Stellingen behorende bij het proefschrift

“Selective Oxidations catalyzed by Chloroperoxidase”

1. De door Zaks en Dodds gebruikte radicaal-klok om het mechanisme van CPO-gekatalyseerde benzyllische hydroxylering te bewijzen is binnen CPO te zeer sterisch gehinderd voor ringopening om als bewijs voor een concerted mechanisme te kunnen worden aangevoerd.

2. De door M.B. Arnao *et al.* afgelaste formule voor de hoeveelheid katalytische cycles is onjuist, daar de auteurs de afhankelijkheid tussen [R'] en [ABTS] niet meenemen.

3. De opkomst van *combinatorial chemistry* en *directed evolution mutagenesis* voor de ontwikkeling van (bio)katalysatoren toont aan dat rationale katalysatorontwikkeling momenteel nog in de kinderschoenen staat.

4. De door Colonna *et al.* beschreven activiteit van CPO bij hergebruik, nadat het enzym 8 dagen in een 20 mM TBHP oplossing heeft gestaan, is uiterst twijfelachtig.

5. De “barrière” tussen biokatalytici en chemokatalytici zou sneller geslechts kunnen worden als beide groepen eenduidige afspraken zouden maken over het gebruik van katalytische begrippen.

6. Uitspraken over het verval van compound III hebben weinig zin als dit verval wordt onderzocht in aanwezigheid van een overmaat reductant of waterstofperoxide.
7. Als bij kinetische metingen de activiteit van het gebruikte enzympreparaat vermeld zou worden, zou een betere vergelijking tussen de gevonden kinetische constanten mogelijk zijn.


8. Niet-heem, niet-metaal haloperoxidases die alleen in propionaat- of acetaatbuffer werken kunnen beter worden ingedeeld in de enzymklasse hydrolases dan in de enzymklasse oxidoreductases.


9. *Site-directed mutagenesis* voor de verbetering van de oxidatieve stabiliteit van heem-peroxidases heeft weinig nut, aangezien de instabiliteit ten aanzien van oxidanten inherent is aan de heemgroep.


10. Tolerantie is een eng begrip in Nederland.

11. Salami-wetenschap leidt tot verstopping van de wetenschappelijke informatie-kanalen.

12. De commerciële omroepen concurreren zichzelf uit de markt.

13. Het fanatisme waarmee de politie controleert op fietsen zonder licht staat in schril contrast met de lakse houding ten aanzien van fietsdiefstallen.


15. *Virtual reality* veroorzaakt verlies van realiteitszin.


Marion van Deurzen
Selective Oxidations
catalyzed by
Chloroperoxidase
The cover shows a bombardier beetle in attack. The
bombardier beetle uses a mixture of hydrogen
peroxide, phenol, peroxidase and catalase for
"chemical warfare".
Selective Oxidations
catalyzed by
Chloroperoxidase

Proefschrift

ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus Prof. ir. K.F. Wakker,
in het openbaar te verdedigen ten overstaan van een commissie,
door het College van Dekanen aangewezen,
op maandag 16 december 1996 te 13.30 uur

door

Maria Petra Johanna VAN DEURZEN
scheikundig ingenieur,
geboren te Budel.
Dit proefschrift is goedgekeurd door de promotor:

Prof. dr. R.A. Sheldon

Toegevoegd promotor:

Dr. ir. F. van Rantwijk.

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Printed in The Netherlands.
“Il faut avoir beaucoup étudié pour savoir peu”

Montesquieu
(Pensées et Jugements)
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## List of abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>ee</td>
<td>enantiomeric excess</td>
</tr>
<tr>
<td>ton</td>
<td>turnover number</td>
</tr>
<tr>
<td>tof</td>
<td>turnover frequency</td>
</tr>
<tr>
<td>sty</td>
<td>space-time yield</td>
</tr>
<tr>
<td>$k_{\text{cat}}$</td>
<td>catalytic constant</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>maximum reaction rate</td>
</tr>
<tr>
<td>CSTR</td>
<td>continuously operated stirred tank reactor</td>
</tr>
<tr>
<td>EMR</td>
<td>enzyme membrane reactor</td>
</tr>
<tr>
<td>CPO</td>
<td>chloroperoxidase from <em>Caldariomyces fumago</em></td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>MP</td>
<td>microperoxidase</td>
</tr>
<tr>
<td>LPO</td>
<td>lactoperoxidase</td>
</tr>
<tr>
<td>CeP</td>
<td>cytochrome c peroxidase</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>LiP</td>
<td>lignin peroxidase</td>
</tr>
<tr>
<td>GiP</td>
<td><em>Coprinus cinereus</em> peroxidase</td>
</tr>
<tr>
<td>SBPO</td>
<td>soybean peroxigenase</td>
</tr>
<tr>
<td>SBP</td>
<td>soybean peroxidase</td>
</tr>
<tr>
<td>TBHP</td>
<td><em>tert</em>-butyl hydroperoxide</td>
</tr>
<tr>
<td><em>t</em>-BuOH</td>
<td><em>tert</em>-butyl alcohol</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)</td>
</tr>
<tr>
<td>HMF</td>
<td>5-hydroxymethylfurfural</td>
</tr>
<tr>
<td>HFCA</td>
<td>5-hydroxymethyl-2-furancarboxylic acid</td>
</tr>
<tr>
<td>FDC</td>
<td>furan-2,5-dicarboxaldehyde</td>
</tr>
<tr>
<td>FFCA</td>
<td>5-formyl-2-furancarboxylic acid</td>
</tr>
<tr>
<td>FDA</td>
<td>furan-2,5-dicarboxylic acid</td>
</tr>
<tr>
<td>BHF</td>
<td>2,5-bis(hydroxymethyl)furan</td>
</tr>
<tr>
<td>Neu5Ac</td>
<td>$N$-acetyl-neuraminic acid</td>
</tr>
<tr>
<td>L</td>
<td>ligand</td>
</tr>
<tr>
<td>T</td>
<td>template</td>
</tr>
</tbody>
</table>
Introduction

General Introduction

Selective catalytic oxidation is an important industrial technology both in bulk chemistry and in fine chemistry. Selective catalytic oxidations have been used to convert oil and natural gas to bulk chemicals like propylene oxide and terephthalic acid for several decades [1-4]. In fine chemistry the need for cleaner and more selective processes has induced intensive research for selective catalytic oxidations [5-9]. Traditional fine chemical processes using stoichiometric amounts of metal oxidants like permanganate or dichromate are increasingly being replaced by catalytic processes, thus decreasing the amount of inorganic waste streams. Selective catalytic oxidation processes are nowadays widely applied for the production of fine chemicals like resorcinol and hydroquinone [5]. There are several methods available for performing selective catalytic oxidations, these include heterogeneous vapor and liquid phase oxidations and homogeneous oxidations in the liquid phase. There is also a wide choice of catalysts. These include metals, metal ions, metal oxides, metal species encapsulated in inorganic matrices like zeolites and heteropolyacids, metal complexes, enzymes and antibodies.
Oxidations in fine chemistry

In contrast to oxidations in bulk chemistry, oxidations in fine chemistry are generally restricted to the liquid phase. Fine chemicals are often susceptible to thermal degradation which limits their manufacture to moderate temperatures in the liquid phase. Moreover, the processing is preferably multi-purpose and batch-wise, unlike the continuous and dedicated processes in bulk chemistry. Whereas most oxidation processes in bulk chemistry are performed with molecular oxygen as the oxidant, fine chemical processes allow the use of other oxidants such as hydrogen peroxide. In the liquid phase several oxidation mechanisms may occur with oxygen or hydroperoxides as the oxidant. A short description is given below.

Free radical autoxidation

Liquid phase autoxidations proceed via metal induced decomposition of alkyl hydroperoxides according to the Haber-Weiss mechanism, which is followed by chain propagation (Fig. 1.1).

Initiation

\[ M^{(n+1)^+} + ROOH \rightarrow M^{n^+} + ROO^- + H^+ \]

\[ M^{n^+} + ROOH \rightarrow M^{(n+1)^+} + RO^- + OH^- \]

Chain propagation

\[ ROO^- (RO^-) + RH \rightarrow R^- + ROOH (ROH) \]

\[ R^- + O_2 \rightarrow ROO^- \]

Fig. 1.1. Autoxidation.

Under normal conditions chain termination occurs via recombination of two alkyl peroxy radicals. In fine chemistry oxidations with molecular oxygen are generally performed with simple molecules containing only one reactive position. This restriction is due to the non-selective autoxidation, which is most pronounced in the liquid phase as the concentration of organic reactant is high. Contamination by peroxidic impurities may initiate free radical
autoxidation in any liquid system in which metal catalysts, oxygen and hydrocarbons are present.

**Metal oxidation of coordinated substrates**

In the first step the organic reactant is oxidized by a metal ion. The reduced metal is subsequently reoxidized by another oxidant, like for example molecular oxygen. An example of such a reaction is the Wacker process [10], which is the first industrialized homogeneous oxidation in the liquid phase. In the Wacker process (Fig. 1.2) ethylene is oxidized to acetaldehyde using oxygen and a CuCl₂/PdCl₂ catalyst. The organic reactant reduces Pd(II) to Pd(0) which is reoxidized by Cu(II). The formed Cu(I) is converted to Cu(II) by molecular oxygen.

\[
\text{CH}_2=\text{CH}_2 + \text{Pd(II),Cu(II)} \xrightarrow{\text{O}_2} \text{CH}_3\text{CHO}
\]

*Fig. 1.2. Wacker process.*

**Oxygen transfer**

Oxygen transfer reactions are reactions in which an organic reactant reacts with the oxygen donor in the presence of a metal catalyst. The reaction proceeds according to the following scheme:

\[
\text{S + XOY} \xrightarrow{} \text{SO + XY}
\]

XOY= H₂O₂, TBHP, ClO⁻, R₃NO, R₂SO etc.

*Fig. 1.3. Oxygen transfer.*

In contrast to autoxidations, catalytic oxygen transfer can proceed selectively. Selective oxygen transfer may yield interesting fine chemicals. Chiral synthons, like epoxides and sulfoxides, can for example be produced via an oxygen transfer reaction.

The call for low salt technologies has confined the oxygen transfer oxidants in the liquid phase to hydrogen peroxide or to peroxides which can easily be regenerated, like tert-
butyl hydroperoxide (TBHP) or amine oxides. Oxygen transfer may proceed via two different mechanisms: the peroxometal pathway and the oxometal pathway (Fig. 1.4).

![Peroxometal and Oxometal Pathways](image)

**Fig. 1.4. Peroxometal pathway vs. oxometal pathway.**

Selectivity and especially stereoselectivity is an important issue in fine chemistry. There are several approaches for obtaining a suitable oxidation catalyst which can perform (stereo)selective oxidations. One can use enzymatic oxidation catalysts provided by Nature, the so-called oxido-reductases, or develop new catalysts. These new oxidation catalysts include antibodies, which are a special kind of biocatalysts, and synzymes, synthetic oxidation catalysts.

**Synzymes**

Synzymes, or artificial enzymes, are being developed with a view to imitating the effective catalytic properties exerted by enzymes. Three approaches for the development of biomimetic catalysts will be discussed in this section: supramolecular metal complexes, ship-in-the-bottle complexes of zeolites and molecular imprinting of polymers.

**Supramolecular metal complexes**

Numerous metal complexes have been designed for catalytic oxidations. Selective oxidations may occur when the right ligand is chosen. There are several examples of rather small ligands which upon complexation to the metal are capable of performing highly selective oxidations, for example the Sharpless epoxidation catalyst in which the ligand is
tartrate [11]. In recent years a considerable research effort has been focused on supramolecular systems, in which a large host molecule provides interactions with the reactant (guest). The host is connected to a catalytic metal center (Fig. 1.5). Examples of such host systems are functionalized cyclodextrins, porphyrins and calixarenes. These structures provide cavities in which selective catalysis may occur through host-guest interactions.

![Supramolecular metal-catalyst for epoxidations](image)

**Fig. 1.5. Supramolecular metal-catalyst for epoxidations [12].**

**Ship-in-the-bottle complexes**

Another approach to obtain artificial enzymes is to synthesize molecular sieves in which metal complexes have been encapsulated. The metal complex is introduced into the zeolite during the synthesis and is too large to diffuse out of the zeolite cages. The zeolite cage provides an environment in which shape-selective catalysis may occur. An example of an oxidation catalyzed by such a ship-in-the-bottle complex is the zeolite encapsulated Mn(II) bipyridine complex, synthesized by Knops-Gerrits et al. [13], which catalyzes the oxidation of olefins with hydrogen peroxide as the oxidant. The oxidative destruction of the catalyst was suppressed by encapsulation of the metal-complex into the zeolite.

**Molecular imprinting**

Molecular imprinted polymers are based on strongly crosslinked polymers. The polymerization occurs around a “template” molecule which is removed after polymerization
(Fig. 1.6). The remaining tailor made cavities may exert catalytic activity when a transition state analogue is used as a template. Catalytic groups may be connected to a part of the monomer thus providing cavities which contain catalytic groups, comparable to the active center of enzymes. There are several means for organizing these catalytic groups in the cavities. These include orientation by covalent interaction with the template, by non-covalent interaction with the template (Fig. 1.6) and by metal complexation [14]. After polymerization the template is selectively removed and the catalytic groups remain attached to the polymeric network. Although currently the acceleration of reactions by imprinted polymer catalysts is not very high, they have the advantage of being very stable and exerting selectivity during reaction. For example a high regio-and stereoselectivity was observed in the polymer/LiAlH₄ reduction of androstan-3,17-dione [15].

![Diagram](Image)

**Fig. 1.6. Synthesis of a molecular imprinted polymer (MIP) via non-covalent interaction; T=template.**

**Catalytic Antibodies**

Antibodies, which can be induced in living organisms by the introduction of a transition state analogue, are proteins which can function as catalysts [16]. In principle every reaction can be catalyzed by antibodies if a suitable transition state analogue is made. Antibody catalysis proceeds selectively under mild conditions. However, the use of antibodies as oxidation catalysts suffers from several limitations. The practical application of catalytic
antibodies is hampered by their low turnover number which in turn is caused by the low turnover frequencies as well as by the inherent instability of antibodies. Furthermore, it takes considerable effort to obtain a good catalytic antibody: the reaction mechanism has to be known and the right transition state analogue has to be synthesized. Moreover, the isolation of antibodies is elaborate. An example of an oxidation catalyzed by antibodies is the epoxidation of unfunctionalized olefins as shown in Fig. 1.7 which proceeded with an enantiomeric excess of over 98% [17].

![Diagram](attachment:diagram.png)

*Fig. 1.7. Antibody catalyzed epoxidation of unfunctionalized olefins.*

**Oxidoreductases**

Nature has developed enzymes for performing selective catalytic redox reactions: the so-called oxidoreductases. The oxidoreductases can be divided into four subclasses: oxidases, oxygenases, dehydrogenases and peroxidases (see also chapter 2). Oxidations may be performed with isolated enzymes or with whole cells in fermentation processes. Research in the field of redox biocatalysis is focused on discovering new enzymes and organisms which perform a desired transformation as well as on exploring the possibilities of known enzymes to perform “unnatural” reactions. An example of an industrial process which makes use of an oxidative enzyme is the regioselective hydroxylation of nicotinic acid catalyzed by the microorganism *Achromobacter xylosoxidans* (Fig. 1.8). Thus 6-hydroxynicotinic acid is obtained, which is an insecticide intermediate [18]. This hydroxylation process has been commercialized by Lonza.

![Diagram](attachment:diagram.png)

*Fig. 1.8. Microbial oxidation of nicotinic acid.*
Catalyst Performance

Before a catalytic oxidation becomes industrially attractive a few characteristics of the process concerning the catalyst have to be considered. Important parameters are the activity of the catalyst for the desired reaction, the turnover number, the space-time yield and the final product concentration.

Activity

The activity of the catalyst determines the amount of catalyst needed to obtain a certain production. It is defined as the amount of product obtained per amount of catalyst during a period of time. There are several means for expressing the catalyst's activity. For chemocatalysts, the catalytic turnover frequency is most frequently used to express the activity. The turnover frequency is defined as mol of product obtained per mol of catalyst during a certain time. The activity of biocatalysts is commonly expressed in μmol of product obtained per mg biocatalyst per minute (=U/mg, specific activity). Clearly, turnover frequencies of biocatalysts can be calculated from their activities if the molecular mass of the biocatalyst is known. A minimum value for the activity of biocatalysts to be applicable in industry is an activity of 1 μmol mg⁻¹ min⁻¹.

When all the enzyme is saturated with substrate the activity of the biocatalyst is given by the $k_{cat}$-value in the Michaelis Menten equation (Equation 1.1.). This value is an important parameter for determining the reactivity of the biocatalyst.

\[
\text{Biocatalyst's activity (μmol mg}^{-1}\text{min}^{-1}) = \frac{V}{[\text{biocatalyst}]} = \frac{k_{cat} \cdot [\text{substrate}]}{[\text{substrate}] + K_m}
\]

\[
= k_{cat} \quad \text{if } [\text{substrate}] >> K_m \\
(V=V_{max} \quad \text{if } [\text{substrate}] >> K_m)
\]

\[
V = \text{reaction rate} \\
K_m = \text{Michaelis constant} \\
= \text{apparent dissociation constant of biocatalyst-substrate complex}
\]

Equation 1.1. Michaelis Menten kinetics [19].
Introduction

Turnover number

The turnover number (amount of product produced per amount of catalyst) is an important parameter for determining the catalyst costs for a process. If a relatively cheap catalyst is used, such as TS-1 for the production of hydroquinone [20], a low turnover number is acceptable. However, oxidative enzymes are generally more expensive; the commercial price for purified CPO is $5,000,000/kg (Chirazyme, USA). It should be noted, however, that the high price is partly a reflection of the small scale on which these enzymes are produced. If a market existed for larger quantities the production costs could be substantially reduced. Moreover, the price is based on purified enzyme, whereas crude enzyme presumably would be as effective as purified enzyme to perform selective oxidations. This would also considerably reduce the price of an enzyme. For example purified horseradish peroxidase costs about $3,000,000/kg. This price is reduced 10-fold to about $300,000/kg for crude HRP (Boehringer Mannheim). Because of the high catalyst’s price, either the turnover number of an enzymatic process has to be very high or the desired product must be very expensive, before an enzymatic process becomes economically feasible.

Space-time yield

A third important parameter for developing a process is the space-time yield: how much product can be produced in a certain reactor volume per unit of time? In Table 1.1 some space-time yields for processes in fine chemistry are shown.

Again, the space-time yield which is acceptable in terms of economics depends on the price of the product. A rule of thumb is a space-time yield of 100 g L\(^{-1}\) d\(^{-1}\) for a product with a value of $100/kg.
Table 1.1. Space-time yield and price of some finechemical processes.

<table>
<thead>
<tr>
<th>Product</th>
<th>Space-time yield (g L⁻¹d⁻¹)</th>
<th>Price ($ kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroquinone [20]</td>
<td>1400</td>
<td>5</td>
</tr>
<tr>
<td>DL-methionine [21]</td>
<td>360</td>
<td>3</td>
</tr>
<tr>
<td>Enzymatic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartic acid [21]</td>
<td>480</td>
<td>3</td>
</tr>
<tr>
<td>Phenylalanine [21]</td>
<td>360</td>
<td>10</td>
</tr>
<tr>
<td>Fermentation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Carnitine [18]</td>
<td>234</td>
<td>170</td>
</tr>
<tr>
<td>Vitamin B₁₂ [21]</td>
<td>0.024</td>
<td>8000</td>
</tr>
</tbody>
</table>

*Final product concentration*

The final product concentration is also an important parameter as this will directly influence the costs associated with isolation of the product. Thus, it is conceivable that a rapid reaction has an acceptable space-time yield but that the final product concentration is very low. In fermentation processes this is generally referred to as the titer and is expressed in gram per liter. An acceptable concentration is of the order of 10%, *i.e.* 100 g L⁻¹ but, of course, this is dependent on the price of the product. For some industrial applications like steroid production even low final product concentrations are acceptable.

*Comparison of asymmetric epoxidation catalysts*

In this section a comparison will be made of three different catalysts for asymmetric epoxidation [22], based on the characteristics explained in the preceding section. These catalysts include an enzyme [23], a chemocatalyst [24] and a catalytic antibody [17] (Fig. 1.9). It is obvious from Table 1.2 that catalytic antibodies cannot yet compete with enzymes or chemocatalysts in view of activity, turnover number, space-time yield and final product concentration. Both the enzymatic and the chemocatalytic pathway yield a high conversion to
the desired product and the epoxide is obtained in high enantiomeric excess with good space-time yields. The biocatalyst has the advantage that the turnover number and activity are high, whereas for the chemocatalyst it is possible to obtain the opposite enantiomer by synthesizing the mirror image of the catalyst. Moreover, the chemocatalyst is cheap (<$ 1,000/kg in bulk) compared to CPO ($ 5,000,000/kg for purified enzyme) and the oxidation by the chemocatalyst yields a higher final product concentration. A disadvantage of the chemocatalyst is the oxidant used: sodium hypochlorite yields one equivalent of sodium chloride as byproduct whereas TBHP, the oxidant of the enzymatic process, can easily be regenerated electrochemically from the obtained tert-butyl alcohol.

The substrate scope of both catalysts is complementary. The manganese salen complex efficiently catalyzes the epoxidation of almost all classes of conjugated olefins. However, non-conjugated, unfunctionalized olefins are epoxidized poorly by the manganese salen complex, whereas CPO can not only epoxidize conjugated olefins of limited size but also a range of unfunctionalized olefins in good yield. Which catalyst system is best for an asymmetric transformation will depend on relative activities and catalyst costs for that transformation.

Fig. 1.9. Catalytic asymmetric epoxidation: reactions catalyzed by the various catalysts.
Table 1.2.
Comparison of epoxidation catalysts.

<table>
<thead>
<tr>
<th></th>
<th>Enzyme</th>
<th>Chemocatalyst</th>
<th>Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPO</td>
<td>(salen)Mn</td>
<td>20B11</td>
</tr>
<tr>
<td>conversion (%)</td>
<td>95</td>
<td>96</td>
<td>32</td>
</tr>
<tr>
<td>ee (%)</td>
<td>93</td>
<td>97</td>
<td>71(^a)</td>
</tr>
<tr>
<td>ton (mol/mol)</td>
<td>6800</td>
<td>26</td>
<td>3.5</td>
</tr>
<tr>
<td>ton (g/g)</td>
<td>23(^b)</td>
<td>8(^c)</td>
<td>0.004</td>
</tr>
<tr>
<td>tof (h(^{-1}))</td>
<td>4500</td>
<td>3</td>
<td>0.2</td>
</tr>
<tr>
<td>sty (g L(^{-1})d(^{-1}))</td>
<td>145</td>
<td>93</td>
<td>0.02</td>
</tr>
<tr>
<td>[product] (g L(^{-1}))</td>
<td>9</td>
<td>35</td>
<td>0.013</td>
</tr>
<tr>
<td>catalyst costs ($/kg)</td>
<td>2000(^d)</td>
<td>135(^e)</td>
<td>astronomical</td>
</tr>
</tbody>
</table>

\(^a\) The uncatalyzed reaction is significant compared to the catalyzed reaction (about 30% of the product is due to the uncatalyzed epoxidation). Thus, the calculated enantiomeric excess of the antibody catalyzed reaction is over 98%.

\(^b\) M\(_w\) of catalyst ~42,000

\(^c\) M\(_w\) of catalyst ~635

\(^d\) Assuming a bulk price for the catalyst of $50,000/kg

\(^e\) Assuming a bulk price for the catalyst of $1,000/kg. Recently [25] the epoxidation with salen(Mn) was improved by addition of 4-(3-phenylpropyl)pyridine N-oxide and the catalyst costs could be reduced to $13/kg

Scope of this thesis

The theme of this thesis is the use of chloroperoxidase from Caldariomyces fumago (CPO) as a catalyst for selective oxidations. In view of the already mentioned environmental and selectivity issues peroxidases have potential as biocatalysts for selective oxidations. Peroxidases are enzymes which use hydroperoxides, preferably hydrogen peroxide, as the oxidant. Unlike dehydrogenases and monooxygenases, peroxidases do not need a cosubstrate such as NADH. Furthermore, they are capable of catalyzing dehydrogenation as well as oxygen transfer reactions. CPO is a versatile heme peroxidase with unusual properties (see also Chapter 2). This heme enzyme contains a thiolate as the fifth ligand and the heme iron of CPO is rather accessible for reactants, thus providing a suitable environment for direct oxygen transfer reactions. Other peroxidases have a more restricted active site which hampers direct
oxygen transfer. Therefore, the emphasis of this thesis is on CPO as a catalyst for selective oxidations.

In chapter 2 a review is given of selective oxidations catalyzed by peroxidases. Synthetic and mechanistic aspects of selective oxidations catalyzed by peroxidases are discussed. Furthermore, attention is paid to the catalytic center of peroxidases, the deactivation of peroxidases and to potential commercial applications.

Chapter 3, 4 and 5 of this thesis deal with synthetic transformations catalyzed by CPO. Enantioselective oxidation of sulfides to sulfoxides is described in chapter 3. Sulfoxidations are performed in water as well as in tert-butyl alcohol/water mixtures. The results in both media are compared. In chapter 4 the selective oxidation of substituted indoles to the corresponding 2-oxindoles is studied. Substituted oxindoles are interesting intermediates for pharmaceuticals. Kinetic experiments are performed for several substituted indoles and a reaction mechanism is proposed. The oxidation of 5-hydroxymethylfurfural is described in chapter 5. Synthetic as well as mechanistic aspects of the reaction are discussed.

The use of a hydrogen peroxide-stat for controlling reactions with CPO is demonstrated for the oxidation of indole to oxindole in chapter 6. Mechanistic aspects of the catalase activity of CPO and deactivation of this heme enzyme are also studied. In chapter 7 several reactor concepts for CPO-catalyzed oxidation of indole are compared with regard to turnover number and space-time yield. Finally, in the appendix a simple and rapid purification method for CPO is described.

References

Chapter 2

Selective oxidations catalyzed by peroxidases

Abstract

A review is given of selective oxidations catalyzed by peroxidases. This review focuses on synthetic and mechanistic aspects of selective oxidations catalyzed by peroxidases. Furthermore, deactivation of peroxidases and potential commercial applications of peroxidases are discussed.
Chapter 2

Introduction

General introduction

The current interest in catalytic oxidative transformations has been stimulated by two major environmental issues. The first one is the need for replacement of oxidations which use stoichiometric amounts of heavy metal salts by cleaner catalytic alternatives using hydrogen peroxide or oxygen as the oxidant. The second major issue is the increasing demand for high chemo-, regio- or stereoselectivity in chemical reactions in order to improve chemical yields, to minimize waste streams and to avoid enantiomeric ballast. Redox enzymes are potentially suitable for meeting these two goals. Redox enzymes (i.e. oxidoreductases) can be classified into the following categories according to the oxidant they use and the reactions they catalyze:

Dehydrogenases/Reductases

\[ \text{RH}_2 + D \rightleftharpoons R + \text{DH}_2 \]

Oxidases

\[ \text{RH}_2 + O_2 \rightarrow R + \text{H}_2\text{O}_2 \]

Oxygenases

Monooxygenases

\[ \text{RH} + O_2 + \text{DH}_2 \rightarrow \text{ROH} + D + \text{H}_2\text{O} \]

Dioxygenases

\[ \text{RH} + O_2 \rightarrow \text{ROOH} \]

Peroxidases

\[ \text{RH}_2 + \text{H}_2\text{O}_2 \rightarrow R + 2\text{H}_2\text{O} \]

D, DH\textsubscript{2} = cosubstrate
Selective oxidations catalyzed by peroxidases

Dehydrogenases are the most studied redox enzymes for synthetic applications. However, they suffer from the disadvantage of requiring stoichiometric quantities of an expensive cosubstrate such as NADH. Monoxygenases, which mediate the introduction of an oxygen atom into the substrate, also have a cosubstrate requirement. Furthermore, they are relatively unstable and difficult to isolate. Hence, monoxygenases are often used in whole cell systems which are, however, prone to competing reactions which can lower the enantioselectivity and yield of a reaction. Processes using dioxygenases are, like monoxygenase-mediated processes, carried out as precursor fermentations and have the same limitations. Oxidases have as a drawback that they generally exhibit a narrow substrate range which limits their synthetic utility. They are often used in biosensors for selective detection of components like glucose and cholesterol. Peroxidases, on the other hand, are interesting catalysts for performing selective oxidations using clean oxidants. They catalyze oxidative transformations of organic substrates with a peroxide, usually hydrogen peroxide. Unlike monoxygenases and dehydrogenases, peroxidases have no requirement for expensive cosubstrates. Moreover, most peroxidases are relatively stable extracellular enzymes that can accommodate a broad range of substrates in a diversity of reactions. These include reactions characteristic of both dehydrogenases as well as monoxygenases.

Peroxidases

Peroxidases are ubiquitous in nature [1-8]. Some peroxidases like ascorbate peroxidase act as hydrogen peroxide scavengers. Others, such as horseradish peroxidase, catalyze free radical oligomerizations and polymerizations of electron-rich aromatics. Lignin peroxidase catalyzes the oxidative degradation of lignin. Although the exact role of haloperoxidases (peroxidases which can halogenate organic substrates, vide infra) is not yet clear the halometabolites they produce probably play an important role in the defense system of organisms.
Fig. 2.1. Reaction cycle of monoxygenases.

Fig. 2.2. Reaction cycle of peroxidases.

