Structure, function and operational stability of peroxidases

Promotoren: dr. ir. I.M.C.M. Rietjens
Persoonlijk hoogleraar, Laboratorium voor Biochemie

dr. ir. N.C.M. Laane
Voormalig hoogleraar in de Biochemie

Structure, function and operational stability of peroxidases

Marjon Joanne Helene van Haandel

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Voor mijn ouders
& mijn W's

Stellingen

1. Het feit dat voor ieder type substraat en voor ieder type reactie een nieuwe QSAR moet worden opgesteld bemoeilijkt de toepassing als industrieel voorspellend hulpmiddel.
   (Dit proefschrift, hoofdstuk 5)

2. Sojaboon peroxidase is een goede biokatalysator voor de productie van de geur- en smaakstof methylanthranilaat.
   (Dit proefschrift, hoofdstuk 6)

3. Het probleem van operationele stabiliteit beperkt de commerciële toepassing van heemperoxidasen.
   (Dit proefschrift, hoofdstuk 7)

4. Het gebruik van cyano-horseradish peroxidase (HRP-CN^-) voor de bepaling van de diamagnetische bijdrage aan de longitudinale relaxatietijd, is onjuist, omdat deze HRP-CN^- low spin Fe^{3+} (S=1/2) bevat en dus paramagnetisch is.
   (Sakurada, Takahashi, Hosoya (1986) The Journal of Biological Chemistry, vol. 261, No. 21, pp. 9657-9662)

5. De vorm van het onderwijs heeft een grote invloed op het al of niet tot stand komen van zelfontplooiing.

6. Het is gemakkelijk genoeg om kwaad te worden. Maar kwaad zijn op de juiste persoon, in de juiste mate, op de juiste tijd, om de juiste reden en op de juiste manier, dat is niet makkelijk.
   (Aristoteles, Ethica Nicomachea)

7. Voor goede arbeidsomstandigheden is de instelling van het management belangrijker dan de wettelijke bepalingen.

8. Communicatie kan bijdragen aan structuur, maar evenzeer leiden tot chaos.

9. Iemands uiteindelijke plek in de maatschappij wordt niet bepaald door het IQ, maar door factoren die uiteenlopen van emotionele intelligentie (EQ), sociale klasse tot geluk.

10. Hoe meer kennis je vergaart, des te groter wordt je gevoel zo weinig te weten.

11. Personen die zichzelf wijs vinden zijn meestal eigenwijs.

12. Motivatie katalyseert de omzetting van inzet tot prestatie.

Stellingen behorende bij het proefschrift:

Structure, function and operational stability of peroxidases

Marjon J.H. van Haandel
Wageningen Universiteit, 21 november 2000

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Introduction

1.1 General introduction

In present industrial research the interest for using enzymes for catalytic purposes is increasing. This is related to the fact that enzymes are supposed to offer various economical and environmental advantages as compared to chemical catalysts. One of the main advantages is that enzymes, better than chemical catalysts, can perform stereo- and regioselective reactions. It is well recognised that the biological effect of stereoisomers can be very different. The production of optically pure compounds is, especially for compounds to be used in drugs and food, of importance. Another reason to use biocatalysts instead of chemical catalysts is that consumers prefer naturally and biologically produced compounds, especially with respect to food additives such as, for example, flavours. To explore the differences between bio- and chemocatalysis and the potential advantages of enzymatic processes, biocatalytic projects were included in the Innovation Oriented Programme (IOP) on catalysis.

1.2 Innovation Oriented research Programmes: IOP

The Dutch Ministry of Economic Affairs supports the collaboration between industry, research institutes and universities through "Innovation Oriented research Programmes" (IOPs), providing subsidiary funding to universities and non-profit research institutes for research projects adapted to the needs of the Dutch industry. Promoting innovative research in promising fields in this way is done in order to maintain and to improve the competitive position of the Dutch industries in the world-wide economic scene. The main objective here is "providing an essential step on the road from fundamental science to novel applicable technology" (Oelderik, J.M. (1997) IOP Catalysis factsheets).

Catalysis is of great interest for the Dutch industry since catalytic conversions are involved in many chemical production routes. On the other hand, Dutch universities are well known for their catalysis research. Therefore, one of the IOPs is called "IOP catalysis". The

main goal of "IOP catalysis" is to obtain clean and more efficient technologies, to improve the quality of the Dutch fine chemistry. "IOP catalysis" consists of several clusters including a cluster on "enzymatic oxidation".

1.3 IOP-cluster "enzymatic oxidation"

The research described in this thesis was part of the research within the IOP cluster "enzymatic oxidation". In this cluster the following five projects were embedded:

IKA94002: Peroxidase mediated biotransformations useful in the biocatalytic production of vanillin, carried out by ir. R. ten Have and supervised by dr. J.A. Field and prof.dr. J.A.M. de Bont.

IKA94013: Application of redox enzymes in the synthesis of fine chemicals, carried out by ir. F. van de Velde and supervised by dr.ir. F. van Rantwijk and prof.dr. R.A. Sheldon.

IKA94045: Microperoxidases as biocatalysts in regioselective oxygen-transfer reactions with clean oxidants, carried out by ir. M.J.H. van Haandel and supervised by prof.dr. I.M.C.M. Rietjens and prof.dr. N.C.M. Laane.

IKA94047: Peroxidases as natural catalysts in the production of (enantiomerically pure) alcohols and epoxides, carried out by drs. A. Tuynman and supervised by dr. R. Wever and prof.dr. H.E. Schoemaker.

IKA94052: Application of vanadium peroxidases as novel biocatalysts, carried out by drs. H.B. ten Brink and supervised by dr. R. Wever and prof.dr. H.E. Schoemaker.

In this way researchers of different, but complementary disciplines (i.e. enzymology, biochemistry, organic chemistry, microbiology and molecular biology), were brought together. The common objective of these projects was the development of biocatalytic processes for the production of fine chemicals via oxidation of industrially accessible starting material. IKA94002 and IKA94045 focused on the development of peroxidases as natural catalysts for the production of flavours and fragrances, whereas IKA94013, IKA94047 and IKA94052 focused on the development of biocatalytic processes for (enantio)selective oxygen transfer reactions.

1.4 Objective and justification of this thesis

The objective of the IOP project IKA94045 and of this thesis, has been presented at several IOP-meetings as "the definition of an optimised (micro)peroxidase system to mimic cytochrome P450 like oxygen transfer reactions using hydrogen peroxide as a clean oxidant, for the catalysis of industrially relevant conversions". Within the IOP cluster this project aimed at providing insight into the mechanistic aspects of these reactions as well as into the possibilities for the application of peroxidases as natural biocatalysts for synthesis of flavours. (Micro)peroxidases were chosen as cytochrome P450 mimics because they are able to operate under mild, controlled conditions using cheap and clean oxidants (hydrogen peroxide). A description of (micro)peroxidases of interest for this thesis is given in chapter 2.

In this thesis especially the natural production of food flavours was of interest. After initial screening of reactions of potential interest, the N-dealkylation of methyl-*N*-methylanthranilate (MNMA) to methylanthranilate (MA), a concord grape flavour, was chosen as the model reaction (Figure 1.1). This model reaction can be justified as follows: MNMA from citrus leaves is readily available and a relatively cheap source whereas MA is more expensive than MNMA. Therefore, the investigated reaction (*i.e.* selective N-demethylation and not O-demethylation) provides an industrially relevant route for the natural production of an important topnote flavour in concord grape.

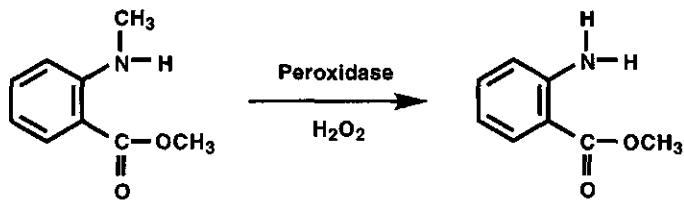


Figure 1.1 The model reaction of this thesis: N-dealkylation of methyl-N-methylanthranilate to methylanthranilate, a concord grape flavour.

1.5 Outline of this thesis

In order to obtain better insight in the industrial applications of peroxidases it is important to understand the relationship between structure, function and operational stability of these versatile catalysts. On the basis of this knowledge the behaviour and functional

characteristics of peroxidases can be understood and even changed, in order to optimise their potential application in industrially relevant conversions. Therefore, this thesis starts with fundamental studies on peroxidases, as described in chapters 3, 4 and 5, using horseradish peroxidase (HRP) as the model peroxidase, since this enzyme is the best studied enzyme of all peroxidases.

These investigations include a study on the reaction mechanism of peroxidases resulting in the revaluation and extension of the kinetic model for horseradish peroxidase as described in Chapter 3.

In additional studies this kinetic model could be used to define quantitative structure-activity relationships (QSARs) for the conversion of a series of model compounds by peroxidases (Chapter 4 and 5). Chapter 4 describes QSARs for the rate limiting oxidation of phenol derivatives by HRP compound II. And chapter 5 describes QSARs for the overall conversion of phenol and aniline derivatives by HRP. Based on these QSARs not only fundamental information on structure and function of enzymes can be obtained, but they also provide an interesting tool for industrial application. The latter because QSARs may predict the outcomes of enzyme catalysed substrate conversions on the basis of calculated chemical parameters of the substrates, thereby saving time and money.

Whereas chapters 3, 4 and 5 describe fundamental research, chapter 6 focuses on a possible industrial application of peroxidases. In this chapter, the catalytic potency of different peroxidases is investigated for the industrially relevant N-demethylation of methyl-*N*-methylanthranilate (MNMA) to produce the food flavour methylanthranilate (MA). Improvement of stability and turnover was an important issue, because poor operational stability limits the large scale use of peroxidase catalysis.

Finally, the summary and conclusions are presented in chapter 7.

2

Structure, function and operational stability of peroxidases

A theoretical background

2.1 Peroxidases: classification, sources & biological function

The family of peroxidases is large and well studied and consists of mammalian and non-mammalian peroxidases. In table 2.1 the different classes of peroxidases are listed.

Examples of mammalian peroxidases are thyroid peroxidase, eosinophil peroxidase, lactoperoxidase, myeloperoxidase and glutathione peroxidase. They contain either heme or selenium as a cofactor (Dunford and Stillman, 1976). Mammalian peroxidases have a function in various tissues. Mammalian thyroid peroxidase, for example, is involved in the production of the hormone thyroxin (Poulos & Fenna, 1994).

Table 2.1 Classification of peroxidases.

Type	Class	Source	Cofactor	Reference
Mammalian		Mammalian	Heme or Selenium	Flohé, 1989; Zeng & Fenna, 1992
Non-mammalian	I	Bacteria	Heme or Flavin	Patterson & Poulos, 1995
	II	Fungi	Heme or Manganese	Sundaramoorthy et al., 1994/1996
	III	Plant	Heme or Vanadium	Hendriksen et al., 1998
				Messerschmidt et al., 1997

The non-mammalian peroxidases are divided into three classes. Class I peroxidases are derived from bacteria and contain no sulphur bridges or structural Ca^{2+}. They are not glycosylated, and do not contain endoplasmatic reticulum signal sequences (Welinder, 1992). They have either heme or flavin as a cofactor (Patterson & Poulos, 1995). Class II peroxidases are derived from fungi and contain heme and/or manganese as a cofactor

(Poulos et al., 1993; Sundaramoorthy et al., 1994/1996). Their substrates vary extensively. Many of the class II peroxidases have been proven to have good potential for the degradation of aromatic pollutants. Class III peroxidases are the "classical" plant peroxidases. Either heme or vanadium is the cofactor in these peroxidases. This thesis focuses on class III peroxidases containing a heme. In each plant many peroxidases occur. Class III peroxidases are heavily glycosylated and contain conserved structural calcium ion-sites and disulphide bridges. Classical plant peroxidases have a broad substrate specificity and are thought to play important roles in plant metabolism. For example, they are involved in the neutralisation of hydrogen peroxide (a product of oxygen metabolism) by reducing hydrogen peroxide to water and in the oxidation of a variety of substrates. Furthermore, they are assumed to be the key catalysts in catabolism of a number of plant hormones, and in plant cell wall biosynthesis, including lignin formation (Gross, 1977; Edwards et al., 1993).

2.2 Structure

An important aspect of peroxidase catalysis is the mode of binding of the substrate to the active site. Therefore, the understanding of the structure of peroxidases, especially the structure of the active site, is of great importance. For example, the crystal structure of HRP, recently resolved at 2.15 Å resolution (Gajhede et al., 1997), has enabled the prediction of the enzyme region that binds aromatic substrates. Much research has been done to understand how enzyme structure influences enzyme function. The final catalytic activity of a metal-containing enzyme, including the heme containing peroxidases, is reported to be determined by: 1) the structure of the metal complex, including the geometry of the complex, the nature of the ligands attached to the metal, and the valency of the metal, and 2) the environment of the metal complex, including the polarity of the immediate surroundings and steric constraints on the accessibility of substrates to the metal and of the metal to the solvent. Especially, the identity of the axial ligand, the metal valency, the nature of the heme environment, and the steric accessibility of the heme iron and heme edge play major roles in determining the reactivity of heme-based enzymes, like the peroxidases (Dawson, 1988). In the following paragraphs these aspects are discussed in more detail.

2.2.1 Prosthetic group of heme peroxidases

In classical heme peroxidases the prosthetic group is ferric protoporphyrin IX (Figure 2.1). The redox potential of the heme protein is largely determined by the ligands which coordinate the heme iron and by the surrounding peptide chain. In addition to the binding to four nitrogen atoms in the centre of the protoporphyrin ring, the iron atom in the heme can also form two axially coordinated bonds on either side of the heme plane. On the proximal side of the heme moiety the iron atom can form a bond to an amino acid residue from the peptide chain and on the distal side of the heme the iron atom can form a bond to an external ligand(s) (see Figure 2.1b). These binding residues are referred to as the fifth and the sixth ligand respectively.

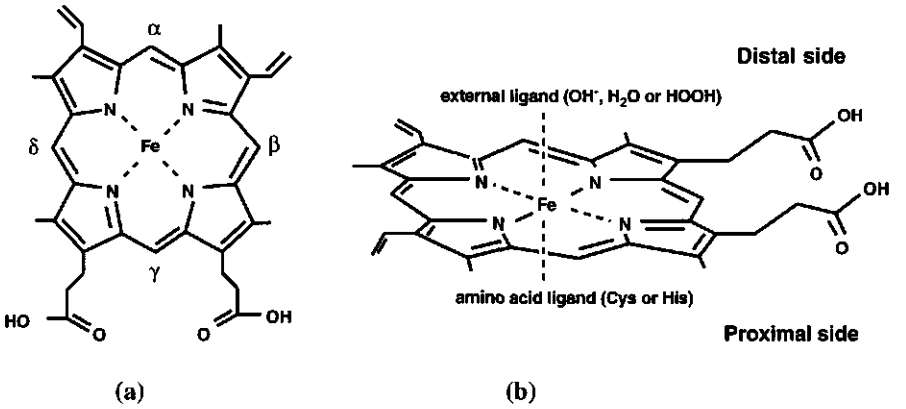


Figure 2.1 Ferric protoporphyrin IX, the prosthetic group of classical heme peroxidases and its axial ligands (b). α, β, γ and δ indicate the *meso* positions (a).

2.2.2 Role of the axial ligands

For peroxidases the distal side of the heme moiety is basically vacant, because the heme-iron is essentially five-coordinated (Smulevich, 1995), which allows the heme iron to be free to bind to peroxides and other external ligands as a sixth ligand.

The fifth ligand is a strong axial ligand on the proximal position. In most peroxidases the fifth ligand is a histidine residue provided by the peptide chain (Dunford & Stillman, 1976; Hewson & Hager, 1979; Poulos & Kraut, 1980; Marnett et al., 1986). An exception is chloroperoxidase containing a cysteinate as the fifth ligand (Sundaramoorthy et al., 1995). Catalase contains a tyrosine as the fifth ligand (Fita & Rossmann, 1985). Cytochromes P450 also contain a cysteinate residue as the fifth ligand (Guengerich, 1993). Cytochromes P450

constitute a family of enzymes that play a key role in the oxidative and peroxidative biotransformation of a large variety of xenobiotic chemicals.

The fifth ligand plays a role in different steps of the reaction cycle, but predominantly in the formation of compound I (see also section 2.2.3). Compound I formation involves cleavage of the O-O bond of the iron bound peroxide moiety. For thiolate ligated enzymes it has been proposed that the thiolate ligand serves as a strong electron donor to facilitate the O-O cleavage. In cysteinate containing cytochromes P450, for example, both the proximal and the distal sides of the heme are non-polar and lack groups capable of hydrogen bonding either to the proximal base (the thiolate) or to the bound peroxide (two electron reduced molecular oxygen). The 'push' of the axial thiolate ligand in cytochrome P450 is sufficient to cleave the O-O bond without need for a 'pull' from charged residues on the distal side (Figure 2.2) (Dawson, 1988; Sono et al., 1996). The electron density distribution required for the 'electron-push' in histidine ligated enzymes is provided by the fact that the proximal histidine is strongly hydrogen bonded to neighbouring groups. At the same time, the distal histidine serves as a proton acceptor/donor and works together with a charged residue to make the distal side of the dioxygen moiety more polar and to 'pull' apart the O-O bond of the bound peroxide by stabilising the separating charge (Dawson, 1988; Choudhury et al., 1994).

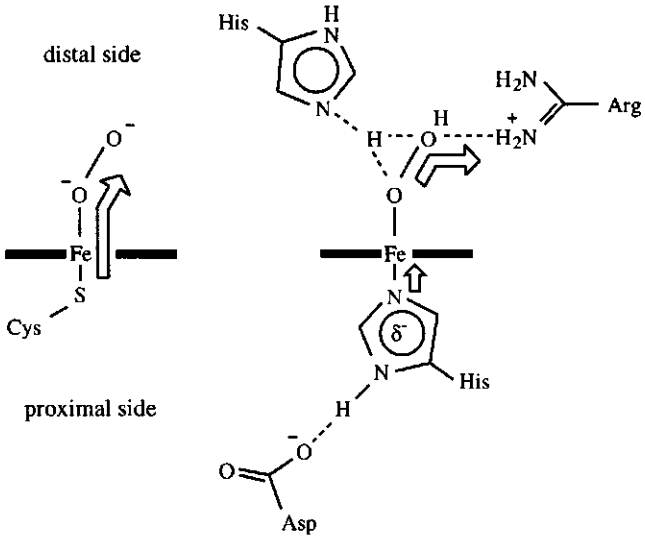


Figure 2.2 Schematic view of the O-O bond cleavage of an iron bound peroxide in thiolate-ligated (left) and histidine ligated (right) systems, such as cytochromes P450 and horseradish peroxidase, respectively (adapted from: Sono et al., 1996)

The fifth ligand is believed to account for the different reaction pathways described for the different families of heme enzymes (Du & Loew, 1992). However, results of Osman et al. (1996) imply that the type of ligand is not decisive for the type of reaction chemistry that can occur, but may only influence the ratio between the possible different reaction pathways. This is in agreement with conclusions from other studies which suggest that a physical barrier between the substrate and the oxygen of the high-valent iron-oxo species of horseradish peroxidase (HRP) is responsible for the fact that HRP, and other classical peroxidases, differ from the monooxygenases in that they do not incorporate the oxygen of their high-valent iron-oxo intermediate into the product (see section 2.2.4) (Ortiz de Montellano et al., 1987; Ortiz de Montellano, 1992; Ozaki, 1995).

2.2.3 Nature of the heme environment

Once compound I has been formed, the heme environment plays a crucial role in controlling enzyme reactivity. The heme environment influences the accessibility of the heme centre. The heme environment of peroxidases is charged and polar, whereas the heme environment of cytochromes P450 is non-polar.

Several mutagenesis and crystallisation studies have provided insight in the mode of catalysis and the structure-function relationships of the important active site residues. Table 2.2 presents several reported crystal structures of peroxidases. Although amino acid homologies are low, those studies are of important value for other studies on peroxidases. This is because, beside mechanistic similarities and similarities in the overall three-dimensional structures of the three classes of peroxidases, also the positioning and identity of the important active site residues show high similarity (Table 2.3). The access channel to the δ-*meso* heme-edge (Figure 2.1) and to the distal side of the heme appears to be the most variable part of the known peroxidase structures (Hasemann et al., 1995; Gajhede et al., 1997).

Table 2.3 shows that the distal arginine and histidine are highly conserved, as well as the proximal histidine hydrogen-bonded with a buried asparagine side chain, which are structurally and functionally important (Welinder, 1992; Welinder & Gajhede, 1993). In addition, the crystal structures of cytochrome *c* peroxidase (CcP) (Finzel et al., 1984), lignine peroxidase (LiP) (Poulos et al., 1993), ARP (Kunishima et al., 1994), *Coprinus cinereus* peroxidase (CiP) (Petersen et al., 1994) and HRP (Gajhede et al., 1997) show that an extended H-bond network connects the proximal to the distal side of the heme cavity through the distal water molecules, the distal arginine, the proprionate groups of the heme, and the proximal histidine hydrogen bonded to the oxygen of an aspartic residue.

Table 2.2 Several reported crystal structures of peroxidases. Most structures can be found using their pdb codes on http://www.pdb.rcsb.org.

Peroxidase	pdb code	Resolution	Source	Reference
Mammalian peroxidases:				
GPX	1gp1	2.00 Å	*Bovine erythrocyte*	Epp. et al., 1983
MPX	1myp	3.00 Å	*Dog (Canis familiaris)*	Zeng & Fenna, 1992
MPX	1mhl	2.25 Å	*Homo sapiens*	Fenna et al., 1995
Non-mammalian peroxidases:				
Class I				
CcP	2cyp	1.70 Å	*Saccharomyces cerevisiae*	Finzel et al., 1984
CcP	1cca	1.80 Å	*Saccharomyces cerevisiae*	Goodin & McRee, 1993
APX	1apx	2.20 Å	*Pisum sativum*	Patterson & Poulos, 1995
Class II				
BPO	1bro	2.05 Å	*Streptomyces aureofaciens*	Hecht et al., 1994
LiP	1lga	2.03 Å	*Phanerochaete chrysosp.*	Poulos et al., 1993
LiP (Pi=4.15)	1llp	1.70 Å	*Phanerochaete chrysosp.*	Choinowski et al., 1999
LiP (Pi=4.65)	1qpa	1.80 Å	*Phanerochaete chrysosp.*	Choinowski et al., 1999
MnP	1mnp	2.06 Å	*Phanerochaete chrysosp.*	Sundaramoorthy et al., 1994
ARP	1gza	2.06 Å	*Arthromyces ramosus*	Fukuyama et al., 1997
CiP	-	2.60 Å	*Coprinus cinereus*	Petersen et al., 1994
CPO	2cpo	2.16 Å	*Caldariomyces fumago*	Sundaramoorthy et al., 1995
CPO	1a88	1.90 Å	*Streptomyces aureofaciens*	Hofmann et al., 1998
Class III				
BPX	1bgp	1.90 Å	*Hordeum vulgare*	Hendriksen et al., 1998
PNP	1sch	2.70 Å	*Arachis hypogaea*	Schuller et al., 1996
HRP	1atj	2.15 Å	*Armoracia rusticana*	Gajhede et al., 1997
HRP	2atj	2.00 Å	*Armoracia rusticana*	Hendriksen et al., 1998
HRP	6atj	2.00 Å	*Armoracia rusticana*	Hendriksen et al., 1999

Abbreviations: GPX = glutathione peroxidase; MPX = myeloperoxidase; CcP = cytochrome *c* peroxidase; APX = ascorbate peroxidase; CAT = catalase; BPO = bromoperoxidase; LiP = lignine peroxidase; MnP = manganese peroxidase; ARP = peroxidase from *Arthromyces ramosus*; CiP = *Coprinus cinereus* peroxidase; CPO = chloroperoxidase; BPX = barley grain peroxidase; PNP = peanut peroxidase; HRP = horseradish peroxidase.

Table 2.3 Essential active site residues for peroxidases of which the structure is known.

Class I		Class II				Class III	
CcP	APX	LiP	MnP	ARP	CiP	PNP	HRP
Arg-48	Arg-38	Arg-43	Arg-42	Arg-52	Arg-51	Arg-38	Arg-38
Trp-51	Trp-41	Phe-46	Phe-45	Phe-55	Phe-54	Phe-41	Phe-41
His-52	His-42	His-47	His-46	His-56	His-55	His-42	His-42
His-175	His-163	His-176	His-173	His-184	His-183	His-169	His-170
Trp-191	Trp-179	Phe-193	Phe-190	Leu-201	Leu-200	Phe-213	Phe-221
Asp-235	Asp-208	Asp-238	Asp-242	Asp-246	Asp-245	Asp-239	Asp-247

In the following, the heme environment is discussed with special emphasis on the reaction mechanism and on the essential active site residues. Starting in the resting state, there is a network of hydrogen bonds on the distal side of the heme.

The distal Phe residue in class II and III peroxidases corresponds to a Trp-51 in CcP, and is positioned directly above the heme ring adjacent to the distal catalytic histidine. Trp-51 is thought to maintain this hydrogen bonding network and to prevent binding of water molecules and/or of His-52 to the heme-iron (Smulevich et al., 1988). In addition, Newmeyer et al. (1995) suggested, based on mutation studies with HRP, that Phe-41 and His-42 shield the iron centred catalytic species, and that His-42 plays a key catalytic role in formation of compound I (see section 2.2.2, Figure 2.2).

The distal histidine (Figure 2.3) is thought to facilitate formation of the initial iron-peroxide complex by deprotonating the peroxide when the peroxide enters the heme cavity (Figure 2.2 and 2.3). Histidine acts as a general base catalyst which, by accepting a proton from H_2O_2, allows the peroxide to bind the heme-iron. A hydrogen bond between the His imidazole ring and Asn maintains the basidity of the histidine enabling the histidine to abstract the proton from the peroxide (Nagano, 1996), and keeps the histidine correctly oriented (Figure 2.3). Subsequently, the cleavage of the oxygen-oxygen bond is promoted by protonating the distal oxygen (Poulos & Kraut, 1980; Erman et al., 1993). Welinder et al. (1994) suggested that the conserved hydrogen bonding networks between the distal His-Asn as well as the proximal His-Asp residues serve to delocalise charges during peroxidase reactions, thereby reducing the free energy and stabilising both resting state and intermediates.

Thus, after binding of the peroxide to the heme-iron, the O-O bond is heterolytically cleaved, producing water and Compound I. The following residues are also thought to be important during cleaving of this O-O bond.

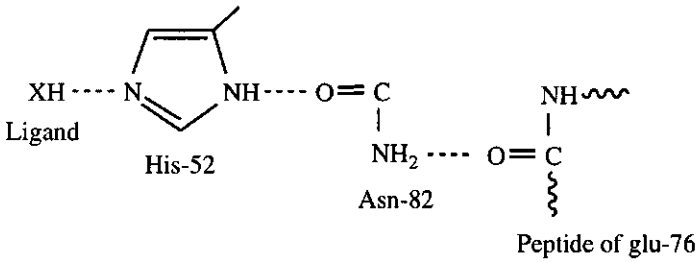


Figure 2.3 The hydrogen bonding network in CcP to His-52, enabling His-52 to deprotonate H_2O_2.

The distal arginine probably stabilises the ferryl centre of compound I during the catalytic cycle (Poulos & Fenna, 1994; Smulevich, 1995), and is thought to facilitate dioxygen bond cleavage (Ortiz de Montellano et al., 1995). The interaction between the guanidinium group of arginine and the peroxide oxygen destabilises the peroxide O-O bond, and stabilises the transition state of the heterolytic peroxide O-O bond cleavage (Rodriguez-Lopez et al.; 1996) (see section 2.2.2, Figure 2.2).

The distal histidine delivers its proton to the newly formed hydroxide anion after cleavage of the peroxide O-O bond, upon which H_2O leaves the heme cavity (Poulos & Krout, 1980). The distal histidine is also proposed to modulate the reactivity of compound I (Rodriguez-Lopez et al., 1996).

