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Journal of Molecular Catalysis B: Enzymatic 6 (1999) 453–461

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

# Selective oxidations with molecular oxygen, catalyzed by chloroperoxidase in the presence of a reductant

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Received 10 December 1997; accepted 11 June 1998

**Abstract**

Chloroperoxidase (CPO) catalyzes the oxidation of various substrates with molecular oxygen as the primary oxidant, in the presence of dihydroxyfumaric acid (DHF) as a sacrificial reductant. For example, indole is oxidized to 2-oxindole with up to 77% selectivity and thioanisole to the corresponding *R*-sulfoxide (e.e. $>99\%$). To our knowledge, these are the first examples of (enantio)selective aerobic oxidations catalyzed by peroxidases. A mechanism is proposed which involves initial formation of hydrogen peroxide via autoxidation of DHF. CPO subsequently uses the hydrogen peroxide for the selective oxidation of the substrate, via an oxygen transfer mechanism. In contrast, horseradish proxidase (HRP) primarily catalyzes the oxidation of DHF via a classical peroxidase mechanism and oxidations of added substrates are aselective. 

*Keywords:* Chloroperoxidase; Aerobic oxidation; Hydroxyl radicals

## 1. Introduction

Chloroperoxidase (CPO: EC 1.11.1.10) from *Caldariomyces fumago* catalyzes oxidative chlorination in vivo. In vitro, in the absence of $Cl^-$, CPO catalyzes a variety of synthetically useful oxidations with $H_2O_2$ [1], e.g., asymmetric epoxidation of olefins [2,3], benzylic and allylic hydroxylation [4,5], asymmetric sulfoxidation [6–8], and oxidation of indoles to oxindoles [9,10].

These (asymmetric) oxidations are generally assumed to proceed via an oxygen transfer reaction between the active enzyme intermediate (an iron(IV) oxo porphyrin radical cation, compound I) and the substrate. In contrast, a classical peroxidase reaction involves two separate electron transfer reactions from two substrate molecules to compound I (Fig. 1). The peroxidase high-valent iron oxo intermediate is formed upon oxidation of the native enzyme with $H_2O_2$. In the formation of the analogous active species in the monooxygenase catalytic cycle, reduction with a cofactor and oxidation with molecular oxygen are involved (Fig. 2) (see Ref. [11]).

A serious shortcoming of CPO and all heme-containing peroxidases is their low operational stability, which is caused by rapid deactivation by hydrogen peroxide [12], even at low concentrations (30 μM). Deactivation involves oxidation of the porphyrin ring, but its precise

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PII: S1381-1169(99)00059-X

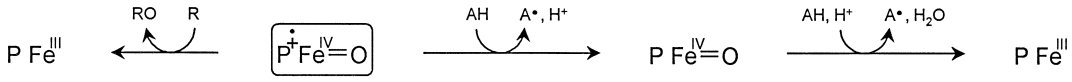



Fig. 1. The two reaction modes of compound I: oxygen transfer (left side), and electron transfer (right side) (P = protoporphyrin IX).

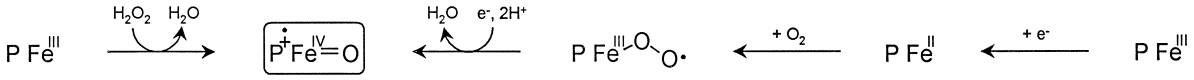



Fig. 2. Two ways of compound I formation: the peroxidase route (left side), and the monooxygenase mechanism (right side) (P = protoporphyrin IX).

mechanism is not clear. The life-time of the catalyst can be extended by maintaining a low $H_2O_2$ concentration, either by continuous addition [13] or, preferably, by *feed-on-demand* [12]. To avoid high *local* concentrations $H_2O_2$ can be generated in situ, for example by the oxidation of glucose mediated by glucose oxidase [14].