Many peroxidases contain heme (iron(III)protoporphyrin IX) as the prosthetic group, although some peroxidases have different catalytic centers (vide infra). During catalysis heme peroxidases are oxidized by a peroxide to a (formally) iron(V)oxo species which is predominantly present as an iron(IV)oxo porphyrin radical cation species in most peroxidases (Fig. 2.2). This so called compound I is comparable to the iron(V)oxo intermediate in the
monooxygenase catalytic cycle (Fig. 2.1). Instead of molecular oxygen and a cosubstrate which transfers 2 hydrogen atoms, peroxidases can directly form the iron(V)oxo species from native enzyme and hydrogen peroxide (pathway 1 in Fig. 2.2). Compound I can be reduced to native enzyme by several mechanisms depending on the type of reaction. Peroxidase catalyzed reactions can be divided into the following four categories:

1. Oxidative dehydrogenation
   (classical peroxidase reaction, pathway 2a and 2b in Fig. 2.2)
   \[ 2SH + H_2O_2 \rightarrow 2S^- + H_2O \]

2. Oxidative halogenation
   (pathway 3 in Fig. 2.2, R = X^-)
   \[ SH + H_2O_2 + H^+ + X^- \rightarrow SX + 2H_2O \]

3. \( H_2O_2 \) disproportionation
   (catalase reaction, pathway 4 in Fig. 2.2)
   \[ 2H_2O_2 \rightarrow 2H_2O + O_2 \]

4. Oxygen transfer reactions
   (pathway 3 in Fig. 2.2, R = organic substrate)
   \[ R + H_2O_2 \rightarrow RO + H_2O \]

Reactions

Oxidative dehydrogenation

This type of reaction is mainly restricted to heme peroxidases. Oxidative dehydrogenation involves one-electron transfer processes with radical cations and radicals as intermediates (pathway 2a and 2b in Fig. 2.2). The iron(V)oxo species is reduced to native enzyme via two one-electron transfers. The intermediate iron(IV)oxo species which is one oxidation equivalent above the native enzyme is called compound II. Peroxidases catalyze a variety of one-electron oxidations of electron-rich aromatics resulting in inter- or intramolecular radical coupling products. This type of reaction is called the classical
peroxidase reaction as it was the first type of reaction of peroxidases discovered. Examples of such reactions are shown in Fig. 2.3 and Fig. 2.4.

![Fig. 2.3. Oxidation of guaiacol, standard assay [9].](image)

![Fig. 2.4. Aldoxime oxidation to iminoxy dimers by HRP [10].](image)

A useful classical peroxidase reaction which deserves to be mentioned is the peroxidase catalyzed polymerization of phenols and anilines (Fig. 2.5) under mild conditions [11-14].

![Fig. 2.5. Polymerization of aniline by a peroxidase [11].](image)

$N$- and $O$-dealkylation are a special case of electron transfer processes catalyzed by peroxidases. These are interesting reactions from a synthetic point of view because oxidative dealkylation is difficult to perform in organic chemistry. Dealkylation of tertiary amines, which is relatively facile, requires stoichiometric amounts of oxidants, like permanganate, chlorine dioxide or alkaline potassium ferricyanide. Dealkylations of secondary amines are more difficult to perform and often harsh reaction conditions are necessary. Heme proteins, peroxidases included, can catalyze the dealkylation of hetero-atoms [15]. An example is shown in Fig. 2.6.
Selective oxidations catalyzed by peroxidases

![Chemical structure](image)

Fig. 2.6. O-demethylation of 9-methoxyellipticine catalyzed by HRP [16].

As this review is primarily concerned with the use of peroxidases for selective oxygen transfer processes we will not deal in detail with the classical peroxidation reaction.

**Oxidative halogenation**

A special class of peroxidases comprises the so-called haloperoxidases which mediate the halogenation of organic substrates. Once thought to be rare — until 40 years ago only 3 examples were known [3] — it has since then become clear that haloperoxidases occur widely in nature. Since the 1970’s over 100 sources of haloperoxidases have been discovered [6]. Oxidative halogenation is not limited to heme peroxidases, but is also catalyzed by vanadium haloperoxidases and other non-heme haloperoxidases (*vide infra*). Oxidative halogenation is believed to proceed via an active halide species. In the case of vanadium and heme peroxidases (pathway 3 in Fig. 2.2, R = halide) this active species is probably hypohalite [7]. Metal-free haloperoxidases are also known which presumably involve the formation of a peroxycarboxylic acid intermediate analogous to the lipase-catalyzed formation of peroxycarboxylic acids from hydrogen peroxide and a carboxylic acid (*vide infra*).

One of the most versatile peroxidases is a chloroperoxidase from *Caldariomyces fumago* (CPO) which was first isolated in 1961 by Hager [17]. *In vivo* CPO is involved in the production of the chlorometabolite caldariomycin (1,1-dichloro-2,5-dihydroxy cyclopentane, Fig. 2.8). A standard assay for CPO involves the chlorination of monochlorodimedone to dichlorodimedone (Fig. 2.7).
Fig. 2.7. Halogenation of monochlorodimedone by CPO (standard assay).

Peroxidase-catalyzed oxidative halogenation reactions are generally non-selective and halogenation of the substrate is believed to take place outside the active site. There is one example, however in which regioselectivity is observed: the metal-free chloroperoxidase from Pseudomonas pyrrocinnia selectively converts indole to 7-chloro-indole [18]. We note that in order to exclude that this reaction proceeds via an enzyme-halide intermediate rather than via a peroxyacetic acid intermediate, a blank reaction with peroxyacetic acid and chloride should be carried out.

Oxidative halogenation will not be further considered in detail in this paper. Reviews concerning halogenation reactions catalyzed by peroxidases can be found in [6,7,8].

\textbf{H}_2\text{O}_2\text{-disproportionation}

Hydrogen peroxide decomposition to water and oxygen is catalyzed by some peroxidases, either directly (via pathway 4 in Fig. 2.2) [19,20] or by production of hypohalous acid which subsequently reacts with hydrogen peroxide to produce singlet oxygen [21, 22]. Especially CPO exhibits substantial catalase activity when hydrogen peroxide is the only reductant present in the reaction mixture [20]. During the last decade a new class of peroxidase related enzymes has been discovered, the catalase-peroxidases. They exhibit in addition to catalase activity also a substantial classical peroxidase activity [23].

\textbf{Oxygen transfer reactions}

Selective oxygen transfer is the fourth type of reaction which is catalyzed by peroxidases (pathway 3 in Fig. 2.2, R = organic substrate). From a synthetic point of view they are the most interesting oxidative transformations catalyzed by peroxidases. The transformations are comparable to those catalyzed by monoxygenases. Hence, many oxygen
transfer reactions described in this review are not only catalyzed by peroxidases but also by
monooxygenases. The oxygenase-type reactions of peroxidases can be divided into the
following categories:

1. **Hetero-atom oxidation:**
   - S-oxidation
   - N-oxidation

2. **Epoxidation**

3. **C-H bond oxidation**
   - Benzylic/allylic hydroxylation
   - Alcohol oxidation
   - Indole oxidation

This review is focused on the synthetic and mechanistic aspects of selective oxygen
transfer reactions catalyzed by peroxidases. Potential commercial applications and the
question of enzyme deactivation are also discussed.

**Oxygen transfer reactions**

**Hetero-atom oxidation**

**Sulfur oxidation**

![Chemical structure](image)

Fig. 2.9. Chiral sulfoxide formation by oxidation of the sulfide.

Chiral sulfoxides are useful auxiliaries in asymmetric synthesis [24,25]. Furthermore,
certain sulfoxides containing a chiral sulfanyl group possess interesting biological properties.
Chapter 2

There are several methods for their preparation, involving both stoichiometric and catalytic oxidation of the corresponding sulfides. Examples include chemical methods [26], biomimetic approaches [27] and enzymatic approaches [28]. For some recent reviews on the preparation of chiral sulfoxides see [24,25,28,29,30].

In the last decade it has been shown that peroxidases are also capable of catalyzing sulfoxidation reactions [31-34]. The first enantioselective sulfoxidation using CPO was described by Kobayashi et al. [35] (methyl p-methylphenyl sulfide yielded R-sulfoxide in 13% ee). The fairly low enantioselectivity in this report was probably due to substantial non-enzymatic oxidation caused by the high concentration of hydrogen peroxide used. More recently, CPO-catalyzed enantioselective sulfoxidations were investigated in more detail by Colonna et al. [36,37].

Table 2.1. Oxidation of alkyl aryl sulfides by CPO and H₂O₂ [37].

<table>
<thead>
<tr>
<th>Sulfide</th>
<th>Conversion (%)</th>
<th>ee (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R₁-S-R₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R₁</td>
<td>R₂</td>
<td></td>
</tr>
<tr>
<td>p-CH₃-C₆H₄</td>
<td>CH₃</td>
<td>98</td>
</tr>
<tr>
<td>p-CH₃-C₆H₄</td>
<td>C₂H₅</td>
<td>50</td>
</tr>
<tr>
<td>p-CH₃-C₆H₄</td>
<td>C₃H₇</td>
<td>53</td>
</tr>
<tr>
<td>o-CH₃-C₆H₄</td>
<td>CH₃</td>
<td>27</td>
</tr>
<tr>
<td>p-CH₂O-C₆H₄</td>
<td>CH₃</td>
<td>72</td>
</tr>
<tr>
<td>o-CH₂O-C₆H₄</td>
<td>CH₃</td>
<td>24</td>
</tr>
<tr>
<td>C₆H₅</td>
<td>CH₃</td>
<td>100</td>
</tr>
<tr>
<td>o-Cl-C₆H₄</td>
<td>CH₃</td>
<td>33</td>
</tr>
<tr>
<td>2-pyridyl-</td>
<td>CH₃</td>
<td>100</td>
</tr>
</tbody>
</table>

The best results were obtained with hydrogen peroxide as the oxidant (Table 2.1). Substantial uncatalyzed oxidation of the sulfides was observed in blank reactions (10-33%), therefore many of the obtained sulfoxides in the enzymatic procedure were not completely enantiopure. When we investigated the influence of tert-butyl alcohol on the reactivity and selectivity of CPO for sulfoxidation we found essentially no blank reaction in water and enantiopure sulfoxides were obtained in water as well as in tert-butyl alcohol/water mixtures.
Selective oxidations catalyzed by peroxidases

[38]. The reason for the blank reactions of Colonna et al. might well be the presence of trace amounts of metal oxides which are known to catalyze the oxidation of sulfides to the corresponding sulfoxides [30].

In a subsequent study the group of Colonna investigated the influence of chloride on the sulfoxidation by CPO [39]. Although the reaction rate increased, the enantiomeric excess of the sulfoxides decreased suggesting that oxidation partially proceeded via the intermediate formation of hypochlorite. Fu et al. investigated the chloroperoxidase catalyzed oxidation of p-substituted alkyl phenyl sulfides by hydrogen peroxide or racemic alkyl hydroperoxides as the oxidant in aqueous buffer [40]. Slow addition of the hydrogen peroxide to the reaction mixture afforded nearly enantiopure sulfoxides (ee=97-99%). When racemic alkyl hydroperoxides were used as the oxidant, optically active alcohols and alkyl hydroperoxides were obtained (Fig. 2.10, ee up to 89%).

Fig. 2.10. Concomitant oxidation of sulfides and reduction of hydroperoxides catalyzed by CPO [40].

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Oxidation of sulfoxide to sulfone was reported to be catalyzed by CPO for dimethyl sulfoxide to dimethyl sulfone [41]. However, overoxidation of the obtained sulfoxides to the sulfones was not observed in the CPO-catalyzed enantioselective sulfoxidations.

Several other heme-peroxidases were found to catalyze the enantioselective sulfoxidation of alkyl aryl sulfides. These include horseradish peroxidase (HRP) [31,42-44], cytochrome c peroxidase (CcP) [45], microperoxidase (MP) [46] and lactoperoxidase (LPO) [31,32,47]. However, their turnover numbers (ton) as well as enantioselectivities (ee) were much lower than those observed with CPO (Table 2.2).

Table 2.2.
Comparison of peroxidases for the sulfoxidation of methyl phenyl sulfide.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>reaction time (min.)</th>
<th>Yield (%)</th>
<th>ee (%)</th>
<th>ton$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPO</td>
<td>60</td>
<td>100</td>
<td>98 [R]</td>
<td>6.3 * 10⁴</td>
</tr>
<tr>
<td>HRP</td>
<td>60</td>
<td>95</td>
<td>46 [S]</td>
<td>29</td>
</tr>
<tr>
<td>LPO</td>
<td>105</td>
<td>40</td>
<td>52 [R]</td>
<td>57</td>
</tr>
<tr>
<td>CcP</td>
<td>not reported</td>
<td>not reported</td>
<td>2 [S]</td>
<td>&lt;300</td>
</tr>
<tr>
<td>MP-11</td>
<td>45</td>
<td>45</td>
<td>3 [S]</td>
<td>3</td>
</tr>
</tbody>
</table>

$^a$ Turnover number= mol of product produced per mol of enzyme used

The differences in reactivity and enantioselectivity of the peroxidases can be explained by the difference in the environment of the active site. Especially heme peroxidases in which the heme-iron is less accessible give a lower selectivity and reactivity (vide infra). In order to facilitate direct oxygen transfer, the active site of several heme peroxidases was enlarged using site-directed mutagenesis. Several mutants of HRP and CcP were made and the influence of the mutation on reaction rate and enantiomeric excess of thioether sulfoxidation was determined [48,49,50,45]. Replacement of the bulky tryptophan-51 in CcP by an alanine still resulted in nearly racemic sulfoxide (ee=10%) although nearly all the oxygen in the sulfoxide was derived from hydrogen peroxide (98%). Modifications of HRP were made at two amino acids in the distal pocket of HRP, His-42 and Phe-41. Phe-41 was replaced by the amino acids leucine or threonine which are of intermediate size and by the small amino acid alanine. Also the distal histidine was replaced by alanine and by valine. The sulfoxidation of alkyl aryl sulfides proceeded faster for the mutants than for the native enzyme (Table 2.3).
Selective oxidations catalyzed by peroxidases

Moreover, in the case of F41L the enantioselectivity was increased. The results indicated that the alkyl chain of the alkyl aryl sulfides binds in a restricted site of HRP, and the reaction rate can be increased by replacing Phe-41 or His-42 with smaller amino acids. Furthermore, the stereospecificity of the reaction was shown to be dependent on the size as well as on the polarity of the amino acid on position 41.

Table 2.3.
Effect of site directed mutagenesis of HRP on rate and enantioselectivity [50].

<table>
<thead>
<tr>
<th>Sulfide</th>
<th>native HRP</th>
<th>F41L</th>
<th>F41T</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rate (nmol s⁻¹)</td>
<td>ee (%)</td>
<td>rate (nmol s⁻¹)</td>
</tr>
<tr>
<td>R₁S-R₂</td>
<td>μmol⁻¹</td>
<td></td>
<td>μmol⁻¹</td>
</tr>
<tr>
<td>R₁</td>
<td>R₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C₆H₅</td>
<td>CH₃</td>
<td>56.4</td>
<td>77</td>
</tr>
<tr>
<td>C₆H₅</td>
<td>cyclopropyl</td>
<td>8.5</td>
<td>7</td>
</tr>
</tbody>
</table>

a The absolute stereochemistry of the dominant isomer is S except where indicated.
b Rates were calculated from Vₘₐₓ and Kₘ at a sulfide concentration of 5 mM.

A peroxidase-related soybean oxidoreductase [51] is also capable of performing enantioselective sulfoxidations. This enzyme can use cumene hydroperoxide or long chain unsaturated alkyl hydroperoxides such as 13-hydroperoxylinoleic acid as the oxidant but it does not catalyze the classical peroxidase reaction. High enantiomeric excesses to the S-sulfoxide were reported (90% ee for methyl p-methylphenyl sulfoxide).

N-oxidation

A few examples of peroxidase-catalyzed selective N-oxidations have been described. For example Corbett et al. reported in 1979 the oxidation of 4-chloroaniline to 4-chloronitrosobenzene catalyzed by CPO with hydrogen peroxide as the oxidant [52]. Later it was shown that other aryl amines could also be oxidized to the corresponding nitroso compounds [53-56]. The reaction involves direct oxygen transfer from the iron(V)oxo complex to the aniline and proceeds via the hydroxylamine which is further oxidized by a second iron(V)oxo species to the nitroso compound.
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\[
\text{NH}_2 \quad \rightarrow \quad \text{NHOOH} \quad \rightarrow \quad \text{NOCl}
\]

Fig. 2.11. Oxidation of anilines by CPO.

A peroxidase-related enzyme from pea seedlings is also capable of mediating the oxidation of amines to the corresponding nitroso compounds [56,57,58]. When HRP was used as the catalyst for the oxidation of aniline-derivatives a complex mixture of coloured high molecular weight products was obtained [3,52,59]. Recently Kalliney and Zaks [60] discovered that HRP can selectively catalyse the oxidation of the hydroxylamino and nitroso derivative of Everninomicin, an antibiotic (Fig. 2.12). An indication for direct oxygen transfer from the iron(V)oxo to the nitroso compound was derived from labeling experiments which showed that the oxygen in the nitrogroup was predominantly derived from hydrogen peroxide.

\[
\text{H}_3\text{CO} \quad \rightarrow \quad \text{H}_3\text{CO} \quad \rightarrow \quad \text{H}_3\text{CO}
\]

Fig. 2.12. N-oxidation catalyzed by HRP.

N-oxidation of anilines to nitrobenzenes is reported to be catalyzed by a couple of non-heme bacterial peroxidases [61,62,63] Whereas the chloroperoxidases from Pseudomonas pyrocina and Serratia marcescens can catalyze the oxidation of 4-chloro-aniline and 3-(2-amino-3-chloro-phenyl)-pyrrole, the bromoperoxidase from Pseudomonas putida catalyzes only the oxidation of aniline to nitrobenzene.

**Epoxidation**

Optically active epoxides are very useful chiral synthons as they can undergo facile stereospecific ring-opening to form bifunctional compounds [64]. They are important as key intermediates in the production of bioactive chiral compounds or as end products with
biological activity. For example chiral epoxides are used for the production of β-blockers, the HIV protease inhibitor Crixivan [65] or calcium antagonists [66] like the drug Diltiazem (Fig. 2.13).

Fig. 2.13. Asymmetric synthesis of Diltiazem.

Because of this important role for epoxides in organic synthesis much research is focused on the development of catalysts which can perform the epoxidation of unfunctionalized olefins with high enantioselectivity [67]. Such catalysts can be divided into three classes: synthetic catalysts [68,69], enzymes [70] and catalytic antibodies [71]. However, the use of catalytic antibodies for enantioselective epoxidation is still in its infancy.

There are several approaches for the use of peroxidases for epoxidation. One approach is indirect epoxidation: i.e. haloperoxidase catalyzed formation of a halohydrin which can be converted chemically or with a halohydrin epoxidase to a (chiral) epoxide. A process for the production of propylene oxide using this approach [72] was developed with in situ generation of hydrogen peroxide by glucose oxidase (Fig. 2.14). However the process has not been commercialized. An important drawback of the halohydrin method is that the produced halohydrins are racemic and the epoxides produced with this method have a low enantiomeric excess.

Another example of the indirect oxidation of alkenes to epoxides catalyzed by peroxidases is the glutathione or 4-methylphenol dependent cooxidation of styrene to styrene oxide catalyzed by HRP. This reaction yields racemic styrene oxide and benzaldehyde [73,74]. The last example of indirect oxidation is the epoxidation of alkenes by metal-free peroxidases yielding racemic epoxides [75]. The reaction can only be performed in acetate or propionate buffer and is believed to proceed via an enzymatically generated peracid, analogous to the lipase catalyzed oxidation of carboxylic acids. The peracid subsequently oxidizes the alkenes to racemic epoxides.
Fig. 2.14. Process for production of epoxides by haloperoxidase-catalyzed halohydrin formation.

A more powerful method for producing epoxides using peroxidases is the direct CPO-catalyzed oxidation of alkenes by hydrogen peroxide or alkyl hydroperoxides [76-79]. CPO catalyzes the epoxidation of \textit{trans}-[1-\textsuperscript{2}H]styrene with retention of stereochemistry [78]. It was demonstrated that all oxygen in styrene oxide is derived from hydrogen peroxide, which implies that the reaction is a true oxygenation reaction. Phenylacetaldehyde is produced as a byproduct in quite large yields (50%). The same effect is observed with the epoxidation of butadiene, where crotonaldehyde is produced in 25% yield [79].
Table 2.4. CPO-catalyzed epoxidation of olefins [82,85].

<table>
<thead>
<tr>
<th>Olefin</th>
<th>yield product/byproducts</th>
<th>turnover number</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Olefin Structure" /></td>
<td>100%, ee 95%</td>
<td>2100</td>
</tr>
<tr>
<td><img src="image2" alt="Olefin Structure" /></td>
<td>40%, ee 95%</td>
<td>840</td>
</tr>
<tr>
<td><img src="image3" alt="Olefin Structure" /></td>
<td>3%</td>
<td>63</td>
</tr>
<tr>
<td><img src="image4" alt="Olefin Structure" /></td>
<td>2%, ee 10%</td>
<td>34</td>
</tr>
<tr>
<td><img src="image5" alt="Olefin Structure" /></td>
<td>23%, ee 95%</td>
<td>1700</td>
</tr>
<tr>
<td><img src="image6" alt="Olefin Structure" /></td>
<td>40%, ee 49%</td>
<td>840</td>
</tr>
<tr>
<td><img src="image7" alt="Olefin Structure" /></td>
<td>24%</td>
<td></td>
</tr>
<tr>
<td><img src="image8" alt="Olefin Structure" /></td>
<td>4%</td>
<td></td>
</tr>
</tbody>
</table>

Although the CPO catalyzed epoxidation of olefins had been known for nearly a decade, it was just recently discovered to be enantioselective [80,81]. Direct epoxidation (Table 2.4) catalyzed by CPO proceeds well, with high yield and excellent enantiomeric excess, for short-chain alkenes (up to C₅) with the double bond close to the chain terminus [81,82,83]. In contrast, epoxidation of trans-alkenes gave very low yields of epoxide. The major reaction was allylic oxidation, accompanied by further oxidation of the allylic alcohols to the corresponding aldehydes. Similarly, allylic hydroxylation was observed with alkenes in
which the double bond was far removed from the chain terminus (cis 3-alkenes). Terminal alkenes gave rise to heme alkylation and subsequent protein deactivation [84]. Consequently, unbranched long-chain aliphatic 1-alkenes are poor substrates for epoxidation. The low turnover numbers for terminal alkenes can be increased by using short-chain prochiral terminal dienes or branched 1-alkenes [82, 85]. Styrenes yielded a mixture of the epoxides and the isomeric phenylacetaldehydes and the obtained enantiomeric excess was moderate.

Microperoxidase-11, an undecapeptide containing a heme unit, can also catalyze the direct oxidation of olefins to epoxides [86]. However, aldehydes were obtained as side-products (71% for styrene and 33% for cis-stilbene), turnover numbers were low (up to 4 for styrene oxide) and enantiomeric excesses were not reported.

Another peroxidase which can catalyze the epoxidation of styrenes is cytochrome c peroxidase [45]. However, again turnover numbers were low (<1 during 20 min. reaction time), enantiomeric excesses were moderate (up to 32% ee) and for cis-styrenes partial rearrangement to the aldehyde or ketone occurred. Moreover, in the case of cis-β-methyl styrene the trans-epoxide was formed as a byproduct, possibly via a protein peroxy radical mechanism.

Native HRP generally does not perform epoxidations, with the exception of trans-β-methyl styrene which was epoxidized in a low enantiomeric excess (ee=6%) [48]. Various mutants of HRP (F41L, F41T, F41A, H42A, H42V) in contrast, catalyze the enantioselective epoxidation of styrene derivatives [48, 49]. Mutants with increased accessibility to the active site were shown to catalyze the oxidation of styrene, cis-β-methyl styrene and trans-β-methyl styrene. However, a large amount of benzaldehyde and the rearranged aldehyde or ketone was obtained. Moreover, in the case of cis-methyl styrene the trans-epoxide was also obtained in low enantiomeric excess. The oxidation appears to involve more than a direct oxygen transfer from the iron(V)oxo species to the olefin as labeling experiments showed that a substantial part of the oxygen in the product is derived from molecular oxygen. Only oxidation of cis-β-methyl styrene, catalyzed by the F41T mutant of HRP, gave complete enantioselectivity to the (1S,2R) enantiomer and in this case all the oxygen in the product was derived from hydrogen peroxide. The synthetic utility of these reactions is questionable, as the rates and enantiomeric excesses are generally low and substantial amounts of byproducts are obtained. Moreover, such mutants are not yet commercially available.
Finally, we want to mention epoxidations catalyzed by a soybean peroxynogenase, which is related to peroxidases (Fig. 2.15). Oxidation of *cis* mono- and polyunsaturated fatty acids with cumene hydroperoxide or long chain unsaturated alkyl hydroperoxides such as 13-hydroperoxylinoleic acid in the presence of soybean peroxynogenase gave the corresponding epoxides in moderate enantiomeric excess [87].

![Fig. 2.15. Oxidation of alkenes by soybean peroxynogenase (SBPO).](image)

**C-H bond oxidation**

**Benzylic/allylic hydroxylation**

Selective hydroxylation of hydrocarbons is difficult to perform chemically. One of the few examples of selective benzylic hydroxylation mediated by a chemical chiral catalyst is shown in Fig. 2.16 [88] in which a chiral manganese salen complex is used as the catalyst.

![Fig. 2.16. Benzylic hydroxylation catalyzed by a chiral salen(Mn) complex.](image)

Nuclear hydroxylation of aromatic compounds is a common peroxidase reaction which generally yields polymerized products. However, selective hydroxylation of benzylic or allylic C-H bonds is rarely observed with peroxidases, although catalysis of this reaction by heme-containing monooxygenases is quite common [89].
Table 2.5.  
Benzylic hydroxylation catalyzed by CPO [82].

<table>
<thead>
<tr>
<th>Substrate</th>
<th>yield product / byproduct</th>
<th>turnover number</th>
</tr>
</thead>
<tbody>
<tr>
<td>[ \text{CH}_3 \text{C}_6 \text{H}_5 ]</td>
<td>[ \text{CHC}_6 \text{H}_4 \text{O} ] 85% [ \text{p-C}_6 \text{H}_4 \text{COO} ] 15%</td>
<td>2100 (1800+300)</td>
</tr>
<tr>
<td>[ \text{CHC}_6 \text{H}_4 \text{OH} ]</td>
<td>[ \text{CHC}_6 \text{H}_4 \text{OH} ] 20%, ee 97%</td>
<td>420</td>
</tr>
<tr>
<td>[ \text{CHC}_6 \text{H}_4 \text{OH} ]</td>
<td>[ \text{CHC}_6 \text{H}_4 \text{OH} ] 20%, ee 88%</td>
<td>420</td>
</tr>
<tr>
<td>[ \text{CH} ] [ \text{CH} ] [ \text{CH} ] [ \text{CH} ]</td>
<td>[ \text{CH} ] [ \text{CH} ] [ \text{CH} ] [ \text{CH} ]</td>
<td>126</td>
</tr>
<tr>
<td>[ \text{CH} ] [ \text{CH} ] [ \text{CH} ] [ \text{CH} ]</td>
<td>[ \text{CH} ] [ \text{CH} ] [ \text{CH} ] [ \text{CH} ]</td>
<td>3.0%</td>
</tr>
</tbody>
</table>

The only peroxidase known so far to give selective hydrocarbon hydroxylation is CPO (Table 2.5). The first study describing CPO-mediated hydrocarbon hydroxylation concerned the oxidation of cyclohexene to cyclohex-2-en-1-ol [76]. Recently two more thorough studies of CPO-catalyzed benzylic and allylic hydroxylation of hydrocarbons appeared [82,90]. The oxygen atom in benzylic alcohol was shown to be derived from hydrogen peroxide consistent with a mechanism involving direct oxygen transfer. O-dealkylation was a major side reaction.
when \( p \)-methyl anisole was oxidized indicating that the reaction probably proceeds via initial one-electron transfer from the aromatic ring. Although in the study of Miller \textit{et al.} [90] benzylic hydroxylation is a very slow reaction yielding total turnover numbers between 0.2 and 5, Zaks and Dodds [82] reported considerably higher turnover numbers (Table 2.5). Remarkable is the reversion in stereochemical preference of the obtained chiral benzyl alcohol when the alkyl chain is enlarged from ethyl to propyl. Inversion of stereochemistry was also observed in CPO-catalyzed epoxidations and it would be interesting to perform docking experiments using the recently resolved crystal structure of CPO [91] to find an explanation for these results.

\textbf{Alcohol oxidation}

Oxidation of alcohols to aldehydes is quite common with dehydrogenases, monooxygenases and oxidases. Peroxidases, on the other hand, are generally restricted to oxidation of phenols to quinones via free radicals intermediates (classical peroxidase reaction). Again chloroperoxidase from \textit{Caldariomyces fumago} is the exception to the general rule and can catalyze the oxidation of primary alcohols to aldehydes [92,93]. CPO has a preference for alcohols which contain an allylic, propargylic or benzylic group. Although Geigert \textit{et al.} [93] reported that the reaction is selective and no oxidation to the corresponding acid occurs, we have shown [94] that reactive aldehydes like 5-hydroxymethylfurfural are oxidized to the corresponding acid (Fig. 2.17). Oxidation of aldehyde to acid proceeds via direct oxygen transfer as complete incorporation of oxygen from \( \text{H}_2^{18}\text{O}_2 \) is observed.

\[
\begin{align*}
\text{CPO} & \quad \text{H}_2\text{O}_2 \\
\text{O} & \quad \text{OH} & \quad \text{O} & \quad \text{OH} \\
\text{CPO} & \quad \text{H}_2\text{O}_2 \\
\text{O} & \quad \text{OH} & \quad \text{O} & \quad \text{OH} \\
\end{align*}
\]

\textit{Fig. 2.17. Oxidation of 5-hydroxymethylfurfural catalyzed by CPO [94].}

\textbf{Indole oxidation}

Substituted oxindoles are interesting compounds due to their biological properties. \( 5 \)-Chloro-oxindole, for example, is an intermediate in the synthesis of Tenidap (1-carbamoyl-5-chloro-3-[hydroxy(2-thienyl)methylene]indole-2-(3\textit{H})-one, Fig. 2.18), an anti-inflammatory
drug [95]. Direct oxidation of indole to oxindole is difficult, as oxidation generally occurs at the more electron-rich 3-position [96], if this position is not substituted, yielding indoxyl, a precursor for indigo [97].

