Enantioselective oxygen-transfer reactions catalyzed by peroxidases

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Chapter 6  Enantioselective epoxidation and carbon-carbon bond cleavage catalyzed by Coprinus cinereus peroxidase and myeloperoxidase

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Abstract

We demonstrate that myeloperoxidase (MPO) and Coprinus cinereus peroxidase (CiP) catalyse 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 towards styrene derivatives. First, in contrast to CPO, MPO and CiP produced the (S)-isomers of the epoxides in enantiomeric excess. Second, for MPO and CiP the \( \text{H}_2\text{O}_2 \) had to be added very slowly (10 eq. in 16 hrs.) to prevent accumulation of catalytically inactive enzyme intermediates. Under these conditions CPO hardly showed any epoxidising activity, only with a high influx of \( \text{H}_2\text{O}_2 \) (300 eq. in 1.6 hrs.) epoxidation was observed. Third, both MPO and CiP formed significant amounts of (substituted) benzaldehydes as side products as a consequence of \( \text{C}\alpha-\text{C}\beta \) bond cleavage of the styrene derivatives, whereas for CPO and cytochrome \( \text{c} \) peroxidase (CcP) this activity is not observed. \( \text{C}\alpha-\text{C}\beta \) cleavage was the most prominent reaction catalysed by CiP, whereas with MPO the relative amount of epoxide formed was higher. This is the first report of peroxidases catalysing both epoxidation reactions and carbon-carbon bond cleavage. The results are discussed in terms of mechanisms involving ferryl oxygen-transfer and electron-transfer, respectively.

Introduction

Haem peroxidases are versatile catalysts that perform oxidative reactions of numerous substrates [1]. In addition to one-electron oxidations and disproportionation of \( \text{H}_2\text{O}_2 \) also oxygen transfer reactions, such as the formation of hypohalous acids from halide ions [2], the oxidation of
Enantioselective epoxidation and C-C bond cleavage

Enantioselective epoxidation and C-C bond cleavage and the enantioselective sulphoxidation of sulphides have been reported [7-10]. Direct epoxidation of styrene and its derivatives, which is believed to be a difficult oxygen-transfer process due to the higher redox potential of alkenes, has only been reported for CPO from 

Caldariomyces fumago [11-19] and cytochrome c peroxidase (CcP) [20]. CPO has an active site, containing a distal glutamate and a proximal cysteine that differs from classical peroxidases that contain both a distal and a proximal histidine [21]. CcP has a more accessible haem iron than the other classical peroxidases and catalyses the enantioselective epoxidation of cis-β-methylstyrene [20]. By site-directed mutagenesis it was possible to convert horseradish peroxidase (HRP), that in the wild type form only generates trace amounts of epoxide, into a species having good peroxygenase activity [22, 26]. However, native HRP will oxidise styrene in the presence of H₂O₂ to form styrene oxide and benzaldehyde, only when 4-methylphenol is present via a cooxidation mechanism [27]. MPO has previously been shown to perform the epoxidation of butadiene [28], but so far no reports about enantioselective epoxidations by this enzyme have been reported.

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, oxidative inactivation of the enzyme during catalysis and the low enantioselectivity sometimes observed in peroxidase catalysed reactions. In the past decade, however, using CPO tremendous progress has been achieved. Thus, styrene derivatives are oxidised to the corresponding (R)-styrene oxides with e.e.'s up to 96%. As side products phenylacetaldehydes are formed [11, 13, 16]. Very high enantioselectivities are obtained with disubstituted olefins [12, 14].

The mechanisms of epoxidations catalysed by haem proteins like cytochrome P450, CcP, 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 [11], comparable to the oxygen-transfer mechanism of cytochrome P450’s. 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 H$_2$O$_2$ via the reduction of compound I by H$_2$O$_2$ itself [39, 40]. Compound II can on its turn react with H$_2$O$_2$ 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 H$_2$O$_2$ should be avoided in the enantioselective sulfoxidation of thioanisole catalysed by CiP and LPO [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 H$_2$O$_2$ influx is slow to such an extent, that accumulation of compound II or compound III due to reaction with H$_2$O$_2$ is avoided. Moreover, we show that both CiP and MPO are also capable of catalysing Cα-Cβ 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 co-oxidation of styrene by HRP and phenols [27]. So far, the only haem peroxidase known to catalyse C-C bond cleavage of non-phenolics has been lignin peroxidase [42,43].

