Enantioselective Epoxidation and Carbon–Carbon Bond Cleavage Catalyzed by Coprinus cinereus Peroxidase and Myeloperoxidase*

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We demonstrate that myeloperoxidase (MPO) and Coprinus cinereus peroxidase (CiP) catalyze the enantioselective epoxidation of styrene and a number of substituted derivatives with a reasonable enantiomeric excess (up to 80%) and in a moderate yield. Three major differences with respect to the chloroperoxidase from Caldariomyces fumago (CPO) are observed in the reactivity of MPO and CiP toward styrene derivatives. First, in contrast to MPO, MPO and CiP produced the (S)-isomers of the epoxides in enantiomeric excess. Second, for MPO and CiP the H2O2 had to be added very slowly (10 eq in 16 h) to prevent accumulation of catalytically inactive enzyme intermediates. Under these conditions, MPO hardly showed any epoxidizing activity; only with a high influx of H2O2 (300 eq in 1.6 h) was epoxidation observed. Third, both MPO and CiP formed significant amounts of (substituted) benzaldehydes as side products as a consequence of Cα-Cβ bond cleavage of the styrene derivatives, whereas for CPO and cytochrome c peroxidase this activity is not observed. Cα-Cβ cleavage was the most prominent reaction catalyzed by CiP, whereas with MPO the relative amount of epoxide formed was higher. This is the first report of peroxidases catalyzing both epoxidation reactions and carbon–carbon bond cleavage. The results are discussed in terms of mechanisms involving ferryl oxygen transfer and electron transfer, respectively.

Heme peroxidases are versatile catalysts that perform oxidative reactions of numerous substrates (1). In addition to one-electron oxidations and disproportionation of H2O2, peroxidases also catalyze oxygen transfer reactions, such as the formation of hypohalous acids from halide ions (2), the oxidation of indole to oxindole (3), the oxidation of amines to nitroso derivatives also catalyze oxygen transfer reactions, such as the formation of hypohalous acids from halide ions (2), the oxidation of indole to oxindole (3), the oxidation of amines to nitroso derivatives, which is believed to be a difficult oxygen transfer process (7–10). Direct epoxidation of styrene and its derivatives is only achieved with disubstituted olefins (12, 14). Very high enantioselectivities are obtained with disubstituted olefins (11, 13, 16). Very high enantioselectivities are obtained with disubstituted olefins (11, 13, 16). Very high enantioselectivities are obtained with disubstituted olefins (11, 13, 16).

Enantiopure epoxides are important building blocks in organic synthesis. The development of practical methods for enantioselective epoxidation of unfunctionalized olefins continues to present important challenges in the field of catalysis. Major progress has been made with synthetic catalysts, although there is still much room for improvement (29–33). Peroxidases perform asymmetric epoxidation under mild conditions, but their application in organic synthesis is hampered by the limited solubility of organic reactants in aqueous systems, the oxidative inactivation of the enzyme during catalysis, and the low enantioselectivity sometimes observed in peroxidase-catalyzed reactions. In the past decade, however, using CPO, tremendous progress has been achieved. Thus, styrene derivatives are oxidized to the corresponding (R)-styrene oxides with e.e. amounts up to 96%. As side products, unsubstituted phenylcetaldehydes are formed (11, 13, 16). Very high enantioselectivities are obtained with disubstituted olefins (12, 14).

The mechanisms of epoxidations catalyzed by heme proteins like cytochrome P450, cytochrome c peroxidase, and mutants of HRP and CPO remain unclear. The mechanism of peroxidases in general comprises two consecutive one-electron transfers in the oxidation of organic compounds at the expense of one equivalent of hydrogen peroxide, involving two intermediate forms of the enzyme, compounds I and II, each of which is able to abstract one electron from the substrate to produce a free radical. The free radical may be subjected to coupling, disproportionation, and reaction with molecular oxygen or with another substrate molecule (34). Apart from this mechanism, a direct oxygen transfer from compound I to substrates will bypass compound II and return the enzyme directly to the native state. This is the putative mechanism for CPO in the epoxidation of alkenes, the so-called ferryl-oxygen transfer mechanism.