Chemical methods available for producing oxindole mostly consist of multi-step procedures [98-101]. Often harsh conditions are necessary or low yields or isomeric mixtures are obtained. The direct oxidation of indole to oxindole, however, is efficiently catalyzed by CPO [102,103]. Indoles with substituents at the 4-, 5- or 6-position yield the corresponding oxindoles in nearly quantitative yield (Fig. 2.19, Table 2.6). Labeling experiments showed that the oxygen in the product is derived from hydrogen peroxide, consistent with a mechanism involving direct oxygen transfer from the iron(V)oxo species to the indole [103].

![Fig. 2.18. Tenidap.](image)

![Fig. 2.19. Oxidation of substituted indoles by CPO.](image)

**Table 2.6.**
Preparative scale synthesis of oxindole derivatives [103].

<table>
<thead>
<tr>
<th>Indole derivative</th>
<th>CPO (kU)</th>
<th>yield (%)</th>
<th>purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>indole</td>
<td>1</td>
<td>96</td>
<td>96</td>
</tr>
<tr>
<td>7-aza-indole</td>
<td>2</td>
<td>97</td>
<td>99</td>
</tr>
<tr>
<td>4-Cl</td>
<td>6</td>
<td>70</td>
<td>76</td>
</tr>
<tr>
<td>5-Cl</td>
<td>2</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>5-Br</td>
<td>3</td>
<td>86</td>
<td>95</td>
</tr>
<tr>
<td>5-CH₃</td>
<td>6</td>
<td>92</td>
<td>94</td>
</tr>
<tr>
<td>5-OCH₃</td>
<td>6</td>
<td>93</td>
<td>95</td>
</tr>
<tr>
<td>6-Cl</td>
<td>2</td>
<td>96</td>
<td>99</td>
</tr>
</tbody>
</table>
Mechanistic aspects

Peroxidases can be broadly divided into three categories based on the nature of the catalytic centre: heme peroxidases, vanadium haloperoxidases and other peroxidases.

**Heme peroxidases**

**Catalytic center**

The prosthetic group of this type of peroxidases is a heme (generally ferriprotoporphyrin IX). For recent literature on structural aspects of peroxidases see ref. [1, 104-106]. In the native enzyme iron(III) is present in the high spin state. The iron is coordinated by the four nitrogen atoms of the heme. The 5th axial ligand of the iron is a histidine in most peroxidases. However, chloroperoxidase from *Caldariomyces fumago*, which has a cysteine as the axial ligand, is an exception. During catalysis the iron(III) species is oxidized by peroxide to a (formally) iron(V)oxo species: compound I. The oxidation of native enzyme is presumed to take place via a push/pull mechanism as depicted in Fig. 2.20 [107,108].

![Diagram of presumed mechanism for heterolytic cleavage of hydroperoxides catalyzed by peroxidases.](image)

**Fig. 2.20.** Presumed mechanism for heterolytic cleavage of hydroperoxides catalyzed by peroxidases.

A base in the active site abstracts a proton from the alkyl hydroperoxide. Charge delocalization of the formed hydroperoxide complex is stabilized by the positive charge on the base and the positive charge on a second amino acid residue in the active site (pull-effect).
Chapter 2

The axial ligand at the proximal position is a good electron donor to the heme thus stabilizing higher oxidation states of the iron (push-effect). This push-pull mechanism facilitates heterolytic cleavage of the hydrogen peroxide yielding an iron(V)oxo species.

Different acid/bases combinations are present in different heme enzymes. For example in horseradish peroxidase and cytochrome c peroxidase the distal base is a histidine and the positively charged amino acid residue is an arginine. A hydrogen bridge between an aspartate and the proximal histidine provides an imidazolate which is a better electron donor and exerts a stronger push effect. In CPO the distal base is presumed to be a glutamate whereas the proximal ligand is a thiolate which is a stronger electron donor than an imidazolate [91]. The heme iron redox potential is believed to be dependent on the stabilization of iron(V)oxo species (compound I). For example in cytochrome c peroxidase the binding of the aspartate to the proximal histidine increases the negative charge of the proximal ligand [109], thus stabilizing higher oxidation states of the heme iron and increasing the stability of compound I. Thus, the redox potential of cytochrome c peroxidase is decreased. In lignin peroxidase a strong hydrogen bonding network of a serine to a aspartate causes a movement of the proximal histidine containing helix, resulting in a weaker ligation of the proximal histidine to the heme iron [110]. Consequently, the heme in lignin peroxidase is more electron deficient than in cytochrome c peroxidase, which is believed to cause the higher oxidation potential of lignin peroxidase. In CPO the thiolate ligand is surrounded by a positive electrostatic environment which decreases the stabilization of compound I and increases the oxidation potential [91].

In most peroxidases, compound I is mainly represent by an iron(IV)oxo porphyrin radical cation species. In some peroxidases like cytochrome c peroxidase, lactoperoxidase and thyroid peroxidase the second oxidation equivalent of compound I is further delocalized to the protein moiety yielding an iron(IV)oxo protein radical cation species [111,112,113].

Compound I can be directly reduced to native enzyme by oxygen transfer (oxygenation, Fig. 2.21.3) or in two one-electron transfer steps (classical peroxidase reaction, Fig. 2.21.2). Oxygen transfer can proceed via a concerted two electron transfer reaction or a two step mechanism (oxygen rebound, vide infra). Electron transfer generally takes place at the heme edge or via protein radicals. Sometimes redox enzymes make use of a mediator to transfer electrons to a substrate which is too bulky to enter the active site. Veratryl alcohol, for example, mediates the oxidative degradation of lignine by lignin peroxidase [114,115].
Selective oxidations catalyzed by peroxidases

Another example is manganese (Mn$^{3+}$) which is oxidized by manganese-dependent lignin peroxidase to Mn$^{4+}$ which subsequently can oxidize phenolic compounds outside the enzyme [116,117]. Oxidation of cytochrome c by cytochrome c peroxidase takes place at the peripheral protein moiety via an electron transfer mechanism through the amino acid residues of the enzyme [118].

![Diagram of reaction cycle of peroxidases](image)

Fig. 2.21. Reaction cycle of peroxidases.

Oxygen transfer from oxidized enzyme to substrate is only possible when the heme iron is accessible for the substrates. Ortiz de Montellano et al. performed several studies to investigate the accessibility of the active site of a range of peroxidases. These authors used phenyl and alkylhydrazines and azide to react oxidatively with the peroxidase and studied the formed adducts. Reaction of HRP with these derivatives yields heme adducts at the δ-meso carbon [119,120,121]. Other peroxidases like Coprinus peroxidase [122], lignin peroxidase [123] and manganese peroxidase [124] yield similar results with phenylhydrazine or azide, azide and ethylhydrazine or azide, respectively. However, addition of phenyl- or alkylhydrazine to more accessible enzymes like catalase and chloroperoxidase results in covalent binding of the alkyl or aryl moiety to the iron and/or nitrogens of the porphyrin [125,126]. With CPO, phenylhydrazine yields a phenyl-iron adduct and azide yields a δ-meso azido heme indicating that CPO can catalyze oxygen transfer reactions as well as the classical peroxidase reaction. Addition of phenylhydrazine to cytochrome c peroxidase similarly yields a phenyl-iron adduct, although the crystal structure of the enzyme shows that the active site is buried in the protein [109]. Probably some small distortion of the active site affords a more
open structure which can accommodate a phenyl group, which also may account for the sulfoxidation and epoxidation activity of cytochrome $c$ peroxidase.

Crystal structures of other peroxidases like lignin peroxidase [110,127], *Coprinus* peroxidase [128] and manganese peroxidase [129] reveal that the active site is buried in the molecule, whereas CPO has a more open active site structure [91] allowing access to the heme iron for substrates with a limited size.

**Mechanism of oxygen transfer**

There are various mechanisms possible to account for direct oxygen transfer from the oxidized heme peroxidase to organic substrates. The oxygenations can be divided into oxygen transfer to alkenes, oxygen transfer to nitrogen or sulfur, C-H bond hydroxylation and indole oxidation.

**Oxygen transfer to alkenes**

![Diagram of oxygen transfer to alkenes]

Fig. 2.22. Possible intermediates in oxygen transfer to alkenes.

Possible mechanisms for alkene oxidation are the shown in Fig. 2.22. We note that the various intermediates are intraconvertible via electron transfer processes.

1. Concerted mechanism (Fig. 2.22.1).

The oxidation proceeds in one, concerted, step.
2. Oxygen transfer via a radical cation (Fig. 2.22.4).

In this mechanism initial one-electron transfer is followed by oxygen transfer from the iron(IV)oxo species to the radical cation intermediate (oxygen rebound). A complex of the iron(IV)oxo intermediate and the styrene radical cation has been trapped for a synthetic iron porphyrin catalyzed epoxidation of styrene [130].

3. The formation of a cationic (Fig. 2.22.3) or a radical (Fig. 2.22.5) intermediate via direct electrophilic attack of the iron(V)oxo-species on the electron-rich substrate or via further reaction of the radical cation.

If the intermediate is a cation and the carbon adjacent to the cation has a hydrogen-atom, an NIH-shift may occur in which a hydride is transferred from the neighbouring carbon to the cationic carbon (Fig. 2.23). This may account for the rearrangement products obtained when styrene derivatives or butadiene are oxidized by chloroperoxidase [78,79].

**Fig. 2.23. Rearrangement to 2-phenylacetaldehyde.**

4. Oxygen transfer via a metalloxetane intermediate (Fig. 2.22.2).

This intermediate has been proposed to be involved in biomimetic alkene epoxidations [131]. However, a metalloxetane intermediate is unlikely because of steric restrictions [132].

**Oxygen transfer to sulfur and nitrogen**

Oxidation at sulfur or nitrogen may proceed concerted, via a radical cation, or via electrophilic attack of the iron(V)oxo species on the electron-rich reactant analogous to the mechanisms described for alkene oxidation (1, 3, 4 and 5 in Fig. 2.22).

Evidence for a radical cation intermediate in sulfoxidation reactions has been obtained for lactoperoxidase [32] and the peroxidase related soybean peroxygenase [51]. There is also support that this mechanism occurs with horseradish peroxidase [133,134]. However, for chloroperoxidase the mechanism is less clear and the sulfoxidation might proceed either via a concerted mechanism or via two very fast subsequent one-electron transfer processes.
In Table 2.7 the source of the oxygen in the sulfoxide is depicted for several peroxidases. Although for all peroxidases shown, the greater part of the oxygen is derived from hydrogen peroxide, indicating that the oxidation is an oxygen transfer reaction, horseradish peroxidase and lactoperoxidase also give oxygen incorporation from another source. Oxygen transfer from water to p-methoxyphenyl methyl sulfide in the case of horseradish peroxidase (10% for p-methoxyphenyl methyl sulfide, Table 2.7) might either be due to reaction of a sulfur dication with water or to exchange of the oxygen of the iron(IV)oxo species (compound II) with water [33,135]. A third possible explanation for oxygen transfer from water to the sulfide is depicted in Fig. 2.24. The intermediate sulfide radical cation reacts with water in the active site. Subsequently the hydroxy-sulfide radical is further oxidized by compound II to yield the sulfoxide. The incorporation of oxygen derived from molecular oxygen in methyl phenyl sulfide for lactoperoxidase might be caused by formation of a protein peroxy radical during catalysis [32].

\[
\begin{align*}
\text{ArSR} & \xrightarrow{-e} \text{ArSR}^+ \\
& \xrightarrow{H^+} \text{ArSR}^{OH} \\
& \xrightarrow{-e} \text{ArSR}^{OH^-} \\
& \xrightarrow{-H^+} \text{ArSR}^0
\end{align*}
\]

Fig. 2.24. Possible mechanism for oxygen transfer from water to sulfide.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>( \text{H}_2^{18}\text{O}_2 ) incorporation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPO [32]</td>
<td>99</td>
</tr>
<tr>
<td>HRP(^a) [32]</td>
<td>90</td>
</tr>
<tr>
<td>LPO(^b) [32]</td>
<td>85</td>
</tr>
<tr>
<td>MP-11 [136]</td>
<td>100</td>
</tr>
</tbody>
</table>

\(^a\) \( \text{H}_2^{18}\text{O} \): HRP yields 10% of oxygen incorporation from water for \( p\)-OCH\(_3\)-phenyl methyl sulfide.

\(^b\) \( ^{18}\text{O}_2 \): LPO yields 22% of oxygen incorporation from molecular oxygen for methyl phenyl sulfide.

We note that hydroxylamine oxidation catalyzed by CPO and HRP might very well proceed via the classical peroxidase reaction (oxidative dehydrogenation) instead of via an
oxygen transfer mechanism. There is no evidence for direct oxygen transfer from the enzymes to the hydroxylamine, contrary to chloroperoxidase-catalyzed oxidation of amines or horseradish peroxidase catalyzed oxidation of nitroso-compounds.

**C-H bond hydroxylation**

![C-H bond hydroxylation diagram](image)

1. concerted
2. hydrogen abstraction

**Fig. 2.25. Possible mechanisms for C-H bond oxidation.**

C-H bond hydroxylation may proceed via a concerted mechanism or via hydrogen abstraction followed by oxygen rebound (Fig. 2.25). Hydrogen abstraction may occur directly or via an intermediate carbocation (Fig. 2.27).

1. Concerted mechanism (Fig. 2.25.1).

Results obtained from experiments with a radical clock support a concerted mechanism for chloroperoxidase-catalyzed benzylic hydroxylation [82]. However, one should keep in mind that steric constraints in the active site of chloroperoxidase might severely retard the rearrangement of the radical clock, so a hydrogen abstraction mechanism cannot be completely ruled out. Deuterium labeling experiments indicate that cleavage of the C-H bond occurs in the rate limiting step [90].

![Rearrangement diagram](image)

**Fig. 2.26. Rearrangement of radical clock if hydroxylation proceeds non-concerted.**
2. Hydrogen abstraction (Fig. 2.25.2).

In this mechanism hydrogen abstraction is followed by hydroxyl transfer from the enzyme to the substrate. The hydrogen abstraction may proceed either directly or in two steps: first electron transfer followed by proton elimination (Fig. 2.27).

![Figure 2.27. Electron transfer followed by proton abstraction.](image)

Oxidation of alcohols to the corresponding aldehydes can either proceed as a dehydrogenation or as an oxygen incorporation into a C-H bond, yielding a *gem*-diol which converts to the aldehyde. Hydrogen abstraction is involved in both mechanisms. When 2-phenylethanol is oxidized (Fig. 2.28) hydrogen abstraction may occur at either carbon. But deuterium labeling experiments rule out the benzylic hydrogen abstraction as subsequent oxidation would lead to a cation which would rearrange to yield 2-deutero-2-phenylacetaldehyde, which was not observed [90].

![Figure 2.28. Oxidation of 2-phenylethanol via a benzylic hydrogen abstraction.](image)

**Indole oxidation**

The oxidation of indole to oxindole is a special case of C-H bond oxidation as the hydroxylated carbon is part of an aromatic system. This is also the case in phenol hydroxylation, a classical peroxidase reaction. A concerted mechanism for indole oxidation is questionable as an epoxide would be expected as intermediate instead of direct C-H bond hydroxylation. The formation of this epoxide intermediate is highly unlikely: the oxidation of alkenes proceeds much slower than the oxidation of indole. Direct electrophilic attack of compound I on indole is not expected to occur at the 2-position as the 3-position is the most
Selective oxidations catalyzed by peroxidases

electron-rich one. However, the active site pocket might provide an environment in which the
2-position becomes the favourite position for electrophilic attack. A metalloxetane
intermediate is unlikely due to the earlier mentioned steric constrains.

By analogy with reactions of peroxidases with other aromatic substrates we propose
[103] a mechanism involving initial one-electron transfer to produce a radical cation
intermediate. Subsequent oxygen rebound followed by electron redistribution and proton loss
yields the hydroxylated indole which tautomerizes to the more stable oxindole (Fig. 2.29).

![Chemical Reaction Diagram]

Fig. 2.29. Proposed mechanism for oxidation of indole to oxindole by CPO.
Chapter 2

Deactivation

Deactivation of heme peroxidases can involve three different pathways: In this review we will discuss oxidation of the porphyrin ring by the oxidant (irreversible), reaction of enzyme with suicide inhibitors (irreversible) and compound III formation (reversible) as deactivation pathways for heme peroxidases.

Oxidation of the porphyrin ring by the oxidant

Heme enzymes are susceptible to oxidative destruction of the porphyrin ring. For example the heme of cytochrome P-450 is rapidly destroyed by oxidants like hydrogen peroxide, alkyl hydroperoxides and iodosobenzene [137]. The oxidative destruction of HRP by hydrogen peroxide or m-chloroperbenzoic acid yields an unstable verdohemoprotein called P-670 [138,139]. Oxidation of heme by heme oxygenase yields biliverdin which involves α-meso-hydroxylation of the porphyrin ring and subsequent fragmentation to verdoheme [140]. The oxidation of the heme moiety may involve various different activated oxygen species, e.g. hydrogen peroxide, superoxide anion, hydroxyl radicals or singlet oxygen. All these activated oxygen species may be produced during the catalytic cycle of peroxidases and may damage the peroxidase irreversibly. Vanadium peroxidases are far more stable towards oxidative destruction than heme peroxidases as they do not contain a porphyrin ring [141,142].

Suicide deactivation

Peroxidases can be deactivated by reaction with so-called suicide inhibitors formed in situ during catalysis. Phenylhydrazine, alkylhydrazines and sodium azide are often used to perform mechanistic and active site topology studies with peroxidases. Reaction of these compounds with peroxidase yields free radicals that form adducts with the peroxidase, either at the meso-heme-edges, the iron or the porphyrin nitrogens, which yields information about the active site topology of peroxidases (vide supra).

In contrast with the above mentioned inhibitors which are deliberately added to the reaction mixture deactivation can also be due to reactive intermediates formed during the reaction. An example is the heme alkylation which occurs when 1-alkenes are oxidized to the corresponding epoxides with CPO [84]. Similarly, resorcinol derivatives can deactivate
Selective oxidations catalyzed by peroxidases

lactoperoxidase and thyroid peroxidase during catalysis [143], probably due to the formation of reactive radicals which covalently bind to amino acid residues specific to these enzymes. Another example of a suicide inhibitor is phenol. During the oxidation of phenol HRP is deactivated. At low phenol concentrations this is due to accumulation of compound III (see next paragraph) and deactivation is reversible. At high phenol concentrations deactivation is irreversible and phenol concentration dependent [144]. This irreversible deactivation might well be due to the formation of phenoxy radicals which react with oxygen to form a reactive peroxy-radical species which destroys the enzyme [145].

**Compound III formation**

A third pathway for deactivation of heme peroxidases is the formation of a so-called Compound III intermediate. Compound III is often referred to as oxyperoxidase as it can be obtained by the addition of oxygen to the ferrous peroxidase [146,147,148]. Other means for obtaining compound III are adding excess hydrogen peroxide to either native enzyme or compound II [148,149] or adding superoxide anion to the native enzyme [150].

![Diagram of Compound III formation](image)

**Fig. 2.30. Formation and mesomeric structures of compound III.**

Compound III can be represented in several isoelectronic structures, including an iron(IV)-η-peroxo complex (Fig. 2.30). This η-peroxo complex is analogous to well-known molybdenum(VI)- and vanadium(V)-η-peroxo complexes [151]. The latter have been proposed as intermediates in the vanadium peroxidase catalyzed oxidation of halides (*vide infra*). Although the precise structure of compound III is not clear yet, its spectroscopic similarity to known oxy-heme proteins [152,153] and X-ray absorption studies favor the iron(II)oxygen assignment for horseradish peroxidase compound III [154]. Accumulation of
compound III during turnover leads to less enzyme available for catalysis and apparent enzyme deactivation, as compound III is not involved in the peroxidase reaction cycle.

The stability of compound III depends on the peroxidase. For CPO it is shown that compound III is very unstable and converts to ferric enzyme by autoxidation without detectable intermediates when no hydrogen peroxide is present [146]. Lignin peroxidase compound III, in contrast, is stable for several days when excess hydrogen peroxide is removed [148,155]. It has been shown recently that radical cations can revert compound III of LiP to native (ferric) enzyme. Thus, the apparent deactivation due to compound III formation is reversible for lignin peroxidase [155,156]. A possible mechanism for the reaction cycle of compound III formation and conversion to native enzyme for lignin peroxidase is shown in Fig. 2.31. The crucial step for converting compound III to native enzyme is oxidation of compound III by a radical cation to an iron(V)-η-peroxo species which spontaneously loses oxygen. This intermediate is comparable to the intermediate which we have proposed for the catalase reaction catalyzed by CPO [157] and which is indicated in the literature [20] as a compound I H2O2-species. Compound III of LiP is not capable of losing oxygen, but when oxidation of compound III takes place by the radical cation the intermediate iron(V)-η-peroxo species spontaneously decays to yield oxygen and native enzyme. Thus, when reductants are present which form stable radical cations, decay of LiP compound III proceeds via a reversible pathway in which hydrogen peroxide is decomposed to oxygen and water. However, with excess hydrogen peroxide and no reductant the decay of compound III of LiP and other peroxidases partially proceeds irreversibly, due to the formation of reactive oxygen intermediates which destroy the enzyme [158,159,160,144]. In this case the decay of compound III to ferric peroxidase is believed to proceed via ferrous enzyme and compound II [160,161].
Selective oxidations catalyzed by peroxidases

![Proposed mechanism for regeneration of native lignin peroxidase from compound III.](image)

**Vanadium haloperoxidases**

Next to heme-containing peroxidases it has recently become clear that a second class of haloperoxidases exists which contains vanadate as the prosthetic group. These vanadium peroxidases are isolated mainly from the marine environment, e.g. a bromoperoxidase from brown seaweed *Ascophyllum nodosum* [162,163], but also from other sources like the terrestrial fungus *Curvularia inaequalis* from which a chloroperoxidase has been isolated [164,141]. These vanadium haloperoxidases show considerable stability towards organic cosolvents [165-167]. Furthermore, in contrast to heme peroxidases, they are very stable under oxidizing conditions [141,142]. The oxidation state of vanadate does not change during catalysis. Reaction of hydrogen peroxide with the vanadium(V) probably affords an η-peroxovanadium(V)complex, comparable to known biomimetic η-peroxovanadium(V)-complexes [151]. This complex subsequently oxidizes the halogen to hypohalite [168,169].

Recently the crystal structure of a vanadium chloroperoxidase from *Curvularia inaequalis* azide complex has been resolved [170] and it was shown that the prosthetic group consists of hydrogen vanadate (V) in a trigonal bipyramidal coordination. The vanadium is coordinated to three non-protein oxygens, one nitrogen from a histidine and one nitrogen from the bound azide. The protein fold is highly α-helical and the helices are packed together in a
compact structure accounting for the high stability of the haloperoxidase. However, accessibility to the active site is limited to small molecules like halide, so far limiting the synthetic utility of vanadium chloroperoxidase.

Other peroxidases

Several bacterial haloperoxidases which contain neither heme nor vanadium as the prosthetic group have been isolated recently [171,172,18,61,63,173]. They catalyze the bromination, but not the chlorination of monochlorodimedone. Furthermore some of these enzymes catalyze the chlorination of indole and the oxidation of anilines to the nitro-compounds [173,61-63]. Selective chlorination of indole to 7-chloro-indole was demonstrated for the chloroperoxidase from Pseudomonas pyrocinia [18]. Only the bromoperoxidase from Pseudomonas putida has been shown to give substrate specificity for aniline in the oxidation of anilines to the nitro-compounds [173].

The non-heme, non-vanadium haloperoxidases exhibit their halogenating properties only when used in acetate or propionate buffer. Furthermore, for the bromoperoxidase from Streptomyces aureofaciens the crystal structure shows that the enzyme contains the same catalytic triad (Asp-His-Ser) as the serine-proteases and lipases [174]. A catalytic mechanism has been proposed involving the oxidation of acetate to peracetic acid which subsequently oxidizes the halide [75]. Whether this oxidation takes place in the active site is not yet clear. The question arises whether the other bacterial non-heme, non-vanadium haloperoxidases have the same catalytic triad and may perform oxidation via the same peracid intermediate as proposed for bromoperoxidase from Streptomyces aureofaciens.

Glutathione peroxidase contains a selenocysteine as the prosthetic group [175]. It catalyzes the oxidative dimerization of glutathione using a hydroperoxide as the primary oxidant. The enzyme has a low specificity towards the hydroperoxide, however the specificity for glutathione is very high and only low activities were observed for thiols other than glutathione. The oxidation presumably proceeds via a selenenic acid derivative as depicted in Fig. 2.32.
Selective oxidations catalyzed by peroxidases

![Reaction cycle of glutathione peroxidase](image)

**Fig. 2.32. Reaction cycle of glutathione peroxidase.**

Recently a novel non-heme, metal containing peroxidase has been discovered which is involved in lignin degradation [176]. The precise structure of the active site and the reaction mechanism of this peroxidase is not yet clear and further research is needed to determine to which class of peroxidases this enzyme belongs. The enzyme showed classical peroxidase activity and could degrade wheat straw in the presence of hydrogen peroxide.

**Commercial applications and conclusions**

Peroxidases have potential commercial applications in many different areas. The most developed field for their commercial application is in analytical diagnostics, for example in biosensors and immunoassays [177,178,179]. Moreover peroxidases are being extensively studied as bleaching inducer in, for example, the detergent and pulp industries [180,181,182,183], in anti-microbial applications [184,185,186], for removing aromatics from waste streams [187,188] and as a synthetic tool for oxidative coupling [189], for example as a catalyst for dough preparation [190] (Fig. 2.33). Recently, attention is focused on the use of peroxidases as catalysts for selective oxygenations (this review) and as mild polymerization catalysts.

Although peroxidases have potential for a variety of commercial applications and techniques are available to produce them on a large scale [191,192,193] commercial processes using peroxidases as catalysts in organic synthesis are still limited. Limited commercial availability and stability and the relatively low productivities (space-time yield) are important...
features which encumber the commercial application of peroxidases. Moreover, heme peroxidases show a low stability towards the oxidant. This problem can be circumvented by using haloperoxidases like vanadium chloroperoxidase which are more stable. However, the only reaction known to be catalyzed by these enzymes is hypohalite production. Other means to avoid destruction of heme peroxidases during catalytic turnover are enzyme modification [194] or process considerations like the mode of oxidant addition [195, 72] or the use of cosolvents which may prevent suicide deactivation [157].

![Chemical structure](image)

**Fig. 2.33. Oxidative coupling of a substituted ferulic acid yielding a carbohydrate gel which can retain water well, R=arobinoxylan. Process developed by Quest Int. Naarden, The Netherlands.**

Summarizing we conclude that peroxidases have potential as catalysts for selective oxygen transfer reactions, however the restricted active site of peroxidases limits their activity and therefore their synthetic utility as oxygen transfer catalysts. One positive exception is CPO which has great potential as a selective oxygen transfer catalyst. However, instability of this heme peroxidase during catalytic turnover encumbers its synthetic utility, although recently a process for the oxidation of indole has been developed in which a catalyst life-time of over 850,000 total turnovers can be reached [196]. We expect that in future new developments in the use of site-directed mutagenesis to design peroxidases with increased accessibility to the metal center, developments to increase the operational stability of peroxidases and the isolation of new peroxidases with novel properties will extend their synthetic utility.
References

Selective oxidations catalyzed by peroxidases

Selective oxidations catalyzed by peroxidases


-57-


Selective oxidations catalyzed by peroxidases


-59-
Chloroperoxidase catalyzed sulfoxidations in tert-butyl alcohol/water mixtures

Abstract

Chloroperoxidase (CPO) catalyzed oxidations of sulfides to chiral sulfoxides were performed in tert-butyl alcohol/water mixtures at ambient temperature. tert-Butyl alcohol/water (50:50, v/v) proved to be a good solvent system for performing synthetic oxidations catalyzed by CPO. The sulfoxidation of alkyl aryl sulfides and related compounds catalyzed by CPO in tert-butyl alcohol/water mixtures (50:50, v/v) was compared to the sulfoxidation in water. In both solvent systems complete enantioselectivity to the R-sulfoxide (ee=99%) was observed with hydrogen peroxide as oxidant when the size of the alkyl moiety was smaller than propyl. The uncatalyzed, racemic sulfoxidation did not proceed under these conditions. This is in contrast to literature data on sulfoxidation in water, where enantioselectivities were lower due to this uncatalyzed reaction. Reactions in water generally
Chapter 3

proceeded faster than reactions in the cosolvent system except for substrates which dissolve poorly in water or for solid substrates for which diffusion becomes an important limiting factor in water. The lower activity in tert-butyl alcohol/water for sulfoxidation is mainly due to an increase of the $K_m$ value (thermodynamically controlled). Also a decrease of $k_{cat}$ (catalytic turnover frequency) is observed, probably caused by a change in structure of the enzyme.

