Flavoenzyme-Catalyzed Oxygenations and Oxidations of Phenolic Compounds

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Dedicated to Roger A. Sheldon on the occasion of his 60th birthday.

Abstract: Flavin-dependent monooxygenases and oxidases play an important role in the mineralization of phenolic compounds. Because of their exquisite regioselectivity and stereoselectivity, these enzymes are of interest for the biocatalytic production of fine chemicals and food ingredients. In our group, we have characterized several flavoenzymes that act on phenolic compounds, including 4-hydroxybenzoate 3-hydroxylase, 3-hydroxyphenylacetate 6-hydroxylase, 4-hydroxybenzoate 1-hydroxylase (decarboxylating), hydroquinone hydroxylase, 2-hydroxybiphenyl 3-monooxygenase, phenol hydroxylase, 4-hydroxyacetophenone monooxygenase and vanillyl-alcohol oxidase. The catalytic properties of these enzymes are reviewed here, together with insights obtained from site-directed and random mutagenesis.

Keywords: aromatic hydroxylation; flavin-dependent; flavoprotein; monooxygenase; oxidase; phenols; regioselectivity; stereoselectivity

1 Introduction

Phenolic compounds constitute one of the largest groups of natural products. Being synthesized by plants, their microbial degradation forms a substantial part of the biogeochemical cycle. Besides natural phenols, many anthropogenic (halo)phenolic derivatives are found in soil. These compounds have been used as building blocks in chemical and pharmaceutical syntheses and as herbicides and pesticides, sometimes causing serious local contamination of the environment.

The mineralization of (halo)phenolic compounds by aerobic microorganisms is initiated by the action of inducible flavoenzymes. These include flavoprotein monooxygenases, which use electrons from NAD(P)H to activate and cleave a molecule of oxygen, enabling incorporation of an oxygen atom into the substrate, as well as flavoprotein oxidases which react with their substrates without the need of external cofactors using only molecular oxygen.

Flavoprotein monooxygenases that are active with phenolic compounds usually contain FAD as prosthetic group. They activate molecular oxygen through the formation of a reactive flavin (hydro)peroxide which can attack the substrate by an electrophilic or nucleophilic process depending on the protonation state of the flavin (hydro)peroxide and the nature and protonation state of the substrate. In aromatic hydroxylation reactions, the flavin hydroperoxide acts as an electrophile. However, in Baeyer–Villiger monooxygenation reactions, the flavin peroxide is active as a nucleophile.[1]

Flavoprotein monooxygenases are of interest for biocatalytic applications, since they are able to perform regioselective and stereoselective oxidations under mild and environmentally friendly conditions.[2] Aspects

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relevant for biotechnological application of flavoprotein monooxygenases are the substrate and cofactor specificity, the regioselectivity and stereoselectivity of the conversions and the efficiency of the reactions including chances of unwanted side reactions and products. Clearly, these important characteristics are highly dependent on the structure-activity characteristics dictated by the active site of the enzymes. So far, relatively little is known about the structure-function relationship of flavoprotein monooxygenases. Except for *p*-hydroxybenzoate hydroxylase (PHBH) from *Pseudomonas fluorescens*[3] and phenol hydroxylase from *Trichosporon cutaneum*,[4] no structural information is available at the atomic level. Furthermore, with the aromatic hydroxylases of known structure it is unclear how the protein interacts with the pyridine nucleotide coenzyme.[5,6]

Based on sequence homology, FAD-dependent monooxygenases belong to the following subclasses:[1,7]

i) Aromatic hydroxylases. These enzymes share a typical dinucleotide binding domain for FAD binding, but lack a common NAD(P) binding fold.[8] PHBH is the prototype of this subclass.

ii) Baeyer–Villiger monooxygenases and *N*-hydroxylating enzymes. Many of these flavoproteins, like cyclohexanone monooxygenase and dimethylaniline monooxygenase, contain two dinucleotide binding motifs ($\beta\alpha\beta$-folds), one for FAD binding and one for NAD(P) binding.[9]

There are also aromatic hydroxylases that use FAD or FMN only as a coenzyme. With these enzymes, the reduced flavin is supplied by a flavin reductase at the expense of NAD(P)H. The protein partners of these aromatic hydroxylases do not tightly interact and contain no common dinucleotide binding motifs. Representatives of these two-component aromatic hydroxylases are pyrrole-2-carboxylate monooxygenase[10] and 4-hydroxyphenylacetate 3-hydroxylase.[11]

In addition to flavoprotein monooxygenases, also flavoprotein oxidases form an important group of flavin-dependent biocatalysts. Flavoprotein oxidases are attractive for synthetic applications because they constitute a group of enzymes that use molecular oxygen as

a clean and cheap oxidant and do not require expensive cofactors. The best characterized flavoprotein oxidase which acts on phenolic compounds is vanillyl-alcohol oxidase (VAO).[12] Unlike other flavoprotein oxidases such as glucose oxidase and D-amino acid oxidase,[13,14] VAO is structurally not related to the flavoprotein aromatic hydroxylases.[15]

In this review we discuss the different classes of flavin-dependent monooxygenases and oxidases that are active with phenolic compounds. For each class, a model enzyme is discussed to illustrate the catalytic potential as well as the limits and opportunities defined by their active site characteristics and mechanism of catalysis. For the flavoprotein aromatic hydroxylases, which form the largest subclass of flavin-dependent monooxygenases, a few other family members are discussed as well.