One intrinsically attractive approach to circumvent deactivation is to replace hydrogen peroxide with the combination of molecular oxygen and a sacrificial reductant, i.e., to use a peroxidase in a monooxygenase reaction mode. It was already shown in 1957 that horseradish peroxidase (HRP), in the presence of oxygen and dihydroxyfumaric acid (DHF) as a cosubstrate (sacrificial reductant), catalyzes the hydroxylation of a number of aromatic compounds [15,16]. Klibanov et al. [17] used this procedure for the synthesis of, for example, L-3,4-dihydroxyphenylalanine (L-DOPA). Two different mechanisms were proposed to account for these results. Both mechanisms involve hydroxyl radicals as the oxidizing species, but differ in the way in which these are generated: (a) by an oxidized species of HRP that results from the oxidation of the native enzyme by superoxide anion [18,19], or (b) non-enzymatically from DHF radicals which are, in turn, formed in a classical peroxidase reaction [20–22].

Our interest in the use of DHF/$O_2$ was two-fold: (a) as a model to test the possibility of using CPO/$O_2$ for selective oxygen transfer reactions and (b) to gain insight into the mechanism of these transformations.

## 2. Experimental

### 2.1. Material and analytical methods

CPO from *C. fumago* was isolated and purified in this laboratory as described in literature [13]. The enzyme preparation (30.6 μM) contained 3000 U $ml^{-1}$ (standard monochlorodimedone assay as described by Morris and Hager [23]) with a purity of $R_z = 1.3$ ($R_z = A_{400}/A_{280} = 1.44$ for pure CPO). Microperoxidase-11 (MP-11), superoxide dismutase (SOD; from bovine erythrocytes), and catalase (from bovine liver) were purchased from Sigma. Lactoperoxidase (LPO) and HRP were received as a gift from Boehringer Mannheim. Soybean peroxidase (SBP) was received as a gift from Enzymol International.

DHF, indole, oxindole, thioanisole, phenol, hydroquinone, resorcinol, catechol, sodium dithionite, copper(I) cloride, chromium(II) chloride and 1,2-diphenylhydrazine were purchased from Aldrich Chemical. L-Ascorbic acid was purchased from Fisher Scientific. Trolox™-C (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was purchased from Fluka. Racemic thioanisole sulfoxide was prepared by chemical oxidation as reported by Drabowicz et al. [24].

Samples for analyzing indole, phenol, and thioanisole oxidation were diluted with a methanol/*tert*-butyl alcohol mixture of 50:50 (v/v) or with methanol, in case of the oxidation of thioanisole. After centrifugation, the samples were analyzed on reversed phase HPLC using a

custom-packed Symmetry $C_{18}$ cartridge (Waters Radial-Pak, 8 × 100 mm, 7 μm) contained in a Waters RCM 8 × 10 compression unit, eluent flow 1.0 ml $min^{-1}$, with simultaneous detection on a Waters 410 differential refractometer and a Waters 486 tunable absorbance detector at 260, 254 and 220 nm, respectively. *tert*-Butyl alcohol or 1,2,3-trimethoxybenzene were used as internal standard. Methanol/water mixtures of 60:40 (v/v), 25:75 (v/v) and acetonitrile/water 45:55 (v/v), respectively, were used as eluent. Samples for analyzing DHF conversion were centrifuged and analyzed on reversed phase HPLC using a custom-packed Nucleosil $C_{18}$ cartridge (Waters Radial-Pak, 8 × 100 mm, 10 μm), contained in a Waters RCM 8 × 10 compression unit, eluent flow 1.0 ml $min^{-1}$, with simultaneous detection on a Waters 410 differential refractometer and a Waters 486 tunable absorbance detector at 280 nm. Acetate was used as internal standard and the eluent was water containing 0.25% trifluoroacetic acid. Samples for analyzing the enantioselectivity of the oxidation of thioanisole were diluted with a hexane/isopropanol mixture of 85:15 (v/v) and dried over $Na_2SO_4$. After centrifugation, the samples were analyzed on chiral HPLC using a Chiralcel OD column (Daicel Chemical Industries, 250 × 4.6 mm), eluent flow 0.5 ml $min^{-1}$, and detected on a Waters 486 tunable absorbance detector at 220 nm. A hexane/isopropanol mixture of 85:15 (v:v) was used as eluent.