Introduction

Chiral sulfoxides are widely applied as synthons for numerous asymmetric transformations. They are, for example, important auxiliaries for the synthesis of natural products and biologically active compounds [1]. There are several methods for obtaining chiral sulfoxides [2]. These include nucleophilic substitution on chiral sulfur derivatives, like the classical Anderson method [3], and direct asymmetric oxidation of thioethers (Fig. 3.1). Direct asymmetric oxidation of sulfides has been induced by chiral oxidants such as oxaziridines [4] or by chiral catalysts, including a modified Sharpless agent [5], chiral porphyrins [6] or salen complexes [7] and chiral “host” molecules [8,9]. Next to these biomimetic approaches, direct asymmetric oxidation of sulfides can also be catalyzed by oxidative enzymes ([10], chapter 2 of this thesis). An example of an effective sulfoxidation catalyst is chloroperoxidase from Caldariomyces fumago (CPO), which catalyzes the oxidation of alkyl aryl sulfides with hydrogen peroxide in moderate to excellent enantiomeric excess [11,12,13].

\[ \begin{array}{c}
\text{R}_1\text{S}\text{R}_2 \xrightarrow{\text{CPO}} \text{H}_2\text{O}_2 \text{R}_1\text{O}\text{S}\text{R}_2
\end{array} \]

Fig. 3.1. Oxidation of sulfides by CPO.
Practical application of CPO in organic synthesis entails the use of organic solvents because the solubility of organic reactants in water is too low. Although there have been earlier reports regarding the use of CPO in aqueous cosolvent mixtures [11,14,15,16] most of the solvents used have some major drawbacks. Methanol and dimethyl sulfoxide are both reported to be substrates for CPO [17,18], acetonitrile is unacceptable due to its toxicity and acetone has been reported to lower the enantiomeric excess in sulfoxidation reactions [11] and is known to form peroxide complexes with hydrogen peroxide [19]. Owing to its bulky structure tert-butyl alcohol is not a substrate for CPO. Moreover, it is environmentally acceptable and is completely water miscible. In this paper we present the results obtained with CPO catalyzed sulfoxidation, using tert-butyl alcohol as a cosolvent and including reactions on a synthetically useful scale. Furthermore, the influence of tert-butyl alcohol on the rate and the selectivity of the reactions is discussed.

**Results and discussion**

*tet-Butyl alcohol as cosolvent*

The influence of the tert-butyl alcohol content on the oxidation of methyl phenyl sulfide by CPO was investigated. The activity of CPO was measured for the sulfoxidation of methyl phenyl sulfide at 10 mM concentration and with hydrogen peroxide as the oxidant. CPO retained its activity in tert-butyl alcohol/water mixtures up to 70% cosolvent (v/v, Fig. 3.2). However, the reactivity decreased with increasing amounts of cosolvent. The final conversion which was obtained was also very much dependent on the mode of addition of hydrogen peroxide (Fig. 3.3). This is due to deactivation of CPO by hydrogen peroxide, hence, the hydrogen peroxide concentration in the reaction mixture should be kept as low as possible. Optimal conditions are achieved when the rate of hydrogen peroxide addition equals the initial reaction rate and hydrogen peroxide is added continuously.
Since organic solvents can influence both the reaction rate [20] and selectivity [21] of enzymatic reactions we investigated the influence of the composition of tert-butyl alcohol/water mixtures on the rate and enantioselectivity of the sulfoxidation of a number of sulfides by CPO. The results were compared to the oxidation in aqueous buffer. Furthermore some new heteroaromatic sulfides and m/p-substituted phenyl methyl sulfides were oxidized to the corresponding sulfoxides. The experiments were carried out at 50 mM sulfide concentration. Reactions performed in tert-butyl alcohol/water mixtures (50:50, v/v) were homogeneous whereas the reactions carried out in buffer were heterogeneous.

Sulfoxidation in tert-butyl alcohol mixtures is facile (Table 3.1), although the final conversion of the sulfide is generally somewhat lower than in aqueous buffer. The observed conversions are final conversions since addition of more hydrogen peroxide did not increase the conversion. Reactions which did not reach completion stopped due to deactivation of CPO by an excess of hydrogen peroxide. The turnover numbers' obtained for the production of R-methyl phenyl sulfoxide were $11 \times 10^4$ in buffer and $8 \times 10^5$ in tert-butyl alcohol/water (50:50, v/v) respectively.

* Turnover number $= \text{mol of product obtained} / \text{mol of CPO used}$
Table 3.1.
Oxidation of sulfides by CPO and H₂O₂.

<table>
<thead>
<tr>
<th>Sulfide</th>
<th>tert-butyl alcohol/buffer</th>
<th>buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(50:50, v/v)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>conversion (%)</td>
<td>ee (%)</td>
</tr>
<tr>
<td>C₆H₅</td>
<td>CH₃</td>
<td>73</td>
</tr>
<tr>
<td>C₆H₅</td>
<td>CH₂CH₃</td>
<td>52</td>
</tr>
<tr>
<td>C₆H₅</td>
<td>CH₂CH₂CH₃</td>
<td>1</td>
</tr>
<tr>
<td>p-CH₃-C₆H₄</td>
<td>CH₃</td>
<td>66</td>
</tr>
<tr>
<td>p-OCH₃-C₆H₄</td>
<td>CH₃</td>
<td>50</td>
</tr>
<tr>
<td>m-OCH₃-C₆H₄</td>
<td>CH₃</td>
<td>19</td>
</tr>
<tr>
<td>o-OCH₃-C₆H₄</td>
<td>CH₃</td>
<td>2</td>
</tr>
<tr>
<td>p-NO₂-C₆H₄</td>
<td>CH₃</td>
<td>17</td>
</tr>
<tr>
<td>p-Cl-C₆H₄</td>
<td>CH₃</td>
<td>73</td>
</tr>
<tr>
<td>m-Cl-C₆H₄</td>
<td>CH₃</td>
<td>50</td>
</tr>
<tr>
<td>p-Br-C₆H₄</td>
<td>CH₃</td>
<td>46</td>
</tr>
<tr>
<td>m-Br-C₆H₄</td>
<td>CH₃</td>
<td>22</td>
</tr>
<tr>
<td>p-Cl-C₆H₄</td>
<td>CH₂CH₃</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>CH₃</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>CH₃</td>
<td>80</td>
</tr>
</tbody>
</table>

*a 50 mM sulfide, 25 mL solvent, 610 U CPO, 1 eq. H₂O₂/2h.*
Chapter 3

The lower conversions to the sulfoxides in the cosolvent system are caused by a lower \( k_{cat}/K_m \) value in tert-butyl alcohol/water mixtures (Table 3.2) compared to aqueous buffer. For methyl phenyl sulfide the main cause of decreased activity in cosolvent mixtures is an increase in \( K_m \) with a factor of 32. A slight decrease in \( k_{cat} \) is also observed. The lower \( k_{cat}/K_m \) values can be caused by several factors [20]:

a) Better stabilization of the ground state of the sulfides in the cosolvent system than in water. This will result in a higher \( K_m \) value for the sulfides.

b) Destabilization of the enzyme-substrate complex causing an increase in \( K_m \).

c) Solvent induced changes in the secondary and tertiary structure of CPO in tert-butyl alcohol/water mixtures leading to a decrease in the catalytic turnover frequency (\( k_{cat} \)).

d) Penetration of the cosolvent in the active site of CPO, which can decrease the local polarity in the active site of CPO. This can have an effect on \( k_{cat} \), on \( K_m \) and on the reaction selectivity.

For some sulfides a higher conversion to the corresponding sulfoxides was obtained in cosolvent mixtures than in pure buffer. We ascribe this effect to the low solubility of the corresponding sulfides in water. Methyl \( p \)-bromophenyl sulfide is nearly 10 times less soluble in buffer (0.1 M acetate, pH 5) than methyl phenyl sulfide (Table 3.2). The reaction rate at sulfide saturation in water is lower than the reaction rate at 50 mM sulfide concentration in tert-butyl alcohol/water mixtures for methyl \( p \)-bromophenyl sulfide (Table 3.2). At saturation the reaction rate in buffer is slightly higher than the rate of hydrogen peroxide addition (reaction rate is 95 mM/h at 400 \( \mu \)M \( \text{H}_2\text{O}_2 \) vs. 25 mM/h \( \text{H}_2\text{O}_2 \) addition). As CPO already is deactivated at low concentrations of hydrogen peroxide (chapter 6) and as some diffusion limitation probably occurs during reaction because methyl \( p \)-bromophenyl sulfide is solid, the reaction rate may be expected to quickly drop below the critical value of 25 mM/h, resulting in deactivation of CPO due to accumulation of hydrogen peroxide. The reaction rate in tert-butyl alcohol/water mixtures is somewhat higher at the start (120 mM/h), but during the course of the reaction the reaction rate declines due to lowering of the sulfide concentration and some enzyme deactivation. Again, the reaction ceases when the reaction rate is lower than the rate of hydrogen peroxide addition of 25 mM/h.
Table 3.2.
Reaction rates and solubilities of sulfides.

<table>
<thead>
<tr>
<th>Sulfide</th>
<th>Solvent</th>
<th>$k_{cat}/K_m$ (s$^{-1}$mM$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>Solubility (mM)</th>
<th>$V_{50}$ mM $^a$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_6H_5$</td>
<td>CH$_3$</td>
<td>730</td>
<td>0.9</td>
<td>650</td>
<td>2.4</td>
<td>480</td>
</tr>
<tr>
<td></td>
<td>H$_2$O/t-BuOH</td>
<td>20</td>
<td>29</td>
<td>580</td>
<td>&gt;50</td>
<td>367</td>
</tr>
<tr>
<td></td>
<td>(50:50)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$p$-Br-$C_6H_4$</td>
<td>CH$_3$</td>
<td>193</td>
<td>&gt;&gt;0.3$^b$</td>
<td>n.d.</td>
<td>0.3</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>H$_2$O/t-BuOH</td>
<td>3</td>
<td>55</td>
<td>150</td>
<td>&gt;50</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>(50:50)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Turnover frequency when 50 mM of sulfide is present in the reaction mixture. The actual sulfide concentration in aqueous solution is the saturated concentration.

$^b$ The $K_m$ value could not be determined as the enzyme could not be saturated with the sulfide in the solubility range.

It is clear from Table 3.1 that tert-butyl alcohol as cosolvent does not negatively influence the stereochemical outcome of the reaction. For all tested sulfides, with the exception of propyl phenyl sulfide, essentially complete enantioselectivity to the corresponding R-sulfoxide was observed in water as well as in tert-butyl alcohol/water. A plausible reason for the observed low enantiomeric excess of propyl phenyl sulfide is its size. From the crystal structure of CPO [22] it has been shown that CPO has a small opening above the heme which allows access of substrates to the iron-oxo complex. The size of propyl phenyl sulfide is presumably too large for this opening, therefore the enzymatic oxidation of the sulfide is very slow. The non-catalyzed racemic oxidation becomes competitive, leading to a lower enantioselectivity of the obtained sulfoxide.

Blank experiments without enzyme present showed less than 1% spontaneous oxidation to the racemic sulfoxide after 2.5 h. This contrasts with results of Colonna [12], who observed substantial oxidation in blank experiments (up to 33%) leading to a lower enantiomeric purity of the obtained sulfoxides in enzymatic reactions. As our experiments were performed under different conditions we repeated the blank reaction with methyl o-methoxyphenyl sulfide using the method of Colonna et al. [12] (Table 3.3).
Table 3.3.
Comparison of blank experiments for methyl o-methoxyphenyl sulfide.

<table>
<thead>
<tr>
<th></th>
<th>Conversion</th>
<th>ee</th>
<th>Conversion [12]</th>
<th>ee</th>
</tr>
</thead>
<tbody>
<tr>
<td>blank</td>
<td>&lt;1</td>
<td>0</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>CPO</td>
<td>3.3</td>
<td>99</td>
<td>24</td>
<td>27</td>
</tr>
</tbody>
</table>

*Experiments performed as described in [12]*

We observed much less blank reaction compared to the results reported by Colonna et al. (Table 3.3), although the reaction conditions were the same. This may be due to the presence of adventitious impurities catalyzing the spontaneous racemic oxidation of the sulfide to the sulfoxide. It is known for example [23] that catalytic amounts of acid or metal oxide enhance the oxidation of sulfides to sulfoxides.

**TBHP as the oxidant**

Sulfoxidations catalyzed by CPO were also performed with tert-butyl hydroperoxide as the oxidant. TBHP (2 eq.) was added at once and after 2 days the results in tert-butyl alcohol/aqueous buffer (50:50, v/v) were compared to reaction in aqueous buffer (Table 3.4).

The obtained conversions in tert-butyl alcohol/aqueous buffer mixtures (50:50, v/v) are much lower than in aqueous buffer when TBHP is used as the oxidant. Another striking difference is the lower enantiomeric excess of the sulfoxides which are obtained in the cosolvent system compared to pure buffer. These low enantioselectivities are merely caused by the relatively high racemic oxidation compared to the enzymatic oxidation. The enzymatic oxidation proceeds very slowly in the cosolvent system, thus the uncatalyzed oxidation of the sulfides to the racemic sulfoxides becomes a competitive reaction.
Table 3.4  
Oxidation of sulfides by CPO and TBHP.

<table>
<thead>
<tr>
<th>Sulfide</th>
<th>tert-butyl alcohol/buffer (50:50, v/v)</th>
<th>buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>conversion (%)</td>
<td>ee (%)</td>
</tr>
<tr>
<td>R1</td>
<td>R2</td>
<td></td>
</tr>
<tr>
<td>C₆H₅</td>
<td>CH₃</td>
<td>14</td>
</tr>
<tr>
<td>C₆H₄</td>
<td>CH₃CH₃</td>
<td>18</td>
</tr>
<tr>
<td>p-CH₃-C₆H₄</td>
<td>CH₃</td>
<td>22</td>
</tr>
</tbody>
</table>

Other enzymes

Other heme enzymes were also tested for performing enantioselective sulfoxidations with hydrogen peroxide as the oxidant. Vanadium chloroperoxidase from *Curvularia inaequalis* and *Coprinus cinereus* peroxidase showed no activity for oxidation of methyl phenyl sulfide. Soybean peroxidase catalyzed the enantioselective sulfoxidation of methyl phenyl sulfide to the S-sulfoxide. Unfortunately, the obtained turnover number and enantiomeric excess were both rather low (maximum ee of 39% at a turnover number of less than 2). Hence, we conclude that this catalyst is not applicable for enantioselective sulfoxidation reactions.

Conclusions

*tert*-Butyl alcohol/water provides an excellent solvent system for performing synthetic oxidations with CPO and hydrogen peroxide as the oxidant. Reactions remain highly (enantio)selective in this cosolvent system. R-sulfoxides are obtained in high optical and chemical yield. Generally the reaction rate in *tert*-butyl alcohol/water is lower than in aqueous buffer. However, the problems caused by the low solubility of substrates and the diffusion limitation, which are inherent to reactions in water, can be circumvented. The lower reactivity in the cosolvent system is mainly due to the higher $K_m$ value of the substrates in this solvent system as compared to water.
Experimental procedure

Materials and analytical methods

Chloroperoxidase from *Caldariomyces fumago* was isolated and purified as described in the literature [19]. The enzyme preparation contained 6100 U/mL according to the method of Morris et al. [24], with a purity of R₂ = 1.3 and an enzyme concentration of 115 μM. Enzyme concentrations were determined using a molar extinction coefficient of 91200 M⁻¹cm⁻¹ at 400 nm [25]. Vanadium chloroperoxidase from *Curvularia inaequalis* was received as a gift from Dr. R. Wever, University of Amsterdam and contained 21 U/mL according to the method of Morris et al. [24]. Microperoxidase-11 was purchased from Sigma. *Coprinus cinereus* peroxidase SP 502 (10 kPU/mL) and SP 676 (2263 kPU/g) were received as a gift from NOVO Nordisk. 1 Peroxidase Unit (PU) is the amount of enzyme which catalyzes the conversion of 1 μmol of H₂O₂ per minute in a system in which 2,2’-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) is oxidized under standard conditions (pH=7, [H₂O₂]=0.88 mM, [ABTS]=1.67 mM, 30°C). Soybean peroxidase was obtained as a gift from Enzymol International and contained 300 kPU/g.

Hydrogen peroxide 35% was obtained from Merck. tert-Butyl alcohol was purchased from Baker. Methyl phenyl sulfide was obtained from Janssen Chimica. Ethyl phenyl sulfide, methyl p-methylphenyl sulfide, methyl p-methoxyphenyl sulfide, methyl p-bromophenyl sulfide and methyl p-nitrophenyl sulfide were obtained from Aldrich Chemical Company. Methyl p-bromophenyl sulfide and methyl p-nitrophenyl sulfide were recrystallized from 60% acetic acid prior to use. Methyl p-chlorophenyl sulfide, 1-chloro-4-ethyl-thiobenzene, propyl phenyl sulfide and methyl m-methoxyphenyl sulfide were synthesized from the corresponding thiophenols according to the method of Herriot and Picker [26]. Methyl o-methoxyphenyl sulfide, methyl m-chlorophenyl sulfide, methyl m-bromophenyl sulfide, 2-methylthiothiophene and 2-methylthio-thiazole were donated by Prof. Dr. Brandsma of the University of Utrecht, and were distilled before use.

HPLC-analysis: Chiral. A Chiralcel OD column (Baker, 25 x 0.46 cm) was used for monitoring reactions. Eluents (flow 0.5 mL/min) used: hexane/2-propanol (75:25, v/v) for methyl p-nitrophenyl sulfide and hexane/2-propanol (85:15, v/v) for all other sulfides. Detection was performed using a Shimadzu SPD-6a UV spectrophotometer at 220 nm. Samples were treated with excess Na₂SO₃ to remove H₂O₂. Subsequently the samples were
diluted with eluent, 1,3,5-trimethoxybenzene was added as internal standard and the samples were dried over MgSO₄. After filtration through a 0.4 μm membrane filter the samples were analyzed by chiral HPLC.

**HPLC-analysis: Reversed phase.** A Novapak C₁₈ column (Waters, 8x10 mm 4 μm) contained in a Waters RCM 8 x 10 compression unit was used for monitoring reactions. Eluent (flow 1.0 mL/min) used for sulfide derivatives: methanol/water (60:40, v/v). Detection was performed using a Shimadzu SPD-6a UV spectrophotometer at 220 nm and an Erma ERC-7510 RI detector. tert-Butyl alcohol was used as internal standard. Samples were diluted with eluent saturated with Na₂SO₃. After filtration through a 0.4 μm membrane filter the samples were analyzed by reversed phase HPLC.

NMR spectra were recorded on a Varian VXR-400S spectrometer, using TMS as an internal standard and CDCl₃ as the solvent. UV measurements were performed on a Cary 3 spectrophotometer from Varian.

**General oxidation procedure**

At room temperature 1.25 mmol of sulfide was dissolved in 25 mL of solvent (0.1 M aqueous acetate buffer pH 5 or tert-butyl alcohol/0.1 M aqueous acetate buffer pH 5 (50:50, v/v)). 610 U CPO was added to the reaction mixture, followed by 5 min. of stirring. The reaction was started by the continuous addition of 1.66 M H₂O₂ at a rate of 1 eq./2h. In total 1.1 eq. of H₂O₂ was added and the reaction was quenched after 2.5 h. by the addition of an excess of Na₂SO₃. Reactions with tert-butyl hydroperoxide were started by the addition of 2 eq. of oxidant at once, and the reactions were stopped after 2 days. The reactions in tert-butyl alcohol/water mixtures were monitored by removing aliquots which were analyzed by chiral HPLC. The products of the reactions in 0.1 M aqueous acetate buffer pH 5 were analyzed after 2.5 h. by adding 25 mL of tert-butyl alcohol to the reaction mixture for homogenization. Subsequently a sample was taken and analyzed by chiral HPLC. Sulfoxides were isolated by addition of 25 mL water to the reaction mixture and saturation of the solution with NaCl. The aqueous solution was extracted with 3x 40 mL of CHCl₃, and the collected organic layers were dried over MgSO₄. After evaporation of the CHCl₃ in vacuo the sulfoxide was obtained. If necessary the sulfoxide was purified by column chromatography (stationary phase: silica; eluent methanol/toluene (10:90, v/v). The structures of the sulfoxides were confirmed by ¹H
and $^{13}$C NMR. The enantiomeric purity of the sulfoxides was determined by chiral HPLC [19]. Reactions concerning the influence of the amount of tert-buty alcohol and the mode of addition on oxidation of methyl phenyl sulfide were performed as described in [19].

**Other heme enzymes**

*Coprinus cinereus* peroxidase: at room temperature 0.5 mmol of methyl phenyl sulfide was dissolved in 50 mL solvent (0.1 M phosphate buffer pH 6.5 or tert-buty alcohol/0.1 M aqueous phosphate buffer pH 6.5 (50:50, v/v)). 4.5 kPU of SP502 or SP676 was added to the reaction mixture. The reaction was started by the continuous addition of 1.66 M H$_2$O$_2$ at a velocity of 1eq./2h. After 2 h. the reaction was stopped by the addition of excess Na$_2$SO$_3$. Reactions with *vanadium chloroperoxidase* (6.3 U) were similarly performed on a 4.2 mL scale (0.1 M aqueous acetate buffer pH 6) with 10 mM of methyl phenyl sulfide.

*Soybean peroxidase*: at room temperature 1 µmol of methyl phenyl sulfide and 4.3 mg soybean peroxidase were dissolved in 1 mL buffer (0.1 M acetate, pH 5). Every 15 min. 1 µL of H$_2$O$_2$ (50 mM) was added to the reaction mixture during 5 h. After 5 h. the reaction was stopped by addition of excess Na$_2$SO$_3$.

All reactions with heme enzymes were quenched with excess Na$_2$SO$_3$ and tert-buty alcohol was added for homogenization. Subsequently samples were analyzed by chiral HPLC.

**Blank reaction according to the method of Colonna [12]**

10 mM sulfide was dissolved in 42 mL 0.1 M citrate buffer pH 5. The reaction mixture was stirred for 5 min. Subsequently 1 eq. H$_2$O$_2$ (0.2 M) was added in 13 aliquots of 330 µL at 5 min. intervals and the reaction was continued for 5 min. After addition of excess Na$_2$SO$_3$ tert-buty alcohol was added for homogenization and samples were analyzed by chiral HPLC.

**Reaction rates**

Reaction rates of methyl phenyl sulfide and methyl $p$-bromophenyl sulfide were determined in buffer pH 5, 0.1 M and in tert-buty alcohol water. In buffer reaction rates were determined by spectrophotometric measurement of the decrease of sulfide by UV. The difference of molar extinction coefficient between the sulfides and the corresponding
Sulfoxidations in tert-butyl alcohol

Solubilities of sulfides in buffer

Solubilities of methyl phenyl sulfide and methyl p-bromophenyl sulfide were determined by dissolving the corresponding sulfide in acetate buffer pH 5, 0.1 M. From the saturated solution a sample was taken and tert-butyl alcohol was added as internal standard. The solubility of the sulfide was determined by analyzing the sample on reversed phase HPLC. The sample was compared to a standard solution of sulfide in tert-butyl alcohol/water (50:50, v/v).

Acknowledgements

A kind gift of sulfides by Prof. Dr. L. Brandsma of the University of Utrecht, The Netherlands, and his coworkers is gratefully acknowledged. Fred van de Velde is acknowledged for the experiments he performed with soybean peroxidase.

References

Chapter 4

Synthesis of substituted oxindoles by chloroperoxidase catalyzed oxidation of indoles

Abstract

Chloroperoxidase catalyzed oxidation of substituted indoles yields the corresponding oxindoles in virtually quantitative yield. These include 5-chloro-oxindole, a precursor for the anti-inflammatory agent Tenidap, which was obtained in 95% yield. The reactivity of the substituted indoles depends on the nature and the position of the substituent. Both electronic and steric effects of substituents appear to be important. All of the oxygen in the product is derived from hydrogen peroxide. A mechanism is proposed for the hydroxylation which is consistent with these observations. Indole oxidation in \textit{tert}-butyl alcohol/aqueous buffer mixtures proceeds slower than in pure buffer, due to an increase of \(K_m\) and a decrease of \(k_{cat}\).
Chapter 4

Introduction

Oxindoles and aza-oxindoles are of interest due to their biological properties [1-7]. Furthermore, some derivatives are pharmaceutical intermediates, for example 5-chlorooxindole is an intermediate in the synthesis of Tenidap (1-carbamoyl-5-chloro-3-[hydroxy(2-thienyl)methylene]indol-2-(3H)-one) [8], an anti-inflammatory drug. There are several known procedures to synthesize (substituted) oxindoles, for example nucleophilic substitution of halonitrobenzenes by dimethyl malonate and subsequent ring closure [9], Friedel-Crafts alkylation of α-chloro acetanilides [10], cyclization of N-acyl phenylhydrazines by CaH₂ [11], Gassman cyclization of azasulphonium salts [12], addition of ketene silyl radicals to nitrobenzenes [13], functionalization of nitrotoluenes [14], photo-induced cyclization of mono and dianions of N-acyl-o-chloroanilines [15], acid-catalyzed cyclization of α-hydroxy-acetanilides [7], photo-induced cyclization of 2-(N-methylanilino)-acetoacetates followed by oxidative rearrangement [16], cyclization of N-(o-bromo-phenyl)-acrylamides with Pd(II) [17] and Wolff-Kishner type reduction of isatin derivatives by hydrazine [18].

Methods for the synthesis of aza-oxindoles are less numerous. These include oxidation of 7-aza-indole to 3,3-dibromo-oxindoles followed by reduction to the corresponding oxindole [19], synthesis from 2-aminonicotinic acid, diazomethane and N,N-dimethylaniline [20] and thermally induced cyclization of aminopyridine-acetic acid [21].

Most of these methods comprise multi-step procedures for the preparation of the oxindoles. Often harsh conditions are necessary or low yields are obtained. Furthermore, starting materials with the substituent meta relative to the reactive group often lead to more than one product. Straightforward oxidation of indoles generally takes place at the electron-rich 3-position, unless this position is substituted [22]. Oxidation of indole at the 3-position leads to indoxyl, an intermediate for the manufacture of indigo [23]. However, the direct oxidation of indole (1) to oxindole (2) is known to be catalyzed by the enzyme chloroperoxidase from Caldariomyces fumago (CPO) [24] (Fig. 4.1). Isolated yields were moderate however, and as the reaction was performed in water the reactant concentration was limited. Furthermore, no indole derivatives with substituents in the benzene ring were tested whilst these are the most interesting ones for pharmaceutical application.
Fig. 4.1. Oxidation of indole to oxindole.

CPO is a versatile catalyst which, in addition to its ability to chlorinate organic substrates [25], can catalyze other useful reactions such as the enantioselective oxidation of sulfides to sulfoxides [26] and alkenes to epoxides [27,28]. We have found [29] that CPO performs well in tert-butyl alcohol - water mixtures containing up to 70% tert-butyl alcohol (v/v) (chapter 3). Therefore, we have investigated the oxidation of indole in this medium and explored the scope of CPO catalyzed oxidation of substituted indoles as a general synthetic method for the synthesis of the corresponding oxindoles.

Results and Discussion

General experiments

The reactivity of a number of substituted indoles was compared in small-scale experiments which were run at a low reactant concentration (10 mM) with 400 U of CPO (Table 4.1). It should be noted that CPO is deactivated by hydrogen peroxide in the micromolar range, therefore its concentration should be kept low, preferably rate-limiting, by continuous addition [30]. Even then slow deactivation of the enzyme still takes place due to non-perfect mixing of the reaction mixture which leads to local high concentrations of hydrogen peroxide. Furthermore, in the course of a batch reaction the reactant concentration declines as the reaction proceeds and in the first order region the reaction rate will decrease proportionally. As soon as a surplus of hydrogen peroxide builds up in the solution it will rapidly deactivate the catalyst and the reaction will stop.
### Table 4.1.
Conversion of substituted indoles to the oxindoles<sup>a</sup>.

<table>
<thead>
<tr>
<th>indole-derivative</th>
<th>conversion (15 min) (%)</th>
<th>conversion (60 min) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>indole</td>
<td>25</td>
<td>96</td>
</tr>
<tr>
<td>7-aza-indole</td>
<td>25</td>
<td>83</td>
</tr>
<tr>
<td>3-COOH</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>3-CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3-CH&lt;sub&gt;2&lt;/sub&gt;-COOH</td>
<td>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4-CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>4-OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4-Cl</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>5-CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>5-OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>5-Cl</td>
<td>19</td>
<td>47</td>
</tr>
<tr>
<td>5-Br</td>
<td>9</td>
<td>19</td>
</tr>
<tr>
<td>6-CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>6-Cl</td>
<td>19</td>
<td>46</td>
</tr>
<tr>
<td>7-CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Indole derivative 0.5 mmol, tert-butyl alcohol-0.1 M acetate buffer pH 4 (50:50, v/v), 50 mL; CPO 400 U; H<sub>2</sub>O<sub>2</sub> (1.66 M in 0.1 M acetate buffer pH 4) 0.5 mmol over 60 min.

<sup>b</sup> a complex mixture of reaction products was observed.

