with a tyrosine residue. The third mutant has the cysteine-29 replaced with a histidine residue. The cysteine mutation in the three mutants is the only amino acid replacement. All other mutations in the three clones were silent mutations. These data suggest that "directed evolution" can be successfully applied to the engineering of chloroperoxidase in the quest for a better industrial biocatalyst.

Keywords: biocatalysis; chlorination; chloroperoxidase (CPO); directed evolution; epoxidation

Introduction

The enzyme chloroperoxidase (CPO, EC: 1.11.1.10) is secreted in large amounts by the filamentous fungus, *Caldariomyces fumago*. To date, it is probably the most versatile heme protein known. CPO performs a plethora of chemical reactions that can be classified into two broad categories, the halide-dependent and halide-independent reactions. The halide-dependent reactions are catalyzed by the acidic enzyme form. These include the halogenation of a large variety of nucleophilic acceptors, for example, the halogenation of β-diketones[1] and halohydration of olefinic compounds.[2,3,4] The chlorination activity of CPO has been used to remove heavy metals from the heavy asphaltene fraction of Castilla crude oil.[5] The halide-independent reactions of CPO include stereoselective epoxidation of alkenes[6,7,8,9] and the oxidation of sulfides to chiral sulfoxides.[10] Chloroperoxidase also catalyzes the oxidation of alcohols to aldehydes,[4,11] aldehydes to acids,[12] amines to nitroso compounds,[13] disproportionation of peroxides,[14] peroxidative coupling of aromatics,[8] *N*-dealkylation of alkylamines,[15] enantioselective benzylic hydroxylations,[12] and propargylic oxidation of 2-alkynes to chiral alcohols.[16,17] Thus, the biocatalytic activities of CPO can be exploited in a variety of biochemical reactions with significant relevance to the fields of agrochemicals, drug discovery, drug manufacturing, and crude oil purification.

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Chiral epoxides are useful chiral synthons because of their ability to generate optically active compounds upon stereoselective ring opening.[18] The demand for the use of enantiopure drugs by the pharmaceutical industry was triggered by what is now known as the "Thalidomide Tragedy" of the 1950's.[19,20] Thalidomide™ was given to pregnant women to alleviate their morning sickness as a racemic mixture of the *R* and *S* enantiomers. The children born to these women had severe abnormalities. It was subsequently discovered that while the (*R*)-(+)-thalidomide is a sedative, the (*R*)-(–)-thalidomide is a teratogen causing fetal abnormalities.[19,21] This tragedy underscored the necessity for the pharmaceutical and agrochemical industries to synthesize pure enantiomeric drugs. Chiral synthons have since found their way in products with significant biological activity. Included are pharmacologically active compounds, antifungals, antivirals, antimalarials, antibiotics, anti-cancer agents, anti-inflammatory drugs, and herbicides, all of which find extensive use worldwide.

While the synthetic utility of enantiopure epoxides is widely recognized,[22,23,24] their production is often limited to traditional organic methods that require multistep syntheses and suffer from high production costs. Quite often product yield is low and the processes are plagued by problems arising from side-product disposal. Chloroperoxidase not only performs chiral reactions with relative ease but also generates products in high yields and high enantiomeric purity.[6,25,26,27,28] The industrial use of CPO for environmentally safe "green biocatalysis" is however marred by its ability to catalyze a broad spectrum of oxidative reactions. All chloroperoxidase-mediated reactions proceed via an oxyferryl porphyrin π-radical cation intermediate, CPO-compound I (CPO-I).[29,30,31,32] This well-characterized intermediate is generated by the reaction between the resting enzyme and a molecule of hydrogen peroxide. The broad spectrum of biochemical reactivities of chloroperoxidase emerges from the different reactions of CPO-compound I (see Figure 1). In its catalase reaction, CPO-compound I reacts with a second molecule of hydrogen peroxide and forms molecular oxygen and water. This catalase reaction competes successfully with all other substrates for the oxidizing equivalents of compound I. This problem especially applies to chiral epoxidations where the k_{cat} for catalase activity ($1.45 \times 10^5\ sec^{-1}$) exceeds the k_{cat} for epoxidation ($3.4 \times 10\ sec^{-1}$) by ~40 fold. Wild-type CPO also exhibits poor reactivity in organic solvents, is not tolerant to thermal changes and does not encompass a wide range of substrate molecules in its small active site.

Work in this laboratory has focused on making chloroperoxidase a viable industrial biocatalyst. "Directed evolution" offers a very important tool in overcoming the inherent limitations of enzymes.[33,34,35,36,37] Previously we have successfully used "directed evolution" for producing chloroperoxidase mutants that are resistant to suicidal inactivation by terminal alkenes.[38] We also have isolated mutants that have higher activity in 40% *tert*-butanol.[39] We now report on the isolation of CPO mutants that exhibit improved epoxidation activity and mutants that have better reactivity in an isopropanol:toluene:aqueous ternary solvent microemulsion.