A Metrohm Dosimat 665 was used for continuous addition of reagents. UV measurements were carried out on a Cary 3 spectrophotometer from Varian.

### 2.2. General experiment with molecular oxygen

The substrate (0.05 mmol indole or thioanisole, or 0.10 mmol phenol) was dissolved in 10 ml of buffer (acetate, 0.1 M pH 5.0). Enzyme solution (100 μl; concentration of CPO: 30.6 μM; MP-11: 1.0 mg $ml^{-1}$; HRP: 5.5 mg $ml^{-1}$; LPO: 5.0 mg $ml^{-1}$; SBP: 8.1 mg $ml^{-1}$) was added to the reaction mixture, followed by 5 min of stirring under 1 atm $O_2$ at room temperature or at 4°C. The reaction was started by the addition of 2.5 eq of reductant (DHF, ascorbic acid, or diphenylhydrazine). Samples for monitoring the reaction were taken every 5 and 15 min, respectively, for phenol and the other substrates and analyzed by HPLC.

### 2.3. Competition experiments

Competition experiments were carried out at 0°C under continuous sparging with argon. Indole (0.11 mmol) was dissolved in 20 ml of buffer (acetate, 0.1 M pH 5.0) and stirred for 10 min. Enzyme solution (200 μl; concentrations see Section 2.2) and DHF (0.17 mmol) were added. After 5 min of stirring the addition of $H_2O_2$ (1.0 M in water; saturated with Ar) was started, at a rate of 1 μl $min^{-1}$; total volume 0.11 ml. Samples were taken every 15 min and analyzed directly for the DHF concentration. Samples for indole conversion were quenched with sodium sulfite and diluted with a 50:50 (v/v) methanol/*tert*-butyl alcohol mixture.

### 2.4. UV-spectra of reduced CPO

A buffer solution (acetate, 0.1 M pH 5.0) was cooled to 4°C, saturated with argon and stored under Ar. A cuvette was charged with 2.5 ml buffer and 0.5 ml of CPO solution and sparged with argon for 3 min. CPO was reduced by adding 200 μl sodium dithionite solution (200 mg in 3 ml, saturated with Ar) followed by 2 min of sparging with Ar. In the same way CPO was treated with 200 μl of a saturated DHF solution. After the spectrum was recorded, argon was replaced by oxygen.

## 3. Results

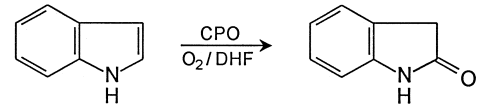
### 3.1. Phenol hydroxylation

We first investigated the hydroxylation of phenol, since most of the published work on the oxygen/DHF system was focused on this reac-

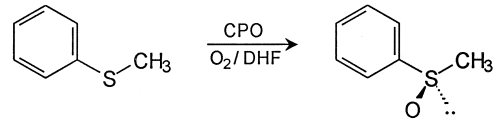
tion. In the absence of any catalyst phenol was slowly converted to a mixture of hydroquinone and catechol. We found that, apart from the known catalysts HRP and lignin peroxidase (LiP), the reaction was catalyzed by CPO, SBP and LPO with initial turnover frequencies in the range of 128–496 $min^{-1}$ (Table 1). The catalytic effect of MP-11 was much less and catalase was inactive. The catalyst also influenced the hydroquinone/catechol ratio (H/C), but there was no effect on the final conversion of phenol.