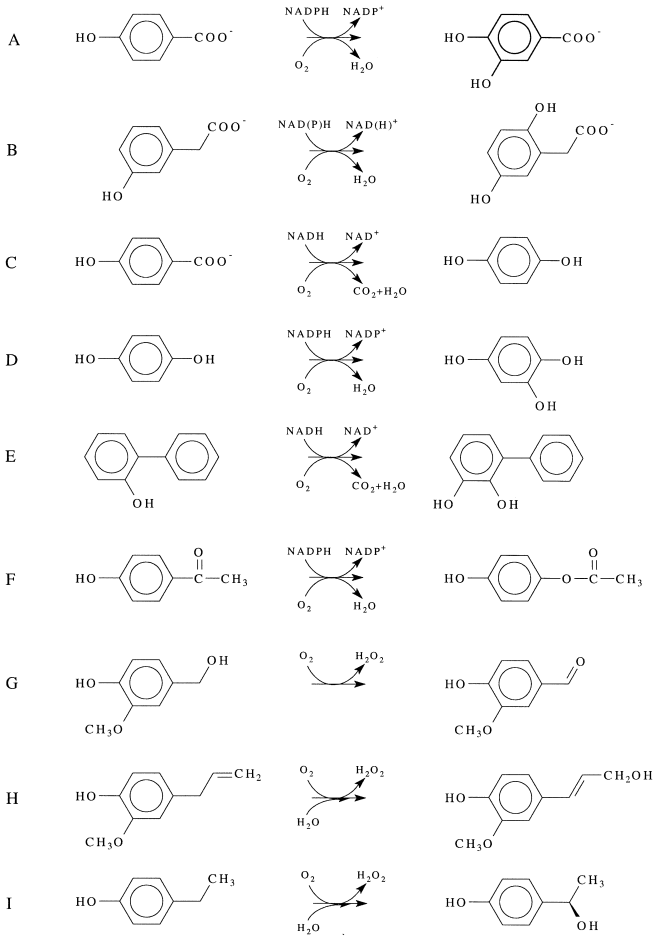
2 Flavoprotein Aromatic Hydroxylases

Flavoprotein aromatic hydroxylases have many catalytic properties in common and their substrate specificity is consistent with an electrophilic aromatic substitution mechanism.[1] To illustrate the mechanism of action of flavoprotein aromatic hydroxylases and their catalytic potential and limitations, we start this review with a detailed overview of the properties of PHBH from *P. fluorescens*. This enzyme is the most well-studied member of this class of flavoenzymes and its catalytic mechanism is representative for the whole family.

2.1 4-Hydroxybenzoate 3-Hydroxylase

PHBH from *P. fluorescens* is a homodimer of about 88 kDa with each subunit containing a non-covalently bound FAD.[16] PHBH catalyzes the conversion of 4-hydroxybenzoate into 3,4-dihydroxybenzoate (Scheme 1), a common intermediate step in the biodegradation of aromatic compounds in soil. The enzyme from *P. fluorescens* is strictly NADPH dependent.[17] However, some PHBH enzymes from other strains prefer NADH as electron donor.[18,19]

PHBH is one of the first flavoenzymes with known three-dimensional structure.[20] Figure 1 presents a ribbon structure of the enzyme-substrate complex as refined at 1.9 Å resolution.[3] The folding topology of PHBH is shared by several other oxidative flavoenzymes, including cholesterol oxidase,[21] glucose oxidase,[22] D-amino acid oxidase,[23] and phenol hydroxylase.[4] Crystal structures have also been obtained of PHBH in complex with substrate analogues and of a number of mutant enzymes.[5,6,24–30] The PHBH structure can be divided in three domains:[3] the FAD binding domain (residues 1 – 175), the substrate binding domain (residues 176 – 290), and the interface domain (residues 291 – 394). However, in recent structural classifications, the FAD binding domain and interface domain are regarded as a single domain, leaving PHBH as a two-domain protein.[13]



Scheme 1. Reactions catalyzed by flavoprotein monooxygenases and oxidases described in this review. (**A**) *ortho*-hydroxylation of 4-hydroxybenzoate, (**B**) *para*-hydroxylation of 3-hydroxyphenylacetate, (**C**) oxidative decarboxylation of 4-hydroxybenzoate, (**D**) *ortho*-hydroxylation of hydroquinone, (**E**) *ortho*-hydroxylation of 2-hydroxybiphenyl, (**F**) Baeyer-Villiger oxidation of 4-hydroxyacetophenone, (**G**) oxidation of vanillyl-alcohol, (**H**) VAO-mediated hydroxylation of eugenol, and (**I**) VAO-mediated hydroxylation of 4-ethylphenol.

The substrate binding site of PHBH is deeply buried in the protein.[3] Crystallographic studies with substrate and flavin analogues have indicated that the flavin ring moves out of the active site to allow substrate binding and product release.[24,25,27] Figure 2 presents a close up of the substrate binding site. Arg214 forms an indispensable ionic interaction with the carboxyl group of the substrate.[31] Ser212 and Tyr222 are also involved in binding the carboxylic moiety of 4-hydroxybenzoate. Substrate hydroxylation is facilitated by deprotonation

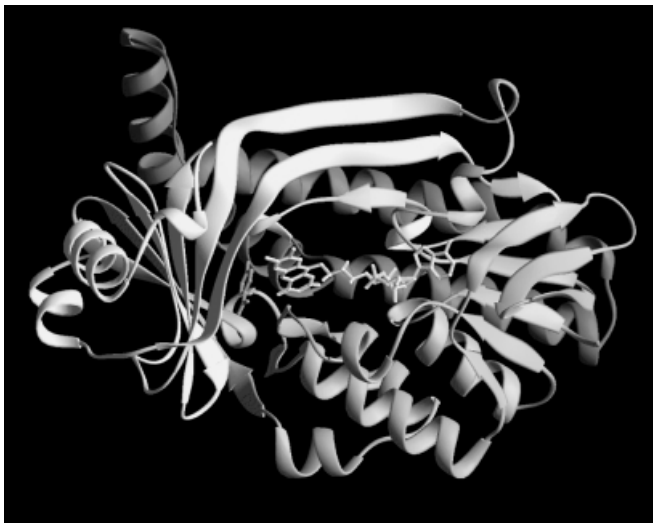


Figure 1. Ribbon diagram of the crystal structure of the enzyme-substrate complex of *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens*.[3] The isoalloxazine ring of the FAD is located close to the aromatic substrate in the interior position in the active site.