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¶ Terms of mechanisms involving ferryl oxygen transfer and carbon–carbon bond cleavage. The results are discussed in terms of mechanisms involving ferryl oxygen transfer and electron transfer, respectively.

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(11), comparable with the oxygen transfer mechanism of cytochrome P450. It has also been suggested that electron transfer from the alkene to compound I may occur first, forming a compound II-like species and an intermediate radical cation that can undergo different fates, leading to the formation of epoxides or aldehydes (11, 35–38).

For Cip and MPO, it has been observed that compound II is also formed in the presence of sufficient H₂O₂ via the reduction of compound I by H₂O₂ itself (39, 40). Compound II can, in its turn, react with H₂O₂ to give compound III, which is believed to be a dead end species and catalytically inactive (41). We recently demonstrated that the presence of compound III and an excess of H₂O₂ should be avoided in the enantioselective sulfonation of thioanisole catalyzed by Cip and lactoperoxidase (10).

In the present work, we show that Cip and MPO are also capable of enantioselective epoxidation forming (S)-styrene oxide derivatives, provided that the H₂O₂ influx is slow to such an extent that accumulation of compound II or compound III due to reaction with H₂O₂ is avoided. Moreover, we show that both Cip and MPO are also capable of catalyzing C-α–C-β bond cleavage of (electron-poor) styrene derivatives without the necessity for the presence of a phenol as a co-substrate, as is the case in the cooxidation of styrene by HRP and phenols (27). So far, the only heme peroxidase known to catalyze C-C bond cleavage of nonphenolics has been lignin peroxidase (42, 43).

**Experimental Procedures**

MPO was purified from human leukocytes as described ($A_{420}$/A₂₈₀ = 0.8) (44). Lactoperoxidase was isolated from whey obtained at the Alida cheese farm (Volerendam, The Netherlands) as described ($A_{110}$/A₂₈₀ = 0.9) (10). CPO was purified as described ($A_{420}$/A₂₈₀ = 1.36) (45). Cip concentrations were kindly provided by Dr. T. T. Hansen, Novo Nordisk A/S, Denmark ($A_{420}$/A₂₈₀ = 2.3). Enzyme concentrations were determined using a molar extinction coefficient of 89 M⁻¹ cm⁻¹ at 428 nm for MPO (44); 114 M⁻¹ cm⁻¹ at 412 nm for lactoperoxidase (46); 109 M⁻¹ cm⁻¹ at 405 nm for GIP (39); and 75.3 M⁻¹ cm⁻¹ at 403 nm for CPO (45).

Absorption spectra in the UV-visible range were recorded on a Hewlett Packard 8452A spectrophotometer.

Typical reactions were carried out in 1.67-ml quartz cuvette sealed with a Teflon cap with two capillaries, one to add the hydrogen peroxide solution and one to dispose of the overflow. The cuvettes were completely filled with the reaction mixture in order to prevent partitioning of the substrate into a gas-phase head space. The contents of the cuvette were stirred continuously. General conditions were 20 μM Cip or 2 μM MPO, 100 mM buffer, and 1 mM substrate. During 16 h, hydrogen peroxide was added continuously via a syringe pump (Cole Parmer model 74900-10) with a 250-μl Hamilton syringe with Teflon luer lock connected to the reaction cuvette via a PEEK Tubing 1/32-inch outer diameter, 0.20-inch inner diameter, that went through a capillary in the Teflon cap that sealed the cuvette. The rate of hydrogen peroxide addition was 1 μmol/h. For CPO, the conditions were as described previously (16) with some modifications regarding the scale of the experiment (10 μM CPO, 100 mM buffer, pH 5.5, and 1 mM substrate).