### 3.2. Oxidation of indole and thioanisole

We next turned our attention to the oxidation of indole and thioanisole, because CPO is unique in its selective $H_2O_2$-supported oxidation of these reactants. CPO also mediated these reactions with oxygen and DHF, but the turnover numbers were reduced by a factor ten. Whereas indole is cleanly oxidized to 2-oxindole (reaction 1) by CPO and $H_2O_2$, the selectivity dropped to 20–70% (depending on the medium, vide infra) in the oxygen/DHF system. Thioanisole (reaction 2) afforded the *R*-sulfoxide (e.e. > 99%) analogous to the $H_2O_2$-supported oxidation. When CPO was replaced by HRP, aerobic oxidation of indole gave only a trace of oxindole and no sulfoxide was formed from thioanisole.




(1)




(2)

### 3.3. Effect of superoxide dismutase

Because the previously proposed mechanisms (vide infra) for aerobic hydroxylation all involve superoxide anion (or its protonated form $HO_2^{\cdot}$) in some way, we investigated the effect of SOD. In agreement with the literature [18] we found that the HRP catalyzed aerobic hydroxylation of phenol was inhibited by SOD. Surprisingly, the CPO catalyzed aerobic hydroxylation of phenol, as well as the oxidation of indole and thioanisole were not influenced by the addition of SOD (Fig. 3).

### 3.4. Effect of radical scavengers

In order to investigate the possible role of hydroxyl radicals in the CPO catalyzed aerobic oxidations, *tert*-butyl alcohol was added as a

Table 1
Oxidation of phenol catalyzed by different peroxidases[a]

| Peroxidase | Initial rate ($\mu$mol $min^{-1}$)[b] | Initial TOF[c] ($\mu$mol $\mu$mol$^{-1}$ $min^{-1}$)[b] | H/C (−) |
|---|---|---|---|
| CPO | 1.18 | 236 | 0.4 |
| – | 0.072 | – | 0.9 |
| SBP | 1.74 | 128 | 1.3 |
| HRP | 1.36 | 195 | 1.4 |
| LPO | 1.58 | 496 | 0.4 |
| MP-11 | 0.18 | 7 | 2.0 |
| Catalase | 0.073 | – | n.d. |

[a] A total of 10 ml acetate buffer pH 5.0; 10 mM phenol; 25 mM (2.5 eq) DHF; 1 atm $O_2$ at 4°C; 100 $\mu$l enzyme solution.
[b] In duplicate at 5 ml scale, samples were taken every minute and quenched with an equal volume of DMSO.
[c] Turnover frequency.

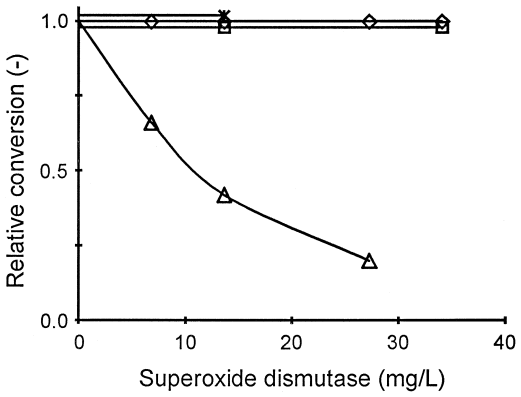



Fig. 3. Effect of SOD on aerobic oxidations catalyzed by peroxidases: CPO-catalyzed oxidation of phenol ◇; indole □; thioanisole ★; HRP-catalyzed oxidation of phenol △.

radical scavenger. *tert*-Butyl alcohol is only moderately reactive towards hydroxyl radicals (rate constant, $k = 2.5$ to $4.2 \times 10^8$ $M^{-1}$ $s^{-1}$ [25]), but has the advantage that it is inert towards CPO [8]. Hence, any effect is due only to its radical scavenging properties and not to enzyme inhibition.

The hydroxylation of phenol was inhibited by *tert*-butyl alcohol (Fig. 4), whereas the sulfoxidation of thioanisole was not influenced. The effect of *tert*-butyl alcohol on the aerobic oxidation of indole was two-fold. The relative conversion decreased by a factor of 10, whereas the selectivity increased from 25% (0% *t*-BuOH) to 60% (20% *t*-BuOH). Mannitol had a similar effect (Table 2).