Indole and 7-aza-indole proved to be the most reactive substrates. Their conversion into oxindole and 7-aza-oxindole, respectively, nearly kept pace with the addition of hydrogen peroxide (Table 4.1). Indoles bearing a substituent at the 3-position either did not react with CPO (3-COOH) or gave rise to a complex mixture of products which probably result from non-selective radical reactions (3-CH<sub>3</sub> and 3-CH<sub>2</sub>COOH). This deviation from the general pattern is ascribed to steric hindrance of the 2-position by substituents at the 3-position and, in the case of 3-carboxy-indole, its deactivation by the electron withdrawing substituent.
Indoles which were substituted at the benzene ring were less reactive than indole itself; their conversion into the corresponding oxindole ranged from less than 1% to 47% (Table 4.1). This is mainly ascribed to unfavourable steric interactions of the reactants with the restricted active site of CPO. In a similar way the enantioselective sulfoxidation of alkyl aryl sulfides is negatively influenced by the size of the alkyl group: increasing the size of the substrate from methyl phenyl sulfide to propyl phenyl sulfide gives a large decrease in enantiomeric excess of the (R)-sulfoxide (chapter 3). Also the enantioselective epoxidation of cis-2-alkenes is limited to smaller alkenes (<C₆) [28]. In order to study the effects of the substituents on binding and catalysis, kinetic experiments were performed.

**Kinetic experiments**

By analogy with the oxidation of N,N,N',N'-tetramethyl-p-phenylenediamine by CPO the oxidation of indole presumably proceeds through a ping-pong mechanism when the reactant concentration exceeds the hydrogen peroxide concentration [31]. If the concentration of the oxidant is not significantly lower than the concentration of the reactant, enzyme catalyzed hydrogen peroxide decomposition to molecular oxygen and water also occurs [31].

![Fig. 4.2. Ping-pong mechanism of CPO.](image)

In the first step, the enzyme is oxidized by hydrogen peroxide to compound I, which is two oxidation equivalents above the resting state of the enzyme. In step 2 compound I is reduced by the reactant to native enzyme and the oxidized product is obtained (Fig. 4.2). A reliable measurement of the kinetic parameters of the indole oxidation requires that the experiments are carried out in an oxidant concentration range within which the enzyme remains saturated with hydrogen peroxide. A high oxidant concentration (millimolar range) is undesirable, because then the catalase activity, deactivation of CPO and formation of compound III interfere and the oxidation rate of indole will decrease [24]. The dissociation
constant of CPO for hydrogen peroxide is reported to be very low (in the micromolar range [31]), therefore a high hydrogen peroxide concentration would seem unnecessary. Preliminary experiments confirmed that the oxidation rate of indole is independent of the hydrogen peroxide concentration between 200 µM and 400 µM (Table 4.2). Hence kinetic experiments were carried out in this concentration range (Table 4.3).

<table>
<thead>
<tr>
<th>[H₂O₂] (µM)</th>
<th>V_{initial} (µmol mg⁻¹ min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td>30</td>
</tr>
<tr>
<td>1000</td>
<td>38</td>
</tr>
<tr>
<td>500</td>
<td>57</td>
</tr>
<tr>
<td>400</td>
<td>62</td>
</tr>
<tr>
<td>200</td>
<td>62</td>
</tr>
</tbody>
</table>

\^a for details see experimental section

Table 4.3.
Kinetic parameters of substituted indoles\(^a\).

<table>
<thead>
<tr>
<th>Indole-derivative</th>
<th>V_{max} (µmol mg⁻¹ min⁻¹)</th>
<th>k_{cat} (s⁻¹)</th>
<th>K_m (mM)</th>
<th>k_{cat}/K_m (mM⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>indole</td>
<td>360</td>
<td>250</td>
<td>22</td>
<td>11.4</td>
</tr>
<tr>
<td>4-Cl</td>
<td>220</td>
<td>160</td>
<td>200</td>
<td>0.8</td>
</tr>
<tr>
<td>5-Cl</td>
<td>125</td>
<td>84</td>
<td>24</td>
<td>3.5</td>
</tr>
<tr>
<td>5-OCH₃</td>
<td>680</td>
<td>475</td>
<td>400</td>
<td>1.2</td>
</tr>
<tr>
<td>6-Cl</td>
<td>375</td>
<td>263</td>
<td>75</td>
<td>3.5</td>
</tr>
</tbody>
</table>

\(^a\) for details see experimental section
Synthesis of substituted oxindoles

The kinetic data confirm our observation that indole is the most reactive substrate. An electron donating substituent at the 5-position (5-methoxy-indole) increases the catalytic turnover frequency ($k_{cat}$), whilst an electron withdrawing substituent (5-chloro-indole) at this position decreases the turnover frequency. The electron withdrawing property of the 6-chloro substituent does not affect the turnover frequency.

For all substituents the $K_m$ value is increased. This strongly suggests that the binding of the indole derivative to the enzyme is negatively influenced by substituents. This effect is very pronounced at the 4-position where the $K_m$ value for chloro-substituted indole is highest. 5-Chloro-indole is still bound well (in the same range as indole) whilst 5-methoxy-indole binding is much weaker. 6-Chloro-indole also has a larger $K_m$ value than indole, suggesting that the binding of this reactant to the enzyme is weaker than for indole. The observed low reactivity of 7-methyl-indole (Table 4.1) similarly suggests that a substituent at this position exerts an important steric effect.

Preparative scale

Preparative scale experiments were carried out with indoles which showed sufficient reactivity (Table 4.1). The amount of CPO was adjusted to the reactivity of the substrate. Nearly all oxindoles were obtained in quantitative yield with a high purity (Table 4.4). Only in the case of 4-chloro-indole did the reaction stop before completion resulting in contamination of the product with starting material. All products were coloured due to the presence of trace amounts (< 2%) of oligomeric products.
Table 4.4.
Preparative scale synthesis of oxindole derivatives$^a$.

<table>
<thead>
<tr>
<th>indole derivative</th>
<th>CPO (kU)</th>
<th>yield (%)</th>
<th>purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>indole</td>
<td>1</td>
<td>96</td>
<td>96</td>
</tr>
<tr>
<td>7-aza-indole</td>
<td>2</td>
<td>97</td>
<td>99</td>
</tr>
<tr>
<td>4-Cl</td>
<td>6</td>
<td>70</td>
<td>76</td>
</tr>
<tr>
<td>5-Cl</td>
<td>2</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>5-Br</td>
<td>3</td>
<td>86</td>
<td>95</td>
</tr>
<tr>
<td>5-CH$_3$</td>
<td>6</td>
<td>92</td>
<td>94</td>
</tr>
<tr>
<td>5-OCH$_3$</td>
<td>6</td>
<td>93</td>
<td>95</td>
</tr>
<tr>
<td>6-Cl</td>
<td>2</td>
<td>96</td>
<td>99</td>
</tr>
</tbody>
</table>

$^a$ for details see experimental section

Other substrates

Other hetero aromatic compounds were also tested as substrates in CPO-mediated oxidations with hydrogen peroxide. Nicotinic acid and quinoline were unreactive, consistent with the lower reactivity of the pyridine ring compared to the pyrrole ring. This is also consistent with the results of the oxidation of 7-aza-indole, where oxidation at the pyridine moiety was not observed.

Benzothiophene and benzofuran were not oxidized by CPO, suggesting that the presence of a nitrogen atom is essential for reactivity. Pyrrole (3) was selectively oxidized at the 2-position to yield 2,5-dihydropyrrol-2-one (4 in Fig. 4.3, the most stable tautomer [32]). In contrast with the oxindoles it was not possible to obtain 4 in quantitative yield (maximum conversion 67%).
Other enzymes

Vanadium chloroperoxidase from *Curvularia inaquata* did not oxidize indole to oxindole. Recently, the crystal structure of vanadium chloroperoxidase has been elucidated [33,34]. It was shown that this enzyme has a narrow channel (5 Å) at the active site entrance. Hence, direct oxidation is restricted to small molecules or ions such as chloride.

As known from the literature, horseradish peroxidase oxidizes indole to a mixture of products, with a trimer as the major product and only traces of oxindole [35]. *Coprinus cinereus* peroxidase which is known to catalyze the same kind of reactions as horseradish peroxidase showed no activity for the formation of oxindole. However, a very slow reaction occurred when a large amount of *Coprinus cinereus* peroxidase (20 kPU of SP676, conversion 17%) was used and unidentified products were obtained, probably due to radical reactions at the δ-heme edge comparable to the reaction of indole with horseradish peroxidase. Microperoxidase-11, which is an undecapeptide containing a heme group, showed no activity for indole oxidation.

Proposed mechanism

To investigate whether the oxygen in oxindole is derived from hydrogen peroxide, a reaction was performed with labeled H$_2^{18}$O$_2$. The reaction mixture was analyzed with GC-MS. The results were compared to a reaction mixture in which H$_2^{16}$O$_2$ was used as the oxidant. It was shown that all oxygen (>97%) in oxindole is derived from hydrogen peroxide. This implies that the reaction is a two electron oxygen transfer reaction proceeding via a concerted mechanism or via two fast consecutive one-electron transfer reactions. The CPO catalyzed
sulfoxidation [36] and epoxidation reactions [37] as well as the oxidation of aryl amines to N-oxides [38] similarly appear to involve direct oxygen transfer.

Fig. 4.4. Putative mechanism for the oxidation of indole to oxindole by CPO.

We propose the mechanism in Fig. 4.4 for the observed hydroxylation. The first step is a one electron transfer between the iron(V)oxo species and indole (1) affording an indole radical cation (I) together with an iron(IV)oxo species analogous to the traditional peroxidase reaction mechanism. The resonance structures (Ia) and (Ib) presumably are the most stable ones, because the positive charge in Ia is stabilized by the adjacent nitrogen atom and because Ia and Ib represent a benzylic radical. Furthermore, this mechanism explains the positive influence of an electron donating group at the 5-position on the catalytic turnover frequency. This electron donating group stabilizes the intermediate radical cation.
Synthesis of substituted oxindoles

A possible explanation for the observation that the oxidations of benzofuran and benzothiophene are not catalyzed by CPO and 1-methyl-indole is oxidized at a much lower rate than indole [24], is that proton abstraction by the enzyme would stabilize the positive charge on the nitrogen.

In the second step of the oxidation the 2-position of the indole radical cation is attacked by the oxyanion, followed by an electron transfer to Fe^{IV} to give Fe^{III}. After deprotonation of (III) at the 2-position and substitution of Fe^{III} by a proton, 2-hydroxy-indole is obtained which spontaneously converts to the more stable oxindole (2).

Influence of cosolvent on reactivity

We studied the influence of tert-butyl alcohol on the catalytic efficiency of CPO for indole oxidation. Kinetic parameters were determined for indole in water and compared to tert-butyl alcohol/water (30:70 and 50:50, v/v). Contrary to the results of Corbett and Chipko [24] we could determine a $K_m$ value for the oxidation of indole in water. The difference between both studies is most likely caused by the difference in the hydrogen peroxide concentration which is used to determine the kinetic parameters. Corbett and Chipko used an oxidant concentration of 4 mM. At such a high hydrogen peroxide concentration other reactions interfere with the oxidation of indole [24,31].

As can be seen from Table 4.5, the cosolvent effects both $k_{cat}$ and $K_m$. A lower $k_{cat}$ value is probably caused by structural changes of CPO induced by the cosolvent, thus changing the structure of the active site and the catalytic turnover frequency. The increase of the $K_m$ value is thermodynamically expected as the ground state of indole is stabilized in cosolvent mixtures. The same effects were observed for methyl phenyl sulfide (chapter 3) for which the $K_m$ value was increased from 0.9 mM in aqueous solution to 29 mM in tert-butyl alcohol water mixtures (50:50, v/v). However the influence of the medium on $k_{cat}$ was less pronounced for methyl phenyl sulfide: a decrease from 650 s^{-1} in aqueous buffer to 580 s^{-1} in tert-butyl alcohol/water mixtures (50:50, v/v) was observed compared to a decrease by a factor of nearly 3 in the case of indole.
Table 4.5.
Kinetic parameters for indole oxidation with various cosolvent amounts.

<table>
<thead>
<tr>
<th>tert-butyl alcohol/water (v/v)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:100</td>
<td>740</td>
<td>3.3</td>
<td>225</td>
</tr>
<tr>
<td>30:70</td>
<td>580</td>
<td>12</td>
<td>48</td>
</tr>
<tr>
<td>50:50</td>
<td>250</td>
<td>22</td>
<td>11</td>
</tr>
</tbody>
</table>

Conclusions

CPO efficiently catalyzes the selective oxidation of substituted indoles to oxindoles and of pyrrole to 2,5-dihydropyrrol-2-one. High yields of substituted oxindoles can be obtained in tert-butyl alcohol/water mixtures, in which a high concentration of reactant is possible.

The reactivity of substituted indoles depends on the position and the nature of the substituent. A substantial variation of the substituent is possible at the 5-position: electron withdrawing substituents have a negative effect on the reaction velocity as does the bulkiness of the substituent. It is possible to obtain 5-chloro-oxindole, a precursor for the drug Tenidap, in high yield using this method. Reactions with 4- and 6- substituted indoles are also possible, however, these positions are more sterically hindered so these substrates are weakly bound to the enzyme. The oxidation of indole to oxindole involves direct oxygen transfer from hydrogen peroxide to the substrate. An initially formed radical cation is proposed to afford the oxindole in a subsequent oxygen rebound reaction with the iron(IV)oxo anion.

Oxidation of indole proceeds slower in cosolvent mixtures than in pure buffer. The lower activity in tert-butyl alcohol/water for indole oxidation is mainly due to an increase of the $K_m$ value (thermodynamically controlled). Also a decrease of $k_{cat}$ (catalytic turnover frequency) is observed, probably caused by a change in structure of the enzyme.
Experimental procedure

Materials and analytical methods

Chloroperoxidase from *Caldariomyces fumago* was isolated and purified as described in the literature [29]. The enzyme preparation (93 μM) contained 8000 U/mL according to the method of Morris *et al.* [39], with a purity of R₉ = 1.3. Vanadium chloroperoxidase from *Curvularia inaequalis* was received as a gift from Dr. R. Wever, University of Amsterdam and contained 21 U/mL according to the method of Morris *et al.* [39]. Microperoxidase-11 was purchased from Sigma. *Coprinus cinereus* peroxidase SP 502 (10 kPU/mL) and SP 676 (2263 kPU/g) were received as a gift from NOVO Nordisk. 1 Peroxidase Unit (PU) is the amount of enzyme which catalyzes the conversion of 1 μmol of H₂O₂ per minute in a system in which 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) is oxidized under standard conditions (pH=7, [H₂O₂]=0.88 mM, [ABTS]=1.67 mM, 30°C)

Hydrogen peroxide 35% was obtained from Merck. All indole derivatives, pyrrole, quinoline, benzofuran, benzothiophene, and nicotinic acid were purchased from Aldrich Chemical Company. Labeled H₂¹⁸O₂ (2.7% solution) was obtained from Campro Scientific. tert-Butyl alcohol was purchased from Baker.

**HPLC-analysis.** Samples were diluted with methanol/water (50:50, v/v) saturated with Na₂SO₃. After filtration through a 0.4 μm membrane filter the samples were analyzed by reversed phase HPLC. A custom-packed 8x100 mm 10 μm Nucleosil C₁₈ column Waters Radial Pak cartridge (quinoline and nicotinic acid) and a 8 x 100 mm 4 μm Novapak C₁₈ cartridge (all other reactants) contained in a Waters RCM 8 x 10 compression unit were used. Eluents (flow 1.0 mL/min) used: for indole derivatives, benzofuran and benzothiophene: methanol/water (50:50, v/v); for pyrrole: methanol/water (10:90, v/v), for quinoline: methanol/0.1 M aqueous ammonium formate buffer pH 3 (40:60, v/v), which contains 2.0 g/l pentadecafluorooctanoic acid, for nicotinic acid: 0.1 M aqueous ammonium formate buffer pH 3. Dual-channel detection was performed using a Shimadzu SPD-6A UV spectrophotometer at 254 nm and an Erma ERC-7510 RI detector. tert-Butyl alcohol was used as internal standard.

NMR spectra were recorded on a Varian VXR-400S spectrometer, using TMS as an internal standard and CDCl₃ or DMSO-d₆ as the solvent.

Mass spectra were obtained using a VG 70-SE spectrometer. GC-MS was performed on a CP-SIL5CB-MS column (25 m x 0.25 mm).
Chapter 4

General experimental

At room temperature 0.5 mmol of reactant (indole derivative, quinoline, nicotinic acid, benzofuran, benzothiophene or pyrrole) was dissolved in 50 mL of solvent, consisting of tert-butyl alcohol/0.1 M aqueous acetate buffer pH 4 (50:50, v/v). 400 U CPO was added to the reaction mixture, followed by 5 min of stirring. The reaction was started by the continuous addition of 1.66 M H₂O₂ over 60 minutes until 1 eq. of H₂O₂ had been added. The reactions were monitored by removing aliquots and analyzing by HPLC. Reactions with indole and pyrrole were repeated with no enzyme present.

The reaction with indole was repeated at the same conditions as above with microperoxidase (0.25 mg). Reactions with peroxidase from Coprinus cinereus (SP502 4 kPU, SP676 20 kPU) were similarly performed in tert-butyl alcohol/0.1 M aqueous phosphate buffer pH 6.5 (50:50, v/v). Reactions with vanadium chloroperoxidase (6.3 U) were performed in 5 ml tert-butyl alcohol/0.1 M aqueous acetate buffer pH 6 (50:50, v/v).

Preparative scale

Indole derivatives: 1.25 mmol of indole derivative was dissolved in 25 mL of solvent, consisting of tert-butyl alcohol/0.1 M aqueous acetate buffer pH 4 (50:50, v/v). To the reaction mixture was added CPO (1-6 kU) and the reaction was started by continuous addition of 1.66 M H₂O₂; 1.1 equivalent of H₂O₂ was added in 160 min. Subsequently the reaction was stopped by addition of Na₂SO₃ and the products were extracted with 3x25 mL of ethyl acetate. The combined fractions were dried over Na₂SO₄, filtered and concentrated in vacuo. All products contained trace amounts (<2%) of coloured side products, probably due to oligomerization of the reaction product.

Pyrrole: 5 mmol of pyrrole was dissolved in 50 mL of solvent, consisting of tert-butyl alcohol/0.1 M aqueous acetate buffer pH 4 (50:50, v/v). To the reaction mixture was added CPO (4 kU) and the reaction was started by continuous addition of 1.66 M H₂O₂; 1.1 equivalent of H₂O₂ was added in 16 h. Subsequently the reaction was stopped by addition of Na₂SO₃, the solvent was evaporated in vacuo and the residue was dissolved in dioxane. After removing the remaining salts by filtration dioxane was removed by evaporation in vacuo and a yellow syrup was obtained.
The conversion and purity of the isolated products were determined by HPLC. All isolated products are known compounds and have been described in literature [9, 12, 14, 19, 40].

**oxindole**

Yield of oxindole in the preparative synthesis: 166 mg, purity (HPLC): 96%, yield: 96%. $^{13}$C NMR (400 MHz, CDCl$_3$): $\delta$ 36.26 (C-3), 109.79 (C-7), 122.31 (C-5), 124.56 (C-4), 125.27 (C-3a), 127.91 (C-6), 142.55 (C-7a), 177.98 (C-2). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 3.55 (s, 2H, H-3), 6.90 (d, J$_{4,5}$=7.9 Hz, 1H, H-4), 7.01 (dd, J$_{4,5}$=J$_{5,6}$=7.2 Hz, 1H, H-5), 7.21 (dd, J$_{5,6}$=J$_{6,7}$=7.5 Hz, 1H, H-6), 7.23 (d, J$_{6,7}$=7.5 Hz, 1H, H-7), 9.09 (s, 1H, H-1). MS: m/e 133 (C$_8$H$_7$NO).

**7-aza-oxindole**

Yield of 7-aza-oxindole in the preparative synthesis: 164 mg, purity (HPLC): 99%, yield: 97%. $^{13}$C NMR (400 MHz, CDCl$_3$): $\delta$ 35.56 (C-3), 117.94 (C-5), 120.08 (C-3a), 132.33 (C-4), 146.27 (C-6), 157.86 (C-7a), 175.67 (C-2). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 3.60 (s, 2H, H-3), 6.95 (dd, J$_{4,5}$=J$_{5,6}$=5 Hz, 1H, H-5), 7.50 (dm, J$_{4,5}$=7 Hz, 1H, H-4), 8.19 (dm, J$_{5,6}$=5 Hz, 1H, H-6), 10.50 (s, 1H, H-1). MS: m/e = 134 (C$_{7}$H$_{6}$N$_{2}$O).

**4-chloro-oxindole**

Yield of 4-chloro-oxindole in the preparative synthesis: 191 mg, purity (HPLC): 76%, yield: 70%. $^{13}$C NMR (400 MHz, DMSO-$d_6$): $\delta$ 35.15 (C-3), 107.83 (C-7), 120.81 (C-5), 124.05 (C-3a), 128.84 (C-6), 129.25 (C-4), 144.98 (C-7a), 175.03 (C-2). $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 3.49 (s, 2H, H-3), 6.79 (d, J$_{5,6}$=8 Hz, 1H, H-5), 6.98 (d, J$_{6,7}$=8 Hz, 1H, H-7), 7.2 (dd, J$_{5,6}$=J$_{6,7}$=8 Hz, 1H, H-6), 10.60 (s, 1H, H-1). MS: m/e = 167 (C$_{7}$H$_{6}$NOCl).

**5-chloro-oxindole**

Yield of 5-chloro-oxindole in the preparative synthesis: 208 mg, purity (HPLC): 99%, yield: 99%. $^{13}$C NMR (400 MHz, DMSO-$d_6$): $\delta$ 36.16 (C-3), 110.55 (C-7), 125.09 (C-4),
126.90 (C-3a), 127.76 (C-5), 127.96 (C-6), 140.91 (C-7a), 176.94 (C-2). $^1$H NMR (400 MHz, DMSO-$d_6$): δ 3.50 (s, 2H, H-3), 6.80 (d, J$_{6,7}$=8 Hz, 1H, H-7), 7.20 (dm, J$_{6,5}$=8 Hz, 1H, H-6), 7.26 (sm, 1H, H-4), 10.50 (s, 1H, H-1). MS: m/e= 167 (C$_9$H$_8$NOCl).

6-chloro-oxindole

Yield of 6-chloro-oxindole in the preparative synthesis: 200 mg, purity (HPLC): 99%, yield: 96%. $^{13}$C NMR (400 MHz, CDCl$_3$): δ 35.74 (C-3), 110.36 (C-7), 122.34 (C-5), 123.51 (C-3a), 125.49 (C-4), 133.65 (C-6), 143.49 (C-7a), 177.50 (C-2). $^1$H NMR (400 MHz, CDCl$_3$): δ 3.52 (s, 2H, H-3), 6.91 (d, J$_{5,7}$=2 Hz, 1H, H-7), 7.00 (dd, J$_{4,5}$=8 Hz, J$_{5,7}$=2 Hz, 1H, H-5), 7.14 (d, J$_{4,5}$=8 Hz, 1H, H-4), 8.78 (s, 1H, H-1). MS: m/e= 167 (C$_9$H$_8$NOCl).

5-bromo-oxindole

Yield of 5-bromo-oxindole in the preparative synthesis: 240 mg, purity (HPLC): 95%, yield: 86%. $^{13}$C NMR (400 MHz, DMSO-$d_6$): δ 35.66 (C-3), 110.77 (C-5), 112.69 (C-7), 127.12 (C-4), 128.41 (C-3a), 127.91 (C-6), 142.92 (C-7a), 175.77 (C-2). $^1$H NMR (400 MHz, DMSO-$d_6$): δ 3.51 (s, 2H, H-3), 6.76 (d, J$_{6,7}$=8 Hz, 1H, H-7), 7.34 (dm, J$_{6,5}$=8 Hz, 1H, H-6), 7.38 (sm, 1H, H-4), 10.50 (s, 1H, H-1). MS: m/e= 211 (C$_{9}$H$_{9}$NOBr).

5-methyl-oxindole

Yield of 5-methyl-oxindole in the preparative synthesis: 183 mg, purity (HPLC): 94%, yield: 93%. $^{13}$C NMR (400 MHz, DMSO-$d_6$): δ 20.60 (CH$_3$), 35.71 (C-3), 108.78 (C-7), 125.02 (C-4), 125.77 (C-3a), 127.53 (C-6), 129.83 (C-5), 141.16 (C-7a), 176.24 (C-2). $^1$H NMR (400 MHz, DMSO-$d_6$): δ 1.11 (sm, 3H, CH$_3$), 2.28 (s, 2H, H-3), 5.57 (d, J$_{6,7}$=8 Hz, 1H, H-7), 5.83 (dm, J$_{6,5}$=8 Hz, 1H, H-6), 5.88 (s, 1H, H-4), 9.14 (s, 1H, H-1). MS: m/e= 147 (C$_9$H$_9$NO).

5-methoxy-oxindole

Yield of 5-methoxy-oxindole in the preparative synthesis: 197 mg, purity (HPLC): 95%, yield: 92%. $^{13}$C NMR (400 MHz, CDCl$_3$): δ 36.71 (C-3), 55.81 (OCH$_3$), 110.02 (C-4), 1
Synthesis of substituted oxindoles

111.81 (C-7), 112.54 (C-6), 126.65 (C-3a), 136.01 (C-7a), 155.72 (C-5), 177.70 (C-2). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 3.52 (s, 2H, H-3), 3.78 (s, 3H, OCH\(_3\)), 6.74 (dd, \(J_{6,7}=8\) Hz, \(J_{4,6}=2.6\) Hz, 1H, H-6), 6.79 (d, \(J_{6,7}=8\) Hz, 1H, H-7), 6.84 (sm, 1H, H-4), 8.93 (s, 1H, H-1). MS: \(m/e = 163\) (C\(_9\)H\(_8\)NO\(_2\)).

2,5-dihydropyrrol-2-one

Conversion (HPLC) of pyrrole: 61%. Yield of 2,5-dihydropyrrol-2-one in the preparative synthesis: 226 mg, purity (HPLC): 92%, yield: 50%. \(^1\)C NMR (400 MHz, DMSO-\(d_6\)): \(\delta\) 48.20 (C-4), 127.08 (C-3), 146.81 (C-2), 173.65 (C-1). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta\) 3.95 (m, \(J_{3,5}=J_{4,5}=1.7\) Hz, 2H, H-5), 6.04 (m, \(J_{3,4}=5.7\) Hz, \(J_{3,5}=1.9\) Hz, \(J_{1,3}=1.9\) Hz, 1H, H-3), 7.29 (m, \(J_{3,4}=5.7\) Hz, \(J_{4,5}=1.9\) Hz, \(J_{1,4}=1.9\), 1H, H-4), 8.18 (s, 1H, H-1).

\(H_2O_2\) dependence

The dependence of the reaction velocity on the hydrogen peroxide concentration was measured at room temperature in 5 mL tert-butyl alcohol/0.1 M aqueous acetate buffer pH 4 (50:50, v/v) containing 5 mM indole and 11 U CPO. The hydrogen peroxide concentration was varied between 0.2 and 2 mM. The reaction was monitored for 90 seconds. Samples were analyzed on reversed phase HPLC as described under analysis and initial reaction rates were calculated.

Kinetic experiments

The kinetic experiments were carried out at 20°C with 400 \(\mu\)M \(H_2O_2\). The substrate concentration was varied between 0.5 and 400 mM. The reaction was started by addition of a certain amount of CPO (0.5 to 4 U CPO per mL, depending on the reactivity; CPO-batch: 0.102 mM, 6000 U/mL). Every 30 seconds a sample was taken which was added to a methanol/water solution containing Na\(_2\)SO\(_3\) to stop the reaction immediately. The reaction was monitored for 90 seconds. Samples were analyzed on reversed phase HPLC as described under analysis. All experiments were carried out in triplicate. Initial reaction rates were calculated and kinetic parameters were determined using the Scientist program. Enzyme
concentrations were determined using a molar extinction coefficient of 91200 M\(^{-1}\)cm\(^{-1}\) at 400 nm [39].

**\(H_2^{18}O_2\) studies**

To a 8 mM solution of indole (2.5 mL acetate buffer, 0.1 M, pH 4) was added CPO (100 U). Oxidation was started by the stepwise addition of \(H_2^{18}O_2\) (2.7%). Every minute, 5 μl of \(H_2^{18}O_2\) was added to a total of 50 μl. 5 Minutes after the last addition the reaction mixture was extracted with dichloromethane and the the reaction products of the labeling experiments were analyzed with GC-MS.

**References**

Synthesis of substituted oxindoles


Chapter 5

Chloroperoxidase-catalyzed oxidation of 5-hydroxymethylfurfural

Abstract

Chloroperoxidase catalyzes the oxidation of 5-hydroxymethylfurfural (HMF) with hydrogen peroxide as the oxidant. The reaction proceeds with 60-74% selectivity to 2,5-furandicarboxaldehyde (FDC). The main byproduct is 5-hydroxymethyl-2-furancarboxylic acid (HFCA); a minor amount of 5-formyl-2-furancarboxylic acid (FFCA) was also detected. When H$_2^{18}$O$_2$ was used a virtually quantitative incorporation of $^{18}$O was observed in the HFCA product, whereas no $^{18}$O was incorporated from H$_2^{18}$O. Hence, the CPO-catalyzed oxidation of aldehydes to acids proceeds with direct oxygen transfer from the iron-oxo complex of CPO. Controlling the H$_2$O$_2$-addition with a hydrogen peroxide-stat facilitated the reaction procedure and a conversion of 87% of HMF was reached within 21 min.
Chapter 5

Introduction

The oxidation of 5-hydroxymethylfurfural (HMF) yields products of potential interest as industrial monomers [1-5]. Possible oxidation products of HMF are 2,5-furandicarboxaldehyde (FDC), 5-hydroxymethyl-2-furancarboxylic acid (HFCA), 5-formyl-2-furancarboxylic acid (FFCA) and 2,5-furandicarboxylic acid (FDCA), (Fig. 5.1).