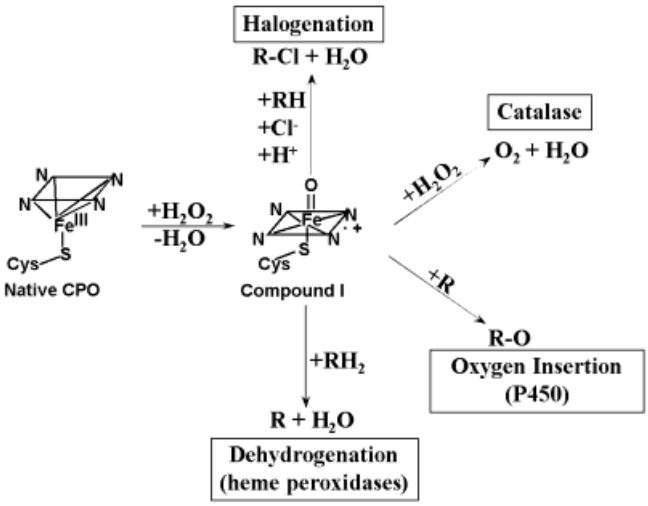

Figure 1. Chloroperoxidase-catalyzed reactions.

Results

Improved Epoxidation Activity

Mutant CPO genes were generated by random PCR mutagenesis using a bacterial plasmid containing a hygromycin resistance marker. Linear plasmid DNA was introduced into *Caldariomyces fumago* spheroplasts using the calcium chloride-polyethylene glycol technique previously adapted for transformation of this fungal host.[40] Hygromycin-resistant clones were isolated and grown in 24-well plate cultures containing a fructose-inorganic salt medium. After a 15 – 21 day growth period, aliquots of the growth media were collected and assayed for CPO halogenation activity using the standard monochlorodimedone (MCD) reaction.[1] After measuring the concentration of CPO in the culture supernatant fractions, 1, 5, and 10 nmoles of mutant CPO were removed for screening the mutant for epoxidation activity using *p*-nitrostyrene as substrate. The culture supernatant fractions also were analyzed for catalase activity using the methylene-blue catalase assay. The activity of the best mutants is depicted in Figure 2 and Figure 3 for the epoxidation reaction, and in Figure 4 for catalase activity. Figure 2 records the ratio of the k_{cat} for the

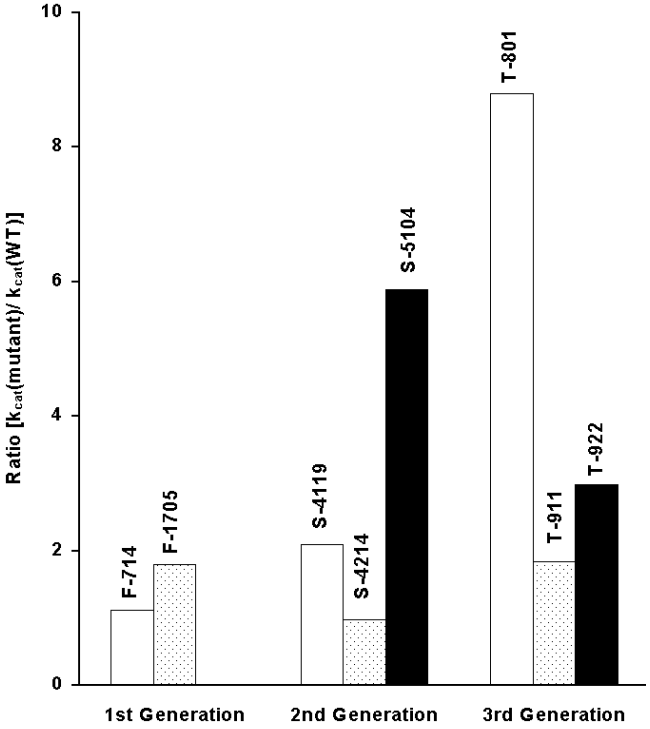

Figure 2. *p*-Nitrostyrene epoxidation activity of mutant chloroperoxidase. Each bar represents the ratio of k_{cat} for directed evolution transformants with respect to the k_{cat} for the wild-type CPO. The figure depicts the activities of two mutants from the first generation and three each from the second and third generation of directed evolution cycles.