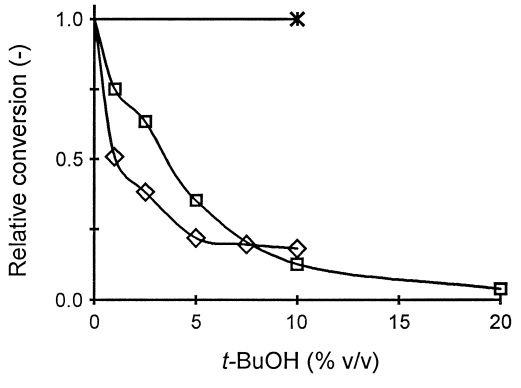



Fig. 4. Effect of *tert*-butyl alcohol on CPO-catalyzed aerobic oxidation of phenol ◇; indole □; thioanisole ★.

Table 2
Effect of different radical scavengers on the aerobic oxidation of indole catalyzed by CPO[a]

| Radical scavenger | Relative conversion (%) | Selectivity (%) |
|---|---|---|
| – | 100 | 25 |
| 10 vol.% *t*-BuOH | 13 | 55 |
| 20 vol.% *t*-BuOH | 4 | 60 |
| 0.12 M mannitol | 52 | 35 |
| 0.25 M mannitol | 21 | 73 |
| 7.5 mM Trolox | 124 | 61 |
| 17.3 mM Trolox | 112 | 77 |

[a]A total of 10 ml acetate buffer pH 5.0; 5.0 mM indole; radical scavenger; 13 mM (2.5 eq) DHF; 1 atm $O_2$ at room temperature; 100 μl CPO.

Trolox™-C, a water soluble analog of α-tocopherol (vitamin E), is a more efficient hydroxyl radical scavenger (rate constant, $k = 6.9 \times 10^9$ $M^{-1}$ $s^{-1}$ [26]) than *tert*-butyl alcohol. Trolox inhibited the CPO and HRP catalyzed aerobic hydroxylation of phenol (Fig. 5) consistent with the intermediacy of hydroxyl radicals. In contrast, both the initial rates and final conversions of indole and thioanisole oxidation increased in the presence of Trolox. Moreover, in the case of indole the selectivity increased up to 77%.

### 3.5. Spectral data

The question whether reduction of the native peroxidase to ferrous enzyme plays any role in

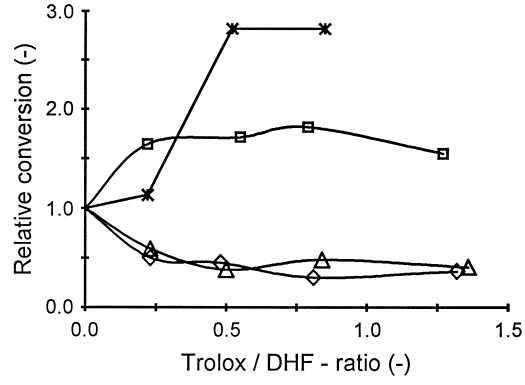



Fig. 5. Effect of Trolox on peroxidase catalyzed aerobic oxidations: CPO-catalyzed oxidation of phenol ◇; indole □; thioanisole ★; HRP-catalyzed oxidation of phenol △.