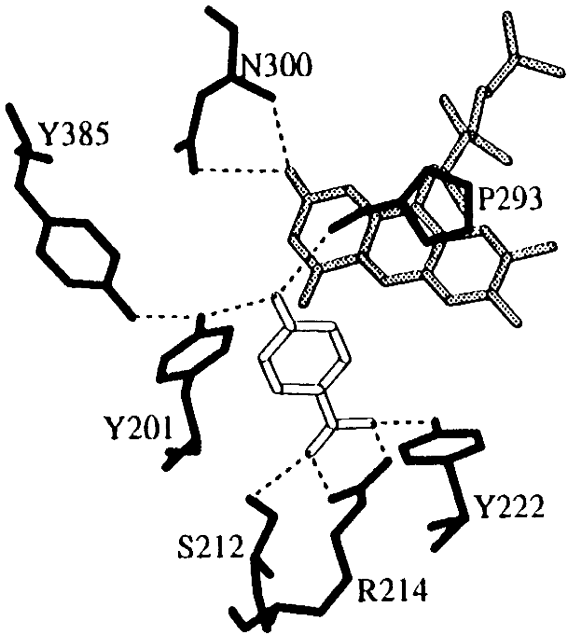


Figure 2. Close-up view of the active site of the enzyme-substrate complex of *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens*.[3] The FAD is gray, the aromatic substrate is white, and the protein residues are black. The *si* face of the flavin is the back of the page in this view; the *re* side is the front, as seen with the substrate phenol pointing out of the page.

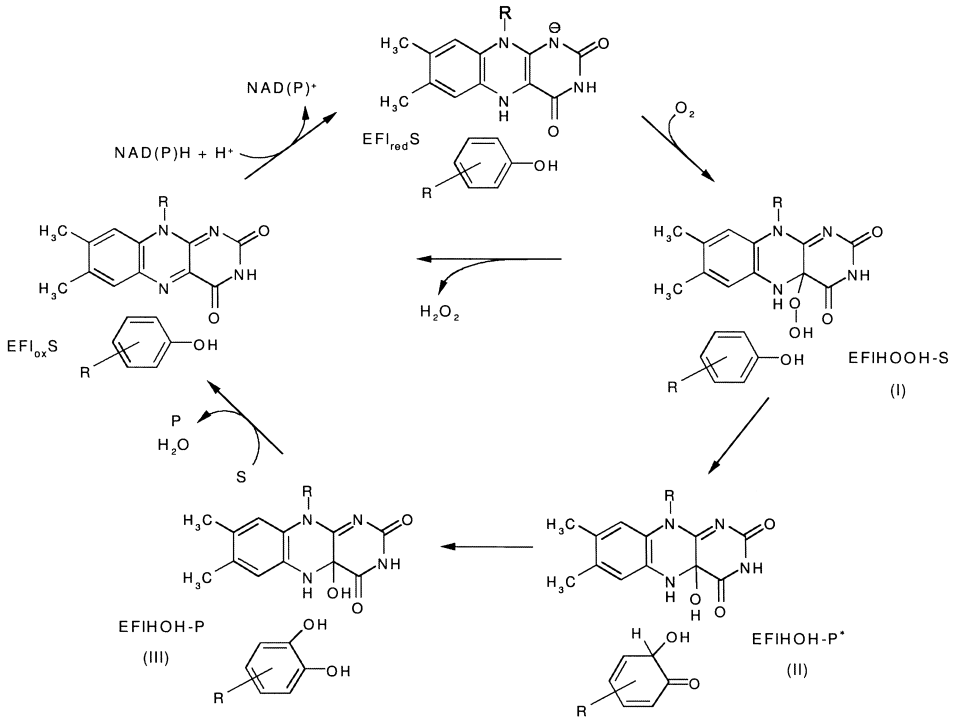
of the phenol.[32–35] The hydroxy group of the substrate is at hydrogen bond distance of Tyr201 which contacts Tyr385. Selective Phe replacements of these tyrosine residues strongly hamper substrate deprotonation and flavin reduction.[36,37] Pro293 is another important active site residue and is located in a highly conserved loop. The peptide carbonyl oxygen of Pro293 is within hydrogen bonding distance of the phenolic oxygen of bound substrate. It has been inferred from site-directed mutagenesis that the backbone rigidity at residue 293 is important for controlling the conformation of the flavin that is central to coordinating the complexities of catalysis.[38]

The catalytic mechanism of PHBH has been studied by stopped-flow absorbance spectroscopy.[39–41] The overall reaction can be divided into two half reactions, the reductive half reaction and the oxidative half reaction, each consisting of several reaction steps (Scheme 2). The reductive half reaction, which can be studied under anaerobic conditions, involves ternary complex formation. Only in the presence of substrate is fast reduction of the FAD cofactor by NADPH observed.[17,42] In the absence of substrate, a binary complex between NADPH and the enzyme results in a reduction rate that is 10^5 times slower. Thus, the substrate is an essential component in the reduction reaction because it stimulates the rate of flavin reduction, without being converted itself.

Only a few substrate analogues can stimulate the rate of flavin reduction.[37,43] Some benzoate derivatives bind to the enzyme and elicit its reduction but are not converted, and are therefore called effectors. The potential substrate 4-aminobenzoate binds to the enzyme in a similar way as 4-hydroxybenzoate,[24] but the reduction reaction in the presence of 4-aminobenzoate is very slow,[39] indicating a fine-tuning of the mechanism by which the substrate exerts its effector action. The rate of reduction does not correlate with the orientation of the flavin ring observed in the crystal structures, and all evidence presently available suggests that the effector specificity is linked to the ionic state of the substrate and the hydrogen bond network connecting the 4-hydroxy group with the protein surface.[24,44]

The fast release of $NADP^+$ from the reduced ternary complex is well established.[28,39,40] Furthermore, it has been demonstrated that the rate of dissociation of the substrate from the reduced enzyme-substrate complex is 5000 times slower than from the binary complex with the flavin in its oxidized state.[39] In addition to the increased reduction rate induced by substrate binding, the slow rate of substrate dissociation from the reduced enzyme is a second mechanism by which the enzyme controls the optimal use of valuable reducing equivalents.