For this enzyme, the rate of hydrogen peroxide addition was 312.5 μmol/h during 1.6 h. To determine a pH optimum, the buffers used were Tris-chloride (pH 7.0, 7.2), Tris-gluconate (pH 7.4), and Tris-phosphate (pH 7.5, 7.6, 7.8, 8.0, 8.15, 8.4, and 8.6) solutions. The Tris-sulfate (pH 8.4), and sodium carbonate (pH 9.5 and 10.0). Sodium acetate (pH 5.0 and 5.5), potassium phosphate (pH 6.0, 6.5, 7.0, and 7.8), Tris-sulfate (pH 8.4), and sodium carbonate (pH 9.5 and 10.0).

To determine the substrate specificity, the reactions were carried out in a potassium phosphate buffer, pH 7.0. After 16 h, 1 μmol of anisaldehyde was added as an internal standard, and then the reaction mixture was extracted twice with 3.4 ml of CH₂Cl₂. The combined organic phases were dried (Na₂SO₄) and concentrated under a stream of nitrogen. To prevent excessive evaporation of the products, the organic phase was not evaporated further than to a volume of about 100 μl. To measure the conversion, the concentrated extracts were analyzed by gas chromatography on a Varian 3400 instrument equipped with a BP X-35 column (0.25 mm × 30 m) (SGE) programmed to run at 80 °C for 17 min and to rise at 15 °C/min to 200 °C. The enantiomeric excess of the epoxides was determined with a Hewlett-Packard 5890 gas chromatograph, equipped with a FID detector, using a Chiraldex G-TA capillary column (0.25 mm × 50 m) programmed to run at 110° for 45 min. The retention times are summarized in Table I. Absolute configurations were determined previously (47). The enantiomeric excesses were double-checked on a CP-cyclodextrin-β-2,3,6-M-19 capillary column (0.25 mm × 50 m) programmed to run at 100 °C for 90 min as described previously (47). Gas chromatography-mass spectrometry measurements were carried out with a HP5973 MSD bench top gas chromatography-mass spectrometer using a CP Wax 52 CB column (25 m × 0.25 mm, phase film thickness = 1.2 μm, Chrompack) programmed to run at 30 °C for 5 min, to rise to 5 °C/min to 180 °C and to run at this temperature for 40 min at a 1 μl/min flow using a 1:40 split ratio. A Programmed Temperature Vaporization, (cis4) injector was used, starting at 50 °C and rising at 12 °C/s to 180 °C. Retention times for the different components were 24, 33.5, and 35 min for styrene, benzaldehyde, and styrene oxide, respectively.

Catalase activities were determined by measuring the rate of oxygen evolution with an oxylight. To calculate the relative amounts of the different compounds present during turnover, a multicomponent analysis method present in the HP 8422 A software was used, which is based on an extension of Beer’s law to m components as follows,

$$A = \sum_{i=1}^{m} E_i c_i d_i \quad \text{(Eq. 1)}$$

where E, and c, denote, respectively, the extinction coefficient and concentration of component i at wavelength λ. The program estimates the parameters by nonlinear least squares. The time-resolved spectra during the epoxidation of styrene by Cip and MPO are considered to be a superposition of the contributions of the spectra of native enzyme, compound I, compound II, and compound III. For MPO, the amount of compound I present during turnover is assumed to be zero, since the formation of compound II from compound I is very fast (48). Therefore, the spectrum of this component was not used in the method of deconvolution. Since the porphyrins are degraded during the oxidative processes and the enzyme thus loses its absorption in the Soret and visual bands, an additional equation was added to this analysis. This equation states that the sum of the relative amounts of the compounds should add up to 1. H₂O₂ solutions were freshly prepared by dilution of a 30% stock solution (Merck). The concentration was determined spectrophotometrically, using an absorption coefficient of 43.6 M⁻¹ cm⁻¹ at 240 nm (49). All other chemicals were of the highest purity. Styrene, styrene oxide, 4-methoxyxystyrene, 4-methylstyrene, α-methylstyrene, benzaldehyde, 2-chlorobenzaldehyde, 3-chlorobenzaldehyde, 4-chlorobenzaldehyde, anisaldehyde, and p-tolualdehyde were from Fluka, and 4-chlorostyrene, 3-chlorostyrene, 2-chlorostyrene, trans-β-methylstyrene, and acetaldehyde were from Aldrich. cis-β-Methylstyrene was from TCI. Epoxides were synthesized as described previously (47).
RESULTS

Determination of Conditions for Epoxidation—In earlier work, we demonstrated the enantioselective sulfoxidation of thioanisole by lactoperoxidase and Coprinus cinereus peroxidase under very special conditions, i.e. a continuous influx of \( \text{H}_2\text{O}_2 \) such that an accumulation of compound III was prevented (10).