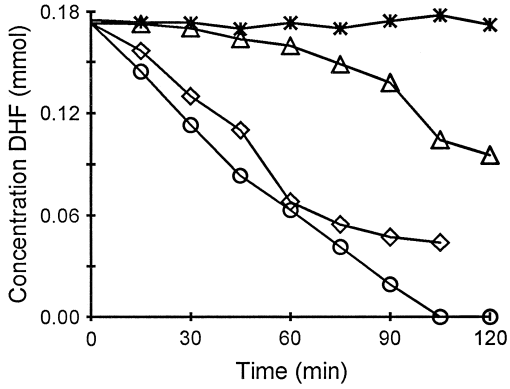



Fig. 6. Amount of DHF during competition experiments: no oxidant ★; $H_2O_2$ without catalyst ◇; CPO with $H_2O_2$ △; HRP with $H_2O_2$ □.

the $O_2$/DHF-driven oxidations still is unresolved. In agreement with the literature [23] the reduction of native CPO with sodium dithionite was facile and could easily be monitored by the shift of the Soret band from 403 to 409 nm. However, treatment of CPO with DHF under argon or DHF and $O_2$ did not induce any change in the UV spectrum. Under the same conditions HRP was converted into its compound III, as reported [18].

### *3.6. The role of hydrogen peroxide in the catalytic cycle*

Solutions of DHF under 1 atm $O_2$ were found to undergo facile autoxidation at room temperature ($t_{1/2} = 30$ min), whereas DHF is stable in the absence of oxygen. The addition of catalase or SOD decreased the rate of DHF autoxidation. During autoxidation, even in the presence of SOD and catalase, hydrogen peroxide was detected with a titanium(IV) chloride reagent [27], which is a very sensitive test. The hydrogen peroxide concentrations are low, since they cannot be detected with the less sensitive chromium pentoxide test [27] or by standard titrations. In apparent contradiction with the detection of $H_2O_2$, catalase had no effect the aerobic oxidation of indole catalyzed by CPO. Similarly, Halliwell [28], Dordick et al. [18], Schmall et al. [19], Durliat et al. [20] and Courteix and Bergel [22] reported that the addition of catalase did not totally stop the HRP-catalyzed aerobic hydroxylation of phenol.

To investigate the role of hydrogen peroxide in the catalytic cycle, competition experiments between substrate (indole) and reductant (DHF) were carried out in a hydrogen peroxide-driven oxidation. CPO and HRP, which both catalyze the oxidation of indole by hydrogen peroxide [10,29] were used as catalysts. The plots of DHF concentration vs. time (Fig. 6), show that DHF is oxidized by $H_2O_2$ in the absence of any enzyme. When a mixture of indole and DHF is allowed to react with $H_2O_2$ in the presence of CPO the indole is oxidized preferentially. DHF is oxidized only at low indole concentrations. In contrast, HRP catalyzes only the oxidation of DHF and no indole was converted.

### *3.7. Other reductants*

Dordick et al. [18] and Durliat et al. [20] reported that only DHF acts as a sacrificial reductant in the HRP catalyzed aerobic hydroxylation of phenol. Other reductants, such as ascorbic acid or NADH, did not give rise to hydroxylated products. To our surprise we found that CPO catalyzes the aerobic oxidation of thioanisole and indole also in the presence of ascorbic acid or 1,2-diphenylhydrazine, although, analogous with HRP, ascorbic acid does not support the hydroxylation of phenol by CPO. On the other hand, phenol is hydroxylated in the presence of 1,2-diphenylhydrazine and CPO, but not HRP (Table 3).

Table 3
Ability of different reductants to support aerobic oxidations catalyzed by peroxidases[a]

| Reductant | Phenol | CPO indole | Thioanisole | HRP phenol |
|---|---|---|---|---|
| DHF | + | + | + | + |
| Ascorbic acid | − | + | + | − |
| 1,2-Diphenylhydrazine | + | + | + | − |

[a]A total of 10 ml acetate buffer pH 5.0; 5 mM reactant; 12.5 mM (2.5 eq) reductant; 1 atm $O_2$ at room temperature; 100 μl enzyme solution.

Strong reducing agents, such as sodium dithionite, copper(I) chloride, and chromium(II) chloride, that are known to reduce the native peroxidases to their ferrous form, did not afford any detectable amounts of oxidized products.