The oxidative half reaction includes the reaction steps that lead to the hydroxylation of the substrate.[39,41] Upon reaction of the reduced enzyme-substrate complex with molecular oxygen the flavin C(4a)-hydroperoxide intermediate is formed. Several authors have discussed the actual way in which this reaction occurs, since in principle the reaction between singlet reduced flavin and triplet molecular oxygen is spin forbidden.[45]



Scheme 2. Catalytic cycle of flavoprotein aromatic hydroxylases.[39–41] $EFl_{OX}S$, oxidized enzyme-substrate complex; $EFl_{red}S$, reduced enzyme-substrate complex; EFlHOOH-S, flavin C(4a)-hydroperoxide enzyme-substrate complex (intermediate I); EFlHOH-P*, flavin C(4a)-hydroxide enzyme-hydroxycyclohexadienone product complex (intermediate II); EFlHOH-P, flavin C(4a)-hydroxide enzyme-product complex (intermediate III), S, substrate; P, product.

The flavin C(4a)-hydroperoxide species is generally referred to as intermediate I and has a typical absorption spectrum.[39,41] Protonation of the distal oxygen of the peroxide moiety increases the electrophilic reactivity of the flavin peroxide and facilitates its subsequent attack on the nucleophilic carbon centre of the substrate. In the hydroxylation step, the product 3,4-dihydroxybenzoate is formed together with the flavin C(4a)-hydroxide (intermediate III). The aromatic product initially is formed in its keto isomeric form (intermediate II)[46,47] which isomerizes to give the energetically favored dihydroxy isomer. Evidence for the initial formation of the quinoid form of the aromatic product comes from experiments with 2,4-dihydroxybenzoate as the substrate.[39,41,48]

In PHBH, the electron density on the C3 reaction centre of the substrate is increased by deprotonation of the phenol. This is supported by spectroscopic binding studies,[32,33,49] by molecular orbital calculations on the reactivity of the substrate,[34] and by reaction pathway calculations.[35] Moreover, the role of Tyr201 and Tyr385 in substrate activation is well established.[50]

In the presence of non-substrate effectors, the flavin C(4a)-hydroperoxide decays to oxidized enzyme with release of hydrogen peroxide.[43] Uncoupling can also occur with true substrates. The extent to which this uncoupling occurs is not only dependent on the nature and possibilities for activation of the substrate but is also influenced by the microenvironment of the active site. Other flavin-dependent monooxygenases as well as mutant PHBH enzymes may vary in the efficiency of substrate hydroxylation.[50]

The final steps in the catalytic cycle are the elimination of water from the flavin C(4a)-hydroxide and product release. Studies with 2-fluoro-4-hydroxybenzoate[51] and with the related phenol hydroxylase[47,52] have indicated that in this step a dead-end complex can be formed between the flavin C(4a)-hydroxide form of the enzyme and the substrate leading to substrate inhibition.

Studies from active-site variants have revealed that the scope of reactions catalyzed by PHBH cannot easily be changed by site-directed mutagenesis. Nevertheless, replacement of Tyr385 by Phe affects the regiospecificity of hydroxylation[36] and turns the enzyme into a more efficient dehalogenase.[53] Studies with tetrafluoro-4-hydroxybenzoate revealed that the hydroxylation of this fluorinated substrate is not restricted to the C3 centre of the aromatic ring but rather involves sequential oxygenation steps. ^{19}F NMR analysis showed that the initial aromatic product 2,5,6-trifluoro-3,4-dihydroxybenzoate is further converted to 5,6-difluoro-2,3,4-trihydroxybenzoate and that this reaction is most efficient with mutant Y385F. The initial dihydroxy product is not bound in a unique regiospecific orientation as also 2,6-difluoro-3,4,5-trihydroxybenzoate is formed. Due to elimination of the halogen substituent as a fluoride anion and the electron balance of the reaction, hydrox-

ylation of tetrafluoro-4-hydroxybenzoate involves the formation of quinone intermediates as primary products of oxygenolytic dehalogenation.[51] Ascorbate competes favorably with NADPH for the non-enzymatic reduction of these reactive intermediates and prevents the accumulation of non-specific oxidation products.[53]

PHBH does not contain a common dinucleotide fold for NADPH binding.[8] Studies from site-directed PHBH mutants have provided some insight into the mode of coenzyme recognition. Based on the properties of His162 and Arg269 variants, an interdomain binding mode for NADPH was proposed, where His162 and Arg269 interact with the pyrophosphate moiety of NADPH.[5] Furthermore, by changing several amino acid residues in the solvent exposed helix H2 region, it was possible to switch the PHBH coenzyme specificity.[54] This is the first coenzyme reversion of a member of a superfamily of flavoenzymes where the exact binding mode of the cofactor is unknown. The coenzyme switch suggests that in PHBH, an arginine is important in the recognition of the 2′-phosphate moiety of NADPH and that an acidic group (Asp/Glu) is required for the recognition of the 2′-OH group of NADH. This strengthens the idea that in NAD(P)H-dependent enzymes the main determinants for coenzyme recognition are conserved[55] and is in keeping with the hypothesis that biological specificity is caused to some extent by hydrogen bonding but is best mediated by charged residues.[56]

Recently, the first crystal structure of PHBH with NADPH was obtained.[6] In this structure of the R220Q mutant, the NADPH binds in an extended conformation at the enzyme surface in a groove that crosses the binding site of FAD. The pyrophosphate moiety is indeed situated in between the FAD binding domain and substrate binding domain but especially the nicotinamide moiety is located in a region not expected to be relevant for catalysis. Therefore, a role was proposed for protein and ligand dynamics with multiple movements involving the protein, the two cofactors, and the substrate.[6] However, the R220Q mutation changes the interdomain interaction, possibly influencing the mode of NADPH binding.