The next key catalytic residue is the Asp on the proximal side of the heme. This Asp residue is one of the neighbouring groups (as mentioned in 2.2.2), which strongly hydrogen bonds to the proximal histidine (fifth ligand). In this way the basicity of the proximal histidine is increased, helping to stabilise high oxidation state intermediates by a charge relay system, while helping to maintain the heme in a five-coordinate state.

Now compound I is formed, small substrates can be oxidised at the δ-*meso* edge of the heme moiety (Figure 2.1), while prevented from reacting with the ferryl oxygen, as proposed by Ator (1987) and Poulos (1995) (see section 2.2.4).

The conserved calcium ions in peroxidases may serve structural roles. Hasemann et al. (1995) suggested that they are involved in stabilising or destabilising the diverse oxidation states of peroxidases by exerting a kind of electrostatic control over the heme cavity. Smith et al. (1990) reported that calcium ions are essential for peroxidase activity of HRP. But the exact roles of the conserved calcium ions are still a matter of debate.

As mentioned before, the mode of binding of the substrate to the active site also determines peroxidase catalysis. It is unclear whether distal heme-residues participate in binding of aromatic electron donors. His-42 of HRP has been proposed to be involved in the binding of aromatic substrates (Veitch, 1995). However, results of Bhattacharyya et al. (1993) did not support this observation of Veitch (1995). Yet, Rodriguez-Lopez et al. (1996) reported that Arg-38 in HRP is involved in cleavage of the O-O bond of the peroxide, but also in modulating the apparent affinity of HRP for reducing substrates such as guaiacol and *p*-cresol. Arginine often serves as a cationic site for the binding of a negatively charged group in a substrate or cofactor (Riordan et al., 1977). Evidence for Arg-38 being directly involved in the binding of aromatic substrates is presented by Rodriguez-Lopez et al. (1996). They gave the following explanation: the role of arginine in substrate binding is a direct electrostatic interaction between the positively charged guanidinium group of arginine and the partial negative charge developed on the oxygen of the phenolic group of substrates such as *p*-cresol and guaiacol, directing the orientation of the substrate in the active site prior to electron transfer. In addition, Rodriguez-Lopez et al. (1996) suggest that interaction between the guanidinium group of arginine and the ferryl oxygen of HRP compound I would be expected to withdraw electron density from the porphyrin radical cation and, thus facilitate the electron transfer from the reducing substrate by increasing the redox potential.

2.2.4 (Steric) Accessibility of the heme centre

Peroxidases are thought to preferentially catalyse one electron substrate oxidations, maybe due to their axial histidine ligand and, especially, the supposed inaccessibility of the oxygen atom in their high-valent-iron-oxo porphyrin form (Ortiz de Montellano, 1992; Ozaki & Ortiz de Montellano, 1995). It is proposed that HRP, and possibly other classical peroxidases, differ from the monooxygenases in that substrates are prevented from reacting with the ferryl oxygen and are only allowed to react with the edge of the prosthetic heme group (Ator, 1987; Poulos, 1995). In HRP this is achieved by imposing a physical barrier, i.e. the protein environment in which the heme cofactor is almost totally embedded, preventing direct interaction between the substrate and the oxygen of the ferryl species. Critical peripheral Phe residues guard the substrate access channel, the access to the reactive heme edge, forming a distinctive hydrophobic path near the exposed heme edge (Gajhede et al., 1997).

In contrast, cytochrome P450 enzymes, which have a cysteinate as the fifth ligand, generally transfer oxygen from their reactive high-valent iron-oxo intermediate directly to their substrates (Ortiz de Montellano, 1986; Poulos, 1985; Black, 1986; Collman, 1975).

This is possible because of a tunnel type of tertiary structure of the peptide chain through which the oxygen can be transferred from the heme-iron to the substrate (Kunze et al., 1983). Thus, cytochromes P450 have a buried heme-edge, but an accessible heme iron, allowing oxygen transfer. Figure 2.4 presents a schematic view of the difference in substrate access to the heme compound I in peroxidases and cytochromes P450, respectively.

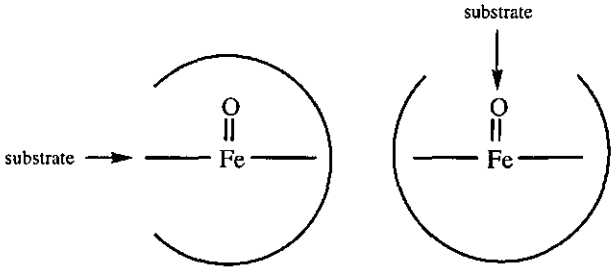


Figure 2.4 A schematic view of the difference in substrate access to the heme compound I in peroxidases (left) and cytochromes P450 (right) (adapted from: Poulos, 1995).

2.3 Reaction cycle

The understanding of catalytic mechanisms and the structure-function relationships of peroxidases is largely based on work with HRP (Dawson, 1988; Dunford, 1991; Ortiz de Montellano, 1992). The characteristic activity of HRP and other classical heme based peroxidases, being the H_2O_2-dependent one-electron oxidation of organic and inorganic substrates (Marnett et al., 1986; Hewson & Hager, 1979; Dunford & Stillman, 1976), proceeds through the mechanism depicted in Figure 2.5 (route a-c-d). The catalytic cycle involves two high-valent intermediate forms of the enzyme, compounds I and II. These active species are able to abstract one electron from a substrate (one electron substrate oxidation) to produce a free substrate radical which undergoes coupling, disproportionation, and/or reaction with molecular oxygen (Yamazaki, 1977).

Compounds I and II of HRP, which have been studied extensively, are spectroscopically and kinetically distinct intermediates (Job & Dunford, 1976). The intermediates have characteristic, intense absorption spectra, which allow the individual reaction steps to be monitored by stopped-flow with spectrophotometric detection (Candeias et al., 1997). The electronic structures of these compounds have been well characterised by various physical methods. Many literature data indicate that both compounds I and II have

high-valent iron-oxo [Fe^{IV}=O] structures and that compound I contains a π cation radical (Adeniran & Dunford, 1983; Chance et al., 1986; Edwards et al., 1987).

Compound I is formed by two-electron oxidation of the enzyme by hydrogen peroxide (= two oxidation equivalents above the Fe(III) state) (Figure 2.5: a). This formation of compound I is reported to occur through formation of an intermediate called compound 0 (Jones & Suggett, 1968a/b; Adams, 1990; Rodriguez-Lopez et al., 1996; Loew & Depuis, 1996; Harris & Loew, 1996). The two electrons required for peroxide reduction originate from Fe(III) and from the porphyrin, producing a high-valent-iron-oxo porphyrin radical cation (Ortiz de Montellano, 1992). Cytochrome *c* peroxidase (CcP) is an exception producing a protein radical instead of a porphyrin radical cation (Bosshard et al., 1991). Compound I then reverts to the ferric resting state via two successive one-electron reactions with reducing substrate molecules. The first one-electron oxidation (Figure 2.5: c) yields a second enzyme intermediate, compound II (Dunford and Stillman, 1976), in which the Fe^{IV}=O species remains intact and the porphyrin is reduced (Schultz et al., 1984; Penner-Hahn et al., 1986). Reduction of compound II (Figure 2.5: d) to native enzyme is often rate limiting in the peroxidase catalytic cycle (Chance, 1951; Dunford & Stillman, 1976; Job & Dunford, 1976; Yamazaki et al., 1981; Dunford & Adeniran, 1986), caused by the fact that compound I behaves as a stronger oxidant than compound II (Yamazaki et al., 1981). However, under certain conditions (limiting concentrations of H_2O_2 and a large excess of reducing substrate), it is possible to make compound I formation the rate-controlling step (Dunford & Stillman, 1976). Kinetics of HRP-catalysed reactions have been excessively discussed (Jones & sugget, 1968a/b; Critchlow & Dunford, 1972; Adams, 1990; Dunford, 1991; Rodriguez-Lopez et al., 1996).

Under certain conditions, compound I is considered to be reduced to the ferric form without producing compound II: 1) via decomposition of hydrogen peroxide to water and oxygen (Figure 2.5: b), called dismutase or catalase activity (Thomas et al., 1970; Nakajima & Yamazaki, 1987; Arnao et al., 1990; Griffin, 1991), or 2) via a so-called mono-oxygenation reaction (Figure 2.5: route h) in which the oxygen of the high-valent iron-oxo intermediate is transferred to the substrate (Dawson, 1988). The actual nature of the reaction catalysed varies, depending on the substrate (Ohtaki et al., 1982; Nakamura et al., 1985), and for example the presence of ascorbic acid.

Peroxidases reacting in large excess of H_2O_2 give compound III (Figure 2.5: g), an inactive form of the enzyme (Arnao et al., 1990).

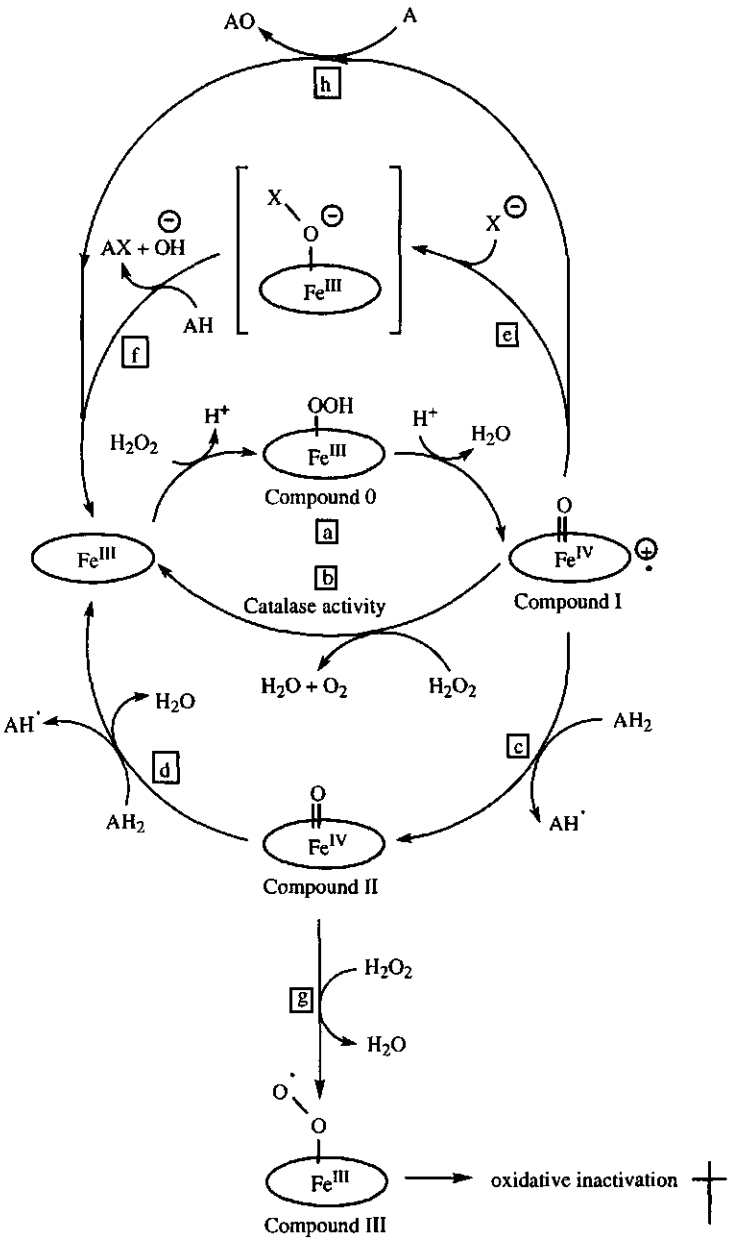


Figure 2.5 Catalytic cycle of peroxidases (adapted from Colonna et al., 1999). AH_2 represents the substrate, such as guaiacol (peroxidative pathway) or monochlorodimedone (halogenation pathway), and the oval represents the heme. Compounds I and II represent the ferryl intermediates. X represents Cl, Br or I involved in the halogenation pathway.

2.3.1 Modification of peroxidase catalysed reaction chemistry by ascorbate

The scavenging characteristic of ascorbic acid (vitamine C) can be used for efficiently blocking all peroxidase type of reactions (Potter et al., 1984; Subrahmanyam & O'Brien, 1985; Osman et al.; 1996), whereas cytochrome P450 type of oxygen transfer reactions are not blocked (Osman et al.; 1996). This difference is caused by a different mechanism through which both type of enzymes react. A peroxidase type of aromatic hydroxylation, for example, is initiated by initial one-electron oxidation of the substrate by compound I followed by oxygen incorporation through a reaction of the substrate radical with O_2 or H_2O (Nakamura et al., 1992). On the other hand, the mechanism of cytochrome P450 catalysed aromatic hydroxylation proceeds by an initial attack of the high-valent-iron-oxo intermediate of the enzyme on the π-electrons of the aromatic ring of the substrate leading to product formation by σ-O-addition or by an initial $H^{\bullet}$ abstraction by the high-valent-iron-oxo intermediate followed by oxygen rebound leading to oxygen incorporation into the substrate (Guengerich & MacDonald, 1990). Ascorbic acid may inhibit peroxidase-type of reactions by chemically reducing the initial substrate radical formed by initial one-electron oxidation, to the parent substrate (Potter et al., 1984; Subrahmanyam & O'Brien, 1985), or by reducing compound I or compound II back to the ferric form of peroxidase. If such a reduction of compound I and II is the reason underlying the inhibition of catalysis, remaining reactions may be catalysed through different type of reactive heme intermediates such as for example the porphyrin-OOH adduct (Compound 0) formed prior to compound I formation (Primus et al., 1999). Ascorbic acid is interesting from an industrial point of view for reactions with peroxidases which are able to react through mechanisms other than peroxidase-type of mechanisms, because formation of side-products resulting from reactions by radical type of peroxidase activity can be eliminated using ascorbic acid (Boersma et al., submitted). Microperoxidase 8 (MP8) is an example of such a peroxidase (Osman et al., 1996), being able to react through mechanisms other than peroxidase-type of mechanisms, i.e. cytochrome P450 type of oxygen transfer reactions, still observed in the presence of ascorbate.

2.4 Reactions

Heme peroxidases can catalyse many types of reactions, allowing possible use in the manufacture of fine chemicals, such as natural flavours, drugs and agrochemicals. Typical examples of heme peroxidase-catalysed reactions are shown in Table 2.4.

Generally, peroxidase-catalysed reactions can be grouped into four categories:

1) Oxidative dehydrogenation: (Figure 2.5: a-c-d)
   $2AH + H_2O_2 \rightarrow 2A\cdot + 2H_2O$
2) H_2O_2 dismutation: (Figure 2.5: b)
   $2H_2O_2 \rightarrow 2H_2O + O_2$
3) Oxidative halogenation: (Figure 2.5: a-e-f)
   $AH + H_2O_2 + H^{(+)} + X^{(-)} \rightarrow AX + 2H_2O$ [X=Cl,Br,I]
4) Oxygen-transfer reaction: (Figure 2.5: a-h)
   $A + H_2O_2 \rightarrow AO + H_2O$

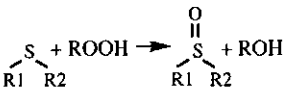
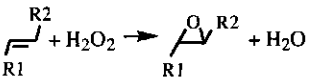
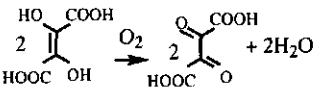
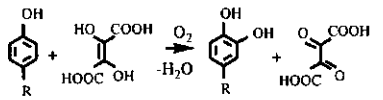
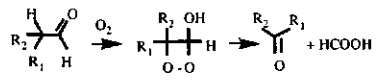
Oxidative dehydrogenation

Oxidative dehydrogenation is referred to as the classical peroxidase reaction. HRP is able to produce a great variety of useful compounds and is therefore an example of a peroxidase with a broad substrate specificity for oxidative dehydrogenation. In a typical peroxidase reaction, electron-rich organic substrates such as phenols, aromatic amines (Whitaker, 1972), as well as NADPH and NADH (Yokota & Yamazaki, 1977), are oxidised by compound I or II through one-electron transfer to form radical products (Figure 2.5: a-c-d). These radicals may give rise to a variety of products due to subsequent dimerisation, dismutation, water and/or oxygen addition reactions. In the peroxidase catalysed oxidation of phenols, phenolic radicals are produced, which rapidly dimerize in a non-enzymatic step. On prolonging the reaction times, a complex mixture of oligomeric oxidative products results (Colonna et al., 1999). The peroxidase-catalysed polymerisation of anilines also occurs under mild conditions and produces polymeric amines (Zemel & Quinn, 1993). Peroxidase type catalysis of the N-dealkylation of alkylamines and hydroxylation of a benzylic methyl group has been described by Kedderish et al. (1986) and Ortiz de Montellano (1987).

H_2O_2 dismutation

Peroxidases, in particular catalase (CAT), can decompose hydroperoxides and peracids in the absence of an electron donor (Schonbaum & Chance, 1976)(Figure 2.5: b). Chloroperoxidase (CPO) and to a lesser extent HRP can catalyse the decomposition of hydrogen peroxide to water and oxygen (Thomas et al., 1970; Nakajima & Yamazaki, 1987; Arnao et al., 1990; Griffin, 1991).

Table 2.4 Typical examples of heme peroxidase-catalysed reactions (AH or A represents the substrate; R represents rest groups; X represents Cl, Br or I) (from Adam et al., 1999).

Type of reaction		Typical substrates	Peroxidase	Ref.
electron transfer	$2AH + ROOH \rightarrow A\text{-}A + ROH + H_2O$	H_2O_2, ROOH, phenols, aromatic amines	HRP, LiP	1,2
disproportionation	$2H_2O_2 \rightarrow 2H_2O + O_2$	H_2O_2	CAT, HRP, CPO	3-7
halogenation	$AH + HX + H_2O_2 \rightarrow AX + 2H_2O$	tyrosine, H_2O_2	CPO, LPO	7-11
sulfoxidation		thioanisole, ROOH, H_2O_2	CPO, HRP LPO, CcP, MP	7, 10-17
epoxidation		alkenes, H_2O_2	CPO, HRP CcP	16, 18-19
demethylation	$ROOH + R_1R_2NCH_3 \rightarrow ROH + R_1R_2NH + HCHO$	MNMA, ROOH	CPO, HRP LPO	18-24
dehydrogenation		dihydroxyfumaric acid	HRP	25
hydroxylation		L-tyrosine, adrenaline	HRP	26
α-oxidation		aldehydes	HRP	27

Abbreviations: Ref.= references, HRP = horseradish peroxidase, LiP = lignin peroxidase, CPO = chloroperoxidase, LPO = lactoperoxidase, CAT = catalase, MP = microperoxidase, CcP = cytochrome *c* peroxidase.
References: 1) Whitaker, 1972; 2) Yokota & Yamazaki, 1977; 3) Schonbaum & Chance, 1976; 4) Thomas et al., 1970; 5) Nakajima & Yamazaki, 1987; 6) Arnao et al., 1990; 7) Griffin, 1991; 8) Neidleman & Geigert, 1986; 9) Colonna et al., 1999; 10) Dawson, 1988; 11) Ortiz de Montellano, 1992; 12) Colonna et al., 12a= 1992, 12b= 1994, 12c=1995, 12d=1997; 13) Kobayashi et al., 1987; 14)Harris et al., 1993; 15) Gajhede et al., 1997; 16) Miller et al., 1992; 17) Osman et al., 1996; 18) Ortiz de Montellano, 1987; 19) Allain et al., 1993; 20) Kedderis et al., 1980; 21) Griffin et al., 1978; 22) Kedderis & Hollenberg, 1983; 23) Hollenberg et al., 1985; 24) Miwa et al., 1983; 25) Halliwell & Ryker, 1978; 26) Adam et al., 1999; 27) Campa, 1984.

Oxidative halogenation

Several heme-containing peroxidases (CPO and myeloperoxidase) use hydrogen peroxide to oxidise halide ions (Cl^-, Br^-, I^-, F^-) to reactive halogenating species (Neidleman & Geigert, 1986)(Figure 2.5: a-e-f). CPO, for example, can catalyse the conversion of alkenes to α,β halodrins, and the halogenation of a range of aromatic compounds, including anilines, phenols and heterocycles (Colonna et al., 1999). In the halogenation reaction, the halide ion may interact directly with the oxo ligand of compound I to produce a ferric hypochlorite adduct (Dawson, 1988; Griffin, 1991; Ortiz de Montellano, 1992). It is still controversial whether the chlorinating agent is an enzyme-bound (Fe-O-Cl) species, or the catalytically generated hypochlorous acid. In general, enzymatic halogenation reactions do not show the typical features of enzymatic reactions, such as narrow substrate range and high product selectivity.

Oxygen-transfer reaction

Peroxidases may also catalyse P450-like monooxygenation reactions including sulfoxidation, epoxidation, hydroxylation and N-dealkylation (Dawson, 1988; Griffin, 1991; Ortiz de Montellano, 1992).

Oxidation of sulfides (Figure 2.5: a-h) are catalysed by several heme peroxidases, such as HRP (Kobayashi et al., 1987; Colonna et al., 1992; Harris et al., 1993; Gajhede et al., 1997), cytochrome *c* peroxidase (CcP) (Miller et al., 1992), MP8 (Colonna et al., 1994; Osman et al., 1996), CPO (Colonna et al., 1997) and lactoperoxidase (LPO) (Colonna et al., 1995). Especially CPO is of interest for sulfoxidation reactions because of its high enantioselectivity and versatility (Colonna et al., 1997). Furthermore, epoxidations catalysed by HRP (Ortiz de Montellano, 1987), CPO (Allain et al., 1993) and CcP (Miller et al., 1992) have been reported. Nitrogen oxidation is described for HRP (Boucher et al., 1996) and CPO (Doerge et al., 1991). Examples of catalysis of carbon-hydrogen-bond oxidation is mainly described for CPO (Geigert et al., 1983; Miller et al., 1995), whereas one example is described for HRP (Klibanov et al., 1981).

2.4.1 Oxidation of phenols and aromatic amines

As oxidation of phenols and aromatic amines are important peroxidase reactions in this thesis, these reactions are shortly discussed in this paragraph. Phenol and aromatic amines are typical substrates for peroxidases (Dunford & Stillman, 1976; Dunford & Adeniran, 1986). Oxidation of these substrates by peroxidases in the presence of hydrogen peroxide results in substrate radicals. After diffusion from the active centre of the enzyme

these substrate radicals react further with aromatic substrates to form dimeric, oligomeric or polymeric products (Figure 2.6) (Simmons et al., 1988; Zemel & Quinn, 1993; Huixian & Taylor, 1994).

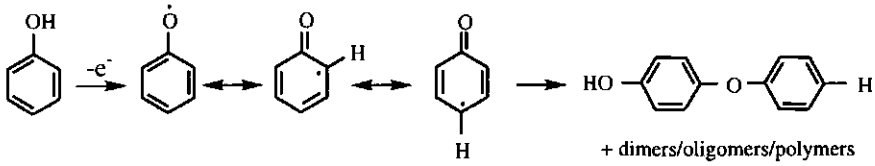


Figure 2.6 Example of a reaction pathway for phenol oxidation by HRP in presence of H_2O_2.

Kinetics of the oxidation of phenols and aromatic amines has been extensively studied (Critchlow & Dunford, 1972a; Job & Dunford, 1976; Dunford & Adeniran, 1986; Sakurada et al., 1990). Many studies have been done directed at elucidation/characterisation of the aromatic donor molecule binding sites of heme peroxidases, of which an overview has been reported by Veitch (1995). Another tool to study peroxidase catalysis of phenols and aromatic amines is the description of quantitative structure activity relationships (QSARs) for these conversions as reported by Sakurada et al. (1990) and Brewster et al. (1991).

Polymerisation of phenols and aromatic amines has been reported to be applied in the manufacture of resin (Dordick et al., 1987; Ryu et asl., 1989) and in the removal of phenols from waste water (Klibanov et al., 1980; Klibanov et al., 1983).

2.4.2 Heteroatom dealkylation

Peroxidase-mediated heteroatom dealkylation has been reported to be especially efficient for N- and not for O-dealkylations. Only a few reports demonstrate peroxidase-catalysed O-dealkylations (Meunier & Meunier, 1985; Ross et al., 1985). Generally, peroxidase mediated O-dealkylations are observed so far only for methoxybenzenes substituted with an additional amino (Meunier & Meunier, 1985; Haim et al., 1987) or a methoxy group (Haim et al., 1987). In this paragraph the peroxidase catalysed N-demethylation of N-substituted aromatic amines is described in some more detail, because the N-demethylation of methyl-*N*-methylanthranilate (MNMA) to methylanthranilate (MA), a concord grape flavour, was chosen as the model reaction in this thesis. In the literature N-demethylation has been reported to be catalysed by CPO (Kedderis et al., 1980), HRP (Griffin & Ting, 1978; Kedderis & Hollenberg, 1983; Hollenberg et al., 1985), and other peroxidases like lactoperoxidase (Miwa et al., 1983). During N-demethylation of N-

substituted aromatic amines formaldehyde is generated and the amine residue is recovered as secondary amine (Table 2.4). Kinetic values and turnover numbers for the N-demethylation of N,N-dimethylaniline by several heme proteins were determined by Kedderis et al. (1980) and Kedderis & Hollenberg (1983a). Detailed kinetic studies of this demethylation reaction indicate a so called "ping-pong" mechanism as kinetic model (Kedderis & Hollenberg, 1983a/b).

The mechanism of peroxidase-catalysed N-demethylation has been investigated in studies using physico-chemical methods including electron spin resonance (ESR), in studies with kinetic isotope effects and in studies using labelled oxidants (Northrop, 1975; Griffin, 1978; Griffin & Ting, 1978; Griffin et al., 1981; Guengerich et al., 1996). The mechanism of peroxidase-catalysed N-demethylation has long been and still is a matter of debate. Figure 2.7 shows several mechanisms for N-demethylation by heme proteins.

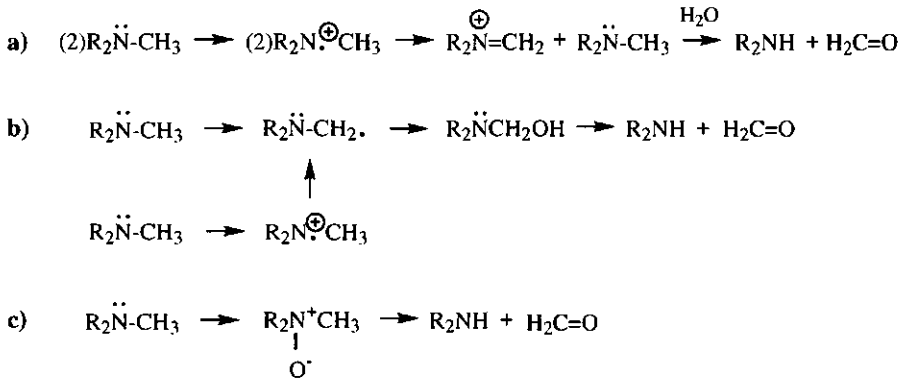


Figure 2.7 Heteroatom dealkylation catalysed by a peroxidase type of mechanism (a), a cytochrome P450-type of mechanism (b), or a non-radical-type of mechanism (c).

The first mechanism (Figure 2.7a) is generally accepted to be used by peroxidases, because activated peroxidases are known to perform one electron abstractions rather than oxygen transfer reactions (see section 2.3). Thus, peroxidase-mediated N-dealkylation proceeds by a radicaloid type of mechanism (Griffin & Ting, 1978; Guengerich et al., 1996) and the oxygen of formaldehyde is derived from H_2O. The formation of the two-electron oxidised substrate occurs by means of two subsequent one-electron oxidation steps of one substrate molecule, or by dismutation of two one electron oxidised substrate radicals,

but this is still a matter of debate. Altogether, the mechanism of peroxidase-catalysed N-demethylation is still a matter of discussion and might be dependent on the compound studied (Meunier, 1991).