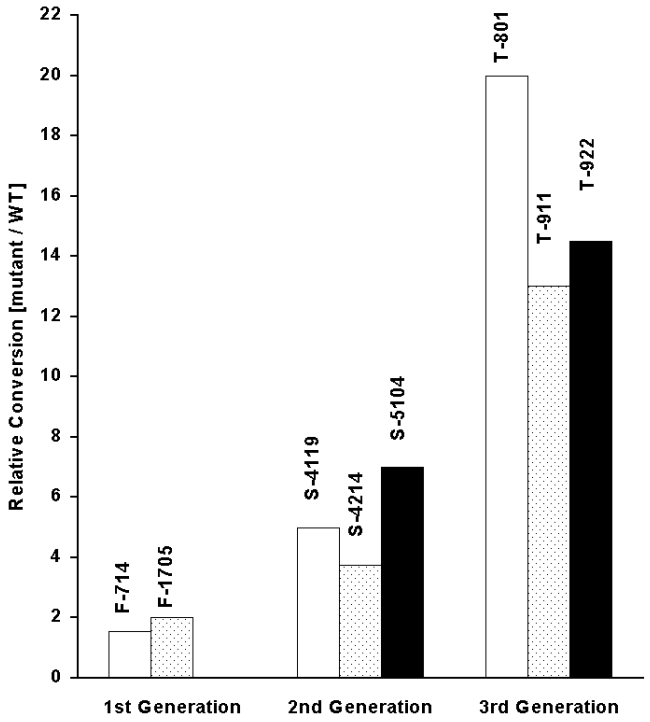

Figure 3. Conversion of *p*-nitrostyrene to *p*-nitrostyrene epoxide by mutant chloroperoxidase. Each bar represents the ratio of the epoxide yields for each mutant with respect to that of the wild-type enzyme, prior to inactivation of the enzyme by the alkene. Under the assay conditions, 100 nM wild-type CPO produced 4.2 μM *p*-nitrostyrene epoxide prior to inactivation by *p*-nitrostyrene.

mutant epoxidation activity to the k_{cat} for wild type epoxidation. Figure 3 records the ratio of mutant to wild-type activity for the same mutants in terms of the total product yield prior to inactivation of the enzyme. Figure 4 records the ratio of the k_{cat} of mutant to wild-type enzyme in terms of their catalytic activity. Table 1 shows the amino acid mutations incorporated in each of these mutants during each cycle of PCR-induced random mutagenesis.

It is important to note that the increase in epoxidation activity of two mutants, mutant F-714 from the first generation and mutant S-5104 from the second generation, was accompanied by a reduction in their catalase activity. However, all the mutants obtained in the third round had an increase in both the epoxidase and the catalase activities. Mutant T-801 from the third round of random mutagenesis was the best by far, having an 8-fold increase in the epoxidation activity and a 20-fold increase in the conversion of *p*-nitrostyrene to *p*-nitrostyrene oxide, when compared with the wild-type CPO (Figures 2 and 3). This same mutant also showed an enhancement of the catalase activity by a remarkable 12-fold excess over the wild-type enzyme (Figure 4).

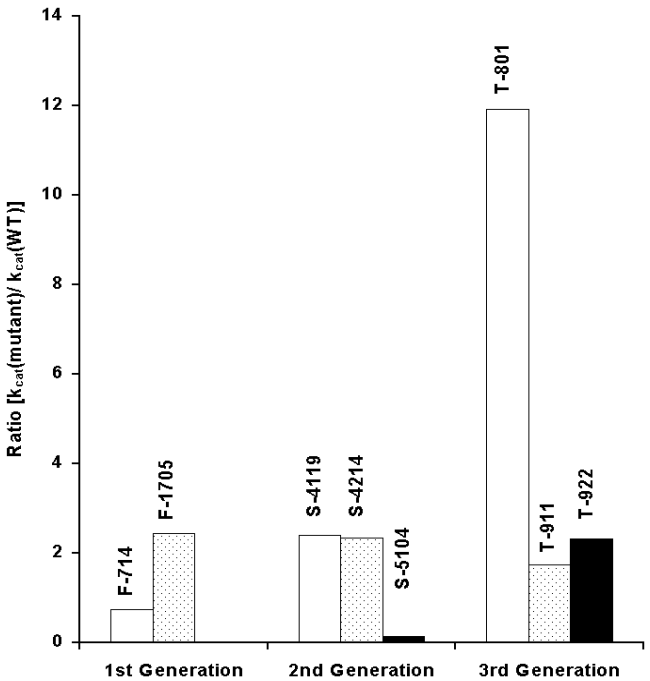

Figure 4. Catalase activity of mutant chloroperoxidase. Each bar represents the ratio of the k_{cat} for the eight mutant enzymes obtained in rounds 1, 2, and 3 of directed evolution, with respect to the k_{cat} for the wild-type enzyme.