## 4. Discussion

On the basis of our results a monooxygenase catalytic cycle, involving initial reduction of the native iron(III) enzyme to its ferrous form, can be ruled out. Thus, native CPO was not reduced by DHF and sodium dithionite did not support aerobic oxidation.

Therefore, we propose a mechanism for the CPO-catalyzed aerobic oxidations which involves initial formation of hydrogen peroxide via autoxidation of DHF (Fig. 7). Detection of $H_2O_2$ in aerated DHF solutions confirmed the formation of hydrogen peroxide. CPO subsequently utilizes the hydrogen peroxide ($K_M = 1.7\ \mu M$; $k = 2.3 \times 10^6\ M^{-1}\ s^{-1}$ [30]) in the selective oxidation of the substrate via an oxygen transfer mechanism. This mechanism cannot be operative with HRP, because it is predominantly present as its inactive compound III (see Section 3.5). Consequently, only non-enzymatic oxidation is observed in the presence of HRP.

Hydroxyl radicals are likely reactive species in the non-enzymatic oxidation. They can be formed during the autoxidation of DHF, for example by bimolecular termination of $HO_2^{\cdot}$ radicals (reaction 3). SOD suppresses reaction 3 by catalyzing the conversion of $HO_2^{\cdot}$ to $H_2O_2$ and $O_2$ (reaction 4). Hence, the CPO-catalyzed aerobic oxidation of indole and thioanisole is not inhibited by SOD, consistent with the proposed oxygen transfer mechanism. By the same token, the increase in selectivity observed in the CPO-catalyzed aerobic oxidation of indole (Table 2) in the presence of hydroxyl radical scavengers (Trolox or *tert*-butyl alcohol) is consistent with hydroxyl radicals being involved in competing aselective oxidation of indole. Since indole itself is an efficient hydroxyl radical scavenger ($k = 3.2 \times 10^{10}\ M^{-1}\ s^{-1}$) [31] selectivities will probably never reach 100%.

$$2\,HO_2^{\cdot} \rightarrow O_2 + 2\,HO^{\cdot} \quad (3)$$

$$2\,HO_2^{\cdot} \xrightarrow{SOD} O_2 + H_2O_2 \quad (4)$$

Similarly, the CPO-catalyzed aerobic oxidation of thioanisole was not inhibited by Trolox or *tert*-butyl alcohol. The rate acceleration observed in the presence of Trolox suggests that it either accelerates hydrogen peroxide formation, by scavenging $HO_2^{\cdot}$ radicals, and/or it protects the enzyme from deactivation. In contrast, phenol hydroxylation with DHF/$O_2$ in the presence of CPO and HRP was inhibited by radical scavengers (Trolox or *tert*-butyl alcohol) consistent with the involvement of hydroxyl radicals as previously proposed [18–22].

A mechanism for aromatic hydroxylation via hydroxyl radicals is described by Walling and

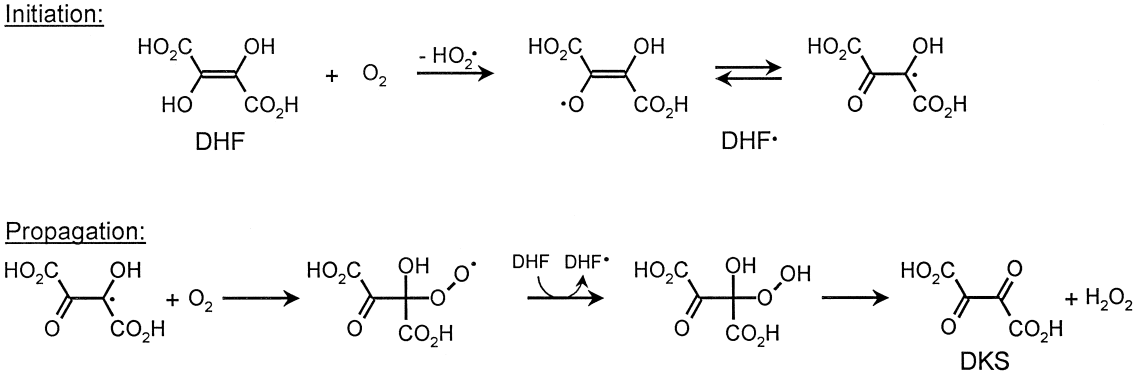



Fig. 7. Autoxidation of DHF.