**Experimental procedures**

MPO was purified from human leukocytes as described ($A_{428\text{ nm}/280\text{ nm}} = 0.8$) [44]. LPO was isolated from whey obtained at the Alida cheese farm in Volendam (The Netherlands) as described ($A_{412\text{ nm}/280\text{ nm}} = 0.9$) [10]. CPO was purified as described ($A_{403\text{ nm}/280\text{ nm}} = 1.36$) [45]. CiP was
Enantioselective epoxidation and C-C bond cleavage

kindly provided by Dr. T.T. Hansen, Novo Nordisk A/S, Denmark. \((A_{405\text{ nm}}/280\text{ nm}) = 2.3\). Enzyme concentrations were determined using a molar extinction coefficient of 89 \(\text{mM}^{-1}\text{cm}^{-1}\) at 428 nm for MPO [44]; 114 \(\text{mM}^{-1}\text{cm}^{-1}\) at 412 nm for LPO [46]; 109 \(\text{mM}^{-1}\text{cm}^{-1}\) at 405 nm for CiP [39] and 75.3 \(\text{mM}^{-1}\text{cm}^{-1}\) at 403 nm for CPO [45]. Absorption spectra in the UV-Vis range were recorded on a Hewlett Packard 8452 A spectrophotometer.

Typically reactions were carried out in 1.67 ml quartz cuvet sealed with a Teflon cap with two capillaries; one to add the hydrogen peroxide solution and one to dispose of the overflow. The cuvets were completely filled with the reaction mixture in order to prevent partitioning of the substrate into a gas-phase headspace. The contents of the cuvet were stirred continuously. General conditions were 20 \(\mu\text{M}\) CiP or 2 \(\mu\text{M}\) MPO, 100 mM buffer and 1 mM substrate. During 16 hours hydrogen peroxide was added continuously via a syringe pump (Cole Parmer 74900-10) with a 250 \(\mu\ell\) Hamilton syringe with Teflon luer lock connected to the reaction cuvet via a PEEK Tubing 1/16” OD, 0.20”ID, that went through a capillary in the Teflon cap that sealed the cuvet. The rate of hydrogen peroxide addition was 1 \(\mu\text{mol} / \text{hr}\). For CPO the conditions were as described previously [16] with some modifications regarding the scale of the experiment (10 \(\mu\text{M}\) CPO, 100 mM, buffer pH 5.5 and 1 mM substrate). For this enzyme the rate of hydrogen peroxide addition was 312.5 \(\mu\text{mol}\)/hr. during 1.6 hrs. To determine a pH optimum the buffers used were 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 pH 10.0). To determine the substrate specificity, the reactions were carried out in a potassium phosphate buffer pH 7.0. After 16 hours 1 \(\mu\text{mol}\) of anisylaldehyde was added as internal standard and then the reaction mixture was extracted with two times 3.4 ml \(\text{CH}_2\text{Cl}_2\). The combined organic phases were dried (\(\text{Na}_2\text{SO}_4\)) and concentrated under a stream of nitrogen to a volume of about 100 \(\mu\ell\) to prevent excessive evaporation of the products. 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 x 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 x 50 m) programmed to run at 110°C for 45 min. The retention times are summarised in Table 1. 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° for 90 min as described before [47]. GC-MS measurements were carried out with a HP5973 MSD bench top GC-MS using a CP Wax 52 CB column (25 m × 0.25 mm, df = 1.20 µm, Chrompack) programmed to run at 30°C for 5 min., to rise at 5°C/min. to 180°C and to run at this temperature for 40 min. at a 1 ml/min. flow using a 1 : 40 split ratio. A Programmed Temperature Vaporisation, (cis 4) injector was used, starting at 50°C and rising at 12°C/sec to 180°C. Retention times for the different components were 24 min., 33.5 min. and 35 min. for styrene, benzaldehyde and styrenoxide, respectively.