Table 1. Amino acid mutations incorporated in each cycle of PCR-induced random mutagenesis of mutants screened for improved epoxidation of *p*-nitrostyrene.

Amino Acid Position	Directed Evolution Cycles							
	First Round		Second Round			Third Round		
	F-714	*F-1705*	*S-4119*	*S-4214*	*S-5104*	*T-801*	*T-911*	*T-922*
17								$Tyr_{17} \rightarrow His$
18	$Val_{18} \rightarrow Ala$			$Val_{18} \rightarrow Ala$	$Val_{18} \rightarrow Ala$			
28					$Pro_{28} \rightarrow Ser$			
80							$Glu_{80} \rightarrow Asp$	
131						$Asp_{131} \rightarrow His$		
147		$His_{147} \rightarrow Arg$	$His_{147} \rightarrow Arg$			$His_{147} \rightarrow Arg$	$His_{147} \rightarrow Arg$	$His_{147} \rightarrow Arg$
203						$Pro_{205} \rightarrow Pro$[a]		
225				$Trp_{225} \rightarrow Gly$				
248					$Ser_{248} \rightarrow Phe$			
249			$Val_{249} \rightarrow Ala$			$Val_{249} \rightarrow Ala$	$Val_{249} \rightarrow Ala$	$Val_{249} \rightarrow Ala$
251			$Ser_{251} \rightarrow Pro$			$Ser_{251} \rightarrow Pro$	$Ser_{251} \rightarrow Pro$	$Ser_{251} \rightarrow Pro$
263					$Pro_{265} \rightarrow Asp$			
265					$Ala_{265} \rightarrow Ile$			
267						$Ala_{267} \rightarrow Ala$[b]	$Ala_{267} \rightarrow Ala$[b]	$Ala_{267} \rightarrow Ala$[b]
282	$Ala_{282} \rightarrow Thr$							
284			$Asn_{284} \rightarrow Ser$			$Asn_{284} \rightarrow Ser$	$Asn_{284} \rightarrow Ser$	$Asn_{284} \rightarrow Ser$
292			$Pro_{292} \rightarrow Ser$	$Pro_{292} \rightarrow Ser$				

[a] Codon change from CCT to CCC.
[b] Codon change from GCT to GCC.

Improved Chlorination Activity

The chlorination activity of mutant CPO was monitored at 278 nm in a ternary solvent system microemulsion used by the Colombia group for removal of heavy metals from crude oil.[5] Three of the first generation transformants showed a two-fold improvement for k_{cat} in chlorination activity in the ternary solvent system (Figure 5). Surprisingly, all three of these recombinant proteins had a mutation in the cysteine residue (cys29) that functions as the proximal ligand to heme group in the wild-type enzyme (Table 2). Mutant 2745 has a cysteine-29 to histidine mutation while in the case of the other two random mutation transformants, a tyrosine residue replaced cysteine-29. The carbon monoxide binding spectra of dithionite-reduced 2745 showed that the transformed mycelia produced both the recombinant and wild-type enzyme. After acetone fractionation, most of the recombinant enzyme precipitated at 30% acetone concentration while the 70% acetone fraction contained relatively pure wild-type enzyme. The absorption spectra of the carbon monoxide bound ferrous complex of mutant 2745 had the Soret peak shifted to 418 nm illustrating the change of the proximal thiolate heme ligand. Wild-type CPO, if not stabilized by

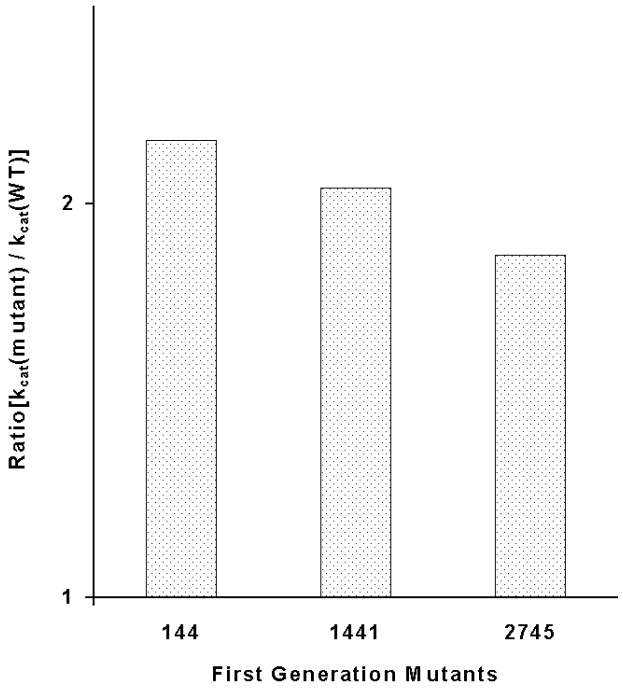

Figure 5. Chlorination activity of mutant chloroperoxidase in ternary solvent. Each bar represents the ratio of the k_{cat} for the three mutant enzymes obtained in the first cycle of directed evolution, with respect to the k_{cat} for the wild-type enzyme in the isopropanol:toluene:aqueous ternary solvent system.