Under the same incubation conditions in the presence of styrene, only a minor amount of styrene epoxidation (3% yield) was detected after 3 h, and the enzyme (CiP) was fully present as compound III throughout the reaction and rapidly inactivated (\( t = 1.6 \text{ h} \)). However, when the \( \text{H}_2\text{O}_2 \) influx at pH 7 was lowered 10 times to 1 \( \mu\text{mol}/\text{h} \) and the reaction time was prolonged to 16 h, 18% of the styrene was converted into the epoxide with 20 \( \mu\text{M} \) CiP as a catalyst, and 18% was converted with 2 \( \mu\text{M} \) MPO. For both enzymes, the S-oxide was formed in enantiomeric excess, (35 and 20%, respectively). Incubation of styrene with lactoperoxidase, manganese peroxidase, or HRP under the same conditions generated only very low amounts (yield \( \leq 1\% \)) of the epoxide with low e.e. amounts. It should be noted that styrene oxide is not stable in aqueous solutions but slowly hydrolyzes (\( t \approx 11\text{ h} \) at pH 7) to yield the diol. Therefore, if no hydrolysis would have occurred in the incubations, the amounts of styrene oxide produced would have been higher.

An important side product is benzaldehyde, which is even the major product in the reaction catalyzed by CiP. Another minor side product for MPO, but not for CiP, is phenylacetaldelyde (up to 9% yield at pH 6). Gas chromatography-mass spectrometry provided us with a positive identification of the products present and excluded the formation of phenethylalcohol, which has a retention time identical to phenylacetaldelyde on the BP-X35 column. Monitoring (in time) the enzyme intermediates of CiP and MPO spectroscopically as described (10) during these incubations showed that for CiP the enzyme was present as 89% compound I and 11% compound II or compound III, between which the multicomponent analysis could not discern. For MPO the enzyme was present as 44% native enzyme and 56% compound II. No compound III was formed during these incubations. For CPO, the conditions to obtain epoxidation were very different. This enzyme epoxidizes styrene under a much higher influx of \( \text{H}_2\text{O}_2 \), i.e. 312.5 \( \mu\text{mol}/\text{h} \) (14). Even under these extreme oxidative conditions, the enzyme was fully present in its native state during catalysis. Conversely, CiP hardly produced any epoxides under the mild conditions that were necessary to obtain conversion of styrene by MPO and GP; nor did MPO and CiP form any epoxide under the high \( \text{H}_2\text{O}_2 \) influx conditions as applied for CPO, and under these conditions both CiP and MPO were fully present as compound III and were inactivated within 1 h as deduced from the decrease of the absorption of the Soret bands. A similar experiment to follow the enzyme intermediates of CiP and MPO under the mild conditions that provide styreneoxide in the presence of styrene, but now conducted in the absence of styrene, showed an accumulation of compound III for both enzymes and a more rapid inactivation of the enzymes, as deduced from the decrease of the absorption of the Soret band. This indicates that the presence of styrene protects the enzymes against oxidative inactivation. From the catalase activity of the enzymes, measured in a separate experiment, the concentration of \( \text{H}_2\text{O}_2 \) during turnover could be estimated, as described in a previous paper (10). Thus, for MPO the \( \text{H}_2\text{O}_2 \) concentration was estimated to be 2 \( \mu\text{M} \), and for CiP it was estimated to be 1.6 \( \mu\text{M} \). From our data, it is possible to calculate that for MPO 91% of the total \( \text{H}_2\text{O}_2 \) is lost due to catalase activity, whereas for CiP this is 94%. When the incubations with CiP were quenched after 3, 6, 16, and 24 h, the amount of epoxide formed was linear in time for 6 h for the epoxide, and the amount of benzaldehyde was linear for 16 h (not shown). For MPO, it was observed that the conversion reached completion after 5 h, after which formation of styreneoxide and benzaldehyde stopped (not shown). Moreover, for both enzymes the formation of benzaldehyde and styreneoxide was proportional to the enzyme concentrations (results not shown). CiP showed a broad pH optimum for the formation of styreneoxide around pH 9 and, for the formation of benzaldehyde, showed an optimum around pH 8 (Fig. 1). The enantioselectivity increases with increasing pH up to 56% at pH 10. For this enzyme, the amount of phenylacetaldelyde was negligible. For MPO, the pH optimum for the formation of styreneoxide and for the formation of benzaldehyde are both pH 6.5 (Fig. 2). Only a minor amount of phenylacetaldelyde (\( \leq 10\% \)) is formed between pH 6 and 7, which decreases at higher pH values and is almost absent above pH 8.5 (Fig. 2). The enantioselectivity of the epoxide reaches a maximum at pH 6 (42%) and remains around 20% at other pH values. A small amount of hydrolysis of the epoxides (47) leading to the formation of diols will not influence the enantioselectivity and is therefore not examined in this research. The addition of \( t\)-BuOH as a cosolvent to the incubations with CiP to increase the solubility of the styrene did not lead to an improved conversion or enantioselectivity.