Table 1: Retention times of the different substrates and products. For the epoxide enantiomers the retention times on the ChiralAx-GTA column are shown (GTA). For the other compounds the retention times on the BP-X35 column are shown (BP).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Substrate</th>
<th>Epoxide</th>
<th>Benzaldehyde</th>
<th>Phenylacetalddehyde</th>
<th>Epoxide</th>
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<tr>
<td>CI</td>
<td>CI</td>
<td>4.0</td>
<td>13.8</td>
<td>7.7</td>
<td>13.1</td>
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<tr>
<td>CI</td>
<td>CI</td>
<td>11.5</td>
<td>22.5</td>
<td>18.9</td>
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<tr>
<td>Cl</td>
<td>Cl</td>
<td>11.9</td>
<td>22.2</td>
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<tr>
<td>Cl</td>
<td>Cl</td>
<td>12.4</td>
<td>22.5</td>
<td>20.0</td>
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<td>CI</td>
<td>CI</td>
<td>6.7</td>
<td>17.8</td>
<td>7.7</td>
<td>n.d.</td>
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Catalase activities were determined by measuring the rate of oxygen evolution with an oxygraph. To calculate the relative amounts of the different compounds present during turnover, the multicomponent analysis method present in the HP 8452 A software was used, as described previously [10]. 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 one. H$_2$O$_2$ 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, styreneoxide, 4-methoxystyrene, 4-methylstyrene, α-methylstyrene, benzaldehyde, 2-chlorobenzaldehyde, 3-chlorobenzaldehyde, 4-chlorobenzaldehyde, anisylaldehyde, p-tolylaldehyde were from Fluka, 4-chlorostyrene, 3-chlorostyrene, 2-chlorostyrene, trans-β-methylstyrene, acetophenone from Aldrich. Cis-β-methylstyrene was from TCI. Epoxides were synthesised as described previously [47].

Results

Determination of conditions for epoxidation

In earlier work we demonstrated the enantioselective sulphoxidation of thioanisole by lactoperoxidase (LPO) and Coprinus cinereus peroxidase under very special conditions i.e. a continuous influx of H$_2$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 hrs and the enzyme (CiP) was fully present as compound III throughout the reaction and rapidly inactivated (t$_{1/2}$=1.6 hrs). However, when the H$_2$O$_2$ influx at pH 7 was lowered 10 times to 1 μmol / hr. and the reaction time prolonged to 16 hrs., 18 % of the styrene was converted into the epoxide with 20 μM CiP as a catalyst and 18 % with 2 μM MPO. For both enzymes the S-oxide was formed in enantiomeric excess, (35 and 20 %,
respectively). Incubation of styrene with LPO, MnP or HRP under the same conditions generated only very low amounts (yield ≤1%) of the epoxide with low e.e.’s.

An important side product is benzaldehyde, which is even the major product in the reaction catalysed by CiP. Another minor side product for MPO, but not for CiP, is phenylacetaldehyde (up to 9% yield at pH 6). GC-MS provided us with a positive identification of the products present and excluded the formation of phenethylalcohol, which has a retention time identical to phenylacetaldehyde on the BP-X35 column. Monitoring the enzyme intermediates of CiP and MPO spectroscopically as described [10] during these incubations in time 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 epoxidises styrene under a much higher influx of H₂O₂ i.e. 312.5 μmol per hour [14]. Even under these extreme oxidative conditions the enzyme was fully present in its native state during catalysis. Conversely, CPO hardly produced any epoxides under the mild conditions that were necessary to obtain conversion of styrene by MPO and CiP. Neither did MPO and CiP form any epoxide under the high H₂O₂ influx conditions as applied for CPO, and under these conditions both CiP and MPO were fully present as compound III and were inactivated within one hour 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 H₂O₂ during turnover could be estimated, as described in a previous paper [10]. Thus for MPO the H₂O₂ concentration was estimated to be 10 μM and for CiP 1.6 μM. From our data it is possible to calculate that for MPO 91% of the total H₂O₂ 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 hrs. the amount of epoxide formed was linear in time for 6 hrs. for the epoxide and the amount of benzaldehyde for 16 hrs. (not shown). For MPO it was observed that the conversion reached
completion after 5 hrs., after which formation of styreneoxide and benzaldehyde stopped (not shown). Moreover, for both enzymes the formation of benzaldehyde and styreneoxide were 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 an optimum around pH 8 (Fig.1).