The second mechanism suggested for heme-based N-dealkylation is a classic cytochrome P450 type of mechanism, proceeding by a so called oxygen rebound mechanism resulting in hydroxylation of the alkyl substituent. In this mechanism the oxygen of formaldehyde is derived from the oxygen donor creating the reactive heme species (O_2 or H_2O_2) (Figure 2.7b). Hydroxyl rebound from the activated species to the radical substrate results in the unstable carbinolamine intermediate which decomposes into the dealkylated substrate and formaldehyde. Formation of the substrate radical may proceed either by direct hydrogen atom abstraction or by subsequent one electron oxidation followed by proton release. In the literature much evidence is provided for the latter (Augusto et al., 1982; Burka et al., 1985; Bordon et al., 1989; MacDonald et al., 1989).

In addition, a non-radical type of mechanism for the N-demethylation is depicted in Figure 2.7c. Here the demethylation proceeds by an N-oxidation. This mechanism for N-dealkylation is often considered to be not very general since most N-oxides are relatively stable. However, the mechanism may become of importance when peroxidase reactions are performed in the presence of the radical scavenger ascorbate. A recent study showed evidence that N-demethylation by MP8 in presence of ascorbate may proceed by such a non-radical type of mechanism because it proceeds efficiently in the presence of ascorbate. The involvement of a (hydro)peroxo-iron heme in the mechanism for heme-based heteroatom dealkylation explains the results (Boersma et al., submitted; Primus et al., 1999).

2.5 Operational stability

Operational stability is important for industrial application of peroxidases. Currently, poor operational stability limits the large scale application of heme peroxidases in the production of fine chemicals. Inactivation of the heme underlying this poor operational stability may occur through several pathways:

Inactivation of the heme can involve irreversible reactions with inhibitors as described by Ortiz de Montellano et al. (1988), DePillis et al. (1990) and Samokyszyn et al. (1991). During oxidation of such inhibitors (for example phenylhydrazine) by peroxidases, radicals are obtained which react with the active site of peroxidases.

During the catalytic cycle of peroxidases activated oxygen species are produced, such as compound I and II, but also, as side products: superoxide anions, hydroxyl radicals

and hydrogen peroxide. Generally, these reactive oxygen species can cause mutations and irreversible modifications of DNA, RNA, proteins and lipids in many ways (Elstner, 1982; Malmstrom, 1982; Farr & Kogoma, 1991), which often result in inactivation of the enzyme. In the case of heme peroxidases oxidative inactivation seems to require formation of high-valent-iron-oxo compounds during catalysis, but mechanisms of inactivation are still not clear. Possible mechanisms for oxidative inactivation are given below in more detail.

2.5.1 Possible mechanisms for oxidative inactivation

Several possible mechanisms for oxidative inactivation, mentioned in the literature, are shortly described here. In the first inactivation mechanism the *meso* positions of the porphyrin ring (Figure 2.1) are oxidised. After subsequent hydrolysis, cleavage of the porphyrin ring with possible loss of the iron occurs. Results from several reports (Cadenas et al., 1980; Rice et al., 1983; Florence, 1985) point at a role for Fe^{IV}-oxo compounds in this degradation mechanism, because scavengers of activated oxygen species had no effect on the degradation rate.

Cross linking is an inactivation mechanism which also involves the *meso* positions of the porphyrin ring and is described for peroxide-treated myoglobins (Catalano et al., 1989) and leghemoglobine (Moreau et al., 1995). The cross links are probably formed by tyrosine residues. It is suggested that ferric leghemoglobine in presence of H_2O_2 gives rise to protein radicals, at least one of which is centered on a tyrosine residue. Such tyrosine radicals may form *meso* adducts with the porphyrin by a mechanism analogous to the mechanism for *meso* addition of radicals to the heme of HRP (Ortiz de Montellano, 1987; Ator et al., 1987). In this mechanism the radical binds to the *meso* carbon of HRP compound II, resulting in reduction of iron from the ferryl to the ferric state. After elimination of a proton the *meso* adduct is obtained.

Other suggested inactivation mechanisms involve oxidation of the fifth histidine ligand to form arginine as described for copper-histidinyl complexes (Cooper et al.; 1985).

Furthermore, Cunningham et al. (1991) showed kinetic evidence for 'substrate-involved' inactivation of MP8 and observed bleaching of MP8 during this inactivation. Such type of inactivation of MP8 has been suggested to proceed through disintegration of the heme moiety (Wiseman et al.; 1982), although the mechanism remains unexplained.

An inactivation mechanism has been proposed that specifically depends on the absence of a protective environment of the heme and involves the formation of μ-oxo dimers as described by Balch (1984).

Finally, formation of compound III (Figure 2.5) has been reported by Arnao et al. (1990). Compound III is formed upon addition of 1) superoxide anion to native peroxidase, 2) molecular oxygen to the ferrous form of peroxidase, and 3) an excess of hydrogen peroxide to native peroxidase or compound II. The accumulation of this compound III during catalysis results in a decrease of the active forms of peroxidases.

2.6 Biotechnological applications

Peroxidases clearly have potential as biocatalysts and many possible industrial applications of peroxidases have been suggested. For example, peroxidases could be used as catalysts for phenolic resin synthesis (Dordick et al., 1987; Pokora and Cyrus, 1987, Akkara et al., 1991), as indicators for food processing (Thompson, 1987; Weng et al., 1991), or as additives in bioremediation, especially for the removal of phenols and aromatic amines from polluted waters (Adler et al., 1994; Arseguel and Baboulene, 1994; Yu et al., 1994; Wu et al., 1993). Most commercial applications of peroxidases are found in analytical diagnostics, like in biosensors and immunoassays (Tamaki, 1995; Yamazaki, 1995). To our knowledge there are no examples of application of peroxidases for the natural production of food flavours. The great diversity of possible applications is mainly due to the wide substrate specificity of peroxidases. However, poor thermal and/or operational stability and/or availability at an acceptable price limit the large scale use of peroxidase catalysis. The problem of (operational) stability of peroxidases is a severe problem for industrial processes and is difficult to solve. On the other hand this thesis will show that peroxidases could represent an interesting tool for industrially relevant reactions, making future research on possibilities and limitations worthwhile.

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Reversible formation of high-valent-iron-oxo porphyrin intermediates in heme-based catalysis: Revisiting the kinetic model for horseradish peroxidase.

Marjon J.H. van Haandel, Jean-Louis Primus, Cees Teunis, Marelle G. Boersma, Ahmed M. Osman, Cees Veeger and Ivonne M.C.M. Rietjens

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Abstract

Many heme-containing biocatalysts, exert their catalytic action through the initial formation of so-called high-valent-iron-oxo porphyrin intermediates. For horseradish peroxidase the initial intermediate formed has been identified as a high-valent-iron-oxo porphyrin π-radical cation, called compound I. A strongly hold concept in the field of peroxidase-type of catalysis is the irreversible character of the reaction leading to formation of this compound I. Results of the present paper, however, point at reversibility of formation of the high-valent-iron-oxo porphyrin intermediate for various heme containing catalysts, including horseradish peroxidase. This results in heme-catalysed exchange of the oxygens of H_2O_2 with those of H_2O. The existence of this heme-catalysed oxygen exchange follows from the observation that upon incubation of ^{18}O labelled $H_2{}^{18}O_2$ with heme-containing biocatalysts significant loss of the ^{18}O label from the $H_2{}^{18}O_2$, accompanied by the formation of unlabelled H_2O_2, is observed. Thus, for the heme biocatalysts studied, exchange of the oxygen of their high-valent-iron-oxo intermediate with that of water occurs rapidly. This observation implies the need for an update of the kinetic model for horseradish peroxidase. Revaluation and extension of the previous kinetic model showed the necessity to include several additional reaction steps, taking both reversible compound I formation and formation of enzyme-substrate complexes into account.

3.1 Introduction

Heme-containing peroxidases and cytochromes P450 are generally considered to exert their catalytic activity through the intermediate formation of so-called high-valent-iron-oxo porphyrin complexes [1-3]. However, the exact nature and reactivity of these high-valent-iron-oxo porphyrin intermediates is far from being fully understood. For horseradish peroxidase and chloroperoxidase the nature of the initial high-valent-iron-oxo species formed upon reaction with H_2O_2 has been characterised as an iron(IV)-porphyrin π-cation radical, generally referred to as compound I [2-6]. In contrast, the high-valent-iron-oxo intermediate of cytochrome c peroxidase has unpaired electron density on a protein residue instead of on the porphyrin [7], whereas the reactive iron-oxo species of cytochromes P450, up to now, could not be detected.

In spite of this uncertainty about the exact nature of the reactive porphyrin species in heme-based peroxidases and cytochromes P450, many studies have focused on the kinetics and reaction mechanisms of these multi-functional enzymes. In general the reactions catalysed proceed through an initial electrophilic attack by the high-valent-iron-oxo porphyrin intermediate on the substrate eventually leading to either one-electron substrate oxidation and/or to a so-called monooxygenation reaction in which the oxygen of the high-valent-iron-oxo intermediate is transferred to the substrate. Peroxidases are thought to preferentially catalyse one electron substrate oxidations, maybe due to their axial histidine ligand and, especially, the supposed inaccessibility of the oxygen atom in their high-valent-iron-oxo porphyrin form [8,9]. Cytochromes P450, on the other hand, might prefer oxygen transfer reactions due to the involvement of their cysteinate proximal Fe ligand, stabilising oxygen transfer reaction pathways [10,11].

A strongly hold general concept in the field of peroxidase catalysis refers to the irreversible character of the reactions leading to formation of the reactive high-valent-iron-oxo porphyrin intermediate. Dunford [12] described the kinetic model for horseradish peroxidase as a modified ping-pong mechanism in which no K_m-values would be obtained due to the irreversible nature of the reactions leading to compound I formation (Figure 3.1a). Recently, however, this model has been corrected at least to some extent, taking into account the reversible formation of an intermediate enzyme-H_2O_2 complex, preceding the formation of the compound I reaction intermediate [13-17] (Figure 3.1b). The existence of such a H_2O_2 intermediate is well accepted nowadays, although the intermediate has never been directly observed. Theoretical quantum physical calculations on the exact nature of this intermediate have been performed [18,19]. Nevertheless in spite of the assumed reversibility

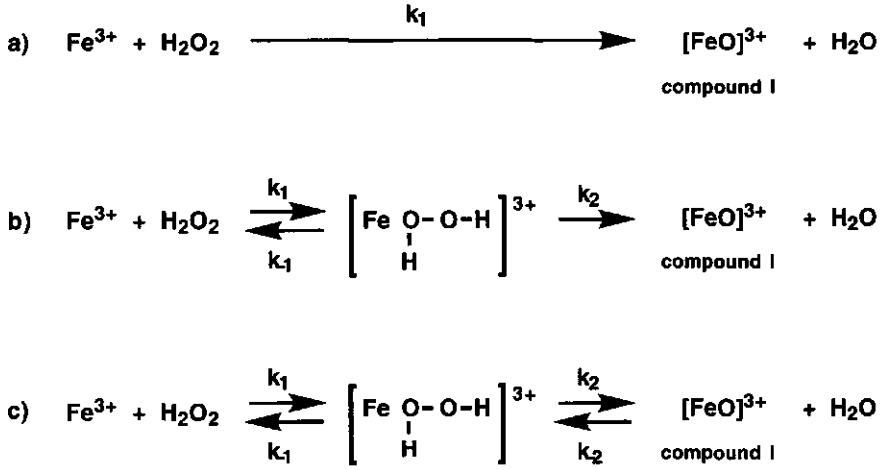

Figure 3.1 *Reaction schemes for the compound I formation from the Fe-porphyrin and* H_2O_2. a) Generally both steps [12] or b) only the second step is taken as essentially irreversible (i.e. k_{-2} and/or k_{-1} are neglected and are much smaller than k_2 and k_1 respectively) [15,16]. c) Compound I formation from the Fe-porphyrin and H_2O_2 through reversible formation of an intermediate porphyrin-H_2O_2 adduct and reversible compound I formation, investigated in more detail in the present study. The notation of the H_2O_2 adduct in figure 3.1b and 3.1c is based on quantum chemical calculations [18,19].

of the formation of the (porphyrin-Fe-H_2O_2)$^{3+}$ intermediate, its subsequent conversion into H_2O and the high-valent-iron-oxo compound I intermediate is still generally assumed to be irreversible [15-17] (Figure 3.1b).

The present study supports the existence of an enzyme-H_2O_2 intermediate and focuses on the concept of the (ir)reversibility of compound I formation in heme-based catalysis. Results are presented that point at the reversibility of not only the first reaction step, leading to formation of the enzyme-H_2O_2 complex, but also the reversibility of the second reaction step in which the enzyme-H_2O_2 complex is converted to compound I and H_2O (Figure 3.1c). An evaluation of the consequences of such a reversible compound I formation and formation of enzyme-substrate complexes for the kinetic models for horseradish peroxidase catalysed reactions is presented.

3.2 Materials and methods

Chemicals

Horseradish peroxidase, cytochrome c from horseheart and catalase were obtained from Boehringer (Mannheim, Germany). Hematin (hydroxyprotoporphyrin IX iron(III)) and hemin (chloroprotoporphyrin IX iron(III)) were purchased from Aldrich (Milwaukee, WI). Microperoxidase-8 was prepared by the proteolytic digestion of horseheart cytochrome c essentially as described previously [20,21]. Heme concentrations were determined by the pyridine-chromogen method [21]. ^{18}O labelled $H_2{}^{18}O_2$ (91% enriched) was obtained as a 2% solution from ICON, Sunnit (New Jersey, USA). Aniline and 4-aminophenol were obtained from Janssen (Beerse, Belgium). L-Ascorbic acid, Fe(II)sulphate and unlabelled hydrogen peroxide (30%) were from Merck (Darmstadt, Germany). Fe(III)chloride was obtained from Sigma (St Louis, USA).

Oxygen exchange experiments

Exchange experiments generally consisted of two subsequent incubations. First a so-called preincubation was performed. In this preincubation the iron-containing catalyst (150 μM, final concentration) was incubated in 0.1 M potassium phosphate pH 7.6 in the presence of 6.7 mM $H_2{}^{18}O_2$ for 5 minutes at 37 °C. During this preincubation the iron-containing catalyst can react with H_2O_2 resulting in formation of its H_2O_2 adduct and subsequently its high-valent-iron-oxo intermediate and H_2O (Figure 3.1b + 3.1c). In case this reaction is reversible (Figure 3.1c) the reverse reaction will lead to incorporation of the unlabelled O from H_2O into the $H_2{}^{18}O_2$, resulting in a gradual loss of the ^{18}O label from $H_2{}^{18}O_2$.

The preincubation was followed by a detection assay, carried out to quantify the residual extent of ^{18}O labelling in the $H_2{}^{18}O_2$. This detection assay was started by adding 6 mM of ascorbic acid, followed by 0.1 M aniline and 150 μM of microperoxidase-8 (final concentrations). Ascorbic acid was added to inhibit any peroxidase-type of chemistry by microperoxidase-8 and/or the heme catalyst of the preincubation during the detection assay. Since P450-type of oxygen transfer reactions are not inhibited by ascorbic acid [11,22], microperoxidase-8, in the presence of ascorbic acid, converts aniline to 4-aminophenol with full transfer of the oxygen from $H_2{}^{18}O_2$ to the aminophenol formed [22]. Thus, this incubation will result in formation of 4-aminophenol reflecting the amount of ^{18}O in the $H_2{}^{18}O_2$, providing a possibility to quantify the extent of (residual) ^{18}O in the $H_2{}^{18}O_2$ at the end of the preincubation period. After 2 minutes of incubation catalase was added (32500 units) to convert all residual H_2O_2. The reaction mixture was then extracted four times with

1 ml ethyl acetate. The collected ethyl acetate fractions were concentrated by evaporation to about 20 µl and the sample obtained was analysed by MS to detect the percentage of ^{18}O labelling of the 4-aminophenol.

Analysis by mass-spectrometry (MS)

MS analysis was performed with a Finnigan MAT 95 mass spectrometer on flat topped peaks at a resolution of 2000. At this resolution the sample peaks are well separated from hydrocarbon impurities. A correction for ^{13}C-content was made by calculation, as the resolution needed to resolve isobaric ^{18}O- and ^{13}C-peaks should be at least 80000. Sample introduction was via a direct probe. The mass spectrometer was operated in the 70 eV EI-ionisation mode, while scanning from mass 24 to 340 at a speed of 4 sec/deg. Labelling results obtained were corrected for the fact that the $H_2{}^{18}O_2$ was only 91% labelled with ^{18}O.

Chemical detection of 4-aminophenol

For the chemical determination of 4-aminophenol, the reaction was terminated by adding 0.8 ml reaction mixture to 0.24 ml 20% (mass/vol) trichloroacetic acid. After centrifugation for 5 min at 13,000 rpm, the supernatant was used for determination of the concentration of 4-aminophenol following the procedure described by Brodie and Axelrod [23]. It is important to notice that the presence of ascorbic acid in the reaction mixtures retarded the normal time of colour development. For this reason, the absorbance at 630 nm was measured after 18-23 h instead of 1 h incubation at room temperature. A calibration curve of 4-aminophenol in the presence of ascorbic acid demonstrated that the absorption coefficient of the indophenol was not affected by the addition of ascorbic acid (the ε value at 630 nm is 30.5 $mM^{-1}cm^{-1}$).

3.3 Results and Discussion

Labelling of aminophenol in the control incubation

Figure 3.2 presents the representative part of the MS spectrum of 4-aminophenol formed in a control sample in which the preincubation mixture contained no heme-protein to catalyse the ^{18}O exchange between $H_2{}^{18}O_2$ and H_2O. From the relative intensities of the peaks at Mz = 111.057 (^{18}O labelled 4-aminophenol) and at Mz = 109.053 (^{16}O containing 4-aminophenol) it can be concluded that, taking into account the necessary correction for the 91% ^{18}O labelling of the $H_2{}^{18}O_2$, all 4-aminophenol formed contains the ^{18}O label

derived from the $H_2{}^{18}O_2$. This result is in accordance with previous data [22]. The oxygen transfer from the H_2O_2 via the histidine co-ordinated microperoxidase-8 to aniline creating 4-aminophenol, is indicative for so-called cytochrome P450- instead of peroxidase-type of reaction chemistry. This microperoxidase-8/H_2O_2-driven 4-hydroxylation of aniline is especially observed under conditions where the presence of ascorbic acid prevents peroxidase-type of product formation but does not affect the P450-type of conversions also catalysed [22,24-28].

Oxygen exchange by different heme-containing proteins

Table 3.1 presents the extent of ^{18}O labelling of 4-aminophenol formed in incubations in which the conversion of aniline by microperoxidase-8/H_2O_2 was preceded by a 5 minutes preincubation of the $H_2{}^{18}O_2$ with either no iron sample, with iron(II)sulphate or iron(III)chloride, with different types of model porphyrins, or with different heme-proteins. The percentages of oxygen exchange present the % of the $H_2{}^{16}O_2$ as compared to $H_2{}^{18}O_2$ in the residual H_2O_2 fraction still available at the end of the preincubation, since this H_2O_2 population is used for the detection of ^{16}O:^{18}O labellling.

As outlined above, in the control incubation with no heme-protein present during the preincubation, the 4-aminophenol formed in the subsequent detection assay, was fully labelled (Table 3.1, Figure 3.2). This also holds for the samples in which the preincubation was carried out in the presence of either Fe(II)sulphate or Fe(III)chloride (Table 3.1). This result indicates that neither ferro- nor ferric cations are capable of catalysing the loss of ^{18}O label from $H_2{}^{18}O_2$. This result excludes that the phenomena observed with the model porphyrins and heme-proteins are due to traces of iron cations in the samples liberated from the heme cofactor upon its inactivation and also implies that Fenton-type of chemistry is not involved in the oxygen exchange observed.

The data presented in Table 3.1 also demonstrate that the model porphyrins hemin or hematin (chloro- and hydroxy-protoporphyrin IX iron(III) respectively) catalyse a partial exchange of the ^{18}O label in $H_2{}^{18}O_2$ resulting in 31/35% and 20/33% loss of label respectively, detected as the formation of respectively 31/35% and 20/33% unlabelled 4-aminophenol. In cases where the preincubation contained one of the heme-proteins tested, namely microperoxidase-8, or horseradish peroxidase, an even larger loss of label is observed. The extent of loss of ^{18}O labelling in the 4-aminophenol formed varies from about 73% for microperoxidase-8 to full (100%) loss of the ^{18}O label in the case of horseradish peroxidase. For horseradish peroxidase, a shorter preincubation period, i.e. 1 minute instead of 5 minutes, results in a smaller loss of label (Table 3.1). In addition, Figure 3.3a shows the time dependence of the loss of label

Table 3.1 Heme-protein catalysed oxygen exchange between $H_2{}^{18}O_2$ and H_2O, represented by the percentage of residual ^{18}O labelling in 4-aminophenol formed from aniline by microperoxidase-8 in the presence of 6 mM ascorbic acid, taken into account the necessary correction for the 91% ^{18}O labelling of the $H_2{}^{18}O_2$. The percentages of oxygen exchange present the % of $H_2{}^{16}O_2$ as compared to $H_2{}^{18}O_2$ in the residual H_2O_2 fraction still available at the end of the preincubation. The preincubation time was 5 minutes unless indicated otherwise. The mechanism proposed for the exchange is schematically presented in Figure 3.1c.

heme protein present in pre-incubation	% of ^{18}O in 4-aminophenol (n=2 or 3)	% of O exchange (n=2 or 3)
none (control)	100 / 100	0 / 0
iron(II)sulphate	100 / 97	0 / 3
iron(III)chloride	100 / 95	0 / 5
hemin (chloroprotoporphyrin IX iron(III))	69 / 65	31 / 35
hematin (hydroxyprotoporphryin IX iron(III))	80 / 67	20 / 33
microperoxidase-8	30 / 23 / 27	70 / 77 / 73
horseradish peroxidase	0 / 0	100 / 100
horseradish peroxidase (1 min)	18 / 18	82 / 82

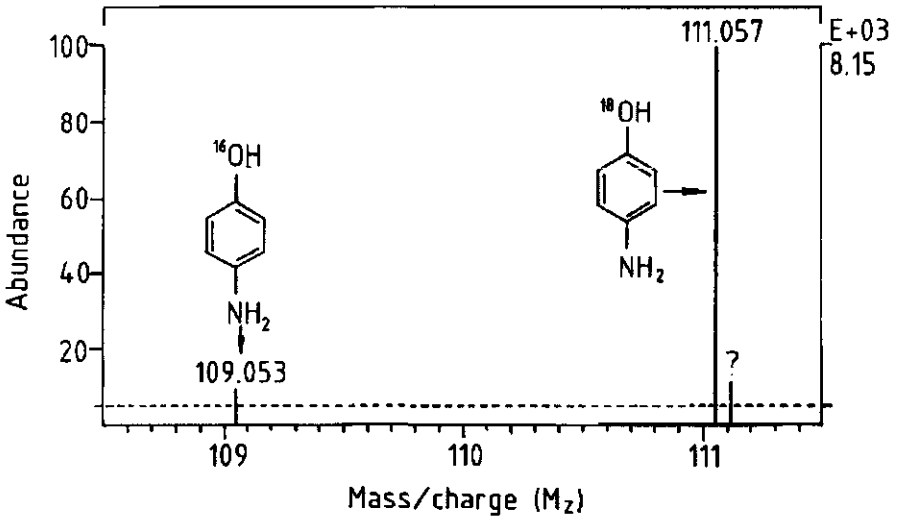

Figure 3.2 The mass spectrum of the molecular region of 4-aminophenol obtained from the extraction of an incubation of aniline with microperoxidase-8 with $H_2{}^{18}O_2$ resulting from 5 minutes preincubation without a heme protein. The unidentified peak is probably a contamination. Only the peaks with an abundancy higher than 5% (dotted line) are presented.

from $H_2{}^{18}O_2$ during the preincubation with microperoxidase-8. The results demonstrate a fast decay in the rate of ^{18}O exchange as catalysed by microperoxidase-8. This observation can be ascribed to three phenomena. First, to the inactivation of the catalyst, in a way comparable to the inactivation observed previously for microperoxidase-8 catalysed peroxidase- and/or aniline-4-hydroxylating activities (Figure 3.3b) [22,29]. Second, after initial exchange of the first of the two ^{18}O atoms of $H_2{}^{18}O_2$ a statistic reduction of the ^{18}O exchange rate can be expected, due to the formation of $H_2{}^{18}O^{16}O$ which can react in two ways: one leading to exchange and the other not leading to exchange. Third, due to full reversibility of the exchange, the accumulation of $H_2{}^{16}O_2$ and $H_2{}^{18}O^{16}O$ results in time-dependent formation of competitive inhibitors, a phenomenon also contributing to a decrease of the rate of ^{18}O exchange in time.

Moreover, comparison of Figure 3.3a to Figure 3.3b leads to the conclusion that, although formation of 4-aminophenol by microperoxidase-8 stops after one minute, still a slow microperoxidase-8 catalysed ^{18}O loss from $H_2{}^{18}O_2$ is observed. This phenomenon could be explained by the hypothesis that microperoxidase-8 looses its aniline 4-hydroxylating activity due to a loss of its axial histidin ligand. However, in spite of this loss in possibilities for oxygen transfer, possibilities for a reaction of the compound I derivative with H_2O leading to ^{18}O exchange may not be affected to the same extent upon loss of the axial histidine ligand. This hypothesis is supported by the observations reported here for hemin and hematin. These porphyrin models also lack the axial histidin ligand and show very limited activity for H_2O_2-driven aniline 4-hydroxylation. In contrast, they appeared to be able to catalyse significant ^{18}O exchange (Table 3.1).

The decline in ^{18}O label incorporation upon preincubation of the $H_2{}^{18}O_2$ with horseradish peroxidase or microperoxidase-8, indicates that, during the preincubation, the $H_2{}^{18}O_2$ looses its ^{18}O label. Since the results obtained under anaerobic conditions were similar (data not shown) it can be concluded that the ^{18}O oxygens in the $H_2{}^{18}O_2$ are exchanged with unlabelled oxygens from H_2O. This could be expected when the reaction between the heme protein and H_2O_2 to form compound I and H_2O, that is, the compound I formation, is reversible (Figure 3.1c). Despite the high driving force of the formation of compound I from the resting enzyme, the reverse reaction is driven by 55.5 M H_2O, which is 10,000 fold in excess of H_2O_2.

The role of Fenton chemistry in the 4-hydroxylation of aniline during the detection assay

Additional experiments were performed in order to exclude a role for Fenton-type of reaction chemistry in the 4-hydroxylation of aniline during the detection assay. Incubation of aniline with 3 or 300 μM of Fe(II)sulphate or 3 or 300 μM Fe(III)chloride, in the presence or absence of ascorbic acid, did not result in formation of 4-aminophenol. Thus, 4-aminophenol formation in the detection assay does not result from Fenton-type of reactions.

H_2O_2 present at the end of the preincubation period is the oxygen donor in the detection assay

Table 3.2 provides the evidence that H_2O_2 is present at the end of the preincubation periods and needed for the incorporation of oxygen into 4-aminophenol in the detection assay, even in the cases of full loss of ^{18}O-label. This can be concluded from the observation (Table 3.2) that in all cases the addition of catalase at the end of the preincubation period fully inhibits the formation of 4-aminophenol in the subsequent detection assay.