Substrate Specificity—Yields and enantioselectivities of epoxidations of different substrates in the CiP- and MPO-catalyzed reactions are shown in Table II. The yield of the benzaldehyde derivative formed is also shown. The turnover numbers of the conversions can be calculated from this table by dividing the yield by the enzyme concentration. From this it becomes clear that MPO is a more potent catalyst in the epoxidation than CiP. Epoxidation of \( p\)-, \( o\)-, and \( m\)-chlorostyrene derivatives catalyzed by CPO were previously shown to provide low yields and moderate enantioselectivities (13). We investigated the epoxidation of the same derivatives by CiP and MPO. Both CiP and MPO give a good conversion of the \( p\)-chlorostyrene but not of the \( m\)- and \( o\)-isomers. Interestingly, an electron-withdrawing substituent such as chlorine led to an increased formation of the chlorobenzaldehyde. This is not observed for CPO catalyzing the same reaction (\(< 1\%\); not shown). The e.e. amounts of the formed epoxides were low for the \( para\)-chloro substituted epoxide and zero for the \( ortho\) substituted epoxides. For CPO, all reactions were carried out at a high influx of \( \text{H}_2\text{O}_2 \), and larger amounts of the epoxide were produced than for CiP and MPO. For CPO, the (\( R\))-epoxide was

![Fig. 1. pH dependence of the formation of styrene oxide, of its e.e., and benzaldehyde by CiP.](http://www.jbc.org/ Downloaded from http://www.jbc.org/)
the isomer preferentially formed, whereas for CiP and MPO the (S)-epoxide predominated. Epoxidations of p-methyl and p-methoxy substituted styrene derivatives were also attempted. Although with these substrates the formation of the epoxide could not be confirmed, the formation of the benzaldehyde derivatives was significant; 20 \mu M CiP produced a 67% yield of p-tolylaldehyde and 10% of anisaldehyde, and 2 \mu M MPO produced 9 and 8%, respectively.

Table II shows that molecules with extensions on the vinyl side of the alkene were hardly epoxidized by MPO. CiP, however, in correspondence to CPO and cytochrome \textit{c} peroxidase, was capable of converting the cis-\beta-methylstyrene with a high e.e. of 80% (1S,2R), but it was not capable of converting the trans-\beta-methylstyrene. For CPO, the presence of terminal alkenes as substrates leads to the formation of green pigments indicative of the formation of an N-alkylated heme (15). This is not observed for CiP or MPO. In the oxidation of 1,1-disubstituted alkenes such as \alpha -methylstyrene, MPO and CiP formed acetophenone as the major product, and hardly any epoxide was formed.