![Graph showing pH dependence of the formation of styreneoxide, its e.e. and benzaldehyde by CiP.](image)

Fig.1. pH dependence of the formation of styreneoxide, its e.e. and benzaldehyde by CiP.

- ■, styreneoxide; ○, e.e.; □, benzaldehyde.

The enantioselectivity increases with increasing pH up to 56 % at pH 10. For this enzyme the amount of phenylacetaldehyde was negligible. For MPO the pH optimum for the formation of styreneoxide and for the formation of benzaldehyde is both pH 6.5 (Fig 2).

Only a minor amount of phenylacetaldehyde (≤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 enantioslectivity 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. 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.

![Graph showing pH dependence of styrene oxide, e.e., benzaldehyde, and phenylacetaldehyde formation by MPO.](image)

**Fig. 2.** pH dependence of the formation of styrene oxide, its e.e. and of benzaldehyde and of phenylacetaldehyde by MPO.

- ■, styrene oxide; ●, e.e.; □, benzaldehyde; Δ, phenylacetaldehyde.

**Substrate specificity**

Yields and enantioselectivities of epoxidations of different substrates in the CiP and MPO catalysed reactions are shown in Table 2. 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-chloro substituted styrene derivatives catalysed by CPO were previously shown to provide low yields and moderate enantioselectivities [12]. 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 could lead to an increased formation of the chlorobenzaldehyde. This is not observed for CPO catalysing the same reaction (<1 %, not
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The e.e.'s 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 H₂O₂ and larger amounts of the epoxide were produced than for CiP and MPO. For CPO the (R)-epoxide was 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 μM CiP produced 67% yield of p-tolualdehyde and 10% of anisyaldehyde and 2 μM MPO produced 9% and 8%, respectively.

Table 2 shows that molecules with extensions on the vinyl side of the alkene were hardly epoxidised by MPO. CiP however, in correspondence to CPO and CcP was capable of converting the cis-β-methylstyrene with a high e.e. of 80% (1S,2R), but it was not capable of converting the trans-β-methylstyrene. For CPO, the presence of terminal alkenes as substrates leads to the formation of green pigments indicative of the formation of a N-alkylated haem[15]. This is not observed for CiP or MPO. In the oxidation of 1,1-disubstituted alkenes such as α-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 catalyse asymmetric epoxidations is rather unique among the peroxidases [16]. This peroxidase differs from other peroxidases in three respects. First, it contains a proximal cysteine ligand 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 stabilise compound I, contributing to the enhanced reactivity of compound I towards alkenes [50]. Third, this enzyme has a more open active site compared to 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-41 Leu and Phe-41 Thr), led to a mutant enzyme that was capable of enantioselective epoxidation of styrene and cis-β-methylstyrere [22]. CcP, which has a more accessible oxoferryl than HRP, could also epoxidise styrene, but no e.e. was reported for this conversion [20]. CcP did however enantioselectively epoxidise cis-β-methylstyrere (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 HRP mutant to epoxidise styrene [24]. This illustrates that
neither one of the discussed properties (the steric and the electronic) is fully responsible for the lack of epoxidising 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 sulphoxidations than HRP, one would on 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 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 carried out 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 one hour. Decreasing the hydrogen peroxide influx in these incubations with styrene to those values that are in general favourable to obtain a good sulphoxidising activity, when a sulphide 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 μmol / hr. and the period of incubation extended to 16 hrs, finally favorable conditions were obtained in which epoxidation was observed and for CiP compound I was the major contributor of the enzyme intermediates and for MPO compound II. Only under these conditions styrene is significantly converted into styreneoxide and benzaldehyde by these enzymes. Nevertheless, in our hands these mild conditions did not lead to significant conversion of styrene by HRP, LPO and manganese peroxidase (MnP) (results not shown).