![Chemical structures]

FDC  
HFCA  
FFCA  
FDCA

Fig. 5.1. Possible oxidation products of HMF.

Although there are several catalytic methods available for obtaining FDCA or FFCA in good yield [6-10], reports on the selective oxidation of HMF to FDC are rare. Oxidation of HMF to FDC occurs selectively with stoichiometric amounts of manganese dioxide [11]. However, the amount of waste produced by this method make it impractical. A mixture of products, including FDC is obtained when platinum is used as catalyst and oxygen as the oxidant [6]. Oxidation of HMF in the presence of 2,2,6,6-tetramethylpiperidinyloxy derivatives (Tempo), oxygen and copper salts, yields 55% FDC [12]. Chloroperoxidase from Caldariomyces fumago (CPO) has been reported [13,14] to catalyze the selective oxidation of primary alcohols using hydrogen peroxide as the oxidant. This prompted us to study the use of CPO as a catalyst for the selective oxidation of HMF.

Results and discussion

The CPO-catalyzed oxidation of HMF proceeded smoothly to a nearly complete conversion of HMF. In view of the known sensitivity of CPO for hydrogen peroxide the oxidant was added over 150 min at a low constant rate of 0.5 eq./h. As expected, oxidation of the alcohol moiety took place with the formation of FDC. Besides this reaction, however, oxidation of the aldehyde function in HMF, yielding HFCA, also took place. This was unexpected, because the CPO-catalyzed oxidation of alcohols by hydrogen peroxide was reported to proceed selectively to the aldehydes without any detection of over-oxidation to the
acids for the alcohols tested, like for example furfuryl alcohol [14]. We repeated the oxidation of furfuryl alcohol and confirmed a virtually 100% selectivity to the aldehyde at a conversion of furfuryl alcohol of 92%. For HMF, however, the maximum selectivity to FDC was 74%, depending on the reaction conditions used, and HFCA was obtained as a major by-product in 25-40% yield (Fig. 5.2 and Table 5.1). A minor amount of FFCA (<5% under the conditions used) was also detected.

![Graph showing the oxidation of HMF, FDC, and HFCA over time.](image)

**Fig. 5.2.** Oxidation of HMF, 50 mM HMF in 25 mL pH 5, 1600 U CPO, 1 eq. $\text{H}_2\text{O}_2$/2 h., * conversion of HMF, ■ [HMF] △ [FDC] ▲ [HFCA].

**Table 5.1.**

<table>
<thead>
<tr>
<th>pH</th>
<th>conversion 120 min. (%)</th>
<th>selectivity to FDC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>25</td>
<td>74</td>
</tr>
<tr>
<td>4</td>
<td>59</td>
<td>68</td>
</tr>
<tr>
<td>5</td>
<td>89</td>
<td>59</td>
</tr>
<tr>
<td>6</td>
<td>83</td>
<td>60</td>
</tr>
<tr>
<td>5, argon</td>
<td>88</td>
<td>60</td>
</tr>
<tr>
<td>5, tert-butyl alcohol$^b$</td>
<td>66</td>
<td>63</td>
</tr>
</tbody>
</table>

$^a$ 50 mM HMF/25 mL, 0.1 M buffer, 1600 U CPO, 1 eq. $\text{H}_2\text{O}_2$/2 h., reaction time: 2.5 h.

$^b$ tert-butyl alcohol/ aqueous buffer 30:70 (v/v).
Optimum activity for oxidation of HMF was obtained at pH 5. Under the conditions used a 92% conversion of HMF to oxidized products was obtained within 2.5 h, which corresponds with a turnover number* of $62 \times 10^3$. Recently [15,16] we have shown that tert-butyl alcohol as a cosolvent stabilizes CPO in the oxidation of indole (chapter 6). Hence, we also performed the oxidation of HMF in tert-butyl alcohol/aqueous buffer (30:70, v/v, citrate buffer 0.1 M pH 5). However, the conversion dropped from 89% to 66% (Table 5.1) thereby also decreasing the turnover number and the selectivity did not improve.

In order to assess the role of CPO in the production of HFCA and FFCA, a number of blank experiments without enzyme were carried out. No blank reaction was observed with either HMF, HFCA or FDC as the reactant. However, all these reactants were oxidized by CPO and hydrogen peroxide (Table 5.2).

| Table 5.2. | Oxidation of HMF, FDC and HFCA with CPO and $H_2O_2$ *.
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting Material</td>
<td>Conversion (%)</td>
<td>Products</td>
</tr>
<tr>
<td>HMF</td>
<td>92</td>
<td>FDC, HFCA, FFCA</td>
</tr>
<tr>
<td>HFCA</td>
<td>68</td>
<td>FFCA</td>
</tr>
<tr>
<td>FDC</td>
<td>22</td>
<td>FFCA</td>
</tr>
</tbody>
</table>

*a 50 mM starting material/25 mL, 0.1 M buffer pH 5, 1600 U CPO for the enzymatic reaction no CPO for the blank, 1 eq. $H_2O_2/2$ h, reaction time: 2.5 h.

Clearly, the formation of HFCA, FDC and FFCA is due to CPO-catalyzed oxidation of HMF with $H_2O_2$. Based on these results the following reaction scheme can be presented for the oxidation of HMF:

*Turnover number = mol HMF oxidized per mol of CPO used
Oxidation of 5-hydroxymethylfurfural

![Chemical structure diagram showing the conversion of HMF to FDC and HFCA to FFCA through the action of CPO and H$_2$O$_2$.]

Fig 5.3. Reaction scheme for CPO catalyzed HMF oxidation.

The observed formation of HFCA is, to the best of our knowledge, the first evidence for CPO-catalyzed oxidation of aldehydes to acids. An indication for the CPO-catalyzed oxidation of aldehydes to acids was previously obtained in benzylic hydroxylation, in which a large amount of benzoic acid was detected as a by-product [17].

To investigate the mechanism of oxygen transfer in aldehyde oxidation, labeling experiments were carried out. Reactions with labeled H$_2^{18}$O$_2$ yielded virtually complete incorporation of $^{18}$O into HFCA, whereas no oxygen from H$_2$O ($^{16}$O:$^{17}$O:$^{18}$O = 50.4:25.7:23.9) was incorporated into the product. Exclusion of oxygen had no effect on the selectivity or conversion for the oxidation of HMF (Table 5.1). From these results we conclude that the CPO-catalyzed oxidation of aldehydes to acids proceeds via direct oxygen transfer from the iron(V)oxo species to the aldehyde, analogous to the corresponding sulfoxidations [18], epoxidations [19], oxidation of arylamines [20] and indole oxidation to oxindole [21].

The oxygen transfer from the iron(V)oxo intermediate to the aldehyde could involve concerted insertion or two discrete one-electron transfers. In the latter case oxygen rebound from the iron(IV)oxo intermediate would result in the observed incorporation of $^{18}$O from H$_2^{18}$O$_2$. Hence we propose that in the oxidation of HMF the initial step involves competing hydrogen abstraction, by the iron(V)oxo species, from the alcohol and aldehyde moieties (Fig. 5.4).
Furfuryl alcohol oxidation catalyzed by CPO shows a selectivity of virtually 100% to aldehyde at a conversion of 92%. Thus, the oxidation of HMF involves more than just a competitive oxidation of alcohol and aldehyde as otherwise the oxidation of furfural to 2-furoic acid should have competed with the furfuryl alcohol oxidation. Hammett parameters indicate that the hydroxymethyl substituent does not exert an electron-donating effect on aromatic systems ($\sigma_m = 0.08$ and $\sigma_r = 0.08$), thus the positive effect of this substituent on the reactivity of the aldehyde group for electrophilic oxidation presumably is negligible. Hence, the observed oxidation of the aldehyde moiety of HMF is not due to a higher intrinsic activity of the aldehyde group of HMF compared to furfural, caused by the 2-hydroxymethyl substituent. Possibly the hydroxyl moiety in HMF is involved in binding the reactant to the enzyme thus directing the aldehyde group into the right direction during catalysis.

The oxidation of 2,5-bis(hydroxymethyl)furan (BHF) yielded, in contrast to the oxidation of furfuryl alcohol, not only the products from alcohol oxidation to aldehyde (HMF and FDC) but also HFCA, comparable to the HMF oxidation (Fig. 5.5). As expected, about the same selectivity to FDC was obtained as for HMF oxidation (67% vs. 63%, both at 60% conversion of HMF).
Fig. 5.5. Oxidation of 2,5-bis(hydroxymethyl)furan (BHF) 50 mM FDM in 25 mL pH 5, 1600 U CPO, 1 eq. H₂O₂/2h. * conversion of BHF, □ [BHF] ○ [HMF] △ [FDC] ▲ [HFCA].

Use of a hydrogen peroxide-stat

CPO is deactivated even by low concentrations of hydrogen peroxide. Therefore, the hydrogen peroxide should preferably be added rate limiting to the reaction mixture. In the reactions described above this was done by slow addition of hydrogen peroxide to the reaction mixture (1 eq./2 h.). We have recently demonstrated [15,16] that a hydrogen peroxide-stat is very useful for controlling CPO-catalyzed oxidation reactions (chapter 6 and 7). The hydrogen peroxide-stat measures the concentration of hydrogen peroxide in solution and keeps it at a constant low level (30 μM). This set-up results in a more efficient use of CPO and it also facilitates the detection of the end point of the reaction. Its advantages in the oxidation of HMF became immediately apparent because the reaction proceeded very rapidly; after 12 min a conversion of 77% was already reached and after 21 min, a final conversion of 87% was obtained (Fig. 5.6), this in contrast to slow addition of hydrogen peroxide in which a conversion of 89% was reached after 2 h. Thus the oxidation of HMF is another example of the practical utility of a hydrogen peroxide-stat for controlling reactions using CPO as the catalyst. The selectivity to FDC was slightly higher than the selectivity obtained with slow addition of hydrogen peroxide (66% vs. 59%).
Fig. 5.6. Oxidation of HMF using a hydrogen peroxide-stat, 50 mM HMF in 25 mL pH 5, 1600 U CPO, [H₂O₂]=30 μM, * conversion of HMF, ■ [HMF] □ [FDC] △ [HFCA].

Conclusions

The oxidation of HMF by CPO and hydrogen peroxide proceeds with a maximum selectivity of 74% to FDC. Virtually quantitative conversion of HMF can be obtained. HFCA is the major by-product of the HMF oxidation and is formed by CPO catalyzed oxidation of the aldehyde moiety of HMF. The CPO catalyzed oxidation of aldehyde to acid proceeds via direct oxygen transfer from the iron(V)oxo intermediate to the aldehyde, presumably via the proposed mechanism. As the oxidation of furfuryl alcohol does proceed selectively to the aldehyde, this in contrast to HMF oxidation, it can be concluded that the oxidation of HMF involves more than competitive oxidation of aldehydes and alcohols. CPO provides an environment to HMF in which oxidation of the aldehyde moiety becomes more favourable than in furfuraldehyde, possibly by interaction of the hydroxyl moiety of HMF with amino acids in the vicinity of the active site of CPO. A hydrogen peroxide-stat facilitates the reaction procedure for CPO-catalyzed oxidation of HMF and leads to lower reaction times.
Experimental procedure

Materials and analytical methods

Chloroperoxidase from Caldariomyces fumago was isolated and purified as described in literature [22]. The enzyme preparation (93 μM) contained 8000 U/mL according to the method of Morris et al. [23]. Hydrogen peroxide 35% was obtained from Merck. H$_2^{18}$O$_2$ (2.7% solution) was purchased from Campro Scientific. 5-Hydroxymethylfurfural was obtained from Süd Zucker. Furfuryl alcohol and 2,5-bis(hydroxymethyl)furan were purchased from Aldrich Chemical Company. tert-Butyl alcohol was obtained from Baker. The hydrogen peroxide-stat, Dulcometer Perox 20/21, was obtained from Prominent Dosierotechnik, Heidelberg, Germany. The measurement of the hydrogen peroxide was based on amperometric measurements at a platinum electrode which is operated as a potentiostat. Two-point calibrations were made before each reaction. A Metrohm Dosimat 655 was used for addition of hydrogen peroxide (1.66 M).

HPLC-analysis: Samples were analyzed on a Phenomenex Rezex Organic Acids column, 60°C, eluent 0.01 M trifluoroacetic acid at 0.6 mL/min. Detection was performed on a Shodex RI SE-51 refractive index detector and on a Shimadzu SPD-6a UV detector (220 nm). Products were identified by comparing the HPLC-retention times of the products to authentic samples and by mass spectroscopy, for which the HPLC column was coupled to a VG70-SE spectrometer operating in plasma spray mode.

General oxidation procedure

At room temperature 1.25 mmol of HMF was dissolved in 25 mL buffer (0.1 M citrate, pH 5). Subsequently, 1600 U CPO was added to the reaction mixture, followed by 5 min. of stirring. The reaction was started by continuous addition of 1.66 M H$_2$O$_2$ in buffer (0.1 M citrate, pH 5). In total 1.25 eq. of H$_2$O$_2$ was added over 150 min. In the case of the hydrogen peroxide-stat controlled reactions the [H$_2$O$_2$] was set at 30 μM. The reactions were monitored by removing aliquots which were analyzed on HPLC. Citric acid was used as internal standard. Oxidations at pH 3 and pH 6 were performed in phosphate buffer (0.1 M) and oxidations at pH 4 were performed in acetate buffer (0.1 M). Blank experiments were carried out under the same conditions as above in which CPO was discarded.
Chapter 5

Labeling experiments

H$_2^{18}$O$_2$: To 1 mL of HMF solution (50 mM in 0.1 M formate buffer pH 5) was added 200 U CPO. H$_2^{18}$O$_2$ (2.7%) was added in 8 portions of 8 µL in 35 min.

H$_2$O ($^{16}$O:$^{17}$O:$^{18}$O = 50.4:25.7:23.9): 1 mg HMF was dissolved in 200 µL labeled H$_2$O. Subsequently 0.5 mg of ammonium formate and 40 U CPO were added to the reaction mixture. H$_2$O$_2$ (2.7%) was added in 8 portions of 2 µL in 35 min. The reaction products of the labeling experiments were analyzed by HPLC-MS as described under materials and analytical methods.

References


Chapter 6

Chloroperoxidase: use of a hydrogen peroxide - stat for controlling reactions and improving enzyme performance*

Abstract

The use of a hydrogen peroxide-stat for controlling the hydrogen peroxide concentration is demonstrated for the chloroperoxidase catalyzed oxidation of indole. It is shown that in tert-butyl alcohol/aqueous buffer mixtures the oxidation can be effectively controlled by a hydrogen peroxide-stat. In this medium chloroperoxidase is stabilized towards oxidative destruction as long as indole is present in the reaction mixture but deactivation occurs in the absence of a reductant. In contrast with the results with tert-butyl alcohol/aqueous buffer mixtures chloroperoxidase is deactivated in aqueous buffer by hydrogen peroxide even when indole is present in the reaction mixture and the oxidant concentration is as low as 30 μM. The hydrogen peroxide-stat is also used to study catalase activity and the deactivation pathway of CPO. The deactivation in aqueous buffer appears to

* In cooperation with K. Seelbach and U. Kragl from Forschungszentrum Jülich.
Chapter 6

proceed via a different intermediate than compound \( \text{H}_2\text{O}_2 \), which is the active intermediate for the catalase activity.

Introduction

Chloroperoxidase (CPO) is a versatile catalyst for numerous interesting oxygen transfer reactions including sulfoxidation [1], epoxidation [2,3,4], oxidation of substituted indoles to the corresponding oxindoles [5,6] and benzylic hydroxylation [7,8]. CPO uses hydrogen peroxide as the oxidant and does not require any cosubstrate. It has a broad substrate range, is easily isolated in large quantities and is relatively stable under non-oxidizing conditions [9]. These properties make the enzyme attractive for synthetic use.

The practical use of CPO has one major drawback: the low turnover numbers which are obtained in industrially interesting applications like benzylic hydroxylation and epoxidation reactions. Besides deactivation of the enzyme by the organic substrate, which is the case with 1-alkenes where heme alkylation by the alkene occurs [10], the low turnover numbers are probably caused by deactivation of CPO by hydrogen peroxide. We have previously shown (chapter 3) that the mode of addition of oxidant is crucial for obtaining high yields in sulfoxidation reactions catalyzed by CPO [11]. It is important to keep the hydrogen peroxide concentration as low as possible, preferably rate limiting, during catalysis. Until now this has usually been done by slow addition of hydrogen peroxide. This requires that the reaction rate is known before starting a reaction so that one can regulate the addition of hydrogen peroxide. This complicates the reaction procedure as it is different for every type of reaction and substrate. A second drawback is that the reaction rate declines when the organic substrate is present in non-saturating concentrations. Hence, the reaction either proceeds under non-optimal conditions because the hydrogen peroxide is added at a much lower rate than the initial reaction rate, or the reaction stops when the rate of hydrogen peroxide addition exceeds the reaction rate because accumulation of hydrogen peroxide leads to complete deactivation of CPO.

\* \text{Turnover number} = \frac{\text{mol of product formed}}{\text{mol of catalyst}}
\text{Turnover frequency} = \frac{\text{mol of product formed}}{\text{mol of catalyst per minute}}
A system which could control the hydrogen peroxide concentration at a constant low level in the reaction mixture would facilitate the reaction procedure. Here we present the use of a hydrogen peroxide-stat for controlling reactions catalyzed by CPO. Recently hydrogen peroxide sensors and dosing systems have become commercially available [12]. Next to reaction control, we also used this technique to study the stability of CPO towards hydrogen peroxide in greater detail. As CPO decomposes hydrogen peroxide to oxygen and water, stability studies of CPO are possible only when the oxidant is kept at a constant concentration by a regulation system like a hydrogen peroxide-stat. The stability of CPO was studied with and without organic substrate present because studies with horseradish peroxidase (HRP) have shown that the enzyme is stabilized by organic substrate as it competes with hydrogen peroxide for the reactive enzyme intermediate [13]. The oxidation of indole to oxindole was taken as the standard reaction because this oxidation is fast and selective. For practical purposes the use of the electrode in aqueous buffer, as well as in tert-butyl alcohol/aqueous buffer mixtures (in which higher organic substrate concentrations are possible) was studied. Earlier studies indicated that CPO performs well in tert-butyl alcohol/aqueous buffer mixtures containing up to 70% tert-butyl alcohol (Chapter 3). The stability of CPO in this cosolvent system towards various denaturing conditions is also presented in this chapter.

**Results and discussion**

*Hydrogen peroxide, alone*

Reaction of native CPO with hydrogen peroxide yields compound I (scheme 6.1) which is two oxidation equivalents above the resting state of the enzyme. Compound I is generally accepted to be a iron(IV)oxo porphyrin radical cation species [14]. If no reductant is present CPO will react with a second molecule of hydrogen peroxide to yield oxygen and water. The reaction is believed to proceed via a compound I•H₂O₂ intermediate [15]. In scheme 6.1 a possible mechanism for the decomposition of hydrogen peroxide is proposed.

Stability experiments for CPO towards hydrogen peroxide were at first carried out without any organic substrate present in the reaction mixture. The effects of the hydrogen peroxide concentration and of the cosolvent (tert-butyl alcohol) were studied (Table 6.1).
Although CPO exhibits catalase activity and decomposes excess hydrogen peroxide to oxygen and water, it is readily apparent that CPO is not stable under oxidizing conditions with no other reductant present. Even at 30 μM hydrogen peroxide concentration the enzyme is rapidly deactivated. Addition of tert-butyl alcohol has a negative influence on enzyme stability towards oxidizing conditions. This may be caused by partial unfolding of the protein, due to exposure of the hydrophobic side-chains of CPO to the solvent [16]. Hence, CPO would be more exposed to the oxidant and more susceptible to oxidative destruction in tert-butyl alcohol/aqueous buffer mixtures.
The deactivation of CPO probably involves internal oxidation of the porphyrin moiety, which is generally believed to occur with heme proteins like cytochrome P450 and HRP [17,18]. Horseradish peroxidase (HRP) is oxidized to a verdohemoprotein called P-670 when reacting with excess hydrogen peroxide. Heme oxygenase catalyzed oxidation of heme to biliverdin with hydrogen peroxide involves α-meso-hydroxylation and subsequent aerobic fragmentation of α-meso-hydroxyheme to verdoheme [19].

Hydrogen peroxide, with indole

If a reductant is present in the reaction mixture it can protect the enzyme from being deactivated by hydrogen peroxide. This is known to be the case for horseradish peroxidase (HRP). 2,2'-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) stabilizes HRP towards hydrogen peroxide deactivation [13]. With no reductant present, HRP is irreversibly deactivated. This deactivation is caused by oxidation of the enzyme, which is presumed to take place via a compound I $\text{H}_2\text{O}_2$-complex [13,20]. Reaction with ABTS prevents formation of the compound I $\text{H}_2\text{O}_2$-complex and results in HRP stabilization. If deactivation of CPO proceeds via the same pathway as for HRP indole should compete with hydrogen peroxide for compound I and so prevent formation of the compound I $\text{H}_2\text{O}_2$ complex. The protection towards oxidative destruction will depend on the relative reaction rates of the relevant reactions and on the hydrogen peroxide concentration.

We studied the use of a hydrogen peroxide-stat for performing indole oxidation in aqueous buffer and in tert-butyl alcohol/aqueous buffer mixtures. The results are depicted in Fig. 6.1 to 6.3. Reactions carried out without CPO in the reaction mixture showed no oxidation of indole, thus indole was stable towards the hydrogen peroxide-stat electrode.
Fig. 6.1. Indole oxidation in aqueous buffer pH 4, 30 μM H$_2$O$_2$ with sensor, ○ conversion 300 U CPO/50 mL, △ conversion 60 U CPO/50 mL, ■ relative activity 300 U CPO/50 mL, ■ relative activity 60 U CPO/50 mL.

Fig. 6.2. Indole oxidation in tert-butyl alcohol/aqueous buffer pH 4 (30:70, v/v), 30 μM H$_2$O$_2$ with sensor, 300 U CPO/50 mL, ○ conversion, △ relative activity.

Fig. 6.3. Indole oxidation in tert-butyl alcohol/aqueous buffer pH 4 (50:50, v/v), 30 μM H$_2$O$_2$ with sensor, 300 U CPO/50 mL, ○ conversion, △ relative activity.
From Fig. 6.1 to 6.3 it becomes clear that CPO is deactivated in the course of the oxidation of indole in water, whereas in tert-butyl alcohol/aqueous buffer mixtures CPO is stable towards oxidative denaturation as long as indole is present in the reaction mixture. This is unexpected since the reaction rate for indole oxidation is higher in water than in tert-butyl alcohol/aqueous buffer mixtures (Table 6.2).

Table 6.2.
Relative reaction rates for indole oxidation.

<table>
<thead>
<tr>
<th>tert-butyl alcohol (%)</th>
<th>[indole] 2 mM</th>
<th>[indole] 4 mM</th>
<th>[indole] 8 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14</td>
<td>20</td>
<td>27</td>
</tr>
<tr>
<td>30</td>
<td>4</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>50</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

* for details see experimental section

The rapid deactivation of CPO in aqueous medium could, in principle, be caused by local high oxidant concentrations resulting from its rapid addition (due to the high reaction rate) combined with imperfect mixing. In order to exclude such artefacts we performed additional experiments in which hydrogen peroxide was added slowly and continuously to the reaction mixture (1 eq./h, Fig. 6.4). The rapid deactivation of CPO which occurred in water and not in tert-butyl alcohol/aqueous buffer (1 eq./0.67 h, Fig. 6.5) proves that the effect of the medium on the stability of CPO is genuine and not an artefact.

![Graph showing conversion and activity over time](image)

**Fig. 6.4.** Indole oxidation in aqueous buffer pH 4, 8 mM indole, 300 U CPO/50 mL, 1 eq. \( \text{H}_2\text{O}_2 \) per 60 min. ○ conversion, Δ relative activity.
Fig. 6.5. Indole oxidation in \textit{tert}-butyl alcohol/aqueous buffer pH 4 (50:50, v/v), 300 U CPO/50 mL, 1 eq. \( \text{H}_2\text{O}_2 \) per 40 min, \( \circ \) conversion, \( \Delta \) relative activity.

In aqueous buffer the deactivation of CPO in the course of the oxidation of indole is faster than deactivation by hydrogen peroxide alone (Table 6.1 and Fig. 6.1). It seems that a suicide mechanism, which is connected to the catalytic cycle, causes destruction of the catalyst. Moreover, this suicide mechanism is suppressed by \textit{tert}-butyl alcohol, as shown in Fig. 6.2 and 6.3. In media containing \textit{tert}-butyl alcohol, indole rather seems to exert a stabilizing effect on the catalyst, similar to the stabilization of HRP by reductant mentioned above.

\textit{Deactivation Pathway}

In order to ascertain the deactivation pathway for CPO we studied in more detail the deactivation of CPO by hydrogen peroxide in aqueous buffer with no organic substrate present. The experiments were performed at pH 5 because oxygen transfer reactions such as sulfoxidation and epoxidation catalyzed by CPO are commonly performed at pH 5 or pH 6. We have considered the following pathways for deactivation of CPO by hydrogen peroxide:

1) Deactivation via a common intermediate in the catalase pathway (e.g. compound I\( \text{H}_2\text{O}_2 \)) as described for HRP [13]. In this case the ratio of \( k_1 \), the initial turnover frequency for hydrogen peroxide decomposition, and \( k_2 \), the deactivation rate, should be independent of the hydrogen peroxide concentration.
2) Deactivation caused by a first-order decay of compound I (Scheme 6.2). The deactivation should be more or less independent of the hydrogen peroxide concentration as the dissociation constant for compound I to native enzyme is very low (micromolar range) for CPO [15]

\[
\begin{align*}
\text{Fe}^{III} & \xrightarrow{k_2} \text{Cys} \\
\text{H}_2\text{O}_2 & \xrightarrow{k_2} \text{Cys} \\
\text{O} & \xrightarrow{k_1} \text{Cys} \\
\text{H}_2\text{O}_2 & \xrightarrow{k_1} \text{Cys} \\
\text{O} \cdot \text{H}_2\text{O}_2 & \xrightarrow{k_1} \text{Cys} \\
\text{H}_2\text{O}_2 \cdot \text{O}_2 & \xrightarrow{k_1} \text{Cys} \\
\text{Fe}^{III} & \xrightarrow{k_2} \text{Cys}
\end{align*}
\]

Catalase activity \(= k_1[^{\text{Compound I}} \cdot \text{H}_2\text{O}_2] = k_1[^{\text{CPO}_{\text{total}}}]\)

Deactivation rate \(= k_2[^{\text{Compound I}}] = k_2[^{\text{CPO}_{\text{total}}}]\)

Scheme 6.2. Possible deactivation pathway for CPO.

Both the initial turnover frequency for catalase activity \((k_1')\) and the deactivation rate \((k_2')\) can be determined by measuring the initial hydrogen peroxide consumption and the decline of CPO activity with time (see Appendix to chapter 6). Fig. 6.6 shows an example for 0.20 mM hydrogen peroxide. The catalase turnover frequency was calculated from the slope of the hydrogen peroxide consumption at \(t=0\). By fitting an \(e\)-function to the plot of the enzyme activity, \(k_2'\) can be determined. The results obtained at different hydrogen peroxide concentrations are given in Table 6.3.
Fig 6.6. Hydrogen peroxide addition and relative enzyme activity, 0.20 mM H₂O₂, 120 U CPO/50 mL, aqueous buffer pH 5, △ relative activity, + addition of H₂O₂.

Table 6.3.
Reaction constants for catalase activity and deactivation of CPO.<

<table>
<thead>
<tr>
<th>[H₂O₂] (mM)</th>
<th>δ (H₂O₂)/δ t (µmol/min)</th>
<th>k₁' (10³ min⁻¹)</th>
<th>k₃' (10³ min⁻¹)</th>
<th>k₁'/k₃' (10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td>7.8</td>
<td>0.8</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>0.20</td>
<td>9.3</td>
<td>2.3</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td>1.00</td>
<td>36.9</td>
<td>9.0</td>
<td>2.2</td>
<td>4.1</td>
</tr>
</tbody>
</table>

a aqueous buffer pH 5, b 300 U CPO/50 mL, c 120 U CPO/50 mL

It is clearly shown in Table 6.3 that k₁'/k₃' varies with the hydrogen peroxide concentration. This implies that the hydrogen peroxide decomposition and enzyme deactivation do not proceed via the same intermediate. This makes deactivation of CPO via pathway 2 more likely. Moreover, the observation that the deactivation is only slightly dependent on the hydrogen peroxide concentration whereas the catalase activity strongly depends on the hydrogen peroxide concentration is consistent with this pathway. However, deactivation of CPO prior to formation of compound I cannot be excluded on the basis of the data. It is for example shown for heme oxygenase catalyzed oxidation of heme that the
reactive intermediate for heme hydroxylation is a ferric peroxide complex (Fe-O-O') and not an iron(V)oxo species [19].

Deactivation and catalase activity were also studied at different pH values (Table 6.4) as the oxidation of indole is performed at pH 4, sulfoxidation and epoxidation proceed well at pH 5 [2,3] and kinetic constants for the catalase activity are known at pH 6.2 [15].

Table 6.4.