**DISCUSSION**

In several studies, it has been suggested that the ability of CPO to catalyze asymmetric epoxidations is unique among the peroxidases (16). This peroxidase differs from other peroxidases in three respects. First, it contains a proximal cysteine instead of a histidine, and its sulfur that is bound to the iron facilitates the cleavage of the oxygen—oxygen bond to form compound I and enhances the reactivity of this compound. Second, this enzyme has a distal glutamate instead of a distal histidine, which is positioned in such a way that it does not stabilize compound I, contributing to the enhanced reactivity of compound I toward alkenes (50). Third, this enzyme has a more open active site compared with many peroxidases (21). Yet the steric and electronic restrictions of peroxidases do not unambiguously account for the lessened oxygen transfer activity to alkenes. The controversy can be depicted as follows.

Originally it was believed that only steric restrictions accounted for lack of oxygen transfer by most peroxidases, since it was shown that enlarging the accessibility to the oxoferryl species in the active site of HRP for aromatic substrates by site-directed mutagenesis (Phe\textsuperscript{41} \rightarrow \text{Leu} and Phe\textsuperscript{41} \rightarrow \text{Thr}) led to a mutant enzyme that was capable of enantioselective epoxidation of styrene and cis-\beta-methylstyrene (22). Cytochrome \textit{c} peroxidase, which has a more accessible oxoferryl than HRP, could also epoxidize styrene, but no e.e. was reported for this conversion (20). Cytochrome \textit{c} peroxidase did, however, enantioselectively epoxidize cis-\beta-methylstyrene (32% e.e. of 1R,2S) (20). Later, it was shown that the mere mutation of the distal histidine into a glutamate or a glutamine, which changes the electronic properties of the enzyme, enabled the mutated HRP to epoxidize styrene (24). This illustrates that neither one of the discussed properties (the steric and the electronic) is fully responsible for the lack of epoxidizing activity of native HRP but that these properties are complementary in providing this activity. For CiP that has a relative open active site and that is much more reactive in sulfoxidations than HRP, one would on the basis of the above considerations expect this enzyme to be capable of the epoxidation of styrene. However, in a recent paper (16) it was shown that \textit{Arthromyces Ramosus} peroxidase, which is identical to CiP in both catalytic properties and crystal structure, was not capable of the epoxidation of cis-2-heptene. These incubations were done under the same conditions as those for CPO. When we repeated these incubations for CiP and MPO with styrene as a substrate and followed the enzyme intermediates spectrophotometrically, it was observed that the enzymes were fully present as the catalytically inactive species compound III and that the enzymes were inactivated within 1 h. Decreasing the hydrogen peroxide influx in these incubations with styrene to those values that are in general favorable to obtain a good sulfoxidizing activity, when a sulfide is the substrate instead of an alkene (10), still led to considerable accumulation of compound III. When the hydrogen peroxide influx was lowered again 10-fold to 1 \mu mol/h and the period of incubation was extended to 16 h, favorable conditions were finally obtained in which epoxidation was observed; for CiP, compound I was the major contributor of the enzyme intermediates, and for MPO, compound II was the major contributor. Only under these conditions is styrene converted into styrene oxide and benzaldehyde by these enzymes. Nevertheless, in our hands these mild conditions did not lead to significant conversion of styrene by HRP, lactoperoxidase, and manganese peroxidase (results not shown).

We therefore put forward the following proposal. Peroxidases that are able to epoxidize styrene should possess an active site with an accessibility to such an extent that the substrate can meander around, permitting the possibility of a correct posi-
A more detailed description of possible reaction pathways and putative intermediates has been given before both for heme models and heme proteins (11, 35–38). From these papers, it becomes clear that in many cases the oxygen transfer is a stepwise process, often involving a ferryl oxygen transfer to form the discrete cation intermediate D or radical intermediate E. Also, an intermediate radical-cation C may be involved. Concerted oxygen transfer A (the oxene mechanism) may occur for cytochrome P450-catalyzed oxidations and partly also for CPO.