Table 3.2 Effect of catalase on the formation of 4-aminophenol by microperoxidase-8 driven by the H_2O_2 resulting from preincubation with different heme biocatalysts. Preincubation time was 5 minutes unless indicated otherwise. 100% = formation of 4-aminophenol in the control (i.e. without heme protein) without catalase.

heme protein present in preincubation	percentage (%) of 4-aminophenol formed	
	- catalase	+ catalase
none (control)	100	0
Fe(II)sulphate	96	0
Fe(III)chloride	99	0
hemin (chloroprotoporphyrin IX iron(III))	56	0
hematin (hydroxyprotoporphryin IX iron(III))	80	0
microperoxidase-8	40	0
horseradish peroxidase	18	0
horseradish peroxidase (1 min)	22	0

From the data presented in Table 3.2 it also follows that the amount of 4-aminophenol formed varies with the type of heme compound present in the preincubation. This can best be ascribed to varying levels of catalase activity of the heme proteins during the preincubation. Using a Clark electrode, N_2 instead of air saturated buffer and concentrations of all reagents 15 times lower than used in the preincubations, the conversion

of H_2O_2 to O_2 could be observed for all heme compounds tested, indicating the possibility for catalase-type of H_2O_2 degradation during the preincubations. Since the H_2O_2/microperoxidase-8 catalysed 4-hydroxylation of aniline is dependent on the H_2O_2 concentration [22], a partial reduction of the amount of H_2O_2 in the preincubation, due to catalase activity of the heme catalyst tested, can explain the differences in 4-aminophenol formation in the subsequent detection assay as following from the data depicted in Table 3.2. Catalase activity appears to be present especially in the incubation with horseradish peroxidase. It is low when compared to the peroxidase activity of the enzyme with for example ortho-dianisidine (i.e. < 1%), but it becomes relevant since our preincubations are performed with relatively high concentrations (150 μM) of the enzyme. Catalase activity of horseradish peroxidase has also been reported by Nakajima & Yamazaki [30] and Arnao et al. [31].

Rate of ^{18}O exchange

In the present study heme-catalysed ^{18}O exchange of the ^{18}O in $H_2{}^{18}O_2$ with that of H_2O was observed maybe via compound I as indicated in Figure 3.1c. However, irrespective of the mechanism of ^{18}O exchange involved, the results of the present study also indicate that the rate of the exchange is fast. Especially when one takes into account that the $t_{1/2}$ of inactivation of microperoxidase-8 under preincubation conditions is 10 seconds [29]. Taking into account an exchange of 28% of the oxygen atoms of 6.7 mM $H_2{}^{18}O_2$ within the first 10 seconds (Figure 3.3a), catalysed by an amount of microperoxidase-8 decreasing from 150 μM to 75 μM in 10 seconds, a rate of exchange of > 100 min^{-1} can be calculated from these data. Thus, the rate of the proposed ^{18}O exchange between the perferryl intermediate and H_2O is much faster than the rate of reaction of this intermediate with the substrate estimated to be about 23 min^{-1} [11,22] for 4-hydroxylation of aniline by microperoxidase-8. Data in the literature [32-34] have reported on the exchange of oxygen between iodosylbenzene and water through the perferryl intermediate of cytochrome P450. The oxygen from water is incorporated into the substrate. Macdonald et al. [33] reported that the rate of oxygen exchange (170 min^{-1}) is much faster than the rate of oxygen incorporation (19 min^{-1}). A result in line with our data. However, Nam and Valentine [35] concludedfrom experiments with iodosylbenzene as oxygen donor that its mechanism of oxygen exchange does not involve metal-oxo intermediates. This, in contrast to the reaction with other oxygen donors for which the high-valent-metal-oxo compound is the suggested intermediate in ^{18}O exchange between (organic) peroxide and water [35].

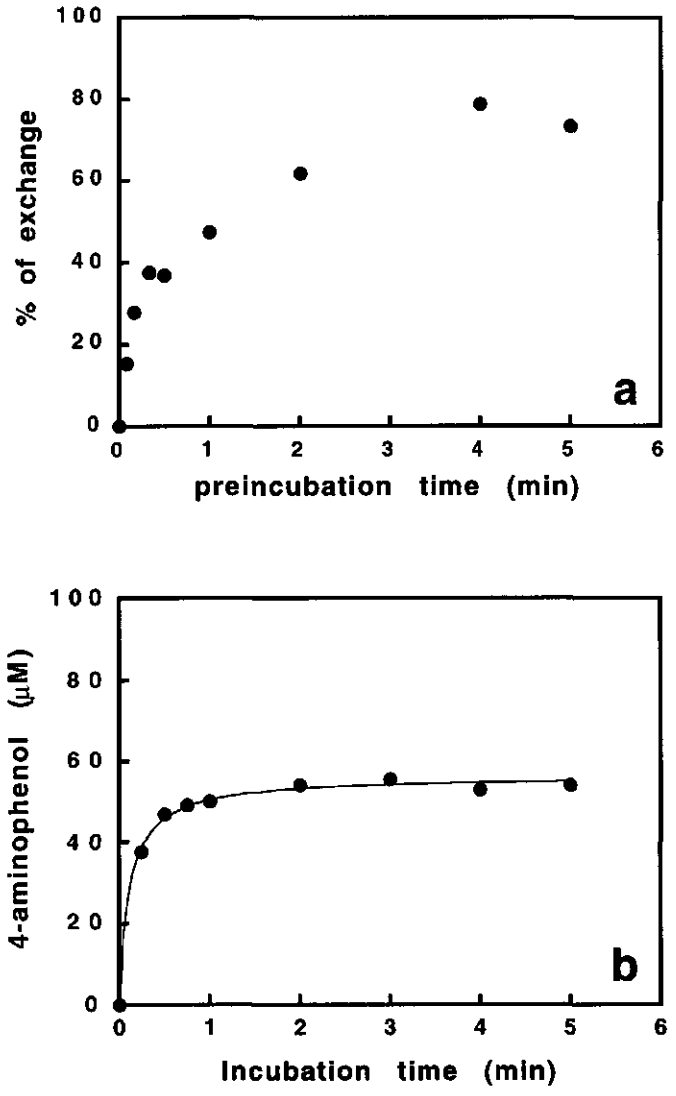

Figure 3.3 a) Time-dependent microperoxidase-8 catalysed oxygen exchange between $H_2{}^{18}O_2$ and H_2O as derived from the residual amount of ^{18}O labelled 4-aminophenol formed in the detection assay described in materials and methods. b) Time-dependent conversion of aniline to 4-aminophenol by microperoxidase-8 in the presence of ascorbic acid. Data points in Figure 3a were not fitted to a kinetic model since, as explained in the text, the mechanism is complex.

However, at this point it also becomes relevant to question the identical nature of the reactive intermediate catalysing either oxygen exchange or aniline-4-hydroxylation. Especially taking into account the above calculated rates for the reaction with H_2O leading to oxygen exchange and that with aniline leading to formation of 4-aminophenol. When in both cases compound I would be involved it is difficult to foresee how 100% ^{18}O transfer to aniline can ever occur when the reaction with H_2O leading to oxygen exchange and loss of ^{18}O label is much faster. Thus, these results could be explained by the reaction scheme already presented by Nam and Valentine [35]. The scheme suggests that the possible heme species involved in oxygen transfer to substrates and in oxygen exchange are different. A definite hypothesis for the exact mechanism underlying the full labelling of 4-aminophenol in spite of the efficient ^{18}O exchange of the compound I with H_2O can at present not be provided.

Furthermore, it is of interest to point out that the observation of hemin and hematin catalysed oxygen transfer seems to be different from previous data reported by Nam and Valentine [35]. Nam and Valentine reported for several model porphyrin catalysts the lack of ^{18}O-incorporation from $H_2{}^{18}O$ into the products of olefin epoxidations, alkane hydroxylation and cyclohexene epoxidation. Since the ^{18}O transfer from $H_2{}^{18}O$ into the monooxygenated products should proceed through exchange phenomena of the compound I-type intermediates, it was concluded that the porphyrin compound I species did not exchange their oxygen atom with that of H_2O. Clearly this seems in contrast to the results of the present study. However, we would like to point out that such controversy may not exist at all, since our experiments were performed in 55.5 M H_2O instead of in 9 M $H_2{}^{18}O$ in 60% CH_2Cl_2/40% CH_3OH. This difference in conditions can affect the labelling in three ways. First, due to mass law the chances for a reaction between compound I and H_2O are 6 fold higher under our conditions. Second, the rate constants for exchange in organic solvents and in water may differ considerably. Third, the high concentration of methanol in the experiments of Nam and Valentine provides a well competing alternative for the reactions between water and compound I. The latter suggestion of a reaction between compound I and methanol is in accord with recent results [36] indicating a role of the methanol in heme-based catalysis mediated through a reaction of compound I with methanol.

Reconsideration of the kinetic model for horseradish peroxidase

The results of the present study point at possibilities for a reaction between compound I and H_2O leading to oxygen exchange between $H_2{}^{18}O_2$ and the solvent H_2O. This leads to the conclusion that formation of the high-valent-iron-oxo intermediate is a reversible reaction. This reversibility of the reaction leading to compound I and H_2O

formation is in contrast to what is generally assumed for peroxidase reaction chemistry and kinetics [12-17] and requires reconsideration of the kinetic models generally accepted for horseradish peroxidase.

Until now the irreversible character of the reaction leading to formation of compound I was a strongly hold concept in the field of peroxidase catalysis. Based on this concept, Dunford described the kinetic model for horseradish peroxidase as a modified ping-pong mechanism in which no K_m-values would be obtained due to the irreversible nature of the reactions leading to compound I (Figure 3.4a, Eq. 3.1) [12].

$$\frac{2E_0}{v} = \frac{1}{k_1[H_2O_2]} + \frac{1}{k_3[AH_2]} \tag{3.1}$$

From this formula it follows that the rate constants k_1 and k_3, but not k_2, can be determined from steady-state kinetics.

Nowadays, the existence of an intermediate enzyme-H_2O_2 complex is well accepted [13-19]. This complex is thought to be converted irreversibly into the high-valent-iron-oxo species (Figure 3.1b). Figure 3.4b shows a modified form of Dunford's reaction scheme taking into account this intermediate enzyme-H_2O_2 complex. Furthermore, the reversibility of the formation of compound I, pointed out in the present study, is also taken into account. Based on this reaction scheme (Figure 3.4b) equation 3.2 can be derived using the schematic method of King and Altman [37].

$$v = \frac{2k_2E_0}{1 + \frac{k_2+k_{-1}}{k_1[H_2O_2]} + \frac{k_2k_4+k_2k_3+k_{-2}k_4}{k_3k_4[AH_2]} + \frac{k_{-1}k_{-2}}{k_1k_3[H_2O_2][AH_2]}} \tag{3.2}$$

According to equation 3.2 the maximum catalytic activity ($k_{cat} = 2k_2$) is not dependent on the nature of the substrate. This is not in line with results previously reported [38,39] which do show dependence on the chemical characteristics of the substrate. To obtain a rate equation in which k_{cat} is dependent on the nature of the substrate a new reaction scheme is proposed taking into account the reversible formation and the existence of enzyme-AH_2 intermediates (Figure 3.4c), as Patel et al. [40] explained the saturation observed for oxidation of phenols by horseradish peroxidase compound II assuming such complex formation. In analogy to previous models, the reactions of compound I and compound II with substrate AH_2 are considered to be irreversible. Based on the reaction scheme in Figure 3.4c a new rate equation (Equation 3.3) can be derived for peroxidase-catalysed reactions using the schematic method of King and Altman [37].

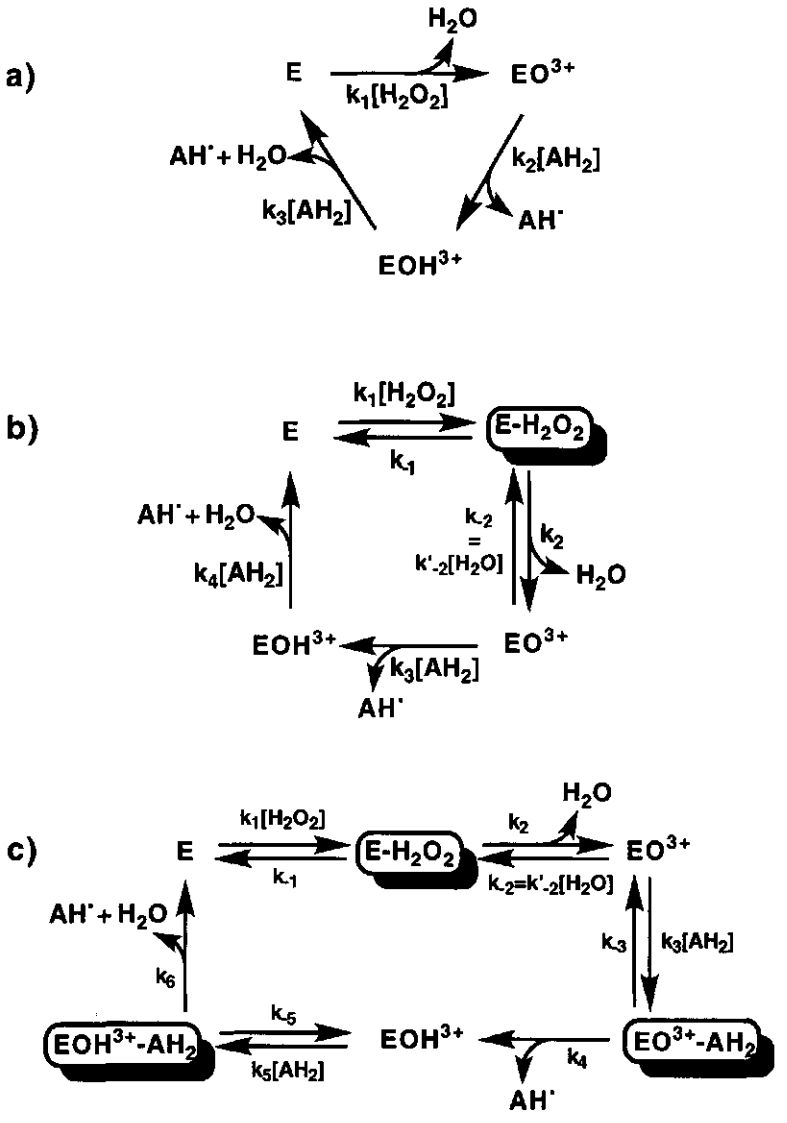

Figure 3.4 Three reaction schemes for peroxidase-type of conversion. a) A reaction scheme for peroxidase-type of conversion as described by Dunford [12]. b) A modification of Dunford's model taking into account an intermediate enzyme-H_2O_2 complex [13-19] and the reversibility of compound I formation as proposed in the present study. c) The modified ping-pong-pang reaction mechanism for peroxidase-type of conversion as proposed in the present study taking into account the intermediate enzyme-H_2O_2 complex [13-19] and the reversibility of compound I formation, as well as the existence of enzyme-AH_2 intermediates. E is the resting enzyme, E-H_2O_2 the enzyme-H_2O_2 intermediate, EO^{3+} compound I and EOH^{3+} the protonated compound II of horseradish peroxidase. EO^{3+}-AH_2 and EOH^{3+}-AH_2 are the enzyme-substrate intermediates.

$$v = \frac{(\frac{2k_2k_4k_6}{k_2k_4+k_2k_6+k_4k_6})E_0}{(\frac{(k_{-1}+k_2)k_4k_6}{k_1(k_2k_4+k_2k_6+k_4k_6)[H_2O_2]} + \frac{(k_{-3}+k_4)k_{-1}k_{-2}k_6}{k_1k_3(k_2k_4+k_2k_6+k_4k_6)[H_2O_2][AH_2]} + \\ + \frac{(k_2+k_{-2})(k_{-3}+k_4)k_5k_6+k_2k_3k_4(k_{-5}+k_6)}{k_3k_5(k_2k_4+k_2k_6+k_4k_6)[AH_2]} + 1)} \quad (3.3)$$

Assuming that binding of the substrate AH_2 to the enzyme is irreversible (i.e. $k_{-3} = k_{-5} = 0$), that $k_2 >> k_4$, and that $k_4 \sim 10 * k_6$ [2,12], eq. 3.3 can be simplified to eq. 3.4.

$$v = \frac{2k_6E_0}{1 + \frac{(k_{-1}+k_2)k_6}{k_1k_2[H_2O_2]} + \frac{(k_2+k_{-2})k_5k_6+k_2k_3k_6}{k_2k_3k_5[AH_2]} + \frac{k_{-1}k_{-2}k_6}{k_1k_2k_3[H_2O_2][AH_2]}} \quad (3.4)$$

From equation 3.4 it can be concluded that the maximum catalytic activity (k_{cat}) = $2k_6$. k_6 is the rate constant for the reaction of the EOH^{3+}-AH_2 complex to give product and native enzyme (E), i.e. the step for substrate oxidation by the so-called compound II. The fact that k_6 determines the maximum rate of conversion is in line with the previously reported observation that substrate oxidation by compound II is the rate limiting step in horseradish peroxidase catalysis [2,38,39,41-44]. The equation supports our previous conclusion [38] that the chemical characteristics (nucleophilicity) of the substrate determines its rate of oxidation. Furthermore, equation 3.4 indicates that plots of 1/v versus $1/[AH_2]$ at various $[H_2O_2]$ or plots of 1/v versus $1/[H_2O_2]$ at various $[AH_2]$ will not give parallel lines due the $[H_2O_2]\cdot[AH_2]$ term. The occurrence of this cross-term in the rate equation originates from the reversibility of the first half reaction, i.e. the contribution of $k_{-2}=k'_{-2}[H_2O]$. Since the reaction is performed in 55.5 M H_2O this backward reaction step can under no circumstances be ignored.

Altogether, we conclude that the reaction mechanism of horseradish peroxidase should not be described by the mechanisms previously proposed [12,15,16]. Based on results of the present study the mechanism as depicted in Figure 3.4c provides an improved alternative. This reaction sequence involves three subsequent cycles of substrate binding followed by product release. This implies that it extends the so-called ping-pong or double displacement mechanism by one additional substrate binding-product release cycle. Therefore, we now suggest to name this revised mechanism for horseradish peroxidase a triple displacement, or, more tempting, a ping-pong-pang mechanism.

3.4 Conclusion

In the present study the (ir)reversibility of compound I formation in heme-based catalysis was investigated. Results are provided that support the existence of an enzyme-H_2O_2 intermediate. In addition, it could be concluded that the reaction between peroxidase-type heme catalysts and H_2O_2, leading to formation of compound I and H_2O, is reversible. The results point at the reversibility of not only the first reaction step leading to formation of the enzyme-H_2O_2 complex but also of the consecutive reaction step in which the complex is converted to compound I and H_2O. Such oxygen exchange with H_2O by the high-valent-iron-oxo porphyrin complex of peroxidase-type heme catalysts seems to be in line with the results published previously by Macdonald et al. [32] for the, as yet unidentified, reactive high-valent-iron-oxo species of cytochromes P450. However, it has to be pointed out that the experiments of Macdonald et al. [33] were performed in the presence of iodosylbenzene.

Upon these findings and results reported previously [38,39] the kinetic model generally accepted for horseradish peroxidase had to be reconsidered. Thus, a possible reversible reaction of compound I formation could best be described using the modified ping-pong-pang type of mechanism described above in which binding of the substrate to compounds I and II is essential.

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4

Computer calculation-based quantitative structure- activity relationships (QSARs) for the oxidation of phenol derivatives by horseradish peroxidase compound II

Marjon J.H. van Haandel, Ivonne M.C.M. Rietjens, Ans E.M.F. Soffers, Cees Veeger, Jacques Vervoort, Sandeep Modi, Madhu S. Mondal, Prasanta K. Patel and Digambar V. Behere

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Abstract

The first-order and second-order rate constants for the oxidation of a series of phenol derivatives by horseradish peroxidase (HRP) compound II were compared to computer calculated chemical parameters characteristic for this reaction step. The phenol derivatives studied were phenol, 4-chlorophenol, 3-hydroxyphenol, 3-methylphenol, 4-methylphenol, 4-hydroxybenzoate, 4-methoxyphenol and 4-hydroxybenzaldehyde. Assuming a reaction of the phenolic substrates in their non-dissociated, uncharged forms, clear correlations (r= 0.977 and r= 0.905) were obtained between the natural logarithm of the rate constants (ln k_{app} and ln k_2 respectively) for their oxidation by compound II and their calculated ionisation potential, i.e. minus the energy of their highest occupied molecular orbital (E(HOMO)). In addition to this first approach in which the quantitative structure-activity relationship (QSAR) was based on a calculated frontier orbital parameter of the substrate, in a second and third approach the relative heat of formation ($\Delta\Delta$HF) calculated for the process of one-electron abstraction and $H^{\bullet}$-abstraction from the phenol derivatives was used as a parameter. Plots of the natural logarithm of the rate constants (k_{app} and k_2) for the reaction and the calculated $\Delta\Delta$HF values for the process of one-electron abstraction also provide clear QSARs with correlation coefficients of -0.968 and -0.926 respectively. Plots of the natural logarithm of the rate constants (k_{app} and k_2) for the reaction and the calculated $\Delta\Delta$HF values for the process of $H^{\bullet}$-abstraction provide QSARs with correlation coefficients

of -0.989 and -0.922 respectively. Since both mechanisms considered, i.e. initial electron abstraction versus initial $H^{\bullet}$-abstraction, provided clear QSARs the results could not be used to discriminate between these two possible mechanisms for phenol oxidation by HRP compound II.

The computer calculation-based QSARs thus obtained for the oxidation of the various phenol derivatives by compound II from HRP indicate the validity of the approaches investigated, i.e. both the frontier orbital approach and the approach in which the process is described by calculated relative heats of formation. The results also indicate that outcomes from computer calculations on relatively unrelated phenol derivatives can be reliably compared to one another. Furthermore, as the actual oxidation of peroxidase substrates by compound II is known to be the rate-limiting step in the overall catalysis by HRP, the QSARs of the present study may have implications for the differences in the overall rate of substrate oxidation of the phenol derivatives by HRP.

4.1 Introduction

The description of quantitative structure activity relationships (QSARs) for the conversion of compounds by either chemical or biochemical catalysts has since long been a tool in studies on enzyme catalysis. Routinely, QSARs have been based on Hammet sigma substituent constants for electronic effects, octanol/water partition coefficients for hydrophobicity effects, Taft substituent constants for steric effects or other empirically determined parameters. More recently, however, the more widespread use of computer programs for ab initio and semi-empirical molecular orbital and chemical calculations, provides an additional tool in studies on the role of chemical reactivity in enzyme catalysis and the description of QSARs for enzyme catalysed reactions.

Three approaches can be chosen for the description of QSARs based on computer-calculated parameters for the reaction studied here. First, the frontier orbital approach can be used, in which the outcome of a reaction is predicted on the basis of the frontier orbital characteristics of the reactants. Frontier orbital theory was originally developed in the field of organic chemistry to explain and predict regioselectivities in nucleophilic and/or electrophilic substitution reactions (1). More recently, frontier orbital calculations were shown to be a useful additional tool in studies on enzyme catalysis as well. Thus, molecular orbital-based QSARs (MO-QSARs) were described for the regioselectivity of the site of cytochrome P450-catalysed aromatic hydroxylation of a series of fluorobenzenes (r= 0.96) (2), for the rate of *para*-hydroxylation of a series of aniline derivatives in an iodosyl

benzene-driven cytochrome P450 reaction (r= 0.96) (3), for the overall rate of conversion of *para*-hydroxybenzoates and phenol derivatives by the flavin-dependent monooxygenases *para*-hydroxybenzoate-3-hydroxylase and phenol hydroxylase (r= 0.99 and 0.85 respectively) (4,5), and for the rate of glutathione S-transferase-catalysed conjugation of a series of fluorinated nitrobenzenes (r= 0.99) (6,7).

In a second approach to describe computer calculation-based QSARs the difference in heat of formation (ΔHF) of reactant(s) and the cation radical product resulting from one-electron abstraction is calculated. This approach is based on a more realistic model for calculating the ionisation potential, because the geometry and orbitals are allowed to relax in response to the removal of the electron. Furthermore, based on the existence of so-called Brønsted relationships (8), which describe the linear relationship between on the one hand the activation energy of a reaction, i.e. the energy difference between reactant(s) and transition state, and on the other hand the energy difference between reactants and products, it can be assumed that this ΔΔHF can also represent a relative measure of the activation energy for the one-electron abstraction. Therefore, this ΔΔHF can be related to the rate constant for the reaction by the Arrhenius equation.

In addition, the oxidation of the phenols by $H^{\bullet}$-abstraction instead of by initial electron abstraction was studied in a third approach, since initial $H^{\bullet}$-abstraction may represent an alternative for the initial electron abstraction for the reaction of phenolic substrates with HRP.

Computer-calculated energy differences between supposed reaction pathway intermediates and the starting compounds are generally calculated in vacuum and do not take into account entropy effects. Therefore, they only represent the free energy of activation ($\Delta G^{\ddagger}$), i.e. the free energy of a reaction, on a relative scale and are not an exact representation of the activation or reaction energy. Nevertheless, the assumption is made that the effect of solvation in the active site of the enzyme and the entropy effect of a similar reaction coordinate will affect the conversion of a series of substrates, converted in a similar reaction, to the same extent. Therefore the calculated energies can be expected to correctly reflect relative differences between the energies required for conversion of series of related compounds. For instance, the rate of the chemical as well as the glutathione S-transferase-catalysed conjugation of a series of fluoronitrobenzenes correlates (r= 0.97) with the relative heat of formation calculated for the conversion of the nitrobenzenes into their corresponding Meisenheimer complex reaction intermediates (6,7).

Up to now, however, computer calculation-based QSARs have mainly been restricted to series of highly related substrates, which varied only in the type, number and position of their halogen substituents. The objective of the present study was to investigate

whether the outcomes of computer calculations can provide a good basis to make QSAR plots for less related phenol derivatives. The phenols studied vary from phenol to 4-chlorophenol, 4-carboxyphenol, 3-hydroxyphenol, 3-methylphenol, 4-methylphenol, 4-methoxyphenol, 4-hydroxybenzaldehyde. The reaction studied is the oxidation of the phenol derivatives by compound II of HRP (EC 1.11.1.7; donor-H_2O_2 oxidoreductase). The oxidation of the phenolic substrates by compound II of HRP, is known to be the rate-limiting step of the multistep reaction cycle of this enzyme (9-11). Thus, any QSAR obtained may not only provide a means to predict the rate constant for the oxidation of the substrates by compound II but might also be indicative for relative changes in the overall rate of conversion of phenolic substrates by HRP.

4.2 Materials and Methods

Determination of k_{app} and k_2

The k_{app} for the oxidation of the different phenol derivatives by compound II from HRP was determined using stopped flow kinetic analysis. Experiments were performed on a "HiTech" microprocessor-controlled stopped flow machine (HiTech-500). The reaction mixture was maintained at 23 ± 0.5 °C by a circulating water bath with a thermostat. Lyophilised powder of crude HRP with $R_z = (A_{403}/A_{275}) = 1.5 - 2.0$ was obtained from Sigma (St. Louis, MO, USA). Hydrogen peroxide was of reagent grade. All other reagents were of analytical grade. Deionised water (PURITE RO 50) was used throughout the experiments. Isozyme HRP-C was purified by repeated ion-exchange chromatography on CM-cellulose (Whatman CM-52) using the procedure reported in the literature (12). The R_z of purified HRP was 3.2. The enzyme concentration was determined spectrophotometrically at 403 nm by using a molar extinction coefficient of 102 $mM^{-1}cm^{-1}$. HRP compound II was prepared from the purified native enzyme by adding 1 equivalent of H_2O_2 and 0.8 equivalent of $K_4Fe(CN)_6$. The concentration of the resultant compound was measured at 416 nm using a molar extinction coefficient of 105 $mM^{-1}cm^{-1}$. In the working syringe, the HRP compound II concentration was maintained at 3 μM. For each run the substrate solution was prepared by diluting a fresh stock solution in 0.01 mM phosphate buffer (pH 6.5 and ionic strength 0.05) to give a final concentration of the range 0.1 - 1.0 mM. A more than 10 fold excess of substrate over the enzyme concentration was always used to ensure that the reaction occurred by a pseudo-first-order process. The reaction was followed by monitoring the disappearance of HRP compound II at 423 nm. Usually 3-4 experiments were conducted for each substrate concentration and the data were stored directly in an on-

line computer memory. Pseudo-first-order rate constants k_{obs} were obtained by fitting the experimental trace to a single exponential function. Measurements were made at different substrate concentrations.