<table>
<thead>
<tr>
<th>pH</th>
<th>$\delta$ ($H_2O_2$)/$\delta$ t (µmol/min)</th>
<th>$k_1'$ ($10^3$ min$^{-1}$)</th>
<th>$k_2'$ ($10^{-2}$ min$^{-1}$)</th>
<th>$k_1'/k_2'$ ($10^9$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>21.9</td>
<td>5.4</td>
<td>2.5</td>
<td>2.2</td>
</tr>
<tr>
<td>5</td>
<td>36.9</td>
<td>9.0</td>
<td>2.2</td>
<td>4.1</td>
</tr>
<tr>
<td>6.2</td>
<td>39.0</td>
<td>9.6</td>
<td>11.0</td>
<td>0.9</td>
</tr>
</tbody>
</table>

$a$ 1 mM $H_2O_2$, 120 U CPO/50 mL

The results for the catalase activity at 0.2 and 1 mM $H_2O_2$ concentration are in good agreement with the kinetic parameters determined by Sun et al. [15]: we found $k_1' = 2.3\times10^3$ min$^{-1}$ at 0.2 mM [$H_2O_2$], pH 5 (literature value 2.4-3.8x10$^3$ min$^{-1}$ at pH 6.2) and $k_1' = 9.6\times10^3$ min$^{-1}$ at 1.0 mM [$H_2O_2$], pH 6.2 (literature value 10.2-15.4x10$^3$ min$^{-1}$ at pH 6.2).

A slight increase in deactivation rate was observed when the pH was increased from 4 to 5. However a large increase in deactivation rate was observed on going from pH 5 to pH 6.2. This suggests that an acid/base couple is involved in the deactivation mechanism. Another possible explanation is that at higher pH values a different mechanism of deactivation becomes effective. This pronounced pH effect is not observed for the catalase activity of CPO.

**tert-Butyl alcohol**

Since CPO was stable during the oxidation of indole in tert-butyl alcohol/aqueous buffer mixtures we investigated the stability of CPO in this medium under various denaturing conditions with a view to scaling up the process for indole oxidation in a fed batch process [21]. The stability of CPO towards the cosolvent, temperature and pH was tested (Table 6.5).
**Table 6.5**

**Stability of CPO**

<table>
<thead>
<tr>
<th>effect of tert-butyl alcohol</th>
<th>tert-butyl alcohol (% v/v)</th>
<th>pH</th>
<th>T (°C)</th>
<th>$t_{1/2}$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>20</td>
<td>5.0</td>
<td>20</td>
<td>&gt;168</td>
</tr>
<tr>
<td>30</td>
<td>20</td>
<td>5.0</td>
<td>20</td>
<td>&gt;168</td>
</tr>
<tr>
<td>40</td>
<td>20</td>
<td>5.0</td>
<td>20</td>
<td>138</td>
</tr>
<tr>
<td>50</td>
<td>20</td>
<td>5.0</td>
<td>20</td>
<td>40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>effect of pH</th>
<th>pH</th>
<th>T (°C)</th>
<th>$t_{1/2}$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>3.2</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>50</td>
<td>4.0</td>
<td>20</td>
<td>&gt;168</td>
</tr>
<tr>
<td>50</td>
<td>5.0</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>50</td>
<td>5.5</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>50</td>
<td>6.0</td>
<td>20</td>
<td>0.2</td>
</tr>
<tr>
<td>50</td>
<td>6.3</td>
<td>20</td>
<td>0.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>effect of temperature</th>
<th>pH</th>
<th>T (°C)</th>
<th>$t_{1/2}$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>5.0</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>50</td>
<td>5.0</td>
<td>25</td>
<td>11</td>
</tr>
<tr>
<td>50</td>
<td>5.0</td>
<td>30</td>
<td>1</td>
</tr>
</tbody>
</table>

$^a$ for details see experimental section

**Cosolvent stability:** CPO is known to be stable for several days in aqueous buffer between pH 3 and pH 6.5. Furthermore, the enzyme can be used in aqueous buffer solutions at temperatures up to 40°C [9]. It is readily apparent from table 6.5 that the stability of CPO declines at higher tert-butyl alcohol content. At tert-butyl alcohol contents up to 30% CPO is relatively stable towards denaturation ($t_{1/2} > 1$ week). The decrease in stability of CPO with increased cosolvent amount may be caused by solvation of hydrophobic side-chains of CPO by the cosolvent. This would lead to partial unfolding of the enzyme in tert-butyl alcohol/aqueous buffer mixtures and partial loss of the hydrophobic contribution to protein stability [16].

**pH-stability:** The pH-range in which the enzyme is stable is narrower in tert-butyl alcohol/aqueous buffer mixtures than in aqueous buffer. In aqueous buffer the enzyme is relatively stable between pH 3 and 7 but it is irreversibly denatured above pH 7. In tert-butyl alcohol/aqueous buffer (50:50, v/v) denaturation already occurs within one hour at pH 6.
Optimal stability of CPO was obtained in buffer pH 4. Under these conditions half life of the enzyme was nearly one week. The lower pH at which CPO remains stable may reflect a change in pK_a of the protein interior due to partial dehydration of the protein by the organic medium. Furthermore, the pH in aqueous buffer usually differs from the actual pH value in cosolvent/aqueous buffer mixtures [22].

**Thermal stability:** In aqueous buffer the enzyme is stable for several days when it is kept below 40°C. In tert-butyl alcohol/aqueous buffer (50:50, v/v), in contrast, rapid deactivation occurs above 25°C. As tert-butyl alcohol as a cosolvent is capable of solvating unfolded hydrophobic parts of the protein, the cosolvent probably enhances the heat-mediated complete unfolding of CPO.

**Conclusions**

CPO is rapidly deactivated by hydrogen peroxide even at low concentrations of hydrogen peroxide, despite its catalase activity. The deactivation in aqueous buffer appears to proceed via a different intermediate than compound IH_2O_2, which is the active intermediate for the catalase activity. Indole can be used to protect the enzyme against oxidative deactivation when tert-butyl alcohol is used as a cosolvent. In aqueous buffer even slow addition of hydrogen peroxide deactivates CPO. In tert-butyl alcohol/aqueous buffer mixtures a hydrogen peroxide-stat can be used to regulate the addition of hydrogen peroxide and CPO is stable as long as indole is present in the reaction mixture. tert-Butyl alcohol narrows the range in which CPO is stable towards denaturation by temperature or pH. Also the oxidative deactivation of CPO is accelerated in tert-butyl alcohol/aqueous buffer mixtures when no other reductant is present.

Finally, we conclude that the use of a hydrogen peroxide-stat to control the rate of addition of hydrogen peroxide significantly improves the performance of CPO and, hence, extends its synthetic utility.
Experimental procedure

Materials and analytical methods

Chloroperoxidase from *Caldarriomycetes fumago* was isolated and purified as described in the literature [11]. The enzyme preparation (0.102 mM) contained 6000 U/mL with a purity of R<sub>2</sub> = 1.3. Hydrogen peroxide 35% was obtained from Merck. Indole was purchased from Aldrich Chemical Company. tert-Butyl alcohol was obtained from Baker.

Samples for monitoring the oxindole formation were diluted with eluent containing excess Na<sub>2</sub>SO<sub>3</sub> for removing H<sub>2</sub>O<sub>2</sub>. After filtration through a 0.4 μm membrane filter, the samples were analyzed by reversed phase HPLC using an 8x100 mm 4 μm Novapak C<sub>18</sub> column contained in a Waters 8x10 compression unit at an eluent flow of 1.0 mL/min, a Shimadzu SPD-6a UV spectrophotometer at 254 nm and an Erma ERC-7510 RI detector. The eluent was methanol/water (50:50, v/v). tert-Butyl alcohol was used as an internal standard.

The hydrogen peroxide-stat (Fig. 6.7), Dulcometer Perox 20/21, was obtained from Prominent Dosierotechnik, Heidelberg, Germany.

![Diagram of hydrogen peroxide-stat](image)

The sensor measures the [H<sub>2</sub>O<sub>2</sub>] in the reactor and is connected to a PID-regulator. This PID-regulator adjusts the [H<sub>2</sub>O<sub>2</sub>] in the reactor to the desired set-point by controlling the pump for H<sub>2</sub>O<sub>2</sub>-addition.

**Fig. 6.7. Hydrogen peroxide-stat.**

The measurement of hydrogen peroxide was based on amperometric measurement at a platinum electrode which is operated as a potentiostat. Two point calibrations were made before each reaction. A Metrohm Dosimat 655 was used for addition of hydrogen peroxide (0.3 M). UV measurements were carried out on a Cary 3 spectrophotometer from Varian.
Enzyme activity

Enzyme activity was measured using the standard monochlorodimedone test as described in literature [23].

Stability

Stability towards hydrogen peroxide was measured at room temperature in a 50 mL solution to which was added 120-300 U CPO. The hydrogen peroxide-stat was set at the desired concentration. A 0.3 M hydrogen peroxide solution was used for titration. Samples were taken and analyzed for enzyme activity. Stability towards tert-butyl alcohol, temperature and pH were measured in 2.5 mL solvent to which was added 120 U CPO. One parameter was varied while the other parameters were kept constant. Samples were diluted with 0.1 M acetate buffer before analyzing enzyme activity.

Indole oxidation

At room temperature 8 mM indole was dissolved in a 50 mL buffer solution (acetate, 0.1 M, pH 4). After addition of CPO (300 U) the reaction was started by operation of the hydrogen peroxide-stat or by slow continuous addition of H₂O₂ (1 eq./h, 0.3 M H₂O₂). In the case of tert-butyl alcohol as a cosolvent (30:70 or 50:50, v/v) 10 mM of indole was used with 300 U of CPO and the reaction was started by operation of the hydrogen peroxide-stat or by slow addition of H₂O₂ (1 eq./0.67 h, 0.3 M H₂O₂). Aliquots were removed from the reaction mixture and analyzed by HPLC as described under analytical methods.

Relative activities

Experiments for comparing the activity for indole oxidation in different solvents were carried out at 20°C with 400 µM H₂O₂ in 5 mL solvent. tert-Butyl alcohol/acetate buffer (pH 4, 0.1 M) with different amounts of cosolvent (0:100; 30:70 and 50:50 tert-butyl alcohol/aqueous buffer, v/v) was used as solvent. Reactivities were measured at indole concentrations of 2, 4 and 8 mM. The reaction was started by addition of a certain amount of CPO (0.6 to 2.4 U CPO per mL, depending on the reactivity). Every 30 seconds a sample was
taken which was added to a methanol/water solution containing excess Na$_2$SO$_3$ to quench the reaction. The reaction was monitored for 90 seconds. Samples were analyzed on reversed phase HPLC as described under analytical methods. Initial reaction rates per mol of enzyme were determined. The reaction rate with 2 mM indole in tert-butyl alcohol/aqueous buffer (50:50, v/v) was set as the standard to which all other reaction rates were related.

References

[16] F.H. Arnold, TIBTECH, 1990, 8, 244.
Appendix to chapter 6.

Catalase activity and deactivation rate.

Both the catalase activity and the deactivation rate can be expressed as a function of $[\text{CPO}_{\text{total}}]$ (= sum of the concentration of all CPO-species, deactivated CPO excluded)

Catalase activity

$$\frac{\partial [O_2]}{\partial t} = \frac{\partial [H_2O_2]}{2 \cdot \partial t} = k_1 \cdot \frac{\text{Compound I} \cdot H_2O_2}{[\text{CPO}_{\text{total}}]} = k_{1^*} \cdot \frac{\text{Compound I} \cdot H_2O_2}{[\text{CPO}_{\text{total}}]} \quad (1)$$

$$f = \text{fraction} \left( \frac{\text{Compound I} \cdot H_2O_2}{[\text{CPO}_{\text{total}}]} \right)$$

Deactivation rate

$$\frac{\partial [\text{CPO}_{\text{total}}]}{\partial t} = k_{2^*} \cdot [\text{CPO}_{\text{total}}] \quad (3)$$

$$[\text{CPO}_{\text{total}}]_t = [\text{CPO}_{\text{total}}]_0 \cdot e^{-k_{2^*}t} \quad (4)$$

If deactivation proceeds via compound $I \cdot H_2O_2$

$$k_{2^*} = k_2 \cdot \frac{\text{Compound I} \cdot H_2O_2}{[\text{CPO}_{\text{total}}]} = k_2 \cdot f \cdot [\text{CPO}_{\text{total}}] \quad (5)$$

Ratio of $k_{1^*}$ and $k_{2^*}$

$$\frac{\partial [H_2O_2]/\partial t}{2 \cdot k_{2^*} \cdot \left[ \text{CPO}_{\text{total}} \right]_0} = \frac{k_{1^*}}{k_{2^*}} = \frac{\text{initial turnover frequency catalase activity}}{\text{deactivation rate}} \quad (6)$$

$$= \frac{k_{1}}{k_{2}} \text{ if deactivation proceeds via compound I} \cdot \text{H}_2\text{O}_2$$
Chapter 7

Improvement of turnover number and space-time yield for chloroperoxidase catalyzed oxidation of indole*

Abstract

Chloroperoxidase catalyzed oxidation of indole was performed under optimized reaction conditions. Different reactor concepts were tested for the CPO catalyzed oxidation of indole to oxindole in order to improve space-time yields and turnover numbers. These concepts include a batch reactor, a fed-batch reactor and a continuously operated stirred tank reactor. An increase in turnover number of over 20 times was reached compared to the traditional oxidation in buffer [1] and the space-time yield increased from 22 g L⁻¹ d⁻¹ to over 200 g L⁻¹ d⁻¹ in a batch reactor.

* In cooperation with K. Seelbach and U. Kragl from Forschungszentrum Jülich

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Chapter 7

Introduction

In the previous chapters it was shown that chloroperoxidase (CPO) catalyzes interesting reactions like enantioselective sulfoxidation (chapter 3) and oxidation of substituted indoles to oxindoles (chapter 4). The main problem associated with the use of CPO in organic synthesis is its rapid deactivation by hydrogen peroxide. As shown in chapter 3 slow, continuous addition of hydrogen peroxide results in a better enzyme performance. Moreover, the use of a hydrogen peroxide-stat (chapter 6) further improves the catalyst performance and facilitates the reaction procedure. In this chapter the effects of the type of reactor (batch, fed batch or CSTR) are investigated. The most important parameters to study the reactor concepts are the turnover number (ton, mol of product formed/mol of CPO used) and the space-time yield (sty; amount of product which can be produced in one liter per day). The oxidation of indole to oxindole was chosen as a model reaction, as the reaction proceeds fast and selective. The merits of the various reactor concepts are discussed and the results are compared to other established enzyme systems for the synthesis of amino acids and carbohydrates.

Results and Discussion

Batch Reactor

As shown in chapter 6 an important factor for increasing the catalyst lifetime and turnover number of CPO is the use of tert-butyl alcohol as cosolvent for indole oxidation. Whereas in buffer the maximum turnover number was 50,000 and there was no remaining activity of CPO, in tert-butyl alcohol-buffer (30:70, v/v) a turnover number of 80,000 was reached, whilst the enzyme still retained about 90% of its activity. Another advantage of tert-butyl alcohol as cosolvent is the higher solubility of the reactant in this medium compared to water, thus potentially providing higher space-time yields. An increase in space-time yield can also be obtained by using a hydrogen peroxide-stat for H₂O₂-addition (Fig. 7.1 and 7.2). In this way the reaction time is cut in half and space-time yield is doubled to about 230 g L⁻¹ d⁻¹. Furthermore, the endpoint of a reaction can easily be detected and if the conversion is not complete additional enzyme can be added to reach complete conversion. As the oxidant
concentration is low the enzyme is not deactivated instantly, this in contrast to continuous addition of hydrogen peroxide.

**Fig. 7.1.** Batch oxidation of indole, H$_2$O$_2$-stat, 50 mM indole, 300 U CPO, 30 μM H$_2$O$_2$.

**Fig. 7.2.** Batch oxidation of indole, continuous addition of H$_2$O$_2$, 50 mM indole, 300 U CPO, 1 eq. H$_2$O$_2$/h.

High turnover numbers, up to 490,000, can be reached in the batch-wise oxidation of indole. As there was still some enzyme active at the end of the reaction we reasoned that it it should be possible to get even higher turnover numbers. These turnover numbers were calculated to be over 700,000 based on results of the batch reaction with 10 mM indole. In this case a turnover number of 80,000 was reached and the remaining enzyme activity was 90% after complete conversion of indole (see chapter 6). Quantitative enzyme activity measurements were not possible for batch reactions containing 50 mM indole as the colouring of the reaction mixture interfered with the activity test, thus the remaining activity of CPO could not be determined.

**Fed-batch reactor**

A fed batch process would be advantageous in respect to obtaining high turnover numbers and space-time yields. A high reactant concentration is maintained during the whole process, thus the relatively high K$_m$ value is no problem. Initial experiments carried out with a high amount of indole in the feedstock (2.3 M in tert-butyl alcohol/water, 90:10, v/v) resulted in rapid enzyme deactivation as soon as either indole or oxindole precipitated in the reaction.
mixture. To avoid precipitation during reaction the fed batch experiments were carried out in such a way that no crystallization in the reaction mixture could occur. The results are shown in Fig. 7.3. The conversion remained constant, around 75%, for a long period of time and the turnover number increased to over 850,000 at the end of the reaction (after 10 h.).

![Graphs showing conversion and turnover number over time.](image)

**Fig. 7.3. Fed-batch oxidation of indole.**

**Enzyme Membrane Reactor**

To demonstrate the versatility of CPO the synthesis of oxindole was performed in a continuously operated stirred tank reactor (CSTR). The CSTR used was an enzyme membrane reactor (EMR, Fig. 7.4) in which CPO was retained by an ultrafiltration membrane (10 K cutoff). As the hydrogen peroxide sensor could not be used in this simple setup the oxidant concentration was limited to 0.87 equivalents to prevent CPO from deactivation. Fig. 7.5 shows that the EMR maintains its performance over a period of 10 residence times. A drawback of the indole oxidation in the EMR is that during the oxidation a small amount of polymeric material is formed which blocks the membrane. This causes an increase in the pressure drop over the membrane, despite the fact that the flow is kept constant. Together with the higher enzyme concentration this results in a turnover number which is lower than that achievable with the batch and fed-batch reactors. Nevertheless a high space-time yield of 120 g L\(^{-1}\) d\(^{-1}\) can be reached due to the high indole concentration which is oxidized continuously.
Conclusions

The turnover numbers and space-time yields obtained using the various reaction conditions and reactor concepts are shown in Table 7.1. The sensor-controlled reactions or continuously operated reactions in tert-butyl alcohol/buffer mixtures generally resulted in high turnover numbers and space-time yields. The best results in respect to ton and sty are obtained in either a batch reactor or a fed-batch process using a hydrogen peroxide-stat for controlling oxidant addition and tert-butyl alcohol as a cosolvent.
Table 7.1.
Ton and sty for various reactor concepts.

<table>
<thead>
<tr>
<th>Reaction medium</th>
<th>Reactor type</th>
<th>Method of H₂O₂-addition</th>
<th>turnover number (10^3)</th>
<th>space-time yield (g L⁻¹ d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>buffer [1]</td>
<td>batch</td>
<td>stepwise</td>
<td>40</td>
<td>21.9</td>
</tr>
<tr>
<td>buffer+tert-butyl alcohol</td>
<td>batch</td>
<td>continuously</td>
<td>&gt;490</td>
<td>114</td>
</tr>
<tr>
<td>buffer+tert-butyl alcohol</td>
<td>batch</td>
<td>H₂O₂-stat</td>
<td>&gt;490</td>
<td>230</td>
</tr>
<tr>
<td>buffer+tert-butyl alcohol</td>
<td>fed-batch</td>
<td>H₂O₂-stat</td>
<td>858</td>
<td>a</td>
</tr>
<tr>
<td>buffer+tert-butyl alcohol</td>
<td>EMR</td>
<td>continuously (limited)</td>
<td>193</td>
<td>120</td>
</tr>
</tbody>
</table>

\(^a\) Due to the way the reaction was performed no value can be given here.

In Table 7.2 some data are given concerning laboratory and industrial scale synthesis of carbohydrates and aminoacids. The results obtained with CPO demonstrate that enzymatic oxidation of indole to oxindole can be compared favorably to other large-scale enzymatic synthesis.

Table 7.2.
Comparison of sty and ton for various enzymes.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reactor type</th>
<th>Product</th>
<th>sty (g L⁻¹ d⁻¹)</th>
<th>ton (10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPO</td>
<td>batch</td>
<td>oxindole</td>
<td>230</td>
<td>&gt;490</td>
</tr>
<tr>
<td>Aminoacylase [2]</td>
<td>EMR</td>
<td>L-methionine</td>
<td>592</td>
<td>253</td>
</tr>
<tr>
<td>Leucine Dehydrogenase [3]</td>
<td>EMR</td>
<td>L-tert-leucine</td>
<td>638</td>
<td>49,000</td>
</tr>
<tr>
<td>Formate Dehydrogenase [3,4,5]</td>
<td>EMR</td>
<td>NADH-regeneration</td>
<td>----</td>
<td>964</td>
</tr>
<tr>
<td>Neu5Ac-aldolase [3,6]</td>
<td>EMR</td>
<td>Neu5Ac</td>
<td>470</td>
<td>373</td>
</tr>
</tbody>
</table>
Improvement of turnover number and space-time yield

Using the described fed-batch process 2.7 kg of oxindole can be synthesized with 1 g of CPO. The commercial price of CPO is prohibitive ($5 per mg for purified CPO, Chirazyme, Illinois, USA) resulting in about $2 catalyst cost per gram of oxindole, which is two to three orders of magnitude too high. However, we presume that the cost price for CPO as catalyst in this reaction can be significantly lowered to less than $0.5 per mg, as it is not necessary to use purified enzyme [7]. Thus about 1 mL of fermentation broth [9] would be sufficient to obtain 1 g of oxindole.

The CPO-catalyzed oxidation of indole to oxindoles is especially cost-effective and environmentally friendly for the production of substituted oxindoles. Recently a new process has been developed which makes 4-, 5-, 6- and 7- substituted indoles readily obtainable from cheap starting materials [8]. The substituted indoles are obtained in good yield by biosynthetic production from substituted anthranilates (Fig. 7.6). Combined with the CPO-catalyzed oxidation of substituted indoles this results in a 2-step method for the production of substituted oxindoles in good yields. Oxidation of 5-chloro-indole by the batch-procedure as described for indole showed that 5-chloro-oxindole could be obtained with a ton of >435,000 and a sty of 80 g L \(^{-1}\) d \(^{-1}\).

\[ \text{r} \text{-recombinant } E.\text{coli} \]

**Fig. 7.6. Biosynthetic production of substituted indoles [8].**

Experimental Procedure

**Materials and analytical methods**

Chloroperoxidase from *Caldariomyces fumago* was isolated and purified as described in the literature [9]. The enzyme preparation (0.102 mM) contained 6000 U/mL with a purity of R\(_x\) = 1.3. Hydrogen peroxide 35% was obtained from Merck. Indole was purchased from Aldrich Chemical Company.
Samples for monitoring the oxindole formation were diluted with eluent containing excess Na₂SO₃ for removing H₂O₂. After filtration through a 0.4 μm membrane filter, the samples were analyzed by reversed phase HPLC using an 8x100 mm 4 μm Novapak C₁₈ column contained in a Waters 8x10 compression unit at an eluent flow of 1.0 mL/min, a Shimadzu SPD-6a UV spectrophotometer at 254 nm and an Erma ERC-7510 RI detector. The eluent was methanol/water (50:50, v/v). tert-Butyl alcohol was used as an internal standard.

The hydrogen peroxide-stat, Dulcometer Perox 20/21, was obtained from Prominent Dosiertechnik, Heidelberg, Germany. The measurement of hydrogen peroxide was based on amperometric measurement at a platinum electrode which is operated as a potentiostat. Two point calibrations were made before each reaction. A Metrohm Dosimat 655 was used for addition of hydrogen peroxide. UV measurements were carried out on a Cary 3 spectrophotometer from Varian.

**Enzyme activity**

Enzyme activity was measured using the standard monochlorodimedone test as described in literature [10].

**Batch Reactor**

At room temperature 50 mM indole was dissolved in a 50 mL solvent (tert-butyl alcohol/aqueous buffer, 30:70, v/v; 0.1 M acetate, pH 4). After addition of CPO (300 U) the reaction was started by slow continuous addition of H₂O₂ (1 eq./h, 1 M H₂O₂) or by operation of the hydrogen peroxide-stat (setpoint for [H₂O₂]: 30 μM).

**Fed batch reactor**

At room temperature the reaction was started in a 200 mL reaction flask containing 50 mM indole and 300 U CPO in 25 mL solvent (tert-butyl alcohol/aqueous buffer, 30:70, v/v; 0.1 M acetate, pH 4). The reaction was controlled by the hydrogen peroxide-stat and the [H₂O₂] was set at 30 μM. To the reaction mixture was added an indole solution consisting of 50 mM indole in tert-butyl alcohol/aqueous buffer (30:70, v/v; 0.1 M phosphate, pH 4) at a
rate of 30 mL/h. After 200 min. the addition of indole solution was stopped and the reaction was allowed to come to a stand-still.

**Enzyme membrane reactor**

For the continuous production of oxindole a 10 mL stainless steel reactor was used, as described elsewhere [11,12]. An ultrafiltration membrane (YM 10, Amicon) was used for retaining CPO in the reactor. The feed solution contained 50 mM indole and 43.5 mM H₂O₂ in tert-butyl alcohol/aqueous buffer (30:70, v/v; 0.1 M phosphate, pH 4). The feed solution was pumped into the membrane reactor at a velocity of 10 mL/h. To start the reaction 680 U of CPO was injected into the reactor. After 6 residence times another 680 U CPO was injected. The typical pressure drop over the membrane increased from 2 bar at the beginning, up to 8 bar at the end of the reaction. The reaction was performed at 20°C.

**References**

A simple purification method for chloroperoxidase

Abstract

A simple two-step purification method for chloroperoxidase from *Caldariomyces fumago* has been developed. After filtration of the mycelium the enzyme was bound to a DEAE Sepharose fast flow column. The enzyme was eluted with a 20-200 mM phosphate buffer, pH=5.8. After gel filtration on a Superose 12 HPLC column pure enzyme was obtained. Instead of gel filtration it was also possible to purify the enzyme by concentration over a membrane filter, 10 K cutoff. Concentration to 8% of the original volume yielded an enzyme preparation with $R_e=1.31$ in 77% yield.

* $R_e = \text{purity standard} = \lambda_{400}/\lambda_{280} = 1.44$ for pure enzyme*
Introduction

Although *Caldariomyces fumago* produces high levels of chloroperoxidase (CPO) [1], the purification of this enzyme as described in the literature is rather laborious [1,2,3]. During production of CPO we discovered a simplified method for its purification, which afforded nearly pure enzyme in good yield.

Results and discussion

Fermentation and Purification

In the course of our studies of oxidations catalyzed by redox enzymes, we performed a 15 L fermentation of chloroperoxidase from *Caldariomyces fumago*. After 7 days, when no sharp increase of enzyme activity was observed the fermentation was stopped. A high level of CPO activity was measured compared to literature data on batch fermentation [4] (Table 1).

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Activity (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>70</td>
</tr>
<tr>
<td>4</td>
<td>300</td>
</tr>
<tr>
<td>6</td>
<td>530</td>
</tr>
<tr>
<td>7</td>
<td>555</td>
</tr>
</tbody>
</table>

Table 1. Enzyme activity in fermenter.

![Purification Scheme](image)

R
\_x<0.10

1. freeze/defrost/filter

R
\_x<0.10

2. DEAE Sepharose

20-200mM phosphate buffer,
pH=5.8

R
\_x=0.90

3a. Concentration

3b. Gel filtration

10 K membrane

R
\_x=1.31

R
\_x=1.44

Fig. 1. Purification scheme for CPO.
Purification procedures as described in the literature are rather laborious [1,2,3]. Gonzalez-Vergara et al. [3] published a method which consisted of PEG precipitation for removing the pigment followed by QAE chromatography and ammonium sulphate precipitation yielding an enzyme preparate with \( R_x = 1.28 \) (89\% yield). Extensive dialysis resulted in pure enzyme (85\% yield). Pickard et al.[1] modified this method to purify the enzyme on a large scale (50 L fermentation). This procedure included freezing and thawing (precipitate is filtered off), concentration by hollow fiber ultrafiltration, PEG precipitation, centrifugation, recentration followed by dialysis and column chromatography on DEAE cellulose (DE-52) resulting in 80\% yield and \( R_x > 1.25 \).

We discovered that the purification of CPO could be simplified (Fig. 1). There was no extra purification step necessary for removing the pigment and enzyme could be directly applied on a chromatography column. Pigment could be removed by washing the column with 20 mM phosphate buffer. Enzyme produced on a large scale had a very high viscosity compared to small scale production (i.e. 900 mL fructose medium in a 2 L flask) and could not be applied on the column directly. The viscosity was lowered by freezing and thawing the enzyme solution, subsequently the black precipitate was filtered off and the filtrate could be applied on the column. Concentration of the enzyme over a 10 K filter gave further improvement of the enzyme purity, because the low molecular weight protein was removed through the filter (Fig. 2). A 600 mL batch of CPO was purified according to this method and yielded nearly pure CPO (\( R_x = 1.31 \)) in good yield (77\%) (Table 2). Pure enzyme (\( R_x = 1.44 \)) could be obtained by gel filtration on a Superose 12 HPLC column (Fig. 2).