In conclusion, PHBH is an enzyme with a rather narrow substrate specificity. Besides the natural substrate (Scheme 1), the enzyme can convert 2,4-dihydroxybenzoate and a series of fluorinated 4-hydroxybenzoates. Most substrates are regioselectively hydroxylated at the C3-postion of the aromatic ring. With some substrate analogues and certain mutant enzymes, hydroxylation at C2 and C5 is feasible as well.

2.2 3-Hydroxyphenylacetate 6-Hydroxylase

3-Hydroxyphenylacetate 6-hydroxylase (HPAH) is an NAD(P)H-dependent FAD-containing monooxygenase involved in the catabolism of phenylacetate in *Flavobacterium* sp. JS-7.[57] The enzyme has a subunit mass of 63 kDa and exists mainly as a homodimer in solution. HPAH from *Flavobacterium* is optimally active around pH 8.3 but is most stable around pH 7.0. Above pH 7.5, the enzyme easily looses FAD and during purification, the presence of FAD and dithiothreitol are needed for high recovery of enzyme activity. Like many other flavoprotein monooxygenases, HPAH is severely inhibited by chloride ions, competitive to the aromatic substrate.

HPAH has a narrow substrate specificity. Besides 3-hydroxyphenylacetate which is stoichiometrically converted to 2,5-dihydroxyphenylacetate (Scheme 1), the only alternative substrate is 3,4-dihydroxyphenylacetate. This compound is exclusively converted to the (red) quinoid form of 2,4,5-trihydroxyphenylacetate. Due to severe uncoupling of hydroxylation (formation of hydrogen peroxide), more than two equivalents of NAD(P)H are needed for the conversion of one equivalent of 3,4-dihydroxyphenylacetate. Therefore, a catalase and glucose-6-phosphate containing NADPH generating system[58] is recommended to generate this product at a large scale.

2.3 4-Hydroxybenzoate 1-Hydroxylase

4-Hydroxybenzoate 1-hydroxylase from *Candida parapsilosis* CBS604 is a monomeric FAD-dependent monooxygenase of about 50 kDa.[59] This enzyme is induced when the yeast is grown on either 4-hydroxybenzoate, 2,4-dihydroxybenzoate, or 3,4-dihydroxybenzoate as the sole carbon source.[60] 4-Hydroxybenzoate 1-hydroxylase is optimally active at pH 8.0 and catalyzes the oxidative decarboxylation of a wide range of 4-hydroxybenzoate derivatives to the corresponding hydroquinones with the stoichiometric consumption of NAD(P)H and oxygen (Scheme 1).[61] Interestingly, nearly no hydroxylation activity is observed with 4-aminobenzoates. 4-Hydroxybenzoate 1-hydroxylase prefers NADH as the electron donor and is inhibited by chloride ions and by 3,5-dichloro-4-hydroxybenzoate, 4-hydroxy-3,5-dinitrobenzoate, and 4-hydroxyisophthalate which are competitors with the aromatic substrate. From the high activity of 4-hydroxybenzoate 1-hydroxylase with tetrafluoro-4-hydroxybenzoate and 4-hydroxy-3-nitrobenzoate and the molecular orbital characteristics of 4-hydroxybenzoate derivatives it was inferred that the phenolate form of the substrates is important for catalysis and that substrate hydroxylation involves the electrophilic attack of the putative flavin hydroperoxide at the C1-atom of the aromatic ring.[61] The resulting benzoquinone species is subsequently converted to the hydroquinone by spontaneous release of the carboxyl side chain.[61]

2.4 Hydroquinone Hydroxylase

Hydroquinone hydroxylase is a homodimeric FAD-dependent monooxygenase of about 150 kDa, which is also involved in the degradation of 4-hydroxybenzoate in *Candida parapsilosis* CBS604.[62] This enzyme is abundantly expressed when the yeast is grown on either 4-hydroxybenzoate, 2,4-dihydroxybenzoate, 1,3-dihydroxybenzene, or 1,4-dihydroxybenzene as sole carbon source. Hydroquinone hydroxylase catalyzes the *ortho*-hydroxylation of a wide range of monocyclic phenols with the stoichiometric consumption of NADPH and oxygen (Scheme 1). The hydroxylation of monofluorinated phenols is highly regiospecific with a preference for C6 hydroxylation. This regiospecificity of hydroxylation resembles that of phenol hydroxylase from *T. cutaneum*,[63] but differs from the regiochemistry experienced with whole cells of the Gram-positive bacterium *Rhodococcus opacus* 1G, where it was found that oxidative dehalogenation of 2-halogenated phenols is preferred to hydroxylation of the aromatic ring at a non-halogenated position.[64] It also differs from the transformation of monofluorophenols in whole cells of *R. opacus* 1cp, where it was found that the initially formed catechols are further converted to the corresponding fluoropyrogallols.[65]

During purification it was noted that hydroquinone hydroxylase is sensitive to limited proteolysis.[62] Proteolytic cleavage does not influence the enzyme's dimeric nature but results in relatively stable protein fragments of 55, 43, 35 and 22 kDa. N-Terminal peptide sequence analysis revealed the presence of two nick sites and showed that hydroquinone hydroxylase from *C. parapsilosis* is structurally related to phenol hydroxylase from *T. cutaneum*. The latter enzyme has been overexpressed in *E. coli*[66] and its three-dimensional structure was recently solved.[4] From the crystallographic data it was argued that the severe uncoupling of hydroxylation in phenol hydroxylase is related to a movement of the flavin ring in concert with a large conformational change of a flexible protein segment which acts as an active site lid. The C-terminal part of this flexible segment is closest to the flavin and its amino acid sequence is not conserved in hydroquinone hydroxylase from *C. parapsilosis*.[62] This suggests that the efficient hydroxylation of phenolic substrates in hydroquinone hydroxylase from *C. parapsilosis* is related to the performance of the active site lid and that an effective closure of this lid sequesters the hydroxylation site from solvent, thereby preventing the unproductive formation of hydrogen peroxide.