Table 2. Random mutations incorporated for mutants having improved chlorination activity in the toluene:isopropanol:aqueous ternary solvent system.

Amino Acid Position	Directed Evolution Cycles		
	First Generation		
	144	*1441*	*2745*
20	$Pro_{90} \rightarrow Pro$[a]		
25	$Ser_{25} \rightarrow Ser$[b]	$Ser_{25} \rightarrow Ser$[b]	$Ser_{25} \rightarrow Ser$[b]
29	**$Cys_{29} \rightarrow Tyr$**	**$Cys_{29} \rightarrow Tyr$**	**$Cys_{29} \rightarrow His$**
254	$Ser_{254} \rightarrow Ser$[b]	$Ser_{254} \rightarrow Ser$[b]	$Ser_{254} \rightarrow Ser$[b]

[a] Codon change from CCT to CCC.
[b] Codon change from TCT to TCC.

other additives, is rendered inactive in the organic ternary solvent within a few seconds (<5 sec). In contrast, all three first generation mutants were approximately 20-fold more stable in the ternary solvent system than the wild-type enzyme (60 – 100 sec).

Discussion

Improved Epoxidation Activity

The epoxidation and the catalase activities of chloroperoxidase proceed via the same CPO-compound I intermediate (Figure 1). Therefore it is not too surprising to expect that a change in the epoxidation activity will also affect the activity of CPO in the catalase reaction. The two mutants, F-714 from the first generation, and S-5104 from the second generation, illustrate this aspect. In both instances, the improvement in the epoxidation activity was accomplished at the expense of a reduced catalase activity. All the mutations in F-714 and S-5104 lie at the periphery of the protein, far away from the heme catalytic site. In the case of mutant S-5104, the catalase activity was reduced drastically while improving the epoxidation activity 7-fold. It is important to note, however, that two of the first and second round mutants with reduced catalase activity (mutants F-714 and S-5104) carried a change of the valine-18 to alanine indicating a possible role for this valine residue in uncoupling the epoxidase and the catalase reactivities. A third mutant from the second generation (S-4214) also carried the val18 to alanine mutation but had increased catalase activity. This could be due to the other accompanying mutations that may eliminate the effect of the val18 mutation. The exact role of this val18 remains to be investigated. Since there is no change at the active site of the enzyme, it is unlikely that the difference in the epoxidation and the catalase activity is due to a change in the geometry of the oxyferryl-compound I species. It seems more likely then that the mutations are affecting the reaction environment at the heme center in such a way that they facilitate the epoxidation reaction by either binding the alkene for a more efficient oxo-transfer or by facilitating the release of the alkene-epoxide intermediate from the heme group, or a combination of both of these factors.

Improved Chlorination Activity

It has been demonstrated for both the metal-free and the heme-containing haloperoxidases that the primary step of the chlorination reaction is the oxidation of the halide ion to the corresponding hypochlorous acid. The hypochlorous acid then targets different nucleophilic acceptors forming a wide array of halogenated compounds ranging from simpler volatile hydrocarbons[41,42] to complex chiral compounds.[43] In the chloroperoxidase reaction, the oxidation of the halide proceeds via the well-characterized oxyferryl porphyrin π-radical cation species called CPO-compound I.[29,30,31,32] Since the amount of mutant protein produced by each clone was rather small the standard MCD assay was used to quantify the concentration of the mutant protein present in the 24-well plate cultures. Site-directed mutagenesis has previously been used to mutate the cysteine residue to histidine.[44] In the "directed evolution" strategy employed here, except for the mutation in cys29, all of the other mutations in the three mutant clones were silent mutations. A mutation in the proximal cysteine ligand to a histidine residue in cytochrome P450 2E1 has been reported to possess no wild-type activity.[45] In contrast, the cys29 to histidine mutant of CPO (mutant 2745) shows 5% chlorination activity of the wild-type enzyme in the standard MCD assay in the aqueous medium. The same mutant exhibits twice as much chlorination activity of the wild-type enzyme in the ternary organic solvent system. Thus, it is apparent that the cys29 mutation facilitates the chlorination reaction in a non-polar reaction environment. It is possible that the improved chlorination activity and the stability of these mutants in the organic solvent is solely due to the inherent improvement of the activity and stability of the mutant proteins in the organic buffer system. Both cysteine and tyrosine are uncharged amino acids at acidic pH values while histidine is positively charged. Therefore, it is unlikely the electronic charge of the mutated amino acid is the cause of the improved reactivity of the mutants. Since both tyrosine and histidine are larger than the wild-type cysteine residue, the differential packing between the mutant ligand and the porphyrin may provide some flexibility to the protein backbone, thereby improving the activity in the non-polar environment. It will be necessary to produce substantial amounts of the mutant proteins to be able to fully characterize their enhanced activity. Future experiments can also pave the way in resolving the controversial nature of compound I in thiolate-ligated heme proteins. Researchers had earlier predicted that, in thiolate-ligated heme proteins, the radical nature of compound I resides on the thiolate sulfur rather than the porphyrin.[46] It is postulated that the thiolate ligand donates the electron to stabilize the Fe(V)-oxo species, thereby generating the sulfur radical.[46,47] Theoretical studies on the B3LYP hybrid functional have been used to argue for the sulfur-based radical species.[48,49] These studies concluded that the Fe-S bond in B3LYP compound I was lengthened while there was a simultaneous reduction in porphyrin spin densities relative to the imidazole-ligated compound I. These theoretical calculations can now be put to an experimental test with the cys29 mutants.