**Table 2. Purification of CPO.**

<table>
<thead>
<tr>
<th></th>
<th>Volume (mL)</th>
<th>( A_{400} )</th>
<th>( R_x )</th>
<th>Activity (U/mL)</th>
<th>Protein (mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtration</td>
<td>600</td>
<td>---</td>
<td>&lt;0.1</td>
<td>555</td>
<td>1320</td>
<td>100</td>
</tr>
<tr>
<td>Freezing</td>
<td>600</td>
<td>---</td>
<td>&lt;0.1</td>
<td>535</td>
<td>900</td>
<td>96</td>
</tr>
<tr>
<td>DEAE</td>
<td>350</td>
<td>0.8</td>
<td>0.90</td>
<td>760</td>
<td>105</td>
<td>80</td>
</tr>
<tr>
<td>Concentration</td>
<td>30</td>
<td>8.8</td>
<td>1.31</td>
<td>8500</td>
<td>77</td>
<td>77</td>
</tr>
</tbody>
</table>
Fig. 2. Gel filtration of CPO samples, a) before concentration b) after concentration over a 10 K filter.

**Experimental procedure**

**Materials and analytical methods**

All materials used were of reagent grade and purchased from commercial suppliers. Spectrophotometric measurements were carried out on a Hewlett Packard 8450 A spectrophotometer.

**Enzyme activity and protein content**

Chloroperoxidase activity was determined by the standard method as described by Morris and Hager [5]. Protein content was determined by the method of Bradford [6] using bovine serum albumin as a standard.

**Fermentation**

*Caldariomyces fumago* IMI 89362 was obtained from the International Mycological Institute and maintained on potato dextrose agar slants at 4°C.

Inocula were prepared by transferring 0.5 cm² mycelium to 50 mL of glucose malt extract in a 100 mL flask. The glucose malt extract medium contained per liter: 40 g glucose, 20
g malt extract, 2 g NaNO₃, 2 g KCl, 2 g KH₂PO₄, 0.5 g MgSO₄, 20 mg FeSO₄·7H₂O (from a solution containing 30 g tricine and 15 g FeSO₄·7H₂O per liter). The flask was rotated at a speed of 200 rpm at room temperature and after 6 days the medium was transferred to 600 mL fructose salts medium in a 3 L flask. In the fructose salt medium the glucose and malt (see above) were replaced by 40 g fructose. After 6 days at 200 rpm fermentation was started by adding the inoculate to 15 L of fructose salts medium in a 20 L stirred fermenter. The fermenter was kept at 23°C, rotation speed: 300 rpm, 3 L air/min. As the best growth takes place in (day)light, an extra lamp was placed near the fermenter. After 7 days the mycelium was harvested by filtering the mycelium over paper.

**Purification**

The filtered medium was stored at -80°C in 1 L batches in plastic buckets. A 600 mL batch containing 3.33 x 10⁵ U was thawed and purified as follows. The black precipitate was filtered over a 1 micron stainless steel mesh filter. The precipitate was washed with 10 mM phosphate buffer, pH 5.8. The combined filtrate was adjusted to pH 5.8 by 6 mM K₂HPO₄ and brought onto a DEAE Sepharose (Pharmacia, 500 mL) fast flow column (Pharmacia, d 5 cm) in 20 mM phosphate buffer, pH 5.8, flow 10 mL/min. The column was washed with 20mM phosphate buffer for 1 h. The enzyme was eluted by a gradient of 20-200 mM phosphate buffer, pH 5.8, during 4 h at a flow rate of 10 mL/min. Fractions with Rₓ>0.6 were collected. A small fraction with Rₓ=0.9 was further purified by gel filtration over a Superose 12 HPLC column (Pharmacia, 1.0 cm x 30 cm), 200 mM phosphate buffer, pH 5.2, flow 0.5 mL/min, to an Rₓ of 1.44. The other fractions with Rₓ> 0.6 were collected and concentrated to 8% of their original volume over a 10 K filter (Centriprep-10 concentrator, Amicon) at a speed of 2000 rpm. The concentrate contained 2.55 x 10⁵ U (77%) with Rₓ=1.31.

**References**

Summary

The theme of this thesis is the exploitation of chloroperoxidase from *Caldariomyces fumago* as a catalyst for selective oxidations in organic synthesis. Chloroperoxidase was chosen because it has unusual properties for a peroxidase which render it suitable as an oxygen transfer catalyst. Enzyme isolation and purification, synthetic and mechanistic aspects of selective oxidations and improvement of catalyst performance are described in this thesis.

An introduction to oxidative transformations in fine chemistry is given in chapter 1. Chapter 2 reviews selective oxidations catalyzed by peroxidases, from both a synthetic and mechanistic viewpoint. Furthermore the different catalytic centers in peroxidases, the deactivation of heme peroxidases and potential commercial applications of peroxidases are discussed.

Chapter 3 describes the chloroperoxidase catalyzed oxidations of sulfides and in tert-butyl alcohol/water mixtures. tert-Butyl alcohol/water (50:50, v/v) proved to be a good solvent system for performing synthetic oxidations catalyzed by chloroperoxidase. The sulfoxidation of alkyl aryl sulfides and related compounds catalyzed by chloroperoxidase in tert-butyl alcohol/water mixtures (50:50, v/v) was compared to the sulfoxidation in water. In both solvent systems complete enantioselectivity to the $R$-sulfoxide (ee $\geq$ 99%) was observed with hydrogen peroxide as oxidant when the size of the alkyl moiety was smaller than propyl. The uncatalyzed, racemic sulfoxidation did not proceed under these conditions. This is in contrast to literature data on sulfoxidation in water, where enantioselectivities were lower due to this uncatalyzed reaction. Reactions in water generally proceeded faster than reactions in the cosolvent system except for substrates which dissolve poorly in water or for solid substrates for which diffusion becomes an important limiting factor in water. The lower activity in tert-butyl alcohol/water for sulfoxidation is mainly due to an increase of the $K_m$ value (thermodynamically controlled). Also a decrease of $k_{cat}$ (catalytic turnover frequency) is observed, probably caused by a change in structure of the enzyme.

In chapter 4 the chloroperoxidase catalyzed oxidation of substituted indoles to the corresponding oxindoles is described. Substituted oxindoles are interesting building blocks for pharmaceuticals. The substituted oxindoles were obtained in virtually quantitative yield. These include 5-chloro-oxindole, a precursor for the anti-inflammatory agent Tenidap, which
was obtained in 95% yield. The reactivity of the substituted indoles depends on the nature and the position of the substituent. Both electronic and steric effects of substituents appear to be important. All of the oxygen in the product is derived from hydrogen peroxide. A mechanism for the hydroxylation is proposed which is consistent with these observations.

Chapter 5 deals with the chloroperoxidase catalyzed oxidation of 5-hydroxymethylfurfural. The reaction proceeds with 60-74% selectivity to 2,5-furandicarboxaldehyde. The main byproduct is 5-hydroxymethyl-2-furancarboxylic acid; a minor amount of 5-formyl-2-furancarboxylic acid was also detected. When $H_2^{18}O_2$ was used virtually quantititative incorporation of $^{18}O$ was observed in the HFCA product, whereas no $^{18}O$ was incorporated when $H_2^{18}O$ was used. Hence, the CPO-catalyzed oxidation of aldehydes to acids proceeds with direct oxygen transfer from the iron-oxo complex of CPO.

In chapter 6 the use of a hydrogen peroxide-stat for controlling the hydrogen peroxide concentration is demonstrated. The chloroperoxidase catalyzed oxidation of indole was chosen as model reaction. It is shown that in tert-butyl alcohol/water mixtures the oxidation can be effectively controlled by a hydrogen peroxide-stat. In this medium chloroperoxidase is stabilized towards oxidative destruction as long as indole is present in the reaction mixture but deactivation occurs in the absence of a reductant. In contrast with the results with tert-butyl alcohol/water mixtures, chloroperoxidase is deactivated in water by hydrogen peroxide even when indole is present in the reaction mixture and the oxidant concentration is as low as 30 $\mu$M. Furthermore, the hydrogen peroxide-stat was used to perform mechanistic studies on the deactivation rate and the catalase activity of chloroperoxidase. Deactivation and catalase activity do not appear to involve the same intermediate.

In chapter 7 a study is described of different reactor concepts for the chloroperoxidase catalyzed oxidation of indole to oxindole in order to improve space-time yields and turnover numbers. These concepts include a batch reaction, a fed-batch reaction and oxidation of indole performed in a continuously operated enzyme membrane reactor. An increase in turnover number of over 20 times was reached compared to the oxidation in buffer and the space-time yield increased to over 200 g L$^{-1}$d$^{-1}$.

Finally, in the appendix a new simple 2-step purification method for chloroperoxidase is described. In the first purification step chloroperoxidase was purified by column chromatography. Subsequently the enzyme was further purified over a 10 K filter yielding 90% pure chloroperoxidase in 77% yield.
Samenvatting

De afgelopen vier jaar heb ik onderzoek gedaan naar "Selectieve Oxidaties gekatalyseerd door Chloorperoxidase". Omdat deze titel voor veel mensen nietszeggend is zal ik in deze samenvatting proberen duidelijk te maken waar ik me tijdens mijn promotieonderzoek mee bezig heb gehouden.

Het maken van producten op een milieu vriendelijke en efficiënte wijze is een belangrijk onderwerp van onderzoek in de chemie. Een manier om aan deze voorwaarden voor een productieproces te voldoen is gebruik te maken van selectiviteit. Selectiviteit in een reaktie kan verkregen worden door een katalysator. Een katalysator is een "hulpsstof" die reakties versnelt en een bepaalde kant op kan sturen. De werking van een katalysator staat symbolisch weergegeven in de figuren 1 en 2.

Om uit een uitgangsstof (A) een product (B) te maken moet een energie-barrière worden overwonnen. Dit wordt voorgesteld in figuur 1 als de berg waar overheen "geklommen" moet worden. Een katalysator kan de "energieberg" verlagen waardoor de weg naar het eindprodukt makkelijker (=sneller) is. Ook is het mogelijk om met een katalysator andere producten te krijgen dan dat je zonder katalysator zou krijgen. In figuur 2.a zien we dat zonder katalysator de energieberg van uitgangsstof A naar product B lager is dan naar (het gewenste) product C. Een katalysator kan de energieberg naar product C verlagen zodat deze barrière lager wordt dan de energieberg naar product B (figuur 2.b). Met een katalysator is het zodoende mogelijk om toch product C te maken zonder dat het (ongewenste) product B wordt gevormd. Een katalysator kan dus niet alleen een reaktie sneller laten verlopen, ook kan een katalysator de reaktie...
“sturen” en dus selectief laten verlopen.

In de scheikunde zijn er verschillende vormen van selectiviteit. Ik wil enkele vormen van selectiviteit die belangrijk zijn geweest voor mijn promotie-onderzoek hier bespreken. Een eerste belangrijke vorm van selectiviteit is atoomselectiviteit. Dit is een maat voor de efficiëntie van een proces. Bij een hoge atoomselectiviteit worden weinig afvalstoffen geproduceerd omdat een hoog percentage van de atomen (=bouwstenen) die worden gebruikt om een reaktie te laten verlopen wordt teruggevonden in het gewenste product. Een hoge atoomselectiviteit levert weinig afval op.

Een tweede vorm van selectiviteit is enantioselectiviteit. Bij sommige reacties kunnen twee producten ontstaan die het spiegelbeeld van elkaar zijn (zoals de linker- en de rechterhand). Het lichaam kan onderscheid maken tussen beide vormen (het ene spiegelbeeld is bijvoorbeeld een slaapmiddel, terwijl het andere spiegelbeeld invloed heeft op ongeboren kinderen zoals bij Softenon). Het is erg belangrijk om in medicijnen en andere chemische producten slechts één van de twee spiegelbeelden te hebben, degene die de gewenste werking heeft. Een katalysator kan ervoor zorgen dat de energieberg naar het gewenste spiegelbeeld wordt verlaagd terwijl de weg naar het ongewenste spiegelbeeld dezelfde barrière houdt.

Een derde vorm van selectiviteit is chemoselectiviteit. Als er twee verschillende chemische groepen in een molekuul aanwezig zijn, die beiden zouden kunnen reageren, kan een katalysator er voor zorgen dat slechts één van beide groepen selectief reageert.

Figuur 3. Enantioselectiviteit.

Figuur 4. Chemoselectiviteit
De laatste vorm van selectiviteit die ik wil bespreken is regioselectiviteit. Daarbij zijn er twee identieke groepen in een molecuul aanwezig die op verschillende plaatsen in het molecuul zitten. Tijdens een regioselectieve reactie reageert slechts één van beide groepen.

Figuur 5. Regioselectiviteit.

Er zijn verschillende soorten katalysatoren om de genoemde vormen van selectiviteit te bewerkstelligen. Zo is het mogelijk om de katalysatoren zelf te synthetiseren (=bouwen). Een andere mogelijkheid is om gebruik te maken van katalysatoren die al in de natuur aanwezig zijn, bijvoorbeeld enzymen. Enzymen zijn eiwitten die een bepaalde reactie in de natuur katalyseren. Deze enzymen kunnen in de scheikunde worden gebruikt om allerlei reacties te katalyseren. In dit proefschrift wordt het onderzoek beschreven dat is verricht naar het gebruik van een enzym, chloorperoxidase, als katalysator voor selectieve oxidaties. Een oxidatie is een type reactie waarbij interessante bouwstenen kunnen worden gevormd. Deze bouwstenen kunnen worden gebruikt om een grote verscheidenheid aan chemische eindproducten te maken, zoals bijvoorbeeld medicijnen of gewasbeschermingsmiddelen. Bij een oxidatie is een oxidant nodig. Oxidanten zijn bijvoorbeeld waterstofperoxide en zuurstof. Zonder katalysator is het vaak noodzakelijk om gebruik te maken van zwaar metaal-oxidanten, waarbij zware (giftige) metalen in het milieu vrijkomen. Met chloorperoxidase als katalysator kan waterstofperoxide worden gebruikt als oxidant. Daarbij wordt het milieuvriendelijke water als bijproduct gevormd en wordt de atoomselectiviteit van de reactie verhoogd.

Tijdens mijn promotie-onderzoek heb ik gekeken naar de selectiviteit van chloorperoxidase voor verschillende soorten oxidaties en naar het precieze verloop van deze reacties. Tevens zijn er manieren onderzocht om de reacties met dit enzym geschikter te maken voor industriële toepassingen. Daarnaast is er tijdens de promotie onderzoek gedaan naar het maken en zuivering van het enzym (Appendix).

In tegenstelling tot verwante enzymen blijkt chloorperoxidase een hoge enantioselectiviteit te vertonen voor sulfooxidatie (hoofdstuk 3). Ook de regioselectiviteit voor oxidatie van gesubstitueerde indolen is erg hoog (hoofdstuk 4). De chemoselectiviteit voor oxidatie van de alcoholgroep in 5-hydroxymethylfurfural is matig (hoofdstuk 5) maar de geconstateerde nevenreactie was nog niet eerder ontdekt. De toepasbaarheid van
chloroperoxidase als katalysator voor oxidatieve reakties is verbeterd door gebruik te maken van een onschadelijk organisch oplosmiddel (tert-butyl alcohol, hoofdstuk 3 en 6) en door een betere en geautomatiseerde manier voor waterstofperoxide-dosering te gebruiken (hoofdstuk 6 en 7).

Concluderend heeft dit onderzoek een aantal ontdekkingen opgeleverd waardoor industriële toepassingen van chloroperoxidase als selectieve oxidatie-katalysator dichterbij zijn gekomen.
Glossary of Terms

Active center  Location in the enzyme where the specific reaction takes place. Also called active site

Antibody  A protein produced by the immune system of an organism in response to a foreign substrate (antigen) and characterized by its specific binding to that substrate

Assay  A procedure for determining the biological activity of a biocatalyst by comparing the reactivity of the biocatalyst to a standard reaction

Asymmetric  Lacking all elements of symmetry

Autoxidation  Oxidation of an organic compound with molecular oxygen, usually via a free radical chain process

Biocatalyst  An enzyme in cell-free or whole-cell form that catalyzes metabolic reactions in living organisms and/or substrate conversions in various chemical reactions

Biomimetic  Refers to a laboratory procedure designed to imitate a natural chemical process or to a compound that mimics a biological material in its structure or function

Catalase  An enzyme which decomposes hydrogen peroxide to molecular oxygen and water

Catalytic antibody  An antibody induced by an transition state analogue of a chemical reaction, which, in theory, catalyzes the conversion of a substrate for that reaction

Chiral  An object or a molecule is chiral when it is not superimposable on its mirror image, that is, when it lacks reflectional symmetry

Chiral auxiliary  An optically active molecule that is used in stoichiometric amounts to orchestrate asymmetric induction at a newly formed stereogenic center without being incorporated into the product

Compound I  Heme-peroxidase which has a formal oxidation state of iron(V). Thus there are two more oxidation equivalents than in the native
enzyme. Oxygen transfer from hydrogen peroxide to the heme group of peroxidase forms compound I

**Compound II**

Heme-peroxidase which has a formal oxidation state of iron(IV). Thus there is one more oxidation equivalent than in the native peroxidase. Compound II is usually formed by a one-electron reduction of compound I

**Compound III**

Heme-peroxidase which has a formal oxidation state of iron(VI). Thus there are three more oxidation equivalents than in the native peroxidase. Compound III is often referred to as oxyperoxidase, the analog of oxyhemoglobin and oxymyoglobin. One of the several methods for the formation of compound III is addition of molecular oxygen to reduced ferrous peroxidase

**Cosubstrate**

Low-molecular-weight, non proteinaceous organic compounds participating in enzymatic reactions as dissociable acceptor or donor of chemical groups or electrons

**Coordination**

A coordination entity is composed of a central atom, usually that of a metal, to which is attached a surrounding array of other atoms or group of atoms, each of which is called a ligand

**Dehydrogenase**

An oxidoreductase which catalyzes the removal of hydrogen atoms from a substrate

**Diastereomers**

Stereoisomers that are not mirror-images of each other

**Dioxygenase**

An enzyme which catalyzes the insertion of two atoms of oxygen, derived from molecular oxygen, into an aromatic or aliphatic compound

**Enantiomer**

One of a pair of molecular entities that are mirror images of each other and non-superimposable

**Enantiomeric excess**

Mol fraction denoting the ratio of enantiomers in a mixture:

\[
ee = \frac{[R]-[S]}{[R]+[S]} \times 100\%
\]

**Enantioselective synthesis**

Preferential formation of one enantiomer from a prochiral substrate

**Enzyme**

A protein that functions as a catalyst in one or more reactions
Fermentation
Growth of microorganisms in the presence of a carbon source and various nutrients. Precursor fermentation denotes a single chemical step effected by subjecting a substrate to growing microorganisms. De novo fermentation, in contrast, refers to the formation of organic substances by multi-step conversion of a carbohydrate feedstock such as glucose.

Haloperoxidase
A peroxidase which catalyzes the oxidative transformation of halide to hypohalite or organic halogen compounds

Heme
A near-planar coordination complex obtained from iron and the dianionic form of porphyrin

High spin
In any coordination entity with a particular $d^n$ ($1<n<9$) configuration and a particular geometry, if some of the higher $d$ orbitals are occupied before all the lower energy ones are completely filled

Inhibition
The decrease in reaction rate brought about by the addition of a substance

Inhibitor
A substance which decreases the reaction rate

Ligand
The atoms or group of atoms bound to the central atom of a coordination complex

Low spin
In any coordination entity with a particular $d^n$ ($1<n<9$) configuration and a particular geometry, if all the lower energy $d$ orbitals are completely filled before some of the higher ones are occupied

Microorganisms
Microscopic living entities. Four classes of microorganisms are of importance for fermentation: actinomycetes (filamentous bacteria), bacteria, molds and yeasts

Molecular imprinted polymer
Polymers obtained by polymerization of a monomer around a template

Molecular sieve
A material which contains regular pores of molecular dimensions

Monooxygenase
An enzyme which catalyzes the insertion of one atom of oxygen, derived from molecular oxygen, into an aromatic or aliphatic compound
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>Mutagenesis</td>
<td>The introduction of permanent inheritable changes (mutations) into the DNA of an organism. When this occurs specifically at one site encoding for a single amino acid in a protein this is referred to as site-directed mutagenesis.</td>
</tr>
<tr>
<td>Mutation</td>
<td>An inheritable change in the nucleotide sequence of genomic DNA which may be induced by chemical mutagens, radiation or the use of <em>in vitro</em> recombinant DNA techniques.</td>
</tr>
<tr>
<td>Optical activity</td>
<td>The unique property exhibited by enantiomers of rotating the plane of polarized light.</td>
</tr>
<tr>
<td>Optical purity</td>
<td>The ratio of the measured specific optical rotation of a substance compared with that of the pure enantiomer under the same conditions.</td>
</tr>
<tr>
<td>Oxidase</td>
<td>An enzyme which catalyzes the oxidation of substrates by molecular oxygen without the need for a cosubstrate.</td>
</tr>
<tr>
<td>Oxidoreductase</td>
<td>An enzyme of E.C. class 1 which catalyzes an oxidation-reduction reaction.</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>An enzyme which catalyzes the oxidation of substrates by hydrogen peroxide or alkyl hydroperoxides.</td>
</tr>
<tr>
<td>Porphyrin</td>
<td>A macrocyclic molecule that contains four pyrrole rings linked together by single carbon atom bridges between the alpha-positions of the pyrrole rings. Porphyrins usually occur in their dianionic form coordinated to a metal ion.</td>
</tr>
<tr>
<td>Prochiral</td>
<td>A molecule is said to be prochiral when addition to a double bond or replacement of one of two equivalent groups at a particular atom leads to the creation of a new stereogenic center in the molecule.</td>
</tr>
<tr>
<td>Prosthetic group</td>
<td>A tightly bound, specific nonpolypeptide unit in a protein essential for catalysis.</td>
</tr>
<tr>
<td>Productivity</td>
<td>Amount of product obtained per reactor volume also referred to as volume yield.</td>
</tr>
<tr>
<td>Racemization</td>
<td>The process whereby an enantiomer is transformed into a racemic mixture. A racemic mixture is composed of equal amounts of a pair of enantiomers.</td>
</tr>
<tr>
<td><strong>Redox potential</strong></td>
<td>The redox potential is the oxidation/reduction potential of a compound measured under standard conditions against a standard reference half-cell: the standard hydrogen electrode. If the half-reaction is written as an oxidation the driving force is the oxidation potential.</td>
</tr>
<tr>
<td>--------------------</td>
<td>----------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>R or S</strong></td>
<td>Absolute configuration of a molecule about a particular stereogenic center according to the Cahn-Ingold-Prelog convention</td>
</tr>
<tr>
<td><strong>Space-time yield</strong></td>
<td>Amount of product obtained per reactor volume per period of time, usually expressed as g L⁻¹ d⁻¹</td>
</tr>
<tr>
<td><strong>Specific activity</strong></td>
<td>A measure for the catalytic power of an enzyme preparation, expressed as units of enzyme per milligram of protein. When an enzyme has a catalytic center whose concentration can be measured the catalytic power can be expressed as catalytic center activity, i.e. the number of molecules of substrate transformed per minute per catalytic center</td>
</tr>
<tr>
<td><strong>Stereochemistry</strong></td>
<td>The part of chemistry which deals with structure in three dimensions</td>
</tr>
<tr>
<td><strong>Stereoselective reaction</strong></td>
<td>A stereoselective reaction is a reaction that yields predominantly one enantiomer of a possible pair, or one diastereomer (or one enantiomeric pair) of several possible diastereomers</td>
</tr>
<tr>
<td><strong>Stereoisomers</strong></td>
<td>Isomers that differ only in the way that the atoms are oriented in the three dimensional space.</td>
</tr>
<tr>
<td><strong>Stereospecific reaction</strong></td>
<td>In a stereospecific reaction stereochemically different molecules react differently</td>
</tr>
<tr>
<td><strong>Substrate</strong></td>
<td>A compound which is transformed under the influence of a catalyst</td>
</tr>
<tr>
<td><strong>Supramolecular</strong></td>
<td>The term supramolecular is used for organized entities of higher complexity which result from the association of two or more chemical species held together by intermolecular forces</td>
</tr>
<tr>
<td><strong>Synthon</strong></td>
<td>A portion of a (target) molecule which is recognizable related to a simple molecule, also: synthetic building block</td>
</tr>
<tr>
<td><strong>Template</strong></td>
<td>Structure directing agent</td>
</tr>
<tr>
<td><strong>Tertiary structure</strong></td>
<td>The overall three-dimensional structure of a biopolymer</td>
</tr>
<tr>
<td>------------------------</td>
<td>-----------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Trapping</strong></td>
<td>The interception of a reactive molecule or reaction intermediate so that it is removed from the system or converted into a more stable form</td>
</tr>
<tr>
<td><strong>Turnover frequency</strong></td>
<td>Amount of product obtained per amount of catalyst used per period of time, usually expressed as mol mol(^{-1}) h(^{-1})</td>
</tr>
<tr>
<td><strong>Turnover number</strong></td>
<td>Amount of product obtained per amount of catalyst used, usually expressed as mol mol(^{-1})</td>
</tr>
<tr>
<td><strong>Unit</strong></td>
<td>Amount of substrate converted per period of time in a standard assay expressed as (\mu)mol min(^{-1})</td>
</tr>
<tr>
<td><strong>Zeolite</strong></td>
<td>A crystalline aluminosilicate containing micropores of molecular dimensions</td>
</tr>
</tbody>
</table>
Nawoord

Voor U ligt het resultaat van ruim vier jaar promotie-onderzoek aan de TU Delft. Ik heb mijn promotie een zeer inspirerende en leerzame tijd gevonden. Alhoewel het niet altijd zo vlot liep als ik gewild had en ondanks mijn (te) idealistische voorstelling van promoveren toen ik nog student was, kan ik tevreden terugkijken op het eindresultaat. Tijdens mijn promotieperiode heb ik van diverse mensen hulp gehad en dit nawoord wil ik dan ook benutten om deze mensen hartelijk te bedanken.

Als eerste een woord van dank aan mijn promotor, Roger Sheldon, die mij gedurende mijn promotie vrij heeft gelaten om zelf mijn onderzoek uit te stippelen. Jouw filosofie om AIO’s (vooral in het eerste jaar) te laten “zwemmen” is erg leerzaam geweest. Met vallen en opstaan heb ik geleerd om zelfstandig onderzoek te doen en ik moet zeggen dat ik de grote vrijheid om zelf mijn lijn te bepalen erg plezierig heb gevonden. Ook Fred van Rantwijk wil ik bedanken. Fred, je bent met name in de periode dat ik als eenling op het peroxidase-onderzoek zat mijn vraag- en klaagbaak geweest. Als ik erg leuke resultaten had of als het juist niet zo wou lukken, kon ik altijd mijn verhaal bij jou kwijt.

Ivar Remkes ben ik dankbaar voor het vele werk dat hij tijdens zijn afstuderen heeft uitgevoerd aan met name de sulfoxidaties van alkyl aryl sulfides, wat voor een groot gedeelte terug te vinden is in hoofdstuk 3. Ivar, ik heb met erg veel plezier met jou samengewerkt (alhoewel ik met schaken mijn meerdere in jou moest erkennen). I would also like to acknowledge the IAESTE-student Sudhasinee Smitra (Ning) for the work she has done on the oxidation of (chiral) alcohols.

Ik vond het prettig toen er een tweede AIO op het peroxidase-onderzoek werd aangenomen en ik denk met veel plezier terug aan de gesprekken en discussies die ik tijdens het laatste jaar van mijn promotie heb gevoerd met Fred van de Velde. Fred, veel succes met de zoektocht naar selectieve oxidaties met peroxidases.

Een speciaal woord van dank wil ik richten aan Karsten Seelbach en Udo Kragl van Forschungszentrum Jülich voor de tijd die ik in Duitsland mocht doorbrengen om met de waterstofperoxide-electrode te experimenteren. Karsten, I’ve learnt a lot during my visit to Jülich (and not only about enzym deactivation!!), and I really enjoyed working with you.

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Barend Groen en Adri Geerlof van Enzymologie ben ik dankbaar voor de hulp bij het kweken en zuiveren van “mijn” enzym. Mijn onkunde op het gebied van onder andere steriel werken en enzymzuivering hebben ze, ondanks wat brandjes, kunnen omzetten in kunde.

Ron Wever van de UvA ben ik erkentelijk voor de gift van vanadium chloorperoxidase. I am grateful to Novo Nordisk for their generous gift of oxidative enzymes.

Verder wil ik het ondersteunend personeel van de TU Delft bedanken: Adri Knol-Kalkman voor de massaspectrometrie, Anton Sinnema, Joop Peters en Anton van Esterik voor de opname van de NMR-spectra, Mieke van der Kooij voor de administratieve ondersteuning, Jan Baas voor de computerberekeningen, Wim Jongeelen voor zijn tekenkunst en niet te vergeten natuurlijk Ernst Wurtz voor alle problemen, wensen en klachten die hij heeft opgelost. Bert van der Hulst en Chris van Drongelen wil ik niet alleen bedanken voor de snelle bestelling van chemicaliën, maar ook voor de onderhoudende uurtjes op de practicumzaal.

Tijdens mijn promotieperiode had ik het geluk om een gezellige groep promovendi als collega’s te treffen. Mijn directe collega’s, en dan met name mijn “hokgenoten” Erwin, Gert, Marian, Anneke en Margeeth, en de “koffietafel” zorgden voor een goede werksfeer, zodat het aangenaam toeven was op het lab.

Mijn ouders wil ik bedanken voor de belangstelling en ondersteuning die ze altijd hebben geboden tijdens mijn lange “studie-loopbaan”. En last but not least, Frank bedankt voor de steun en de relativerende noot.
List of Publications


M.P.J. van Deurzen, F. van Rantwijk and R.A. Sheldon, submitted for publication to *Tetrahedron* (Chapter 2).

M.P.J. van Deurzen, F. van Rantwijk and R.A. Sheldon, submitted for publication to *J. Carbohydr. Chem.* (Chapter 5).


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Curriculum Vitae