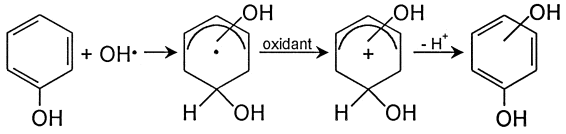



Fig. 8. Mechanism of aromatic hydroxylation by hydroxyl radicals.

Johnson [32] and Walling [33] for Fenton's reagent (Fig. 8). Hydroxyl radicals add very rapidly to aromatics (rate constant, $k = 10^9$ to $10^{10}$ $M^{-1}$ $s^{-1}$) resulting in the formation of hydroxycyclohexadienyl radicals which are subsequently oxidized to the corresponding phenols by iron(III).

In the case of the DHF/$O_2$-system hydroxyl radicals are formed non-enzymatically from DHF and oxygen (see above). The dihydroxycyclohexadienyl radicals are oxidized by the oxidized forms of the peroxidases, such as compound I (in the case of CPO), compound II, or compound III (in the case of HRP). Since the addition of hydroxyl radicals to phenol is a very rapid process, the peroxidase mediated oxidation of the intermediate dihydroxycyclohexadienyl radicals is expected to be the rate limiting step. This accounts for the different H/C-ratios for different peroxidases shown in Table 1. Hydrogen peroxide is formed from DHF and $O_2$ in the presence of SOD, therefore CPO-catalyzed hydroxylation of phenol is not inhibited by SOD. In contrast, HRP compound III is formed from native HRP and superoxide anion. Addition of SOD inhibits HRP compound III formation and therefore aerobic hydroxylation catalyzed by HRP.

Hence, we conclude from our observations that the role of the peroxidases in the aerobic oxidation of phenol is limited to the oxidation of the intermediary dihydroxycyclohexadienyl radicals to the corresponding cation.

## 5. Conclusions

A combination of a sacrificial reductant—DHF, ascorbic acid or 1,2-diphenylhydrazine—and molecular oxygen can replace hydrogen peroxide as the oxidant in CPO-catalyzed oxidations. Indole is oxidized to 2-oxindole and thioanisole to the corresponding (*R*)-sulfoxide in 99% e.e.

The reaction does not involve a monooxygenase pathway, i.e., initial reduction of native iron(III) enzyme to its ferrous form, but rather in situ formation of $H_2O_2$, via free radical autoxidation of DHF, followed by heterolytic, CPO-catalyzed oxygen transfer from $H_2O_2$ to the substrate. An inevitable consequence of the homolytic pathway for $H_2O_2$ formation is the occurrence of side reactions of substrates with reactive radicals ($HO_2^{\cdot}$, $HO^{\cdot}$, etc.) in competition with CPO-catalyzed oxygen transfer. This leads to lower selectivities (and turnover numbers) compared to those observed with CPO/$H_2O_2$. The improvement of selectivities in the presence of radical scavengers is consistent with this notion. In contrast to CPO, HRP predominantly catalyzes the $H_2O_2$ oxidation of DHF, rather than added substrate, via a classical peroxidase pathway.

Finally, another consequence of the reaction involving homolytic, in situ formation of $H_2O_2$ is that the goal of improving the operational stability of CPO was not achieved.

## Acknowledgements

This research was supported by the Dutch Innovation Oriented Program on Catalysis (IOP catalysis; IKA94013). Gifts of enzymes by Boehringer Mannheim (Penzberg, Germany) and Enzymol International (Columbus, OH, USA) are gratefully acknowledged.

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