Conclusion

Despite the wide array of chemical reactions performed by chloroperoxidase, its potential as an industrial biocatalyst is somewhat limited. To overcome some of these restrictions, we have used the strategy of "directed evolution" for the expression of the recombinant CPOs in its natural host, *Caldariomyces fumago*. We have isolated chloroperoxidase mutants that have enhanced epoxidation and chlorination activities. All of the mutants showing improved chlorination activity in the ternary solvent system composed of toluene-isopropanol-water carried a mutation in the cysteine-29 residue which serves as the proximal heme-ligand in wild-type CPO. This is a surprising finding and should prove to be an important factor in further characterization of this biocatalyst.

Experimental Section

General Procedures and Chemicals

Recombinant DNA enzymes were obtained from Boehringer Mannheim (Boehringer Mannheim Corporation, Indianapolis, IN, USA). The DNA purification kits used at various stages of the recombination protocols were from Qiagen (Qiagen Inc. Chatsworth, CA, USA). DNA minipreps were performed using a Promega miniprep kit (Promega Corporation, Madison, WI, USA). All enzymes and DNA isolation and purification kits were used according to the directions supplied by the manufacturer. DNA manipulations, culture growth media, and buffers, unless specified, were as described by Sambrook et al.[50] Where mentioned, chloroperoxidase is purified from the liquid culture medium by –20 °C acetone fractionation. Contaminating proteins and carbohydrate polymer are precipitated by a preliminary 20% acetone fraction. A relatively pure wild-type CPO is obtained by raising the acetone concentration to 70%.

All PCR reactions were carried out in a Perkin Elmer thermal cycler (Perkin Elmer Corporation, Norwalk, CT, USA). The 25 μL reactions were carried out in 0.5-mL GeneAmp PCR tubes (PE Corporation) overlaid with mineral oil. Nonspecific PCR products were eliminated by using "Touchdown PCR" complemented with "Hot Start" PCR technique.[51] The PCR product was purified directly from the PCR mixture using a Qiagen kit.

Chloroperoxidase Activity Assays

MCD Assay: The halogenation activity was determined in the monochlorodimedone (MCD) assay described by Hager et al.[1] Briefly, the CPO catalyzed chlorination of monochlorodimedone (MCD, $\varepsilon_{278} = 1.22 \times 10^4\ M^{-1}\ cm^{-1}$) to dichlorodimedone (DCD, $\varepsilon_{278} = 30\ M^{-1}\ cm^{-1}$), is measured in a 96-well quartz plate containing 0.5 mM MCD, 20 mM potassium phosphate, pH 2.75, 20 mM KCl, and 2 mM H_2O_2 in a total volume of 100 μL. The change in absorbance is recorded over a 1-minute interval at 278 nm. The change in absorbance as a function of time was linear over a 10-fold CPO concentration range in the standard MCD assay (0.001 to 0.01 μM CPO) and in the MCD-ternary solvent system (0.02 to 0.1 μM CPO).