In order to explain the concomitant formation of both epoxide and phenylacetaldehyde in CPO-catalyzed oxidations, the intermediate presence of a cation D has been postulated to rationalize the formation of phenylacetalddehyde via a hydride shift mechanism (11). The epoxide is either formed via direct oxygen transfer A or via a metallooxetane B (11), although the latter is unlikely for enzymatic conversions due to steric restrictions. In the MPO-catalyzed oxidation of the styrene derivative discussed in the present paper, a minor amount of phenylacetalddehyde is also formed, which makes it likely that intermediate cation D is formed here as well.

The origin of the benzaldehyde formed in the enzymatic oxidation of styrene by HRP, H₂O₂, and 4-methylphenol is not known. Here we postulate that the formation of benzaldehyde may be rationalized via the intermediate presence of F, which is either formed as the free species (M=H) or as an enzyme-bound intermediate (M=Fe).

Since no mechanistic details are available as yet, we can only speculate about the formation of F. It may well be that F is formed via the radical cation intermediate C, the cation intermediate D, or the radical intermediate E. Alternatively, F might be formed from the reaction of the styrene oxide with H₂O₂. The latter mechanism, however, appears to be rather unlikely, since the formation of benzaldehyde and styrene (Fig. 1) occur concomitantly. Moreover, in incubations of CIP with H₂O₂ and styreneoxide, no significant formation of benzaldehyde was observed (results not shown).

An ortho substituent is sterically unfavorable, as can be seen from the low enantioselectivity and yield of the formed epoxide for both CIP and MPO. However, they are still oxidized as can be seen from the significant amount of the formed aldehyde. A chloro substituent in the para position leads to a significant formation of the epoxide and thus does not hamper this conversion sterically. With most substrates, there is a striking difference between MPO and CIP in the formation of the epoxide with respect to the amount of benzaldehyde derivative formed. It appears that in the CIP-catalyzed oxidations intermediate F plays an important role compared with the MPO oxidation, which might involve a more concerted oxygen transfer. Molecules with extensions on the vinyl group of styrene cannot be converted by MPO, indicating that on this side of the molecule the enzyme offers a steric restriction. cis-β-Methylstere is, however, a fair substrate for CIP, yielding a high enantioselectivity of 80% of the 1S,2R isomer. Surprisingly, this is the same isomer as the one that is produced by CPO, whereas for all other mentioned substrates CIP and MPO produce the opposite isomer with respect to CPO. These results can be compared with the epoxidation reactions catalyzed by mutants of HRP. F41L HRP gives 46% e.e. (R-configuration) of styrene oxide, and F41T HRP gives 2% e.e. of (S)-styrene oxide. The latter mutant gives 99% e.e. of the 1S,2R configuration in the epoxidation of cis-β-methylstyrene (22). Finally, 1,1-disubstituted alkenes such as α-methylstyrene could be oxidized to acetophenone by MPO and CIP, but not to the epoxide, in contrast to CPO.

Conclusion—We show here that the peroxidases CIP and
MPO are able to perform epoxidation of styrene and derivatives of this molecule, provided that the proper incubation conditions are chosen. This implies a very slow addition of hydrogen peroxide for both enzymes in contrast to CPO. Another important difference is that MPO and CIP preferentially form the S-enantiomer of styrene and para substituted derivatives, whereas CPO produces the R-isomer, with the exception of cis-β-methyl styrene, where the same stereochemistry is observed as with CPO. Remarkably, also carbon–carbon cleavage reactions are catalyzed by both CIP and MPO. So far, with regard to peroxidase catalysis, only for lignin peroxidase has C–C bond cleavage of nonphenolic substrates been reported (43). However, the mechanisms of these cleavage reactions are fundamentally different, despite the fact that the crystal structures of CIP and lignin peroxidase are very similar (51, 52). Further studies are necessary to probe the mechanistic details of these unique conversions. In this respect, the CIP peroxidase promises to be a highly interesting biocatalyst. The enzyme is available on a large scale (from Novo Nordisk), and it has already been shown that mutants may be obtained via directed evolution techniques (53). This may also be the way to obtain an improved biocatalyst for application in epoxidation reactions.

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