The relationship of the experimental pseudo-first-order rate constant (k_{obs}) to the actual apparent second-order rate constant (k_{app}) generally involves the contribution of the dissociation constant of binding of substrate with HRP compound II (13, 14). In short, the process of phenol oxidation by compound II is described by two subsequent steps, i.e. a binding equilibrium of the phenol to the compound II form of the enzyme, followed by oxidation of the phenol by compound II giving rise to the products (oxidised phenolic substrate and H_2O) and HRP-Fe^{3+}. Schematically this can be presented as follows:

$$\text{HRP } Fe^{IV}{=}O + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{HRP } Fe^{IV}{=}O{:}S \xrightarrow{k_2} \text{HRP } Fe^{3+} + \text{ product(s)}$$

$K_D = k_{-1}/k_1$ is the dissociation constant of the enzyme-substrate complex, and the observed reaction rate constant (k_{obs}) follows the expression:

$$k_{obs} = k_2[S]/\{K_D + [S]\} \qquad (4.1)$$

When $K_D >> [S]$ this last term can be approximated by $k_2\,[S]/K_D$. Thus, the apparent second-order rate constant k_{app} ($=k_2/K_D$) can be derived from the initial slope from plots in which the observed rate of the reaction (k_{obs}) is plotted against [S].

Molecular orbital and chemical calculations

Molecular orbital calculations were carried out on a Silicon Graphics Indigo^2 using Spartan version 4.0 (Wave function, Irvine, California). The semi-empirical molecular orbital method was used, applying the AM1 or the PM3 Hamiltonian. Because the results obtained with the AM1 Hamiltonian were in all cases similar to those obtained with the PM3, only the results of the PM3 calculations are presented. Open shell calculations were performed using the unrestricted Hartree Fock method. All calculations were carried out with PRECISE criteria. For all calculations the self-consistent field was achieved. Geometries were optimised for all bond lengths, bond angles and torsion angles using the BFGS criteria.

In this study, the outcomes of the semi-empirical calculations on molecules in vacuum are related to the electronic characteristics of the substrates bound to the hydrophobic cleft of compound II of HRP. Due to solvation effects and a different dielectric constant, the intrinsic properties of the compounds might be influenced upon this binding. However, it is assumed that this phenomenon will not influence the relative differences of parameters between the series of phenolic derivatives to a significant extent. The working hypothesis of the present study therefore includes the assumption that outcomes of the in vacuo computer calculations can be used as an approach to study relative differences within the series of phenolic compounds.

4.3 Results

First-order and apparent second-order rate constants

Second-order rate constants for oxidation of various aromatic substrates by HRP compound II have been obtained with the stopped-flow apparatus by several groups of investigators (10,13-19). However, the results are not always in agreement with each other, due to differences in the reaction conditions and the apparatus used. We have therefore measured the second-order rate constants of a series of phenol derivatives under the same experimental conditions, to use the outcomes for our QSAR plots. Pseudo-first-order traces were observed when the substrate concentration was present in excess over that of the HRP compound II. Figure 4.1 shows a typical pseudo-first-order trace measured at 423 nm, demonstrating the decrease of HRP compound II upon mixing with 4-methoxyphenol. At pH 6.5 and at relatively low substrate concentrations, the plot of k_{obs} vs 4-methoxyphenol was observed to be linear at low substrate concentration (i.e. $[S] << K_D$) with no apparent intercept on the vertical axis, suggesting that k_{app} is related to k_{obs} by the following simple expression:

$$k_{obs} = k_{app}\,[S] \qquad (4.2)$$

where [S] is the substrate concentration. The k_{app} values were determined by the least squares fit of the k_{obs} versus [S] data to equation 4.2 using Sigma Plot routine.

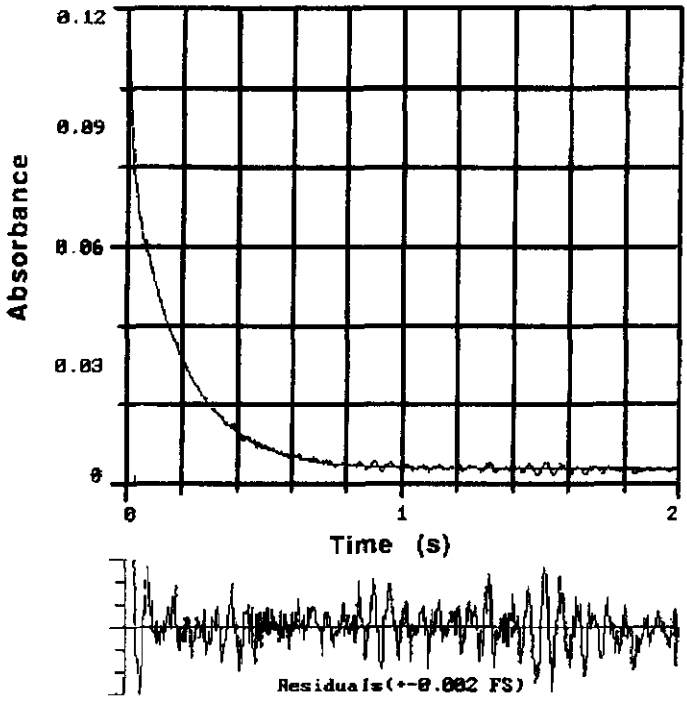

Figure 4.1 A typical stopped flow trace of the relative absorbance at 423 nm for the reduction of compound II of HRP by 4-methoxyphenol in phosphate buffer at pH 6.5 and at 23 °C. Ionic strength was 0.05 M. HRP-II was 1.5 μM and 4-methoxyphenol was 20 μM. The trace was fitted to a single exponential to obtain the pseudo-first-order rate constant (k_{obs}).

Table 4.1 presents the apparent second-order rate constants, k_{app}, for the reduction of HRP compound II in the presence of the different phenolic derivatives, as derived from similar experiments carried out at varying concentrations of the phenol derivatives. Table 4.1 also presents the first-order rate constant, k_2. The apparent second-order rate constants, k_{app}, and the first-order rate constants, k_2, vary by about two orders of magnitude for the different phenol derivatives studied.

The first-order rate constant, k_2, was calculated from k_{app} using the formula $k_{app}=k_2/K_D$. K_D values were deduced from the slope and intercept of the double reciprocal plots of k_{obs}^{-1} versus $[S]^{-1}$ (see equation 4.1) for all substrate concentrations. In the case of 4-methoxyphenol and 4-hydroxybenzoic acid the K_D values could not be deduced in this way because for these substrates $K_D > [S]$ at all substrate concentrations

Table 4.1 Apparent second-order rate constants (k_{app}), dissociation constants (K_D) and first-order rate constants (k_2) for the oxidation of C3 or C4 substituted phenol derivatives by HRP compound II.

compound	substituent	k_{app} (M^{-1} s^{-1})	ln k_{app}	K_D (mM)	k_2 (s^{-1})	ln k_2
phenol	4-H	2.5×10^5	12.43	2.4	6.0×10^2	6.40
4-chlorophenol	4-Cl	1.2×10^6	14.00	11.0	1.3×10^4	9.49
3-hydroxyphenol	3-OH	3.5×10^5	12.77	5.0	1.8×10^3	7.47
3-methylphenol	3-CH_3	4.1×10^5	12.92	5.1	2.1×10^3	7.65
4-methylphenol	4-CH_3	1.2×10^6	14.00	2.4	2.9×10^3	7.97
4-hydroxybenzoic acid	1-COOH	2.3×10^4	10.04	-	2.5×10^2	5.53
4-methoxyphenol	4-OCH_3	5.1×10^6	15.44	-	5.6×10^4	10.93
4-hydroxybenzaldehyde	1-CHO	5.0×10^4	10.82	3.5	1.8×10^2	5.16

tested giving rise to $k_{obs} = [S]k_2/K_D$. Thus, a double reciprocal plot does not provide the possibility to derive K_D separately. Therefore an approximated k_2 was used taking the maximal value for the K_D observed, i.e. the value for 4-chlorophenol (11.0 ± 1.0). The validity of this assumption is based on the observation that most K_D values do not vary by a factor more than 2-fold. In addition, k_2 values were calculated using estimated K_D values for 4-methoxyphenol and 4-hydroxybenzoezuur of 5 or 10 times the average K_D (see hereafter).

Molecular orbital calculations

Table 4.2 presents the energy of the highest occupied molecular orbital (E(HOMO)) as calculated using the PM3 Hamiltonian. Following Koopman's theorem, minus E(HOMO) represents the ionisation potential of the compounds, i.e. the energy required to take an electron from the molecule.

For 4-hydroxybenzoate the respective values for both the neutral and the deprotonated form of the compound were calculated, as these are known to exist in equilibrium in solution at pH 6.5. The results obtained show a variation in the energy of the HOMO for the various compounds from -9.59 for 4-hydroxybenzoic acid in its COOH form, to -8.71 eV for 4-methoxyphenol. Upon deprotonation of the COOH moiety of 4-hydroxybenzoic acid, its E(HOMO) increases, i.e. becomes less negative.

Table 4.2 Computer calculated parameters representative for the one-electron oxidation of C3 or C4 substituted phenol derivatives. $\Delta\Delta HF^{(1)}$ is calculated for the process of one-electron oxidation and $\Delta\Delta HF^{(2)}$ is calculated for the process of H•-abstraction.

compound	substituent	E(HOMO) (eV)	$\Delta\Delta HF^{(1)}$ (kcal/mole)	$\Delta\Delta HF^{(2)}$ (kcal/mole)
phenol	4-H	-9.17	12.08	2.47
4-chlorophenol	4-Cl	-9.01	7.10	1.82
3-hydroxyphenol	3-OH	-9.00	6.46	2.28
3-methylphenol	3-CH_3	-9.09	9.04	2.18
4-methylphenol	4-CH_3	-8.95	6.96	1.34
4-hydroxybenzoic acid	1-COOH	-9.59	19.60	4.39
deprotonated form	1-COO^-	-4.49	-110.58	-3.90
4-methoxyphenol	4-OCH_3	-8.71	0.00	0.00
4-hydroxybenzaldehyde	1-CHO	-9.49	17.77	3.94

Chemical calculations on heats of formation for one electron oxidation

Table 4.2 also presents the relative heats of formation ($\Delta\Delta HF$) calculated for the oxidation of the various phenol derivatives by either one-electron or H•-abstraction. These values were calculated by subtracting the heat of formation of the substrate from the heat of formation calculated for the one-electron oxidised substrate radical or for the H•-abstracted substrate radical, leading to a ΔHF value. The ΔHF values thus obtained were set to a relative scale in which the lowest ΔHF was set to zero, thus providing the relative ΔHF values, i.e. $\Delta\Delta HF$ values.

Computer calculation-based quantitative structure-activity relationships (QSARs)

Figure 4.2a presents the quantitative structure activity relationship (QSAR) obtained when the natural logarithm of the second-order rate constant k_{app} for the reduction of HRP compound II by the various phenol derivatives (Table 4.1) is plotted against their calculated E(HOMO) (Table 4.2). A clear correlation ($r = 0.977$) is observed. Figures 4.2b and 4.2c demonstrate that, when the natural logarithm of the second-order rate constant k_{app} is plotted against the calculated $\Delta\Delta HF$ values for one-electron oxidation and H•-abstraction respectively, clear correlations ($r = -0.968$ and $r = -0.989$ respectively) are also obtained. Since the k_{app} values still contain a contributionof K_D ($k_{app} = k_2/K_D$) one should, from a

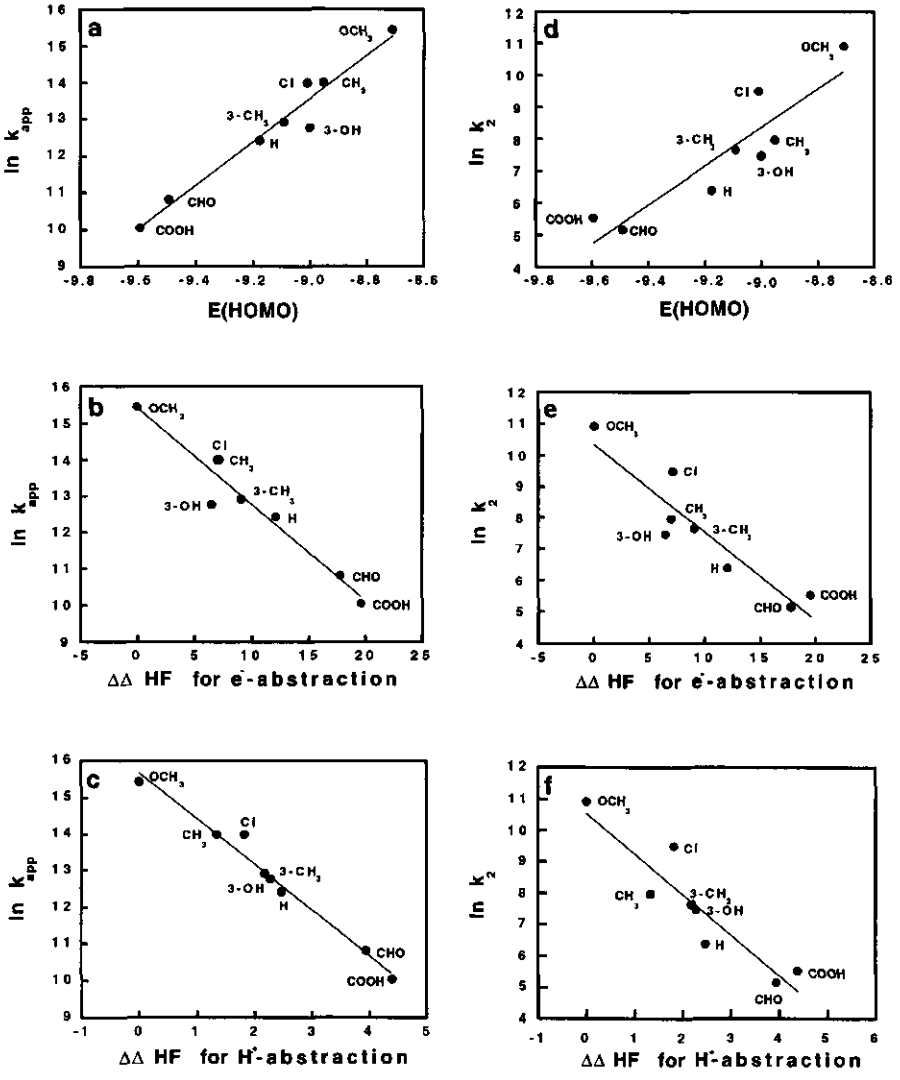

Figure 4.2 Computer calculation-based quantitative structure activity relationships (QSARs) for the apparent second-order rate constants, ln k_{app} (a-c), and first-order rate constants, ln k_2 (d-f), for oxidation of a series of phenol derivatives by HRP compound II. The phenol derivatives were para-substituted with respect to the hydroxyl group, unless differently indicated (i.e. meta-substituted (3-) with respect to the hydroxyl). The parameters used to describe the QSAR were (a,d) E(HOMO) of the phenol, minus E(HOMO) being representative for the ionisation potential, (b,e) the calculated relative heat of formation (ΔΔHF) for conversion of the phenol to its one-electron oxidised cation radical and (c,f) the calculated relative heat of formation (ΔΔHF) for conversion of the phenol to its H•-abstracted radical (c,f). The correlation coefficients of the plots a, b, c, d, e and f are 0.977, -0.968, -0.989, 0.905, -0.926 and -0.922 respectively.

theoretical point of view, make plots of the natural logarithm of k_2 and not k_{app} against the calculated reactivity parameters. Plots of the natural logarithm of the first-order rate constant k_2, calculated as $k_{app}*K_D$, against the computer calculated parameters E(HOMO), $\Delta\Delta$HF for one-electron oxidation and $\Delta\Delta$HF for H$^{\bullet}$-abstraction are presented in Figures 4.2d, 4.2e and 4.2f respectively, and they all show clear correlations ($r = 0.905$, $r = -0.926$ and $r = -0.922$ respectively). When the k_2 values for 4-methoxyphenol and 4-hydroxybenzoic acid were calculated using estimated K_D values of 5 to 10 times the average K_D of all others, QSARs for ln k_2 with correlation coefficients varying from 0.85 to 0.88 and from 0.79 to 0.83 respectively, were obtained.

It is especially interesting that the QSARs thus obtained hold for phenol derivatives with relatively unrelated type of C4 substituents varying from OCH_3 (4-methoxyphenol), to CH_3 (4-methylphenol), to H (phenol), to COOH (4-hydroxybenzoic acid), to CHO (4-hydroxybenzaldehyde), and with a substituent at another position in the aromatic ring, i.e. 3-CH_3 (3-methylphenol) and 3-OH (3-hydroxyphenol). Clearly, the results indicate that 4-hydroxybenzoate is actually reacting in its undissociated form. Using the values of E(HOMO) and $\Delta\Delta$HF for its COO^- form, the data point for 4-hydroxybenzoate would be far off the line.

4.4 Discussion

The present paper describes quantitative structure-activity relationships (QSARs) for the apparent second-order rate constant k_{app} ($M^{-1}s^{-1}$) and for the first-order rate constant k_2 (s^{-1}) for oxidation of a series of phenol derivatives by HRP compound II. The QSARs are based on computer-calculated chemical characteristics of these phenols.

Three types of strategies were applied to obtain the computer calculation-based QSARs. First, an approach based on frontier orbital considerations was used (20). The calculated parameter E(HOMO) of a series of C3 and C4-substituted phenols was taken as representative for the ionisation potential following Koopman's theorem. When E(HOMO) decreases it becomes more difficult to abstract an electron from this highest occupied molecular orbital, i.e. the ionisation potential (represented by minus E(HOMO)) becomes larger. The results of the present study demonstrate that the E(HOMO), i.e. the ionisation potential, indeed provides a good parameter to explain and even predict the actual rate of one-electron oxidation of the phenol model compounds by HRP compound II ($r = 0.977$ and $r = 0.905$ for k_{app} and k_2 respectively).

However, one cannot deny some disparities in the plots of ln k_{app} and ln k_2 versus E(HOMO) and $\Delta\Delta$HF. Calculating E(HOMO) and $\Delta\Delta$HF using different methods (STO-3G, 3-21G*, PM3 and AM1) results in QSAR plots (not shown) which reveal that especially the value of 4-chlorophenol shows a systematic tendency to deviate from the QSAR line in all cases. Interestingly, a deviating behaviour of 4-chlorophenol was also reported by Sakurada et al. (19), whose poor correlation, i.e. r = 0.873, between the k_{app} and the calculated E(HOMO) of similar substrates was mainly due to a deviating E(HOMO) value obtained by the ab initio method for 4-chlorophenol. One explanation for this phenomenon could be that in all calculation methods used chlorine substituents are not parameterised completely correctly. However, because the deviating behaviour of 4-chlorophenol is observed for all calculations this seems unlikely. Perhaps a more likely explanation might be related to the experimental k_{app} values. Because measurement of k_{app} in the present study and in the paper of Sakurada et. al. (19) gave a similar value, i.e. $k_{app} = 1.1 * 10^6$ $M^{-1}s^{-1}$ (19) compared to $1.2 * 10^6$ $M^{-1}s^{-1}$ (present study), it is unlikely that 4-chlorophenol is deviating from the QSAR line due to a wrong experimentally determined value for k_{app}. Therefore one has to conclude that the k_{app} for 4-chlorophenol may deviate from the QSAR because the electron transfer process in a reaction of 4-chlorophenol with compound II is different than for the other phenols studied. This could be, for example, due to a different binding orientation compared to the other phenols. That such a factor may influence the QSAR data points is supported by the observation reported previously (19) that correlations between ln k_{app} and E(HOMO) for anilines are also off line compared to the QSAR for phenols. Thus, difference in the binding orientation and/or binding site of a substrate to HRP, and, as a result, differences in the electron transfer process from the phenol to HRP compound II may cause data points to deviate from a specific QSAR line. Taking this deviating behaviour of 4-chlorophenol into account and leaving these data points from the plots results in correlation coefficients that are even higher, i.e. > 0.927 in all cases, than the ones reported here for the lines including 4-chlorophenol.

Furthermore, in contrast to the study of Sakurada et al. (19), the present study also presents the relationship between the natural logarithm of the first-order rate constant k_2 (s^{-1}) and E(HOMO). From a theoretical point of view, k_2 and not k_{app} is the correct kinetic constant to compare to E(HOMO) since k_2 no longer contains, as k_{app}, the K_D for binding of the substrate to the enzyme.

In a second approach, presented in this study, the relative heat of formation ($\Delta\Delta$HF) for conversion of the parent phenol into its one-electron oxidised cation radical was calculated as a parameter to define the ionisation potential. In addition, following the so-called Brønsted plots, $\Delta\Delta$HF might reflect on a relative scale the activation energy of the

reaction (8). This is the case because Brønsted plots define the correlation between the activation energy of a reaction and the energy difference between reactants and products. The results of the present study demonstrate a good correlation between the calculated ΔΔHF values for one-electron oxidation of the phenol derivatives and the natural logarithm of the rate constants for oxidation of the substrates by HRP compound II ($r = -0.968$ and $r = -0.926$ for k_{app} and k_2 respectively). This observation supports the validity of the assumptions made in this approach. The results also imply that all the phenols of the present study, perhaps except for 4-chlorophenol, are likely to be bound to the same binding site in a similar way, i.e. are indeed all in a similar chemical surrounding when they become oxidised. This is especially interesting because previous studies report that compounds like iodine, thioanisoles and phenols may bind at distinct sites when being converted by HRP (21). The results of the present study support the conclusion drawn by Sakurada et al. (22), who, based on nuclear magnetic resonance (NMR) relaxation studies, concluded that there is little difference in orientation of two other phenol derivatives, i.e. resorcinol and 2-methoxy-4-methylphenol, bound to HRP.

The reaction step investigated so far represents the pathway proceeding by an initial one-electron oxidation of the phenolic substrates by compound II of HRP. However, an alternative mechanism proceeding by an initial H$^{\bullet}$-abstraction has also been suggested. Thus, in contrast to the reports stating that the oxygen of the high-valence-iron-oxo species of HRP is not accessible for substrates (23-27), there is also evidence suggesting that relatively small substrates may interact with this oxygen (28), providing possibilities for H$^{\bullet}$-abstraction. Therefore, in a third approach, relative differences in heat of formation (ΔΔHF) between the parent compounds and the radicals formed by H$^{\bullet}$-abstraction from the phenolic OH were calculated. The plot in Figure 4.2c indicates that plotting ln k_{app} versus these ΔΔHF values results in a QSAR with a correlation coefficient of -0.989, the correlation with ln k_2 was -0.922 (Figure 4.2f). Thus, the QSARs obtained for H$^{\bullet}$-abstraction are comparable to those for initial electron abstraction. This implies that on the basis of these results discriminating between the two possible mechanisms, i.e. initial H$^{\bullet}$-abstraction versus intial electron abstraction, is not possible.

It is also of importance to notice that the reaction step investigated in the present study, i.e. the oxidation of the phenolic substrates by compound II of HRP, is known to be the rate-limiting step in the multistep reaction cycle of the peroxidase reaction (9,10). The HRP reaction proceeds by sequential heterolytic cleavage of the oxygen-oxygen bond of the peroxide leading to the so-called compound I, followed by the one-electron oxidation of the first substrate molecule by compound I leading to formation of compound II, which, in turn, gives rise to the one-electron oxidation of a second substrate molecule leading to

regeneration of the Fe^{3+} resting state of the enzyme. Because this oxidation of the substrate molecule by compound II is known to be the rate-limiting step in catalysis, it can be envisaged that the correlations now described for this rate-limiting step should also hold when the overall rate of conversion of the respective compounds by HRP is determined. Support for this hypothesis is provided by the correlation of the voltametrically determined oxidation potentials of substituted thioanisoles with their overall rates of conversion by HRP (28) or other peroxidases (29), and by Candeias et al. (30), who showed that the rates of reaction of HRP compound I with indole-3-acetic acid derivatives are related to the reduction potential of the respective radicals, determined by pulse radiolysis. Calculation of the E(HOMO) values as well as the ΔΔHF values for the compounds for which k_{cat} values for the overall rate of conversion by HRP were reported by Kobayashi et al. (28) results in the data presented in Table 4.3. The coefficients of correlation between the natural logarithm of the k_{cat} obtained by Kobayashi et al. (28) and the values calculated for E(HOMO) and ΔΔHF of the substrates (Table 4.3) are 0.999 and -0.996 respectively (Figure 4.3). This result corroborates that the theoretical computer-calculated parameters described in the present study to explain the rate constants for the one-electron oxidation of substrates by HRP compound II may also be useful for obtaining insight into overall rates of catalysis by HRP. To what extent QSARs for the overall rate of conversion of substrates like phenols and thioanisoles can be compared, in spite of the different binding sites for these type of substrates in HRP (22), is a matter presently under investigation.

Table 4.3 Calculated E(HOMO) and ΔΔHF values for the one-electron oxidation of sulphur compounds for which k_{cat} values for the overall rate of S-oxidation have been reported in the literature (22).

Substrate	C4-substituent	E(HOMO) (eV)	ΔΔHF (kcal/mole)	k_{cat}[a)] (s^{-1})	ln k_{cat}
4-isopropoxythioanisole	$(CH_3)_2$-CHO	- 8.35	0	0.40	-0.92
4-methoxythioanisole	CH_3O	- 8.37	1.44	0.32	-1.13
4-methylthioanisole	CH_3	- 8.46	5.38	0.07	-2.68
thioanisole	H	- 8.54	8.47	0.02	-3.83

a) k_{cat} (min^{-1}) taken from Kobayashi et al. (23)

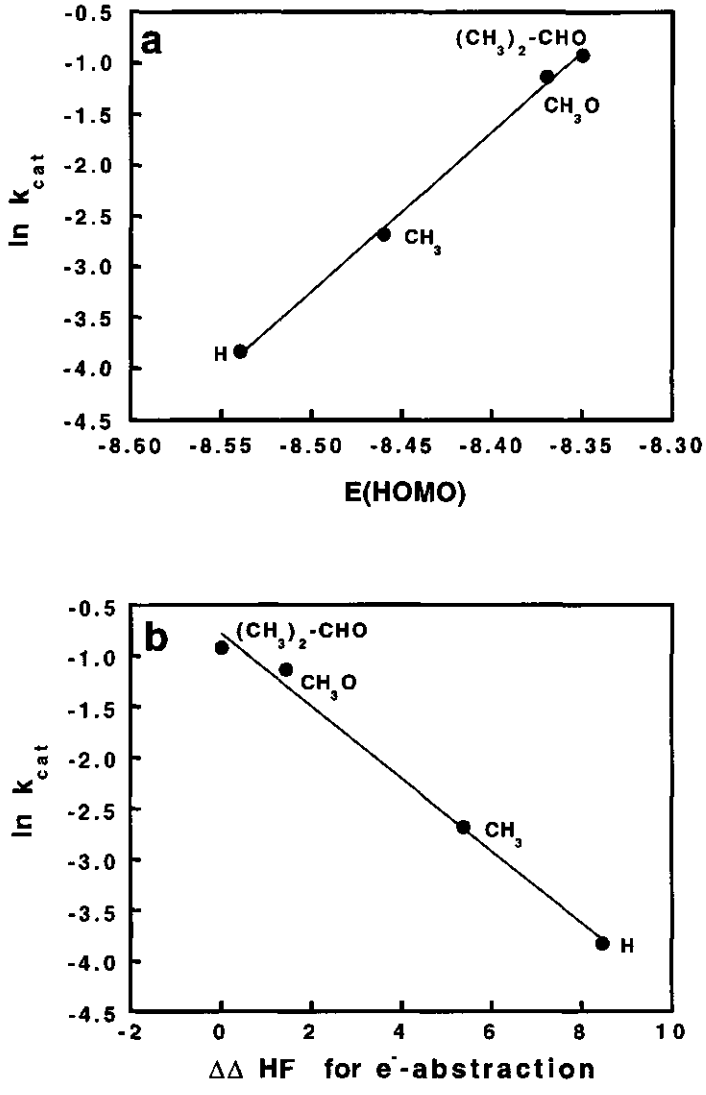

Figure 4.3 Computer calculation-based quantitative structure activity relationships (QSARs) for the k_{cat} values for the overall rate of S-oxidation of sulphur compounds by HRP (22). The parameters used to describe the QSAR were (a) E(HOMO) of the sulphur compound, minus E(HOMO) being representative for the ionisation potential and (b) the calculated relative heat of formation (ΔΔHF) for conversion of the sulphur compound to its one-electron oxidised cation radical. The correlation coefficients of the plots a and b are 0.999 and -0.996 respectively.