We would therefore like to put forward the following proposal. Peroxidases that are capable to epoxidise 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 positioning with regard to the oxoferryl species (CcP, CiP and MPO but not HRP, MnP and LPO). Further, for those peroxidases possessing epoxidising capabilities from a steric point of view, the experimental conditions should be properly chosen i.e. prevent the accumulation of compound III. The fact that MPO is about ten times as active as CiP in the epoxidation of styrene, can probably be accounted for by the higher reactivity or higher redox potential of compound I, that for MPO has a very short lifetime [40].

The formation of benzaldehyde as a side product in the epoxidation of alkenes, which is observed for both CiP and MPO (Table 2) has been described before only for HRP in the co-oxidation of
Chapter 6

styrene with 4-methylphenol [27] and in the direct epoxidation of styrene by some HRP mutants [22,25,26]. Noteworthy, it was specifically stated that both CPO [11] and CcP [20] catalysed oxidations of styrene did not lead to benzaldehyde formation. For the HRP mutants it was stated that benzaldehyde probably was formed via an enzyme-independent mechanism. However, our experiments clearly showed that the amount of benzaldehyde formed is proportional to the enzyme concentration used. In addition, formation of benzaldehyde and styreneoxide occurred simultaneously and the ratio of the products is different for CiP and MPO. These facts are strong indications of an enzyme dependent process in the formation of benzaldehyde. This is interesting since sofar only lignin peroxidase (LiP) [42,43] has been reported to be able to cleave C-C bonds.

Mechanistic considerations

No mechanistic rationale has been formulated yet for the benzaldehyde formation and we have to consider the various mechanisms, which have been proposed for the mechanism(s) of epoxidation by cytochrome P450 and haem peroxidases. In scheme 1 a number of postulated intermediates and transition states are given.

Scheme 1: Possible intermediates of peroxidases in the oxidation of alkenes.
Enantioselective epoxidation and C-C bond cleavage

A depicts a concerted oxygen transfer. B is a metallo-oxetane intermediate. C is a radical-cation intermediate formed by a one-electron abstraction by compound I from the alkene and preceding the oxygen transfer from a compound II-like species. D is a cation intermediate; such an intermediate bearing a positive charge on the Cα and which is attached to an Fe(III)oxo species via its Cβ can be formed from the collapse of the radical-cation intermediate C. E is a radical intermediate; such an intermediate with a radical on the Cα and which is attached to an Fe(IV)oxo species via its Cβ can also be formed from the collapse of the radical-cation intermediate C. F is a “M-O - Cβ-Cα-(R)-OOH” species, which may be formed by the attack of H₂O₂ (and H₂O if necessary) on the intermediates C, D or E or on the epoxide.

A more detailed description of possible reaction pathways and putative intermediates has been given before both for haem models and haem 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 catalysed oxidations and partly also for CPO.

In order to explain the concomitant formation of both epoxide and phenylacetaldehyde in CPO catalysed oxidations the intermediate presence of a cation D has been postulated to rationalise the formation of phenylacetaldehyde 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 catalysed oxidation of the styrene derivatives discussed in the present paper a minor amount of phenylacetaldehyde 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 of 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 
H\textsubscript{2}O\textsubscript{2} and styrene 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 oxidised as can be seen 
from the significant amount of the formed aldehyde. A chloro substituent on 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 
catalysed oxidations intermediate F plays a more important role compared to the MPO oxidation, 
which might involve a more concerted oxygen-transfer. Molecules with extensions on the 
vinylogroup of styrene can not be converted by MPO, indicating that on this side of the molecule 
the enzyme offers a steric restriction. Cis-\(\beta\)-methylstyrene 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 catalysed 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-\(\beta\)-methyl styrene [22]. Finally, 1,1-
disubstituted alkenes such as \(\alpha\)-methylstyrene could be oxidised 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 preferably form the \(S\)-enantiomer of styrene and \(para\) 
substituted derivatives whereas CPO produces the \(R\)-isomer, with the exception of cis-\(\beta\)-methyl 
styrene where the same stereochemistry is observed as with CiP. Remarkably, also carbon-carbon 
cleavage reactions are catalysed by both CiP and MPO. Sofar, with regard to peroxidase catalysis
only for LiP C-C bond cleavage of non-phenolic substrates has been reported [43]. However, the mechanisms of these cleavage reactions are fundamentally different, despite the fact that the crystal structures of CiP and LiP 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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References
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