***p*-Nitrostyrene Assay:** *p*-Nitrostyrene was purchased from TCI-America (TCI-America, Portland, OR, USA). *p*-Nitrostyrene oxide was prepared by *m*CPBA oxidation[52] and the product was characterized by gas chromatography. The UV-VIS spectra of both *p*-nitrostyrene and its oxide were compared with those available in the literature.[53,54] The reaction products of CPO mediated conversion of *p*-nitrostyrene ($\varepsilon_{311} = 1.32 \times 10^4\ M^{-1}\ cm^{-1}$) to *p*-nitrostyrene oxide ($\varepsilon_{311} = 80\ M^{-1}\ cm^{-1}$) was subsequently characterized by both gas chromatography and UV-VIS spectrophotometry. The 100-μL epoxidation assay was carried out for 1 minute in a 96-well UV-transparent plate that contained 0.2 mM *p*-nitrostyrene, 100 mM sodium acetate, pH 4.5, and 2 mM H_2O_2.

Catalase Assay: The dismutation of hydrogen peroxide by CPO generates molecular oxygen that can be monitored by the autoxidizable dye, methylene blue. The molecular oxygen reacts with the reduced colorless form of methylene blue producing the oxidized form that has absorbance maxima at 664 nm.[55] Chloroperoxidase is also known to exhibit *N*-dealkylation activity.[56] The *N*-demethylation of methylene blue by lignin peroxidase causes a hypsochromic effect with a shift of the absorbance maxima by 20 – 25 nm.[57] The CPO mediated color change assay showed an unchanged absorbance maxima indicating a reflection of the catalase activity alone (data not shown). A typical 100-μL catalase assay was performed at 664 nm ($\varepsilon_{664} = 7.41 \times 10^4\ M^{-1}\ cm^{-1}$) for 1 minute in a 96-well plate that contained 0.2 mM dithionite-reduced methylene blue, 100 mM sodium acetate, pH 4.5, and 2 mM H_2O_2.

Biocatalytic Removal of Heavy Metals from Petroporphyrin Fraction: Petroporphyrin concentrations are determined by their absorbance at 410 nm.[5] The biocatalytic removal of nickel and vanadium associated with these petroporphyrin fractions was monitored by following the decrease in absorbance at 410 nm in microemulsion comprising of 15% toluene, 60% isopropanol, and 25% potassium phosphate buffer, pH 2.75 using the Mogollon et al assay.[5] The biocatalytic removal of the nickel porphyrin by mutants was compared to that of the wild-type CPO in the ternary solvent system.

Molecular Biology Protocols

Random Mutagenesis: A 25-μL reaction contained 2.5 μL 10X PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 5 mM $MgCl_2$, 0.4 μM each of forward and reverse primers, 2.5 ng template DNA, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dTTP, and 1 mM dGTP.[38,58] In three separate reactions, 1 mM each dATP, dCTP, and dTTP were used respectively, while maintaining the remainder three dNTPs in each reaction at 0.2 mM concentration each. The reaction mixture was overlaid with mineral oil and denatured at 96 °C for 3 min at which point 0.5 units of *Taq Polymerase* (Gibco BRL) was added and mutagenic PCR performed under the following conditions: 5 cycles (denaturation 96 °C, 30 s; annealing 57 °C, 60 s; elongation 72 °C, 1.2 min), 35 cycles (denaturation 94 °C, 30 s; annealing 52 °C, 60 s; elongation 72 °C, 1.2 min) followed by an incubation at 72 °C for 10 min. Following the PCR reaction, the four reactions were

combined together and purified using Qiagen PCR purification kit (Qiagen Inc. Chatsworth, CA, USA). The sequences of the forward and reverse primers were, 5′-TTC TCT ATC - GAT GTT CTC CAA GG-3′ and 5′-GCG GAT CCT TAA AGG TTG CGG GCC-3′, respectively.

Genomic PCR: A 25-μL reaction contained 2.5 μL 10X PCR buffer (400 mM Tris-HCl, 150 mM potassium acetate, pH 9.3, at 25 °C), 5 mM Mg acetate, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dTTP, 0.2 mM dGTP, 0.4 μM each of forward and reverse primers, 5 ng template DNA and 0.5 μL of *Advantage Genomic Polymerase Mix* (Clonetech Laboratories Inc, Palo Alto, CA, USA). The reaction mixture was overlaid with mineral oil, denatured at 96 °C for 3 min and genomic PCR performed under the following conditions: 2 cycles of "Touch-down" PCR performed for annealing temperatures starting at 72 °C to 48 °C in 2 °C decrements (denaturation 96 °C, 30 s; annealing 72 °C, 60 s; elongation 72 °C, 2 min), 15 cycles (denaturation 94 °C, 30 s; annealing 46 °C, 60 s; elongation 72 °C, 1.2 min) followed by an incubation at 72 °C for 10 min. The sequences of the forward and reverse primers were, 5′-CTA TCG ATG TTC TCC AAG GTC CTT CC-3′ and 5′-GCT GGT GAC GGA ATT TTC AT-3′, respectively.