Acknowledgements

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5

Differential substrate behaviour of phenol and aniline derivatives during conversion by horseradish peroxidase

Marjon J. H. van Haandel, Margo M. J. Claassens, Neline van der Hout, Marelle G. Boersma, J. Vervoort, Ivonne M. C. M. Rietjens

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Abstract

For the first time saturating overall k_{cat} values for horseradish peroxidase (HRP) catalysed conversion of phenols and anilines are described. These k_{cat} values correlate quantitatively with calculated ionisation potentials of the substrates. The correlations for the phenols are shifted to higher k_{cat} values at similar ionisation potentials as compared to those for anilines.

^{1}H NMR T_1 relaxation studies, using 3-methylphenol and 3-methylaniline as the model substrates, revealed smaller average distances of the phenol than of the aniline protons to the paramagnetic Fe^{3+} centre in HRP. This observation, together with a possibly higher extent of deprotonation of the phenols than of the anilines upon binding to the active site of HRP, may contribute to the relatively higher HRP catalysed conversion rates of phenols than of anilines.

5.1 Introduction

Horseradish peroxidase (EC 1.11.1.7, donor-H_2O_2 oxidoreductase) (HRP) is a heme peroxidase that is able to catalyse the oxidation of a wide range of aromatic compounds. The oxidation of the substrates by HRP Compound II is known to be the rate-limiting step of the multistep reaction cycle [1-5]. Linear relationships between the natural logarithm of the second order rate constant for the oxidation of series of phenols and anilines by HRP Compound II and the ionisation potentials of the substrates have been reported [6-10]. These studies already indicate that the rates of oxidation of phenols by HRP Compound II were one to three orders of magnitude higher than those for anilines with similar ionisation potentials [8-9].

In the present study it was investigated whether this difference was still observed when overall rates of conversion (k_{cat}) are measured and also what could be the possible mechanisms underlying this differential substrate behaviour of phenol and aniline derivatives upon conversion by HRP.

5.2 Materials and methods

Chemicals

4-Methoxyphenol and 4-hydroxybenzaldehyde were obtained from Acros Organics (New Jersey, USA), 3-methylphenol and 4-methylphenol from Aldrich-Chemie (Steinheim, Germany), and phenol from Merck (Darmstadt, Germany). Aniline, 3-methylaniline, 4-methylaniline and 4-methoxyaniline were obtained from Janssen (Beerse, Belgium). All substrates were of 98-99% purity. Hydrogen peroxide (30%) was obtained from Merck and was diluted in demineralised water to obtain the required stock solutions. HRP was obtained from Boehringer (Mannheim, Germany). All other reagents used were analytical grade.

Incubation conditions

A typical reaction mixture (total volume of 1.0 ml) contained (final concentrations): 1 - 500 nM HRP depending on reactivity of the substrate (Table 5.1) and 1.0 - 9.0 mM substrate (as indicated) in 0.1 M potassium phosphate (pH 7.6). Phenolic substrates were added as 1% (v/v) of a 100 times concentrated stock solution in dimethyl sulfoxide. The reaction mixture was preincubated at 37 °C for two minutes. The reaction was started by the addition of hydrogen peroxide, carried out for one minute at 37 °C, and then stopped by freezing in liquid nitrogen. The rate of conversion, v (s^{-1}), was determined by measuring the

decrease in the concentration of the substrates after 1 minute of incubation, using high-performance liquid chromatography (HPLC).

Under the reaction conditions described above, activity was demonstrated to be linear with time (up to at least 20 minutes) and with the concentration of HRP (up to at least 1 μM) (data not shown). From kinetic experiments in which the concentration of hydrogen peroxide and the phenol or aniline concentrations were varied, k_{cat} (s^{-1}) at infinite H_2O_2 and infinite phenol or aniline concentrations was determined. This was done by fitting kinetic experimental data to the Michaelis-Menten equation $v = k_{cat} * [S]/(K_m + [S])$ as also described by others studying conversion of guaiacol, *p*-cresol and 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) by HRP [11].

Table 5.1 Experimental conditions in the reaction mixture for incubation of the respective phenol and aniline derivatives with HRP and specification of the λ_{max} and methanol/H_2O eluens for HPLC analysis of these incubations. The final concentration of HRP was chosen in such a way that between 10 and 20% of the substrate was converted in a linear way in 1 minute. The percentages of methanol and H_2O were chosen in such a way that the substrate peak eluted within 5 minutes.

nr.	Substrate	[HRP]	λ_{max}	HPLC eluens	
		(nM)	(nm)	% methanol	% H_2O
1	phenol	5	269	47	53
2	3-methylphenol	10	271	60	40
3	4-methylphenol	10	278	57	43
4	4-methoxyphenol	1	289	45	55
5	4-hydroxybenzaldehyde	500	283	60*	40*
6	aniline	500	280	30 -› 60	70 -› 40
7	3-methylaniline	250	285	50 -› 80	50 -› 20
8	4-methylaniline	50	287	50 -› 80	50 -› 20
9	4-methoxyaniline	10	295	30 -› 60	70 -› 40

* For 4-hydroxybenzaldehyde the eluens was supplemented with 1% of acetic acid, and a reversed phase adsorbosphere C18 column (150 x 4.6 mm) was used.

High-performance liquid chromatography (HPLC)

A volume of 10 μl of defrozen sample was injected into a HPLC system (Waters™ 600 controller) equipped with a reversed-phase lichrosphere RP8 column (150 x 4.6 mm). Ultraviolet detection was performed at the absorption maximum of the respective phenol or aniline (Table 5.1) using a photodiode array detector (Waters™ 996). The substrate concentration was quantified using a calibration curve consisting of the HPLC peak area plotted against the concentration of the compound in an injected series of stock solutions.

For the phenols, isocratic elution of methanol and water at a ratio which was optimised for the respective phenols (Table 5.1) was carried out for 10 minutes at 1.0 ml/min. For 4-hydroxybenzaldehyde the eluens was supplemented with 1% of acetic acid, and a reversed-phase adsorbosphere C18 column (150 x 4.6 mm) was used. For anilines a linear gradient of methanol and water was used decreasing the % of H_2O in 10 minutes as indicated in Table 5.1.

Molecular orbital and chemical calculations

Molecular orbital calculations were carried out on a Silicon Graphics Indigo^2 using Spartan version 4.0 (Wave function, Irvine, California, USA). The ab initio method, applying the 6-31G* basis set was used. Geometries were fully optimised.

In this study, the outcomes of the calculations on molecules in vacuum are related to the electronic characteristics of the substrates bound to HRP. Due to solvation effects and a different dielectric constant, the intrinsic properties of the compounds might be influenced upon this binding. However, it is assumed that this phenomenon will not influence the relative differences between parameters within a series of similar compounds to a significant extent. The working hypothesis of the present study therefore includes that outcomes of the in vacuum computer calculations can be used as an approach to study relative differences within the series of phenol or aniline compounds.

^{1}H NMR T_1 relaxation measurements

^{1}H NMR T_1 relaxation measurements were performed on a Bruker AMX 500 NMR spectrometer at 293 K. The longitudinal relaxation time (T_1) was determined by a 180°-τ-90° inversion recovery method. The T_1 relaxation time could be derived from a plot of the natural logarithm of the peak area at infinite delay time (M_∞) minus the peak area at the specific delay time τ (M_τ) against this delay time τ giving a straight line with a slope of -$1/T_1$. The incremental delay time (τ) for the samples containing aniline and for the samples containing phenol was 0.4 or 0.3 seconds respectively. About 16 delay times (τ) were applied and 32 scans for each τ value were recorded. A 10 seconds relaxation delay was used in between the scans. Samples contained 0.1 M potassium phosphate in D_2O (for locking the magnetic field) (pD 7.6) or a solution of HRP (final concentration 2 μM) in this buffer, 0.1 mM EDTA, 2 mM substrate added from a 110 mM stock solution in dimethyl sulfoxide D6. The total sample volume was 550 μl. Samples were made oxygen-free by four cycles of evacuation and filling with argon.

To correct for the diamagnetic contribution to the longitudinal relaxation time, the T_1 of a control sample containing substrate free in solution in absence of HRP was determined.

The corrected T_1 value ($1/T_{1\ corr} = 1/T_{1\ observed} - 1/T_{1\ blank}$) was used to calculate the distance (r) of the respective proton to the paramagnetic iron essentially as described by Novak and Vatsis [12] using the Solomon-Bloembergen equation, assuming the Fe^{3+} in HRP to be in the high spin conformation [13], and taking a τ_c value of 5×10^{-11} s. This value of 5×10^{-11} s is considered to be suitable for HRP [13-15].

5.3 Results

Overall rates of conversion

In order to determine the overall rate of conversion of phenol and aniline derivatives by HRP, incubations were carried out using a range of aromatic substrate concentrations at several concentrations of hydrogen peroxide. Clear non-infinite k_{cat} values at infinite H_2O_2 and substrate concentrations were obtained.

As an example Figure 5.1a presents the rate of conversion, v (s^{-1}), of 4-methoxyphenol by HRP versus the initial concentration of 4-methoxyphenol at a H_2O_2 concentration of 1.0 mM. The apparent k_{cat} (i.e. $k_{cat,app}$) at infinite 4-methoxyphenol concentration and 1.0 mM H_2O_2 could be derived by fitting the Michaelis-Menten equation to the experimental data. In this way $k_{cat,app}$ values were obtained at different concentrations of H_2O_2. The various $k_{cat,app}$ values thus obtained were plotted versus the concentration of hydrogen peroxide (see for example Figure 5.1b for 4-methoxyphenol) to obtain the final k_{cat} at infinite phenol and infinite H_2O_2 concentration. Table 5.2 presents the k_{cat} values for the overall conversion of the different phenol and aniline derivatives by HRP thus obtained, as well as K_m values, presented as the range of $K_m[AH_2]$ values of a substrate obtained at different H_2O_2 concentrations. According to the recently modified kinetic model for HRP, describing ping-pong kinetics including substrate binding equilibria [16], the K_m $[AH_2]$ values are dependent on the concentration of H_2O_2 at which they are measured. However, the experimental uncertainty in the values for K_m obtained in the present type of assay, where substrate decrease and not product formation can be quantified, is relatively high. Therefore, Table 5.2 indicates only the range of K_m values obtained at different H_2O_2 concentrations. No further kinetic evaluation of the K_m values was undertaken. This also because for the present study especially k_{cat} and not K_m values are of interest.

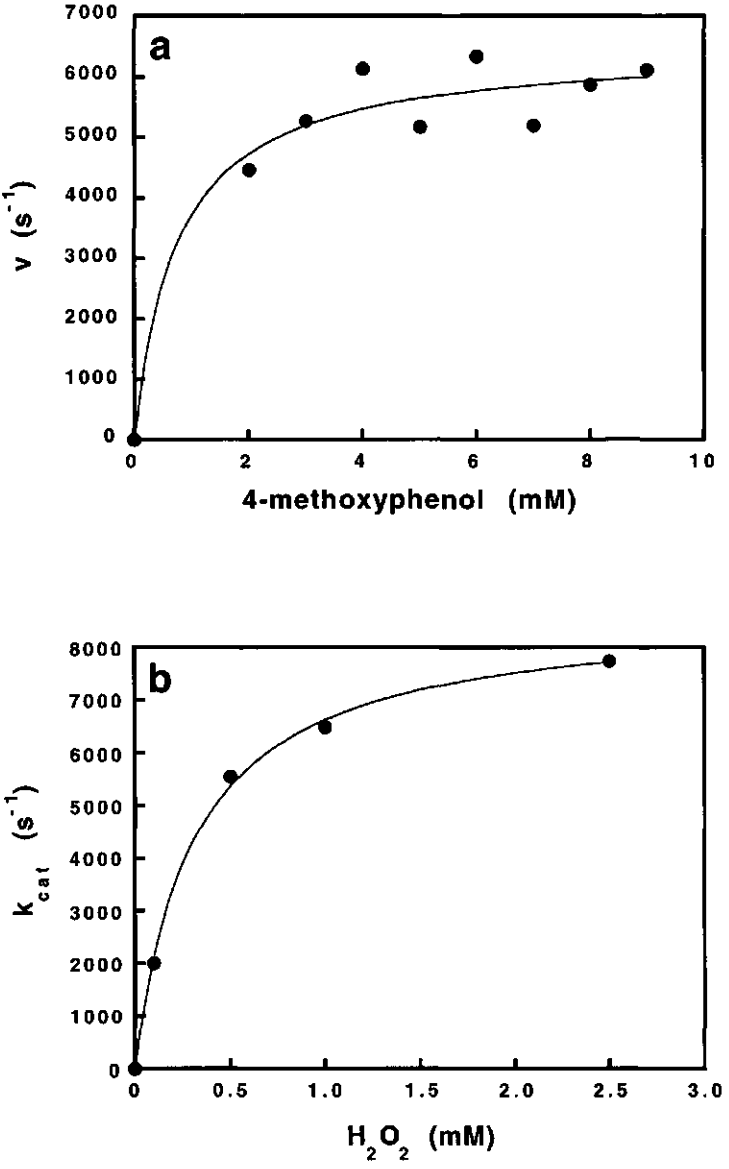

Figure 5.1 Determination of a) the $k_{cat,app}$ at 1.0 mM H_2O_2 and b) the k_{cat} for the conversion of 4-methoxyphenol by horseradish peroxidase. The correlation coefficient of the fit of the data to the Michaelis-Menten equation was 0.975 (Figure 5.1a) and 0.999 (Figure 5.1b).

Table 5.2 The k_{cat} and $K_{m,app}$ values for overall conversion of phenol and aniline derivatives by HRP and computer calculated E(HOMO) values, representing the negative of the ionisation potential of phenol and aniline derivatives. An ab initio method, using the 6-31G* basis set, was applied.

nr.	compound	k_{cat} (s^{-1})	ln k_{cat}	$K_{m,app}(H_2O_2)$ (mM)	$K_{m,app}(AH_2)$ (mM)	E(HOMO) (eV)
1	phenol	5.6×10^2	6.3	0.1 ± 0.1	2.0 - 3.8	-8.41
2	3-methylphenol	1.4×10^3	7.2	0.5 ± 0.3	1.4 - 3.6	-8.28
3	4-methylphenol	2.7×10^3	7.9	0.1 ± 0.1	1.0 - 1.8	-8.15
4	4-methoxyphenol	1.1×10^4	9.3	0.3 ± 0.1	0.7 - 1.7	-7.83
5	4-hydroxybenzaldehyde	6.3×10^1	4.1	0.4 ± 0.1	2.1 - 5.1	-8.88
6	aniline	3.0×10^1	3.4	0.2 ± 0.1	0.8 - 1.0	-7.87
7	3-methylaniline	3.2×10^1	3.5	0.2 ± 0.1	0.2 - 0.4	-7.80
8	4-methylaniline	2.9×10^2	5.7	0.2 ± 0.1	0.3 - 0.7	-7.66
9	4-methoxyaniline	1.8×10^3	7.5	0.5 ± 0.2	0.4 - 0.6	-7.43

Molecular orbital and chemical calculations

Table 5.2 also presents the energy of the highest occupied molecular orbital (E(HOMO)) as calculated using the ab initio method with a 6-31G* basis set. Following Koopman's theorem, minus E(HOMO) represents the ionisation potential of the compounds, i.e. the energy required to take an electron from the molecule.

Computer calculation-based quantitative structure activity relationship (QSAR)

Figure 5.2a presents the QSAR obtained when the natural logarithm of the overall rate of conversion, ln k_{cat}, of the various phenol and aniline derivatives by HRP (Table 5.2) is plotted against their calculated E(HOMO) (Table 5.2). Clear correlations (r= 0.998 and r=0.984 respectively) are observed. The correlations for the phenols are shifted to higher overall k_{cat} values at similar ionisation potentials as compared to those for aniline substrates.

Figure 5.2b presents the QSAR obtained when experimentally obtained half- wave potentials (E1/2) of the respective phenols and anilines [17] are plotted against ln k_{cat} values of the present study. The QSAR lines for the anilines and phenols still deviate by about 2 to 4 orders of magnitude.

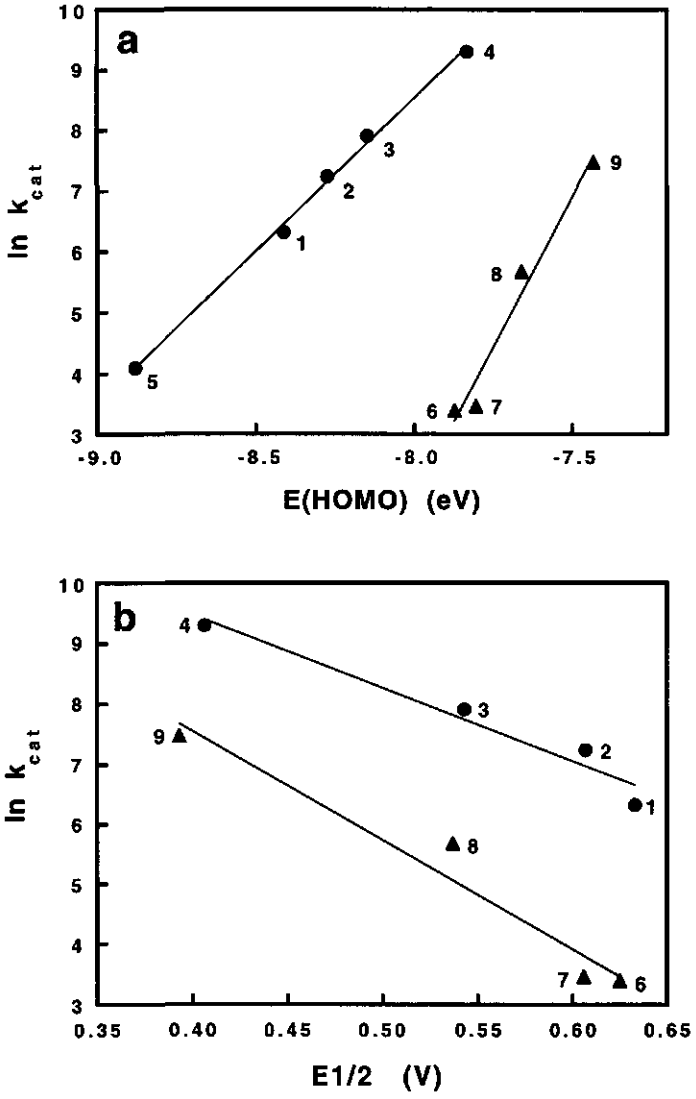

Figure 5.2 Computer calculation-based QSARs describing the relationship between a) the ln k_{cat} for conversion of phenol and aniline derivatives by HRP and their respective E(HOMO) (the correlation coefficients are 0.998 and 0.984 for phenols and anilines respectively), and b) the ln k_{cat} for conversion of a series of phenol and aniline derivatives by HRP and their respective experimentally obtained halve wave potentials (E1/2) [17] (the correlation coefficient is 0.976 for both phenols and anilines). The numbers correlate to the compounds as presented in Table 5.2.

^{1}H NMR T_1 relaxation measurements

T_1 values were obtained for the phenyl protons of 3-methylphenol and 3-methylaniline in the presence of HRP, and T_1 values for the blank samples were also obtained (data not shown). From these T_1 and T_1(blank) values the distances of the respective protons to the Fe^{3+} centre in the active site of HRP were calculated with a τ_c of 5 x 10^{-11} [13-15], and these values are presented in Figure 5.3. The results obtained indicate that the average distances of the various aromatic protons of the phenol derivative to the Fe^{3+} centre are consistently smaller than those observed for the aromatic protons of the aniline derivative.

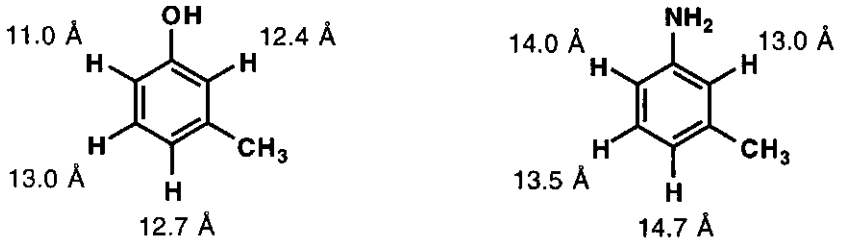

Figure 5.3 Distances between protons of 3-methylphenol (left) or 3-methylaniline (right) and the Fe^{3+} of HRP, obtained from T_1 relaxation measurements, calculated using 5 x 10^{-11} for τ_c.

5.4 Discussion

The present study describes the overall k_{cat} values for the conversion of a series of phenol and aniline derivatives by HRP, illustrating, for the first time, saturating overall kinetic behaviour of HRP. This is especially of interest because previously described models for the overall kinetic behaviour of HRP suggest a modified ping-pong mechanism in which no K_m values, i.e. no saturating kinetics, would be obtained due to the irreversible nature of the reactions leading to Compound I and the absence of substrate-HRP complexes [18]. Recently however, on the basis of the observation of HRP catalysed oxygen exchange between $H_2{}^{18}O_2$ and water, pointing at reversible Compound I formation, we already suggested an alternative kinetic model [16] which would be in line with saturating kinetics, as demonstrated to occur in the present study.

Saturating kinetics have not been observed in previous studies [9,19]. This might be ascribed to the fact that substrate concentrations generally applied were in the 1-100 μM

instead of the mM range. Results of the present study indicate that $K_{m,app}$ values are in the mM range, comparable to the mM range of the K_D values reported by Patel et al. [19] for the binding of substrates to Compound II of HRP.

The overall rates of conversion, k_{cat} (s^{-1}), obtained in the present study are comparable to the second order rate constants, k_2, reported previously for the rate limiting step of the reaction, i.e. the substrate oxidation by HRP Compound II [10]. The correlation between the two data sets is 0.952. Absolute differences are small and most likely due to differences in assay conditions. The fact that the second order rate constants, k_{app}, as reported by Sakurada et al. [8] are three orders of magnitude higher than k_2 [10] and the overall k_{cat} values of the present study, is due to the fact that these k_{app} values still contain the K_D, the dissociation constant for binding of the substrate to HRP Compound II. Correction of k_{app} by the K_D values which are in the mM range results in k_2 values comparable to the k_{cat} data of the present study ($k_{app} = k_2/K_D$ with k_2 being the real second order rate constant [10]).

Comparison of the natural logarithm of the overall k_{cat} values to calculated ionisation potentials or experimental half-wave potentials (E1/2) [17] of the phenol and aniline derivatives reveals clear QSARs (Figure 5.2). The QSAR lines for the anilines and phenols appear to deviate by about 2 to 4 orders of magnitude indicating that the overall rates of oxidation of phenols are about 2 to 4 orders of magnitude higher than those for the anilines of similar ionisation potential. This observation for the overall k_{cat} values is in line with the observation previously reported for the QSAR for the k_{app} data obtained for conversion of phenols and anilines by HRP Compound II [8,9], indicating that the discrepancy between the two QSARs is related to the substrate oxidation step.

^{1}H NMR T_1 relaxation studies were performed, using 3-methylphenol and 3-methylaniline as the model substrates, to investigate whether differences in orientation of phenols and anilines within the active site of HRP may be a factor underlying the higher reactivity of the phenols than expected on the basis of their ionisation potential. ^{1}H NMR T_1 relaxation studies reported so far for binding of substrates to HRP have studied phenols not anilines [13-15,20]. The values obtained for the distances between the protons of the phenols and the paramagnetic Fe^{3+} centre in the present study are in line with literature data for other phenolic substrates and inhibitors [13-15,20]. The results obtained in the present study, however, for the first time report consistently shorter time-averaged distances between the protons of the phenol and the Fe^{3+} of HRP, than between the protons of the aniline and the Fe^{3+} of HRP. This points at a slightly shorter time-averaged distance of the phenolic substrate than of the aniline compound, to the heme of HRP. This difference in orientation may result in differences in the electron transfer process from the aromatic donor

substrate to the Compound II of HRP. A shorter distance between the phenol and the heme than between the aniline and the heme may be a factor contributing to the faster rate of electron transfer with phenol as compared to aniline substrates. However, the actual differences in orientation seem small when the 2-4 orders of magnitude difference in oxidation rate of phenols or anilines at similar calculated ionisation potential are considered.

Recently, the X-ray crystal structure of HRPC with bound benzhydroxamic acid has been reported [21]. Comparison of previous ^{1}H NMR T_1 relaxation measurements on binding of benzhydroxamic acid and phenol substrates like resorcinol and 2-methyl-4-methylphenol [13] to our data on 3-methylphenol point at similar binding orientations for these phenolic aromatic donor molecules. Thus, the 3D model for the binding of benzhydroxamic acid can be taken as a model for the binding of the aromatic substrates of the present study, too. This would imply a binding orientation in which the aromatic ring is positioned in a hydrophobic pocket and the hydroxyl, or amino moiety would be in hydrogen bonding contact with especially the carbonyl of Pro139 and the imidazole nitrogen of His42 [21]. Since the pKa for deprotonation of the hydroxyl moiety of a phenol is lower (pKa phenol = 9.89) [22] than the pKa for deprotonation of an aromatic amino group (pKa > 14) [22], it can be suggested that the extent of deprotonation upon binding to the active site may be higher for the phenol than for the aniline. Since (partial) deprotonation will largely influence, i.e. decrease, the ionisation potential of the aromatic substrate, the relatively higher oxidation rates of phenols may be related to their larger extent of deprotonation upon binding to the substrate pocket of HRP, resulting in lower ionisation potentials than actually expected on the basis of calculations on their non-ionised form. This larger extent of (partial) deprotonation of a phenol than of an aniline could then, together with the somewhat larger distance of the aniline than of the phenol to the heme of HRP, provide an explanation for the differential substrate behaviour of the two series of compounds, and also for the different positions of their QSAR lines for oxidation by HRP when these lines are based on the ionisation potential calculated for the fully protonated forms. The fact that initial products from HRP catalysed phenol oxidation were reported to be phenoxy radicals rather than phenol carbocations [23] would be in line with the requirement for substrate deprotonation preceding and/or accompanying and thereby influencing the rate of electron transfer. Thus, the newly identified substrate binding pocket [21] in combination with the ^{1}H NMR T_1 relaxation data of the present study support the binding of phenols and anilines to the same binding pocket as observed for benzhydroxamic acid [21], and support the hypothesis that the differential substrate behaviour of phenols and anilines may be due to subtle differences in their binding to the active site substrate pocket

of HRP, resulting in i) closer proximity to the heme and ii) larger extent of deprotonation for the phenols than for the aniline substrates.

Acknowledgements

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6

Characterisation of different commercial soybean peroxidase preparations and the use of the enzyme for the N-demethylation of methyl-N-methylanthranilate to produce the food flavour methylanthranilate

Marjon J. H. van Haandel, Florence C.E. Sarabèr, Marelle G. Boersma, Colja Laane, Yvette Fleming, Hugo Weenen, Ivonne M. C. M. Rietjens

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Abstract

The potential of different peroxidase preparations for the N-demethylation of methyl-N-methylanthranilate (MNMA) to produce the food flavour methylanthranilate (MA) was investigated. All tested peroxidase preparations were able to catalyse the N-dealkylation.