2.5 2-Hydroxybiphenyl 3-Monooxygenase

2-Hydroxybiphenyl 3-monooxygenase (HbpA) is an inducible FAD-dependent aromatic hydroxylase involved in the degradation of the fungicide 2-hydroxybiphenyl by the soil bacterium *Pseudomonas azelaica* HBP1[67]. This strain employs a *meta*-cleavage pathway with a broad substrate spectrum for breaking down 2-hydroxybiphenyls.[68] HbpA is a homotetramer of about 250 kDa, is optimally active around pH 7.5 and prefers NADH as the electron donor. 2,3-Dihydroxybiphenyl, the product of the reaction with 2-hydroxybiphenyl (Scheme 1) is a non-substrate effector, strongly facilitating NADH oxidation and hydrogen peroxide formation without being hydroxylated.

HbpA catalyzes the *ortho*-hydroxylation of a wide range of 2-substituted phenols. Besides phenyl rings and aliphatic side chains, halogens are accepted as phenol substituents.[69] The *hbpA* gene has been cloned in *E. coli*[67] and the recombinant strain was used as a whole cell biocatalyst for the large-scale production of 2-substituted catechols.[70] The whole cell approach has the advantage of coenzyme recycling but can be problematic when the substrate and/or product are toxic to the cells. In case of HbpA, the toxic effects of 2-hydroxybiphenyl are minimized by feeding the substrate to the reactor at a rate slightly below the maximum biooxidation rate. Furthermore, the 3-phenylcatechol product can be removed by continuous adsorption on a solid resin.[70] In another biocatalytic process, partially purified recombinant HbpA was used in combination with a formate/formate dehydrogenase cofactor recycling system for the preparative regioselective hydroxylation of 2-hydroxybiphenyl.[71] By using organic/aqueous emulsions, coupled with *in situ* product recovery, volumetric productivities of up to 0.45 g L^{-1} h^{-1} were obtained.

The catalytic mechanism of HbpA has been investigated at 7 °C by stopped-flow absorption spectroscopy.[72] The reaction essentially follows the catalytic cycle depicted in Scheme 2. Binding of 2-hydroxybiphenyl highly stimulates the rate of enzyme reduction by NADH. During this reaction a transient charge-transfer complex is formed between the reduced flavin and NAD^+. Free reduced HbpA reacts rapidly with oxygen to form oxidized enzyme with no appearance of intermediates. In the presence of 2-hydroxybiphenyl (but not in the presence of 3-phenylcatechol), two consecutive spectral intermediates are observed, representing the flavin C(4a)-hydroperoxide and the flavin C(4a)-hydroxide, respectively. These data are consistent with a ternary complex mechanism in which the aromatic substrate has strict control in both the reductive and oxidative half-reaction in a way that reactions leading to substrate hydroxylation are favored over those leading to the futile formation of hydrogen peroxide.

The catalytic scope and performance of HbpA have been improved by directed evolution using error-prone PCR.[73] *In situ* screening of mutant libraries resulted in the identification of the I244V variant, which has an

increased activity towards 2-*tert*-butylphenol, 2-methoxyphenol, and the natural substrate 2-hydroxybiphenyl. The double replacement V368A/L417F was found to improve the efficiency of substrate hydroxylation by reducing the uncoupled oxidation of NADH. Sequence alignments revealed that the primary structure of HbpA shares 20% identity with phenol hydroxylase from *T. cutaneum*. Based on structure homology modelling it was inferred that Ile244 of HbpA is located in the substrate binding pocket and is involved in accommodating the phenyl substituent of the phenol. In contrast, Val368 and Leu417 are not close to the active site and would not have been obvious candidates for modification by rational design. In another study, laboratory evolution of HbpA resulted in an enzyme variant which hydroxylates indole and indole derivatives, such as hydroxyindoles or 5-bromoindole.[74] This indigo-producing HbpA variant contains the amino acid substitutions Asp222Val and Val368Ala. Interestingly, Asp222 is located in the active site and corresponds to Tyr201 of PHBH and Tyr289 of phenol hydroxylase from *T. cutaneum*. Recent results from site-directed mutagenesis have indicated that Tyr289 in phenol hydroxylase is mainly involved in flavin reduction[75] whereas Tyr201 in PHBH is also involved in substrate activation.[36,37] In conclusion, these studies show that directed evolution is a powerful approach for changing the biocatalytic performance of a flavoenzyme.

2.6 Two-Component Aromatic Hydroxylases

Besides the single-component flavoprotein aromatic hydroxylases, there is also a group of flavin-dependent aromatic hydroxylases that consist of two protein components of different size. For 4-hydroxyphenylacetate 3-hydroxylase from *Pseudomonas putida* it was shown that the FAD-containing protein component acts as an NADH oxidase and that the presence of the other non-redox protein component is required for efficient substrate hydroxylation.[76] So far, this is the only example of a flavoprotein hydroxylase where binding of another protein component is needed for the stabilization of the flavin hydroperoxide.

Another family of two-component flavin-dependent aromatic hydroxylases consists of a relatively small flavin reductase which generates reduced flavin at the expense of NAD(P)H and a large protein component which is essential for substrate hydroxylation.[10,11] Besides hydroxylases, also epoxide-forming enzymes such as styrene monooxygenase[77] and tryptophan 7-halogenase[78] belong to this family. The exact mechanism by which the reduced flavin in these enzymes is transferred to the oxygenation site is as yet far from clear but it has been shown that the small reductase from the wild-type strain can be replaced by a flavin reductase from another bacterium.[11,78] In our group, we have characterized a two-component phenol hydroxylase from the thermophilic bacterium *Bacillus thermoglucosidasius* which is active with a range of simple phenols, including chlorophenols, fluorophenols, and cresols.[79] The large dimeric protein component of the recombinant phenol hydroxylase as expressed in *E. coli* is highly unstable in purified form, limiting structure-function relationship studies. The small dimeric flavin reductase on the other hand, is very stable in its recombinant form. Interestingly, each subunit of the reductase contains a tightly bound FAD molecule which is involved in the NADH-dependent reduction of free flavins, including FAD, FMN, and riboflavin.[80] However, like tryptophan 7-halogenase,[78] the large oxygenase component of phenol hydroxylase from *B. thermoglucosidasius* is specific for FAD.