DNA Vectors: Plasmid pTHC contains the CPO gene under the control of its native promoter sequences and a *TrpC* terminator and also contains the hygromycin B phosphotransferase (*hph*) gene[59] under the control of *TrpC* promoter and terminator sequences. The hph gene was obtained as a 3-Kb *EcoR* I/*Xba* I cassette from plasmid pDH25 obtained from Genentech.

***C. fumago* Transformations:** The fungal transformation method is similar to that reported earlier.[38] Briefly, the spheroplasts were generated from a 100-mL culture of finely ground mycelia suspended in 0.8 M sorbitol, 10 mM Bis-Tris buffer, pH 6.5 and 50 mM $CaCl_2$ by digesting the cell walls with Novozym 234 (Sigma Chemical Company, St. Louis, MO, USA). The spheroplasts were collected by differential centrifugation. The concentration of the spheroplasts was adjusted to 1×10^7/mL and a 100-μl aliquot was used to transform a 10-μg preparation of *Sph* I linearized pTHC plasmid. Following a 30 min incubation of the spheroplast-DNA mixture on ice, 0.5-mL aliquots of 30% polyethylene glycol (MW 3325), 10 mM Bis-Tris buffer, pH 6.5 and 50 mM $CaCl_2$ were added three times, at 20 min intervals for a total incubation time of 1 h. After incubation the mixture was suspended in 10 mL of SGMS medium (1 M sorbitol, 4% glucose, 0.5% malt extract and 0.4% each of $NaNO_3$, KH_2PO_4, KCl, and $MgSO_4 \cdot 7\,H_2O$) supplemented with 0.5 mg/mL hygromycin B and 5×10^{-5}% deoxychloate. The transformed spheroplasts were spread on a plastic plate and the hygromycin-resistant cells that grew in 3 – 5 days were collected and subjected to a second round of Novozym 234 digestion and selection in hygromycin medium. This double round of hygromycin selection ensured that the surviving cells contained the *hph* gene integrated into the fungal genome. After the second round of selection, the hygromycin containing SGMS medium was siphoned from the plates, the colonies were washed with 10 mL of SGMS medium and then suspended in the same medium. Aliquots of the suspended cells (500 μL) were spread on potato dextrose agar (PDA) plates (39 g of PDA/L).

Screening of *C. fumago* transformants: Single colony transformants generated after two rounds of hygromycin resistance selection were transferred from the PDA plates to 24-well culture plates containing 3 mL of FS media (10% fructose, 0.2% each of $NaNO_3$, KH_2PO_4, KCl, and $MgSO_4 \cdot 7\,H_2O$, and 0.01% $FeSO_4$). The culture plates were incubated at room temperature with shaking. Aliquots of the recombinant CPO secreted into the culture medium were used directly for MCD chlorination and ABTS [2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)] peroxidation assay.[38] The assays were carried out in a 96-well quartz microplate and the absorbance changes were recorded on a SpectraMax 250 microplate reader (Molecular Dynamics, Sunnyvale, CA). All microplate assays were performed in a total volume of 100 μL. The low concentration of CPO in the 24-well plate culture supernatant fractions necessitated a kinetic assay (MCD chlorination assay) to estimate the concentration of the recombinant enzyme. Based on the activity from the MCD assay, 1, 5, and 10 nmoles of mutant CPO were then used in the subsequent microplate screening assays for the epoxidation and the catalase activities and 1, 5, and 10 pmoles of mutant CPO were used for the chlorination assay in the ternary solvent system. For the screening of the improved epoxidation activity mutants, the data were normalized by the MCD activity slope. We realize that the kinetic assay gave only an approximate enzyme concentration, since any single mutant clone, in theory, is either more active or less active than the wild-type CPO. Nevertheless, the kinetic MCD assay could be used as a guide to approximate the mutant enzyme concentration. For the screening of the improved chlorination activity in the ternary solvent system, any wild-type background is successfully eliminated as the wild-type enzyme is inactivated within a few seconds.

Determination of Random Mutations: The recombinant CPO genes were recovered by PCR amplification. Genomic DNA was isolated from about 50 mg of the mutant fungus using a QiaAmp DNA extraction kit (Qiagen Inc. Chatsworth, CA, USA). The PCR was performed using a set of primers beginning at the start of the CPO coding sequence and terminating in the *TrpC* terminator region of the recombinant DNA. This PCR fragment, approximately 1.4 Kb in length was cloned directly into pPCRScript-Amp vector (Stratagene, La Jolla, CA, USA) for sequencing and used as a template for the next round of PCR-based random mutagenesis.

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