The tested soybean preparations vary widely with respect to their heme content. Furthermore, the operational stability of purified soybean peroxidase (SP) is at least 25-fold lower than that of horseradish peroxidase (HRP) and only 5-fold higher than that of microperoxidase-8 (MP8). Thus, the presence of a large protein chain around a porphyrin cofactor in a peroxidase is, by itself, insufficient to explain the observed differences in operational stability.

Despite its relatively low operational stability, SP proved to be the most efficient biocatalyst for the production of MA with high yield and purity, especially observed at the high temperature and low pH values at which SP appeared to be optimally active.

6.1 Introduction

Heme-based enzymes like the peroxidases and the mammalian cytochromes P450 are among the most versatile enzymes in biochemistry, able to catalyse the conversion of a wide range of substrates in many different types of reactions. Among these are aromatic and aliphatic hydroxylations, heteroatom dealkylations, epoxidations, (de)halogenations and heteroatom oxygenations (Dawson, 1988; Griffin, 1991; Ortiz de Montellano, 1992). From a biotechnological point of view the use of peroxidases is favoured over that of cytochromes P450 because peroxidases can be more easily obtained and use H_2O_2 as a cheap and clean oxidant to create the so-called high-valent-iron-oxo-porphyrin intermediates required for catalysis (Chance, 1952; Dolphin et al., 1971; Groves et al., 1981; Hoffman et al., 1979; Low et al.; 1996; Palcic et al., 1980; Thomas et al., 1970). Many different peroxidases are known (Diehn, 1993; Dunford and Stillman, 1976; Patterson and Poulos, 1995; Schuller et al., 1996; Sessa and Anderson, 1981). Of all these peroxidases HRP is the one best studied from mechanistic and catalytic points of view (Dunford, 1991; Jones and Suggett, 1968a/b; Rodriguez-Lopez et al., 1996a/b; Van Haandel et al., 1998). However, biotechnological application of HRP is seriously hampered by the fact that the enzyme is relatively expensive and has a limited thermostability (McEldoon and Dordick, 1996). In contrast to HRP, SP has been reported to show better temperature behaviour (McEldoon et al., 1995; McEldoon and Dordick, 1996; Toiguchi et al, 1989). Furthermore, SP is more economical for use in biotechnological applications because it can be obtained relatively easy from soybean hulls, a major byproduct of the food industry. Finally, MP8, being a heme-based mini-enzyme with a potentially wide substrate specificity and able to function at relatively high temperatures as well (Aron et al., 1986; Cunningham et al., 1991; Osman et al., 1996) may be another peroxidase of industrial interest.

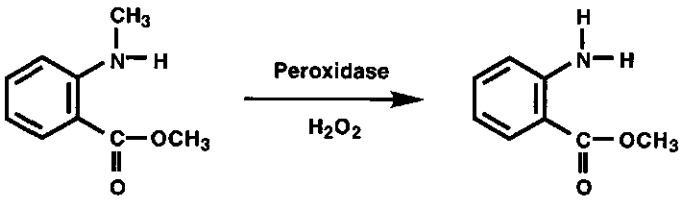

Figure 6.1 N-dealkylation of MNMA to MA, the model reaction of the present study.

On the basis of these considerations, the objective of the present study was to investigate the biocatalytic potential of SP, HRP and MP8 for the production of fine chemicals. Literature data report on a wide range of soybean preparations and isoenzymes (Diehn et al., 1993; McEldoon et al., 1995; Gijzen et al., 1993; Sessa and Anderson, 1981) and, therefore, we compared different commercially available samples with respect to their heme content and number of heme-containing proteins, in order to determine the best source for SP purification. As a model reaction the N-demethylation of methyl-N-methylanthranilate (MNMA), leading to the production of methylanthranilate (MA) (Figure 6.1), was used. This model reaction was chosen for the following reason. MNMA from citrus leaves is readily available and a relatively cheap source, whereas MA is more expensive than MNMA. Therefore, the investigated reaction provides a industrially relevant route for the natural production of an important topnote flavour in Concord grape.

6.2 Materials and methods

Chemicals

Methyl-N-methylanthranilate (MNMA) (ex. citrus leaves), methylanthranilate (MA) (ex. grapes), N-methylanthranilate and anthranilate were obtained from Quest International (Naarden, The Netherlands). H_2O_2 (30% in water) was from Merck (Darmstadt, Germany). HRP and cytochrome c from horse heart were obtained from Boehringer (Mannheim, Germany). MP8 was prepared by proteolytic digestion of horse heart cytochrome c essentially as described before (Aron et al., 1986; Kraehenbuhl et al., 1974). Dried soybeans were obtained from Mervo Products (Hengelo, The Netherlands). Soybean flakes consisting of brown flakes made of soybean hull were obtained from Quest International. Soybean pellets consisting of brown dried pellets from soybean hull, and soybean extract were obtained from ADUMIN (Tel Aviv, Israel). Soybean flour was a pale yellow flour-like powder commercially obtained as provaflor soybean flour from Gargill (Amsterdam, The Netherlands). SP was either purified or obtained from Enzymol International (Ohio, USA). HPLC analysis proved both purified SP preparations to be identical.

Solubilisation of the different soybean samples

The different soybean samples were preincubated at x % (w/v) (x= 1 for extract and flour, 17 for pellets and 10 for flakes) in 0.1 M potassium phosphate (pH 7.6) for 1 h at 0 °C and then homogenised by mixing during 2 minutes using a vortex. The mixture obtained was centrifuged using an eppendorf centrifuge for 10 min at 4 °C (13,000 g). The

supernatant thus obtained was used for further characterisation of the heme content, measurement of peroxidase activity and/or purification of SP. For the dry beans a different procedure was followed. This sample was preincubated at 20 % (w/v) in 0.1 M potassium phosphate (pH 7.6) for 2.5 h and then homogenised during 15 minutes at maximum speed using a blender (Braun, Frankfurt, Germany). The mixture obtained was centrifuged for 10 min at 4 °C (16,000 g). The supernatant obtained was filtered using glasswool, and then centrifuged for 20 min at 4 °C (27,000 g). The supernatant thus obtained was used for further characterisation of the heme content and measurement of peroxidase activity. Based on the solubilisation protocol used, the studied soybean fractions contain especially soluble forms of peroxidases.

Purification of SP

Soybean extract was used as the starting material and prepared as described above only using 20 mM Tris-HCl (pH 7.4). Purification was performed by a single affinity chromatography step on Concavalin A-Sepharose (Pharmacia Biotech, Uppsala, Sweden), performed essentially as described previously (Gillikin and Graham, 1991; Sessa and Anderson, 1981; Toiguchi et al., 1989). The column was equilibrated with 20 mM Tris-HCl (pH 7.4) containing 0.5 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$, and eluted with a gradient of 0 to 100 % 0.5 M methyl α-D-mannopyranoside (Sigma, Missouri, USA) in the same buffer. Fractions were analysed for absorption at A280 and A395 nm and for peroxidase activity using guaiacol as the substrate. Peroxidase-containing fractions were pooled, desalted and concentrated using an amicon 8010 filter apparatus with an YM-10 filter, and dialysed overnight against 20 mM Tris-HCl (pH 7.4). The purity of the final sample was analysed by HPLC and SDS-PAGE (Schägger and von Jagow, 1987), and its specific activity was determined as described below.

Incubations with MNMA

Incubations with MNMA were performed in (final concentrations) 0.1 M potassium acetate (pH 4) for SP or in 0.1 M potassium phosphate (pH 7.6) for HRP and MP8, containing 5 μM heme protein and 1 mM MNMA added as 1% (v/v) of a 0.1 M stock solution in dimethyl sulphoxide. To some incubations ascorbic acid was added at a final concentration of 3 mM. The incubation mixture was preincubated for 2 minutes at 70 °C for SP and at 37 °C for HRP and MP8 (unless indicated otherwise). The reaction was started by the addition of H_2O_2 (2.5 mM final concentration). The incubation was carried out for 1 minute (unless indicated otherwise) and stopped by freezing the sample into liquid nitrogen.

Samples were stored in liquid nitrogen until analysed and defrozen by centrifuging in an eppendorf centrifuge (5 min; 13,000 g; 4 °C) prior to analysis by HPLC.

HPLC

HPLC chromatography of SP samples solubilised as described above and of solutions of purified SP, HRP and MP8, was performed on a Bakerbond wide-pore butyl RP-7116-00 column (4.6 x 250 mm)(J.T. Baker research products, Deventer, The Netherlands). Elution was performed at a flow of 1 ml/min using a linear gradient from 0.1% trifluoroacetic acid in 100% nano pure to 0.1% trifluoroacetic acid in 50% water plus 50% acetonitril in 50 min. Detection was performed at 210 to 450 nm using a photodiode array detector (Waters 996). HPLC analysis of the incubations for N-demethylation of MNMA was performed on a reversed-phase Lichrosphere RP8 column (4.6 x 150 mm)(Alltech, Breda, The Netherlands) using a linear gradient from 0 to 80 % methanol in water in 26 min at a flow of 1.0 ml/min. Detection was at 332 nm using a diode array detector (Waters 996). Products were identified and quantified using commercially available standards. The concentration of MA was quantified using a calibration curve consisting of the HPLC peak area plotted against the concentration of the compound in the injected sample.

Determination of heme and protein content

For the sample containing purified SP, the heme concentration was determined by the pyridine-chromogen method (Aron et al., 1986). The heme content of solubilised SP samples was generally low compared to the other proteins present. This hampered the heme determination by the pyridine-chromogen method. Heme determination for these samples was done using HPLC and a calibration curve made with SP from Enzymol.

Protein was measured according to the method of Bradford (Sigma, Missouri, USA) using bovine serum albumin (Boehringer Mannheim, Germany) as the standard.

Peroxidase activity with guaiacol

Peroxidase activity was detected on the basis of oxidation of guaiacol as described by Gillikin and Graham (1991). The assay was performed in 50 mM potassium acetate (pH 5.5) at 25 °C and contained (final concentrations) 8 mM guaiacol, 0.5 mM H_2O_2, and 2 nM peroxidase. The reaction was initiated by addition of H_2O_2.

6.3 Results

Heme-protein content of different soybean samples

Figure 6.2a presents the HPLC chromatogram of soybean extract with detection at 280 nm for protein content and -more importantly- at 395 nm for heme-containing proteins. The HPLC chromatogram of soybean extract contains one major heme-containing peak with retention time of 49.2 min. Table 6.1 summarises the results from this and similar HPLC analyses of different types of commercially available soybean preparations. The different samples vary considerably with respect to their heme-protein compositions, although especially the peak at 49.2 min and, to a minor extent, the peak at 55.1 min are present in most of the samples. The results obtained indicate either the presence of different heme-containing proteins, or the presence of different degradation or glycosylated products of one major heme protein in the different commercial samples.

Table 6.1 Composition of heme-containing proteins in various commercial soybean preparations as determined by HPLC analysis with detection at 395 nm. Only peaks with an area > 2% of the total intensity are specified.

sample (obtained from)	heme content nmol heme per g sample	retention time of peaks at 395 nm (min)	% of total peak area at 395 nm
soybean extract (ADUMIN)	1291	43.8	2.8
		44.9	8.0
		49.2	80.7
		55.1	4.5
		Σ others	4.0
soybean flakes (QUEST)	2	28.2	18.8
		49.2	28.8
		55.1	52.4
		Σ others	0.0
soybean pellets (ADUMIN)	6	3.0	72.0
		49.2	25.6
		55.1	2.4
		Σ others	0.0
soybean flour (Gargill)	75	33.3	53.6
		55.1	46.4
		Σ others	0.0
dried soybeans (Mervo Products)	8	33.3	24.0
		49.2	76.0
		Σ others	0.0

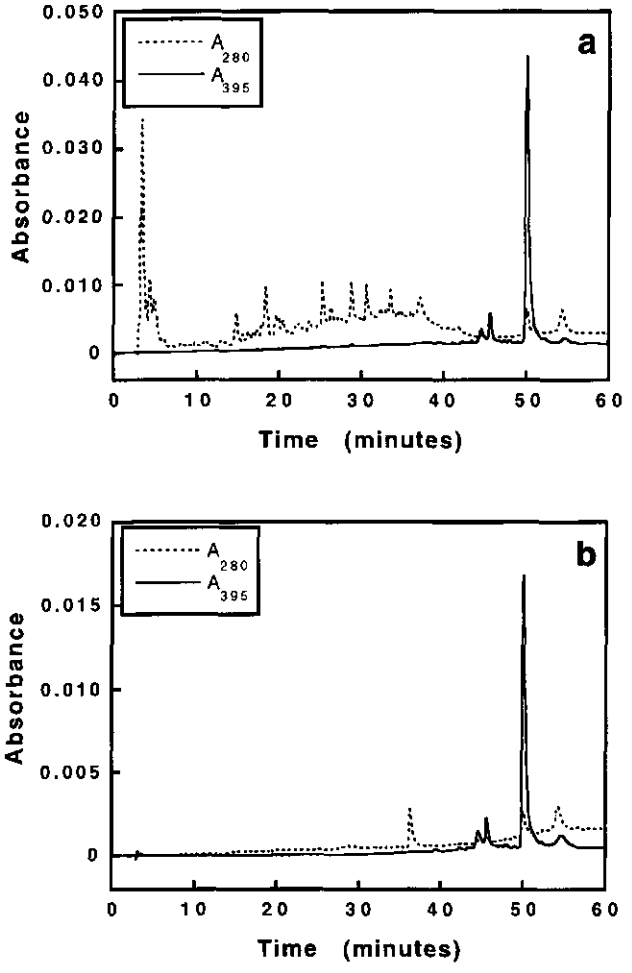

Figure 6.2 HPLC chromatogram of a) soybean extract and b) purified SP from soybean extract. Detection was at 280 nm and 395 nm.

In addition to the heme-protein composition, the amount of heme protein present in the various soybean preparations could be derived from these HPLC data. Since direct measurement of heme-content by spectroscopic methods was hampered by the high amount of other proteins present in the samples, the heme-content was derived from the HPLC data using a calibration curve made on the basis of commercially available SP. Table 6.1 presents the heme contents of the different samples. Marked differences are observed. Especially the

soybean extract contains a relatively large amount of heme proteins, suggesting that the extraction procedure was directed at selective enrichment of soluble and/or heme proteins. Also of interest is that both soybean flakes as well as soybean pellets contain about the same amount of heme as the solubilised fraction from dried soybeans, whereas the soybean flour was relatively rich in heme content.

Altogether the data indicate large differences between the various soybean preparations. On the basis of heme content per g of soybean preparation, the soybean extract shows the highest specific heme content, and, therefore, the best starting material for purification of SP. In addition, one heme-containing peak dominates in the soybean extract, namely, the component with HPLC retention time 49.2 min, also dominant in the least processed sample, the dried soybeans, and present in most of the commercially available SP preparations.

Purification of SP from soybean extract

Figure 6.2b presents the HPLC chromatogram (detection at 280 and 395 nm) of SP purified from soybean extract using affinity chromatography on Concavaline A-Sepharose. Comparison of this chromatogram to the one presented in Figure 6.2a indicates the significant increase in purity of the heme-containing protein. The specific activity of the final SP preparation was 8032 units/mg of protein as measured with guaiacol as the substrate. The purification factor of the final SP preparation was 10. On SDS-PAGE, the purified SP showed one prominent protein band of 37 kD.

Influence of pH and temperature

The pH optimum was determined for the conversion of MNMA to MA by purified SP and for the peroxidase activity of SP assayed with guaiacol. In both cases SP has a pH optimum at 4. At pH 4 the temperature dependence was also investigated. Reaction rates increased with increasing temperature up to an optimum at 70 °C. Blank incubations without H_2O_2 or without SP showed no product formation at all pH values and temperatures tested. Based on these results all subsequent incubations with SP were performed at 70 °C and pH 4.

N-demethylation of MNMA as catalysed by different peroxidases

Figure 6.3 presents the HPLC chromatograms of an incubation of MNMA with purified SP and, for comparison, with similar concentrations of HRP and MP8. All assays were performed at the pH optimum of the respective enzymes (Dunford, 1991;

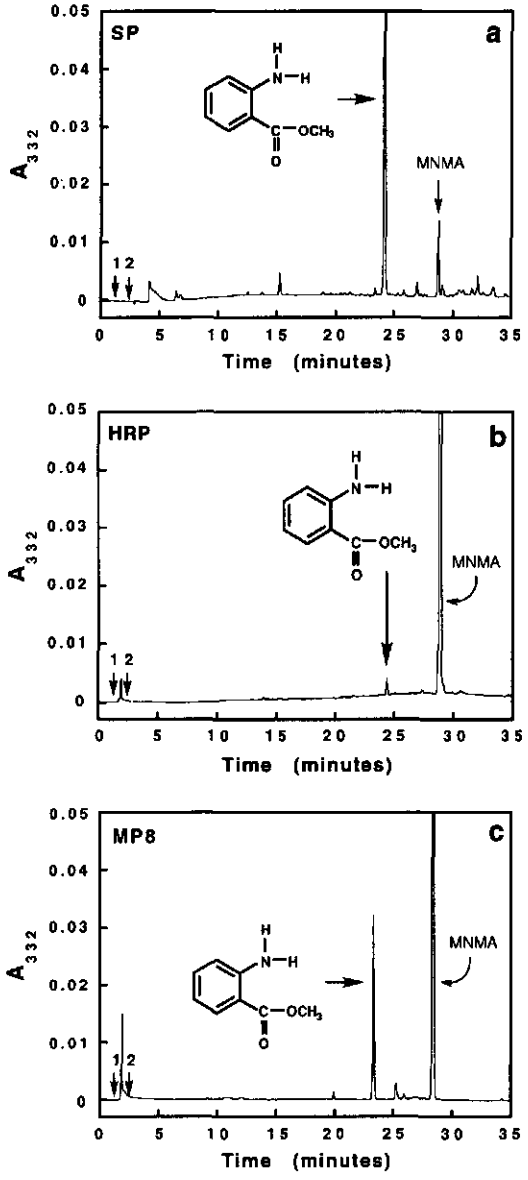

Figure 6.3 HPLC chromatograms of the incubation of MNMA with H_2O_2 driven a) SP purified from soybean extract, b) HRP and c) MP8. The concentration of the enzymes was 5 µM. The incubations were performed during 1 minute at 70 °C and pH 4 for SP, 37 °C and pH 7.6 for HRP and at 70 °C and pH 7.6 for MP8. The substrate MNMA is eluted after 29 minutes whereas the product MA is eluted after 24 minutes. Arrows 1 and 2 indicate where anthranilate respectively N-methylanthranilate should elute, which has not been observed in the present study.

Osman et al., 1996). The temperature used was 37 °C for HRP because of its thermoinstability (McEldoon and Dordick, 1996) and 70 °C for MP8 and SP. Formation of MA is observed in all cases. Formation of N-methylanthranilate (Figure 6.3, arrow 2), or of anthranilate (Figure 6.3, arrow 1) is not observed.

The results obtained with the different peroxidase samples vary not only with respect to the amount of MA formed, but also with respect to the amount and nature of the side products observed. From control experiments we can state that this difference in secondary products composition was not linked to the different pH and temperature conditions used for each enzyme. Comparison of the chromatograms in Figure 6.3 reveals that SP proved to be the most efficient biocatalyst for the N-demethylation of MNMA to MA. Measurement of the MNMA demethylation by SP at 37 °C results in only 17% of the amount of MA formed at 70 °C (data not shown), whereas measurement of the MNMA demethylation by MP8 at 37 °C results in 63% of the amount of MA formed at 70 °C (data not shown). This illustrates that the relatively high potential of SP as compared to MP8 and especially HRP is related to the ability of the enzyme to function at relatively high temperature. Another important observation is that upon conversion of MNMA to MA by SP the amount of side products compared to the amount of MA formed seems low (Figure 6.4a). Upon complete conversion of MNMA the yield of SP catalysed MA production amounted to 82% within 10 minutes under the conditions specified.

Operational stability

Figure 6.4 presents the formation of MA from MNMA as catalysed by the different peroxidases in time. Due to its relatively high activity SP had to be analysed for operational stability at a concentration 10 times lower than MP8 and HRP. However, measurement of SP operational stability at different concentrations of the enzyme (1, 0.5 and 0.2 μM) showed no significant influence of SP concentration on its operational stability.

To describe operational stability in a quantitative way equation 6.1, describing the kinetics of inactivation as reported by Spee et al. (1995), can be applied.

$$d[P] / dt = v = V_{max}\, e^{(-k_i t)} \qquad (6.1)$$

Using this equation to analyse our data provides k_i values of 0.5 ± 0.1 min^{-1}, 0.02 ± 0.004 min^{-1} and 2.6 ± 0.4 min^{-1} for SP, HRP and MP8 respectively. This inactivation constant, k_i, gives information about the stability of the different peroxidases: a high k_i means low operational stability. The operational stability of SP is 5-fold higher than that observed for

MP8, but at least 25-fold lower than that of HRP. Furthermore, the operational stability of SP in soybean extract (before purification) was comparable to that for purified SP (data not shown).

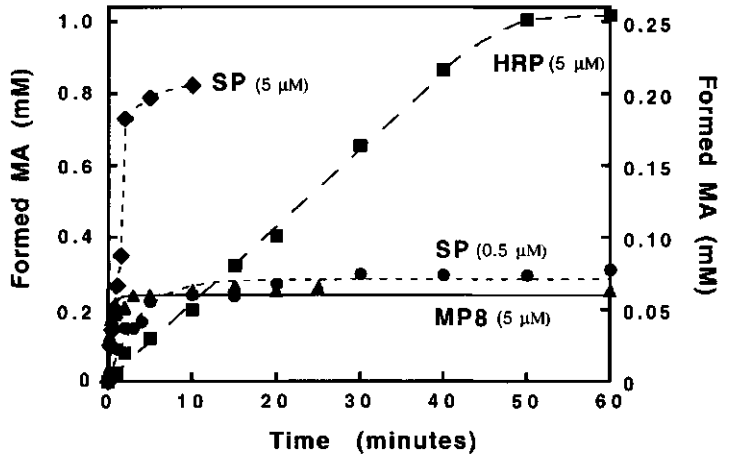

Figure 6.4 Operational stability of purified SP (····) (left Y-axis), HRP (---) (right Y-axis) and MP8 (-) (right Y-axis) in the conversion of MNMA (SP at 70 °C and pH 4, HRP and MP8 at 37 °C and pH 7.6). The enzyme concentration was 5 µM in all cases. As the MNMA conversion by 5 µM SP was hampered by substrate limitation, the operational stability of SP was determined also with 0.5 µM SP (•) (right Y-axis).

Addition of ascorbate

Previous studies reported the mechanism of peroxidase-catalysed heteroatom dealkylation to be either cytochrome P450-like or peroxidase like (Kedderis and Hollenberg, 1983; Walker-Griffin et al., 1978). To investigate the mechanism of the SP-catalysed N-demethylation of MNMA, experiments were performed in the presence of ascorbate, known to effectively block peroxidase-type of reaction chemistry, but not MP8-catalysed cytochrome P450-type conversion of aniline to *p*-aminophenol (Osman et al., 1996). Peroxidase-type of reaction chemistry is defined as H_2O_2-dependent initial one-electron oxidation of the substrate (Dunford et al., 1976).

Upon addition of 3 mM ascorbate to the incubations of the present study, the production of MA by SP as well as the peroxidase activity assayed with guaiacol appeared to be fully inhibited.

6.4 Discussion

The present work describes the possibilities and limitations for using commercially available SP preparations and other peroxidases for the production of the food flavour MA from MNMA.

SP was isolated from soybean extract, purified and characterised. The peroxidase-catalysed conversion of guaiacol and of MNMA to MA at low pH is in line with early reports of peroxidase-catalysed conversions observed at even lower pH values, which supported the notion that SP is resistant to acidic conditions and holds onto its heme more tightly than does HRP (McEldoon et al.; 1995). Comparison of the pH optimum observed in the present study to pH optima reported in the literature (McEldoon et al., 1995; Schmitz et al., 1997; Sessa and Anderson, 1981; Toiguchi et al., 1989) suggests that the actual pH optimum observed for SP may vary between 2 and 6 depending to some extent on the substrate and assay conditions used. Alternatively, this might be due to the fact that SP used in the different studies was obtained from different soybean sources resulting in purification of different isoenzymes. The temperature optimum of 70 °C for the conversion of MNMA to MA by SP and for the conversion of guaiacol by SP is in line with results reported by others (McEldoon et al., 1995; McEldoon and Dordick, 1996; Toiguchi et al., 1989), who found temperature optima of 40-60 °C for other assays, and SP inactivation to occur above 80 °C. The observation in the present study that the pH and temperature effects on demethylation of MNMA are similar to the pH and temperature effects on peroxidase activity assayed with guaiacol, corroborates the conclusion that the demethylation proceeds by a peroxidase-type of reaction mechanism.

Since MNMA from citrus leaves is a relatively cheap source and MA is more expensive then MNMA, the investigated reaction provides a route for the natural production of the Concord grape flavour. The observation that ascorbate fully inhibits the SP catalysed N-demethylation of MNMA also corroborates the hypothesis that the N-dealkylation proceeds by a peroxidase-type of reaction mechanism, and not by a cytochrome P450-type of reaction mechanism (Hollenberg et al., 1985; Kedderis and Hollenberg, 1983; Miwa et al., 1983; Nakamura et al., 1992; Pandey et al., 1989). The observation of peroxidase-catalysed N-demethylation is in line with literature data, which report peroxidase-catalysed N-dealkylations of other N-alkylated substrates (Kedderis and Hollenberg, 1983; Walker-Griffin et al., 1978).

In line with other peroxidases, and especially MP8, SP showed a limited operational stability. This limited operational stability of MP8 is known from previous studies (Osman et al., 1996) and has been ascribed to its open active site, providing possibilities for μ-oxo

dimer formation as well as for intermolecular inactivating reactions occurring once the heme catalyst becomes activated to its high-valent-iron-oxo porphyrin form upon reaction with H_2O_2 (Balch et al., 1984; Bonnet and McDonagh, 1973; Brown et al., 1978; Kaim and Schwederski, 1994). The fact that SP, with its full polypeptide chain, shows a limited operational stability ($k_i = 0.5 \pm 0.1$ min^{-1}) only 5-fold higher than that observed for MP8 ($k_i = 2.6 \pm 0.4$ min^{-1}), suggests that the nature of the inactivation mechanism could be intramolecular. Since HRP ($k_i = 0.02 \pm 0.004$ min^{-1}) showed an operational stability at least 25 times higher than that observed for SP and 130 times higher than that observed for MP8, the present results with SP indicate that the presence of a large protein chain around the porphyrin cofactor in a peroxidase is, by itself, insufficient to explain the observed differences in operational stability.

Despite its relatively low operational stability, SP proved to be the most efficient biocatalyst for the production of MA with high yield and purity. This potential of SP to catalyse the N-demethylation of MNMA to MA more efficient than HRP and MP8 was especially observed at high temperature and low pH values at which SP appeared to be optimally active.

Acknowledgements

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7

Summary and conclusions

This PhD project was started in 1995 and was supported by the dutch Ministry of Economic Affairs through the programme "IOP Catalysis". The main goal of "IOP catalysis" is to obtain clean and more efficient technologies, to improve the quality of the Dutch fine chemistry. Biocatalysis provides a way to minimize non desirable side products, which can have a negative impact on the environment. The objective of this thesis was to investigate the potential of heme-containing peroxidases as natural biocatalysts for industrially relevant conversions. Peroxidases were chosen since they are able to operate under mild conditions using cheap and clean oxidants (hydrogen peroxide).

In this work especially the natural production of food flavours was of interest. After initial screening of reactions of potential interest the N-dealkylation of methyl-N-methylanthranilate (MNMA) (ex citrus leaves) to methylanthranilate (MA), a concord grape flavour was chosen as the model reaction in this thesis.