3 Baeyer–Villiger Monooxygenases

Baeyer–Villiger monooxygenases (BVMOs) are NAD(P)H-dependent flavoenzymes that catalyze Baeyer–Villiger reactions, i.e., the oxidation of ketones to esters or lactones.[81,82] In the enzymatic Baeyer–Villiger reaction, the protein-bound flavin peroxide is active in its deprotonated form thereby performing a nucleophilic attack on the carbon atom of the substrate ketone.[83,84] From rapid reaction studies on *Acinetobacter* cyclohexanone monooxygenase, evidence was obtained that the relatively stable flavin peroxide can undergo acid/base equilibrium.[85] This might explain why BVMOs are also active with certain electron-rich compounds[82] and underlines the ambivalent character of the flavin (hydro)peroxide oxygenation species.

BVMOs are versatile biocatalysts that have been widely used for the regio- and stereoselective transformation of aliphatic ketones.[82,86,87] In view of the need of NAD(P)H-recycling, most of these reactions have been performed with whole microbial cells.[88] Relatively little is known about BVMOs that are active with aromatic compounds. However, several aerobic microorganisms are capable of utilizing aryl ketones for their growth[89] and recently, we succeeded in the purification of a Baeyer–Villiger type flavoenzyme that catalyzes the first step in the degradation of 4-hydroxyacetophenone in *Pseudomonas fluorescens* ACB.[7,90] Characterization revealed that this 4-hydroxyacetophenone monooxygenase (HAPMO) is a homodimer of about 140 kDa with each subunit containing a tightly non-covalently bound FAD. In contrast, HAPMO involved in the degradation of 4-ethylphenol in *P. putida* JD1 purifies as a monomer of 70 kDa.[89]

HAPMO from *P. fluorescens* catalyzes the strictly NADPH-dependent and stoichiometric oxidation of 4-hydroxyacetophenone to 4-hydroxyphenyl acetate (Scheme 1). Besides the natural substrate, HAPMO is active with a wide range of other aryl ketones.[7] The

highest catalytic efficiency is observed with compounds bearing an electron-donating substituent at the *para*-position of the aromatic ring. HAPMO is also active with 4-hydroxybenzaldehyde and 4-hydroxypropiophenone.

Ring-substituted phenyl acetates are valuable synthons for the production of fine chemicals and neuroactive pharmaceuticals.[91] However, the large-scale production of phenyl acetates by whole cells of *P. fluoresens* ACB is limited by the presence of a highly active esterase.[90,92] ^{19}F NMR studies with purified HAPMO at pH 6 and pH 8 showed that the Baeyer–Villiger oxidation of 4-fluoroacetophenones occurs faster at pH 8 but that the fluorophenyl acetates produced are better stabilized at pH 6.[90] Thus, the large-scale biocatalytic production of ring-substituted phenyl acetates requires carefully selected conditions and should either be performed by i) using an engineered microbial strain that lacks esterase activity or ii) by using isolated enzyme coupled with an efficient cofactor recycling system.[93]

With the above considerations in mind, the DNA of *P. fluorescens* ACB was isolated and the operon encoding genes involved in the degradation of 4-hydroxyacetophenone were characterized.[7] The fourth gene of this operon (*hapD*) encodes for 4-hydroxyphenyl acetate hydrolase whereas the fifth gene (*hapE*) encodes for HAPMO. Sequence analysis revealed that except for an N-terminal extension of about 135 residues, HAPMO shares 30% sequence identity with two other characterized BVMOs, cyclohexanone monooxygenase and steroid monooxygenase, classifying the enzyme as a type I BVMO. These BVMOs are FAD and NADPH dependent and have identical subunits. Type II BVMOs on the other hand are FMN and NADH dependent and are composed of $\alpha 2\beta$ trimers.[86] The role of the N-terminal extension in HAPMO is not clear, but studies of truncated forms indicate that the N-terminal domain is important for the protein structural integrity.[7]

Type I BVMOs contain two dinucleotide binding motifs ($\beta\alpha\beta$-folds), one for FAD binding and one for NAD(P) binding. Because no crystal structures of these flavoenzymes are available yet, it was of interest to probe the functional role of these sequence motifs in further detail. Using newly reported BVMO sequences, a BVMO identifying-sequence motif: FxGxxxHxxx-W(P/D) was uncovered that is critically involved in catalysis.[9] The important role of the histidine in this fingerprint sequence was confirmed by the negligible activity of the H296A mutant. The functional role of another sequence motif was assessed by replacement of the conserved Gly490.[7] Analysis of the G490A mutant revealed a dramatic effect on the interaction with NADPH, suggesting that the ATG motif comprising Gly490 is involved in NADPH binding.

4 Flavoprotein Oxidases

Flavoprotein oxidases react with their substrates without the need of external cofactors. Catalysis involves two half-reactions in which first the flavin cofactor is reduced by the substrate, and subsequently the reduced flavin is reoxidized by molecular oxygen.[1] Vanillyl-alcohol oxidase (VAO) from *Penicillium simplicissimum* is the prototype flavoprotein oxidase that is active with phenolic compounds. VAO is a homooctamer of 520 kDa with each subunit containing a covalently bound 8α-(*N*3-histidyl)-FAD.[94] VAO catalyzes the conversion of vanillyl alcohol to vanillin in the presence of molecular oxygen (Scheme 1). Besides vanillyl alcohol, the enzyme is active with a wide range of other phenolic compounds.[95,96] As discussed elsewhere,[97] some of the VAO-mediated reactions are of industrial relevance.