In order to obtain better insight in the industrial applications of peroxidases, it is important to understand structure, function and operational stability of these catalysts. Therefore, this project was started by investigating the reaction mechanism of peroxidases in more detail (***Chapter 3***). Horseradish peroxidase (HRP) was used as the model peroxidase, as this enzyme is the best studied enzyme of all peroxidases. Many heme-containing biocatalysts, exert their catalytic action through the initial formation of so-called high-valent-iron-oxo porphyrin intermediates. For HRP the initial intermediate formed has been reported as a high-valent-iron-oxo porphyrin π-radical cation, called compound I. A strongly hold concept in the field of peroxidase-type of catalysis is the irreversible character of the reaction leading to formation of this compound I. The results of chapter 3, however, indicate that formation of the high-valent-iron-oxo porphyrin intermediate for various heme-containing catalysts, including HRP, might be reversible. This reversible compound I formation results in heme-catalysed exchange of the oxygens of H_2O_2 with those of H_2O. The existence of this heme-catalysed oxygen exchange followed from the observation that upon incubation of ^{18}O labelled $H_2{}^{18}O_2$ with heme-containing biocatalysts, significant loss of the ^{18}O label from the

$H_2{}^{18}O_2$ was observed, accompanied by the formation of unlabelled H_2O_2. Thus, for the heme biocatalysts studied, exchange of the oxygen of their high-valent-iron-oxo intermediate with that of water occurs rapidly. This observation implied the need for an update of the kinetic model for peroxidases. Revaluation and extension of the previous kinetic model for HRP showed the necessity to include several additional reaction steps, taking both reversible compound I formation and the formation of enzyme-substrate complexes into account. As a consequence reactions with HRP are saturable, implying that V_{max} and k_{cat} can be measured. This provides possibilities for investigation of structure-activity relationships.

Investigation of quantitative structure-activity relationships (QSARs) is a way to obtain more insight in the influence of the structure of a substrate on its conversion by an enzyme of interest. Moreover, QSARs could be of interest for industrial applications. With QSARs the outcomes of conversions may be predicted by simply calculating chemical parameters of structurally related substrates. In this way QSARs could be helpful in facilitating screening procedures for biocatalytic productions, saving time and money.

In ***chapter 4*** predictive computer calculation-based QSARs were defined by comparing second order rate constants for the oxidation of a series of model compounds by HRP compound II to computer calculated chemical parameters characteristic for this reaction step. The model compounds studied were a series of structurally related phenols.
For the calculation of the chemical parameters characteristic for the reaction step two approaches were used. In the first approach a frontier orbital parameter of the substrate was calculated being the ionisation potential (i.e. minus the energy of their highest occupied molecular orbital (E(HOMO)). In the second approach the relative heat of formation ($\Delta\Delta HF$) was calculated for the process of one electron abstraction as well as for $H^{\bullet}$-abstraction from the phenol derivatives. Assuming a reaction of the phenolic substrates in their non-dissociated, uncharged forms, clear correlations were obtained between the natural logarithm of the second order rate constants ($\ln k_{app}$ and $\ln k_2$, respectively) for their oxidation by compound II and their calculated parameters.

The computer calculation-based QSARs thus obtained for the oxidation of the various phenol derivatives by compound II from HRP indicate the validity of the approaches investigated, i.e. the frontier orbital approach but also the approach in which the process is described by calculated relative heats of formation. The results also indicated that outcomes from computer calculations on related phenol derivatives can be reliably compared to one another. Since both mechanisms considered, i.e. initial electron abstraction versus initial $H^{\bullet}$-abstraction, provided clear molecular orbital QSARs the results could not be used to discriminate between these two possible mechanisms for phenol oxidation by HRP compound

II. Furthermore, as the actual oxidation of peroxidase substrates by compound II is known to be the rate-limiting step in the overall catalysis by HRP, the QSARs described in chapter 4 may have implications for the differences in the overall rate of oxidation of the phenol derivatives by HRP. As a matter of fact similar QSARs should be obtained when the overall rate of oxidation of the respective compounds by HRP is determined.

As mentioned above, reactions with HRP are saturable, implying that V_{max} and k_{cat} can be measured. Thus, in ***chapter 5*** the overall conversion of phenols by HRP was investigated, resulting in overall k_{cat} values. These saturating overall k_{cat} values indeed correlated quantitatively with calculated ionisation potentials of the substrates. The observation that the rates and QSARs obtained for the overall rate of conversion in chapter 5 are similar to those described for the rate limiting reaction step in chapter 4, corroborates that phenol oxidation by compound II is the rate limiting step in the reaction cycle of HRP, but even more important, it also illustrates that QSARs for oxidation of substrates by compound II can be used for prediction of the overall rate of oxidation of phenol derivatives by HRP and vice versa. Moreover, QSARs for overall k_{cat}, instead of for individual rate constants, eliminate the need for extensive rapid kinetic analysis to make predictions for HRP-based substrate conversions.

Saturating overall k_{cat} values for HRP catalysed conversion of a second series of substrates, i.e. a series of substituted anilines, described in chapter 5, also correlated quantitatively with calculated ionisation potentials of the substrates. However, in the QSAR plots, the correlations for the anilines were shifted to lower k_{cat} values at similar ionisation potentials as compared to those for the phenols. To investigate whether differences in orientation of phenols and anilines within the active site of HRP may be a factor underlying the higher reactivity of the phenols than expected on the basis of their ionisation potential, ^{1}H NMR T_1 relaxation studies were performed, using 3-methylphenol and 3-methylaniline as the model substrates. The ^{1}H NMR T_1 relaxation studies revealed consistently smaller average distances of the phenol than of the aniline protons to the paramagnetic Fe^{3+} centre in HRP, may be resulting in differences in the electron transfer process from the aromatic donor substrate to the Compound II of HRP. A shorter distance between the phenol and the heme than between the aniline and the heme may be a factor contributing to the faster rate of electron transfer with phenol as compared to aniline substrates. However, the actual differences in orientation seem small when the difference in oxidation rate at similar calculated ionisation potential phenols or anilines is considered. Since (partial) deprotonation will largely influence, i.e. decrease, the ionisation potential of the aromatic substrate, the relatively higher oxidation rates of phenols may be related to their larger extent of deprotonation upon binding to the

substrate pocket of HRP, resulting in lower ionisation potentials than actually expected on the basis of calculations on their non-ionised form. Based on the ^{1}H NMR T_1 relaxation data of chapter 4 and literature data of Hendriksen et al. [1], we put forward the hypothesis that the differential substrate behaviour of phenols and anilines may be due to subtle differences in their binding to the active site substrate pocket of HRP, resulting in i) closer proximity to the heme and ii) larger extent of deprotonation for the phenols than for the aniline substrates. An important conclusion following from the results of chapter 5 is that for each type of reaction and substrate, different QSARs have to be obtained. It is also clear that further investigations are nessecary for succesful application of QSARs to industrial prediction of biocatalysis.

Heme-based peroxidases are enzymes with a broad substrate specificity and are capable of catalysing a variety of reactions. However, operational (in)stability limits the use of peroxidases in industrial processes. ***Chapter 6*** describes the possibillities and limitations for using commercially available SP preparations (delivered by QUEST) and other peroxidases (like HRP and microperoxidase-8 (MP8)) for the production of the food flavour methylanthranilate (MA) from methyl-N-methylanthranilate (MNMA). Because MNMA from citrus leaves is a relatively cheap source and MA is more expensive than MNMA, the investigated reaction provides an industrially relevant route for the natural production of an important topnote flavour in concord grape.

The tested soybean preparations varied widely with respect to their heme-content and, as a result, their activity for this reaction. Furthermore, the operational stability of purified soybean peroxidase (SP) was at least 25-fold lower than that of HRP and only 5-fold higher than that of MP8, a small peroxidase with a polypeptide chain of only eight amino acids covalently linked to the protoporphyrin IX cofactor. Therefore, the results with SP indicate that the presence of a large protein chain around a porphyrin cofactor in a peroxidase is, by itself, insufficient to explain the observed differences in operational stability, and that the inactivation mechanism could be intramolecular. In order to find an explanation for the low operational stability of SP and MP8, it would therefore be interesting to investigate, for example, the shielding of the *meso*-positions of the heme cofactors of the different peroxidases.

All tested peroxidase preparations were able to catalyse the requested N-dealkylation. However, SP proved to be a very efficient biocatalyst for the production of MA with high yield and purity, in spite of its relatively low operational stability. This potential of SP to catalyse the N-demethylation of MNMA to MA more efficiently than HRP and MP8, was especially observed at high temperature and low pH values at which SP appeared to be optimally active. Unfortunately, the prices for MA had dropped by the time this efficient SP

catalysed production of MA was developed. As a result the now defined way to produce MA using a peroxidase biocatalyst was no longer of commercial interest for the industrial IOP partner. Nevertheless, the results in chapter 6 clearly define the answer to the initial industrial aim of the present IOP project.

Biotechnological applications

Peroxidases, in principle, have remarkable synthetic possibilities, but commercial processes based on these enzymes have not yet been developed. The inactivation of heme-enzymes by peroxides through oxidation of the porphyrin ring is one of the prime reasons. Attempts to improve the stability have not been very successful yet. The enzyme stability can be improved by stepwise or continuous addition of the oxidant, maintaining a low peroxide concentration [2,3]. Hiner et al. [4] tried to increase the resistance of HRP to H_2O_2 through genetic engineering without satisfying results. Other ways to deal with the problem of poor stability were chemical modification, screening and immobilisation [5].

A second reason why commercial processes based on peroxidases have not yet been developed is that the oxidation of some substrates by peroxidases is in competition with their spontaneous chemical oxidation by peroxides. As a consequence, reduction of the purity of products occurs in case enantioselectivity is the target [6]. Maintaining a low peroxide concentration throughout the reaction period, reducing background oxidation, has also been tried here as a solution.

Furthermore, in the process of one-electron oxidation by peroxidases free radicals are produced which are difficult to control, thereby reducing the purity of products as well.

A fourth reason for the limited scaling up of peroxidase-catalysed reactions is the low water solubility of most of the substrates of synthetic interest. Designing strategies that enhance enzymatic activity in organic solvents [7,8], or the use of hydrophobic matrices that act as a reservoir for both substrates and products are solutions to this general problem.

From the above it can be concluded that the problem of (operational) stability of peroxidases is a severe problem for industrial processes and that this problem is difficult to solve. On the other hand this thesis shows that a peroxidase exists that, in spite of its low operational stability, can be an efficient biocatalyst for the production of a industrially relevant compound with high yield and purity. Altogether, it can be concluded that peroxidases could represent an interesting tool for industrially relevant reactions, making future research on possibilities and limitations worthwhile.

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Abbreviations and symbols

Å	Ångström (10^{-10} m)
AO	atomic orbital
AM1	austin method 1
APX	ascorbate peroxidase
ARP	peroxidase from *arthromyces ramosus*
B	magnetic field
BPX	bromoperoxidase
CAT	catalase
CcP	cytochrome *c* peroxidase
CiP	coprinus cinereus peroxidase
CPO	chloroperoxidase
Cys	cysteine
DMSO	dimethylsulphoxide
E	energy
E(HOMO)	energy of the HOMO
GC-MS	gaschromatography-mass spectrometry
FeO(H)	high-valent-iron-oxo species
H	hamiltonian operator
$H^{\ddagger}$	activation energy
ΔHF	difference in heat of formation
ΔΔHF	relative difference in heat of formation
HPLC	high performance liquid chromatography
HOMO	highest occupied molecular orbital
HRP	horseradish peroxidase
J	Joule
K	kelvin
k	rate constant
k_{cat}	first-order rate constant of enzymatic conversion
K_D	dissociation constant
kDa	kilodalton
K_m	Michaelis-Menten constant, substrate concentration at 1/2 V_{max}
KPi	potassium phosphate buffer
LiP	lignin peroxidase

ln	natural logarithm
LPO	lactoperoxidase
MA	methylanthranilate
MnP	manganese peroxidase
MNMA	methyl-*N*-methylanthranilate
MP8	microperoxidase-8
MO	molecular orbital
MO-QSAR	molecular orbital based quantitative structure-activity relationship
MS	mass spectrometry
$M\tau$	nmr peak area at delay time τ
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electroforesis
PNP	peanut peroxidase
ppm	parts per million
Q	charge
QSAR	quantitative structure-activity relationship
r	distance or correlation coefficient
R	molar gas constant
S	substrate or electron spin (operator)
SDS	sodium dodecyl sulphate
SP	soybean peroxidase
t	time
T	absolute temperature
T_1	longitudinal relaxation time
TCA	trichloroactic acid
Tris	tris(hydroxymethyl)aminoethane
UV	ultra violet light
VIS	visible light
V_{max}	maximal enzymatic velocity
ε	dielectric constant or molar extinction coefficient
λ	wavelength (nm)
λ_{max}	position of absorption maximum
τ	delay time
τ_c	correlation time

Samenvatting

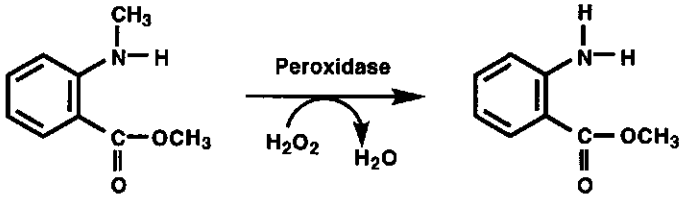
(ook voor niet-vakgenoten)

Dit promotieonderzoek werd gestart in 1995 als onderdeel van het Innovatiegericht Onderzoeksprogramma (IOP) "Katalyse", gefinancierd door de industrie en het ministerie van Economische Zaken. Het hoofddoel van "IOP Katalyse" is om schonere en efficiëntere technologieën te verkrijgen, om zodoende de kwaliteit van de Nederlandse fijnchemicaliën industrie te verbeteren. Fijnchemicaliën zijn over het algemeen complexe chemische stoffen die op kleine schaal gemaakt worden. Er zijn vaak veel stappen nodig om fijnchemicaliën te maken. Voorbeelden van fijnchemicaliën zijn geur- en smaakstoffen, medicijnen en cosmetica.

Het promotieonderzoek dat in dit proefschrift wordt beschreven is uitgevoerd bij de leerstoelgroep Biochemie aan de WUR (Wageningen Universiteit en Research centrum). In de biochemie worden scheikundige processen in biologische systemen bestudeerd. Een belangrijk onderdeel binnen deze wetenschap is het onderzoek aan eiwitten. Eiwitten zijn ketens opgebouwd uit bouwstenen, die aminozuren genoemd worden. Er bestaan 20 verschillende aminozuren, waarmee oneindig veel variaties in lengte en samenstelling gemaakt kunnen worden, en dus oneindig veel specifieke eiwitten. De code voor de volgorde van aminozuren en lengte van de aminozuurketens is opgeslagen in het DNA.

Eiwitten die chemische reacties kunnen katalyseren worden enzymen, of wel biokatalysatoren genoemd. Enzymen katalyseren zeer uiteenlopende reacties. Veel reacties in het menselijk lichaam worden bijvoorbeeld door enzymen gekatalyseerd. Katalyse is het efficiënt, versneld en gecontroleerd laten verlopen van reacties, die onder normale omstandigheden niet of nauwelijks plaatsvinden. Hierdoor kunnen gewenste producten worden verkregen, met een minimum aan bijproducten. Deze bijproducten vormen in het beste geval namelijk ballast, maar zijn ook vaak schadelijk voor het organisme of het milieu. Een reactie met weinig of geen bijproducten is ook voor de industrie milieuvriendelijker en economisch aantrekkelijker. Dit verklaart de industriele belangstelling voor de chemische synthese met behulp van enzymen als katalysator. Katalyse door enzymen kan als volgt worden voorgesteld: De keten aminozuren, waar het enzym uit is opgebouwd, is vaak opgevouwen tot een compact geheel met een holte. Deze holte is het actieve centrum van het enzym waar de reactie plaatsvindt. Een stof (substraat) zal in de holte gaan zitten waarna het door het enzym omgezet kan worden in een andere stof (product). Er zijn veel soorten

enzymen. De enzymen die in dit proefschrift worden beschreven behoren tot de groep van peroxidasen. Peroxidasen zijn enzymen die waterstofperoxide gebruiken voor het omzetten van substraten (Figuur 1). Peroxidasen worden in micro-organismen (bv. schimmels), planten en dieren gevonden. De oorspronkelijke (biologische) functie van peroxidasen loopt zeer uiteen: van het onschadelijk maken van waterstofperoxide (dat gevormd wordt tijdens de stofwisseling) tot het maken van bioverbindingen (bv. hormonen). Peroxidasen staan bekend om hun brede substraatspecificiteit. Dit houdt in dat peroxidasen zeer veel verschillende substraten op verschillende manieren kunnen omzetten. Verder kunnen peroxidasen onder milde, gecontroleerde condities opereren met behulp van een schone en goedkope oxidant, het al genoemde waterstofperoxide (Figuur 1). Deze oxidant wordt hier "schoon" genoemd omdat water het enige afvalproduct is. Genoemde eigenschappen maken peroxidasen als biokatalysator voor industrieel relevante omzettingen interessant. Echter het probleem van (operationele) stabiliteit van de peroxidasen beperkt tot op heden het gebruik van peroxidasen in industriële processen. Peroxidasen kunnen bijvoorbeeld geinactiveerd worden doordat hun structuur tijdens de katalyse onbedoeld veranderd wordt.



Figuur 1 Omzetting van methyl-*N*-methylanthranilaat (MNMA) in de druivensmaakstof methylanthranilaat (MA) door peroxidasen.

Het doel van dit promotieonderzoek is beschreven in ***Hoofdstuk 1*** van dit proefschrift. Het doel was te onderzoeken in hoeverre peroxidasen als natuurlijke biokatalysatoren kunnen worden gebruikt voor industrieel relevante omzettingen. Hiertoe werden de katalytische en structurele eigenschappen, de operationele stabiliteit en de biotechnologische toepassing van peroxidasen bestudeerd. De industrieel relevante omzettingen van belang in dit proefschrift waren gericht op natuurlijke productie van geur- en smaakstoffen. Na het screenen van potentieel interessante omzettingen werd de omzetting van methyl-*N*-methylanthranilaat (MNMA) (uit citrusbladeren) in methylanthranilaat (MA) gekozen als modelreactie tijdens dit promotieonderzoek (Figuur 1). Methylanthranilaat (MA) is een druiven geur- en smaakstof.

Alvorens wordt ingegaan op de resultaten van dit promotieonderzoek worden in ***hoofdstuk 2*** de theoretische achtergronden van dit proefschrift kort beschreven. Allereerst worden de classificatie, de bronnen en de biologische functie van peroxidasen beschreven. Daarna wordt uitgebreid ingegaan op de structuur en het reactiemechanisme van peroxidasen. Vervolgens wordt een overzicht gegeven van de mogelijke reacties die peroxidasen kunnen katalyseren. Tot slot wordt iets verteld over de (operationele) stabiliteit en biotechnologische toepassingen van peroxidasen.

Dit promotieonderzoek is gestart met het onderzoek naar het reactiemechanisme van peroxidasen, zoals beschreven in ***hoofdstuk 3***. De omzetting van stoffen door peroxidasen vindt vaak in deelstappen (deelreacties) plaats. Deze deelstappen tezamen vormen het reactiemechanisme. Een reactiemechanisme geeft dus weer hoe peroxidasen stoffen omzetten. Met behulp van kennis over het reactiemechanisme van peroxidasen kan de werking van peroxidasen beter worden begrepen. Deze kennis is essentieel voor het inzetten van peroxidasen voor industriële doeleinden. In hoofdstuk 3 is aangetoond dat één van de eerste deelreacties in het reactiemechanisme van horseradish peroxidase reversibel (omkeerbaar) is. Dat houdt in dat stof A in B kan worden omgezet en stof B ook weer in A kan worden omgezet. Dit was in tegenstelling tot waar men in de literatuur tot nu toe van uitging: irreversibiliteit (=onomkeerbaarheid) van deze reactiestap. Dit resultaat heeft grote invloed op de beschrijving van de katalytische eigenschappen van peroxidasen (bv. hoe snel zet een peroxidase het substraat om). Het bestaande kinetisch model dat de katalytische eigenschappen van peroxidasen beschrijft is daarom aangepast.

Onderzoek naar de relatie tussen structuur en functionele eigenschappen van eiwittten en enzymen is van groot belang om het gedrag van eiwitten en enzymen te kunnen begrijpen en te sturen. Op basis van deze fundamentele kennis kunnen eiwitten en enzymen gericht toegepast worden in de industrie. Redenen voor de toename van industriële toepassingen van eiwitten en enzymen de laatste jaren zijn:

1) Een toenemende vraag naar natuurlijke functionele ingrediënten voor met name "food", maar ook voor "non-food" toepassingen: de consument wil een grote variëteit aan levensmiddelen, die aantrekkelijk en smakelijk zijn en die weinig niet natuurlijke hulpstoffen bevatten.
2) Een toenemend aanbod: eiwitten en enzymen werden vaak als bijproduct of zelfs als afval beschouwd. Stijgende milieuheffingen zorgen voor een zoektocht naar alternatieven om eiwitten en enzymen zinvol te gebruiken.

3) Een toename van mogelijkheden om de samenstelling (en daarmee de functionele eigenschappen) van eiwitten en enzymen aan te passen aan wensen van de klant.

In ***hoofdstuk 4*** en ***5*** wordt de relatie tussen structuur- en functionele eigenschappen van horseradish peroxidase onderzocht door zogenaamde quantitatieve structuur-activiteit relaties (QSARs) op te stellen. Deze QSARs zijn interessant voor industriële toepassingen omdat met QSARs de uitkomsten van omzettingen voorspeld kunnen worden door eenvoudigweg de chemische parameters van substraten te berekenen met behulp van computerprogramma's. QSARs zouden zo van nut kunnen zijn bij een eerste screening voordat tot experimenteel onderzoek naar mogelijke biokatalytische productie wordt overgegaan, wat tijd en geld bespaard.

De QSARs in hoofdstuk 4 en 5 zijn beschreven voor de omzetting van een reeks modelsubstraten (fenolen en anilines) door horseradish peroxidase. Hiertoe werden parameters voor de omzettingsnelheid van peroxidasen (i.e. eerste en tweede orde snelheidsconstanten) gemeten en vergeleken met computer-berekende chemische parameters van de substraten die karakteristiek zijn voor de reactie. Voor de berekening van deze chemische parameters werden twee benaderingen gevolgd. In de eerste benadering werd de ionisatiepotentiaal van het substraat berekend. De ionisatiepotentiaal is de hoeveelheid energie die nodig is om een elektron van het substraat af te trekken. In de tweede benadering werd de relatieve vormingswarmte berekend. Dit is de warmte die vrijkomt wanneer het (tussen) product gevormd wordt. Er werden duidelijke verbanden (i.e. QSARs) gevonden tussen de gemeten parameters en de computer berekende parameters. De QSARs duiden dus de geldigheid aan van de beide benaderingen. Bovendien kunnen de uitkomsten van computerberekeningen aan substraten van hetzelfde type met elkaar vergeleken worden. Computerberekeningen aan verschillende typen substraten leveren verschillende QSARs op. De QSAR voor anilines was bijvoorbeeld anders dan die voor fenolen. De fenolen bleken reactiever dan de anilines. In ***hoofdstuk 5*** is nader onderzoek gedaan naar de achterliggende reden van dit verschijnsel. Hierbij is met behulp van geavanceerde apparatuur (NMR) onderzocht hoe de oriëntatie van het substraat in het actieve centrum van het enzym is en wat de invloed hiervan is op de reactiviteit van het substraat. Aan de hand van de resultaten is een hypothese opgesteld die het hierboven besproken verschijnsel (van verschil in QSARs voor de omzetting van anilines en fenolen door horseradish peroxidase) kan verklaren. Eén en ander houdt in dat voor ieder type substraat en ieder type reactie een aparte QSAR moet worden opgesteld en/of gebruikt om gedrag te voorspellen. Verder onderzoek is dus noodzakelijk voordat QSARs succesvol toegepast kunnen worden in de voorspelling van (industriële) reacties.

In ***hoofdstuk 6*** staan de mogelijkheden en de beperkingen beschreven van het gebruik van verschillende peroxidasen voor de productie van de druivensmaakstof methylanthranilaat (MA). MA was aan het begin van het onderzoek een relatief dure stof. Methyl-*N*-methylanthranilaat (MNMA) daarentegen kon relatief goedkoop uit citrusbladeren worden verkregen en door peroxidasen worden omgezet in MA (zie figuur 1). De omzetting van MNMA in MA door peroxidasen was dus een industrieel relevante modelreactie voor de natuurlijke productie van een belangrijke smaakstof in Concord druiven. Het resultaat van het onderzoek was dat alle onderzochte peroxidasen deze omzetting in meer of mindere mate konden katalyseren. De potentie van één peroxidase, het sojaboon peroxidase, was hierbij opvallend. Het sojaboon peroxidase bleek een opvallend efficiënte biokatalysator voor de productie van MA met hoge opbrengst en zuiverheid, ondanks de lage (operationele) stabiliteit. Aan het eind van het onderzoek waren de marktprijzen van MA gedaald waardoor de ontwikkelde route om MA te produceren met behulp van een peroxidase biokatalysator niet langer commercieel interessant was voor de industriële IOP partner. Ondanks dat verschaffen de resultaten in hoofdstuk 6 het antwoord op het industriële doel van dit IOP project.

Peroxidasen hebben in principe opmerkelijke synthetische mogelijkheden, maar commerciële processen gebaseerd op deze enzymen zijn nog niet ontwikkeld. Naast de hoge prijs van peroxidasen is de operationele instabiliteit van peroxidasen één van de belangrijkste redenen. Een sterke verlaging van de prijs van peroxidasen of een aanzienlijke verbetering van de (operationele) stabiliteit van peroxidasen zijn nodig om commerciële processen gebasseerd op deze enzymen verder te onwikkelen. Tot nu toe zijn pogingen om de stabiliteit van peroxidasen te verbeteren nog niet erg succesvol geweest. De (operationele) stabiliteit van peroxidasen blijft dus een lastig probleem voor industriële toepassingen. Aan de andere kant laat dit proefschrift zien dat er een peroxidase bestaat (sojaboon peroxidase) dat ondanks een lage operationele stabiliteit kan fungeren als een heel efficiënte biokatalysator voor de productie van een industrieel relevante verbinding met hoge opbrengst en zuiverheid. Dit laat zien dat verder onderzoek naar mogelijkheden en beperkingen van peroxidasen in industriële processen de moeite waard zijn.

List of Publications

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Curriculum Vitae

Marjon Joanne Helene van Haandel werd geboren op 28 maart 1971 te Leiden. Zij behaalde het Gymnasium β diploma aan het Gemeentelijk Gymnasium te Hilversum in juni 1989. Aansluitend startte zij met de studie Moleculaire Wetenschappen aan de Landbouw Universiteit te Wageningen. Tijdens de doctoraalfase verrichtte zij onderzoek bij de Vakgroep Biochemie aan de Landbouw Universiteit te Wageningen (Prof. C. Veeger & Prof. I.M.C.M. Rietjens), bij de sectie Moleculaire Biologie van het Nederlands Kanker Instituut in Amsterdam (Prof. P. Borst) en bij het Eijkman Instituut voor Moleculaire Biologie in Jakarta (Indonesie) (Prof. S. Marzuki). In juni 1995 behaalde zij haar doctoraal diploma. In mei 1995 was zij reeds begonnen met een promotieonderzoek bij het laboratorium voor Biochemie aan de Wageningen Universiteit onder begeleiding van Prof. I.M.C.M. Rietjens en Prof. N.C.M. Laane. De resultaten van het promotieonderzoek zijn beschreven in dit proefschrift. Naast haar promotieonderzoek startte ze in 1998 met scholing op het gebied van arbeidshygiëne aan de Wageningen Universiteit. Vanaf februari 2000 is zij werkzaam als arbeidshygiënist bij de Arbo Unie in Veenendaal.

Dankwoord

Mensen die al vaker een proefschrift hebben doorgelezen weten het al: een proefschrift is nooit het werk van één persoon alleen. Vandaar een dankwoord op deze plek.

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