Stopped-flow kinetic studies[98] have indicated that the reaction of VAO with phenolic substrates involves the initial transfer of a hydride from the Cα-atom of the substrate to the N5-atom of the flavin, resulting in the formation of a complex between reduced enzyme and *p*-quinone methide product intermediate. Next, the reduced flavin is reoxidized by molecular oxygen, yielding hydrogen peroxide, and the *p*-quinone methide intermediate is further converted by water in the enzyme active site. With eugenol as a substrate, this leads to the stoichiometric formation of coniferyl alcohol (Scheme 1), whereas with 4-ethylphenol, the hydroxy group becomes exclusively inserted at the Cα-atom, resulting in the formation of 1-(4′-hydroxphenyl)ethanol (Scheme 1). With short-chain 4-alkylphenols, the protein-bound quinone methide is attacked by water in a stereospecific manner yielding the (*R*)-enantiomer of the alcohol product in high enantiomeric excess.[99] However, with long-chain 4-alkylphenols, the *p*-quinone methide is no longer attacked by water and rearranges to the alkene.[96] A similar observation was made when the reaction with 4-propylphenol was performed in organic solvents,[100] leading to the conclusion that the outcome of products formed from short-chain and medium-chain 4-alkylphenols is due to variations in the intrinsic reactivity of the enzyme-bound *p*-quinone methide, the water accessibility of the enzyme active site, and the orientation of the alkyl side-chain of the substrate. No catalytic activity was observed with 4-alkylphenols when the aliphatic side-chain is longer than seven carbon atoms.[96] This is in line with structural data which show that the VAO active site cavity is completely filled when the inhibitor 1-(4′-heptenyl)phenol is bound.[101]

From the VAO structure,[101] several amino acid residues have been implicated to play an important role in catalysis. Studies from His61 and His422 mutants established that the covalent linkage between the C8α atom of the isoalloxazine ring of the flavin and the N3

atom of His422 raises the redox potential of the FAD cofactor, thereby increasing the oxidative power of the enzyme.[12,102] Catalysis is also facilitated by ionization of the phenolic moiety of the substrate as induced by hydrogen bonding to Tyr108, Tyr503, and Arg504 (Figure 3). Another key residue is Asp170, which is located close to the N5-atom of the flavin and the reactive methylene group of the substrate. With vanillyl alcohol, eugenol, and 4-(methoxymethyl)phenol as substrates, the conserved mutant D170E is up to 100-fold less active than wild-type VAO whereas with other Asp170 variants a more than 1,000-fold decrease in enzyme activity is observed.[103] As no structural changes were found in the mutant enzymes, this indicates that Asp170 is crucial for efficient redox catalysis.

Interestingly, substitution of Asp170 by Ser or Glu strongly influences the hydroxylation efficiency of VAO with 4-alkylphenols.[104] Whereas the D170A and D170S mutants favor the hydration of the *p*-quinone methide intermediate, the D170E mutant favors the formation of alkenes. This suggests that the efficiency of hydroxylation of 4-alkylphenols is regulated by the bulkiness of the side-chain of residue 170 and not by its ionic character.

Studies from site-directed double mutants revealed that the stereochemistry of VAO with short-chain 4-alkylphenols is also related to the function of Asp170.[105] D170A and D170S preferentially hydroxylate 4-ethylphenol to the (*R*)-enantiomer of 1-(4′-hydroxyphenyl)ethanol. However, when the acidic residue is relocated to the other face of the substrate binding pocket (D170A/T457E and D170S/T457E variants), the enzyme exhibits an inverted stereoselectivity with 4-ethylphenol. This high preference for the (*S*)-enantiomer is likely explained by the Glu457 activated attack of a water molecule from the opposite face of the substrate.

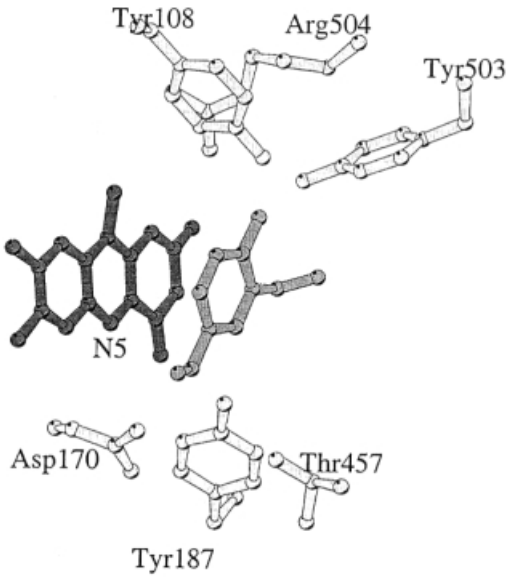


Figure 3. Schematic representation of the active site of the enzyme-isoeugenol complex of vanillyl-alcohol oxidase from *Penicillium simplicissimum*.[101] The isoalloxazine ring of FAD is black, the inhibitor is gray and the protein residues are white. Asp170 is positioned at 0.35 nm from flavin N5 and the Cα-atom of isoeugenol.

5 Conclusion

Flavoprotein monooxygenases and oxidases are useful enzymes for the selective introduction of oxygen atoms in phenolic compounds. Flavoprotein monooxygenases work optimally around pH 8 whereas VAO is most active near pH 10. Flavoprotein aromatic hydroxylases have an unique regiochemistry, allowing the synthesis of a wide range of catechols and hydroquinones. Most flavoprotein aromatic hydroxylases have a narrow substrate specificity and can only be used for the synthesis of a selected range of products. The substrate specificity of PHBH can be broadened by site-directed mutagenesis, but only to a limited extent. Some flavoprotein aromatic hydroxylases like, e.g., 4-hydroxybenzoate 1-hydroxylase and HbpA, have a relaxed substrate specificity, allowing the synthesis of a range of ring-substituted hydroquinones and catechols. Directed evolution has proven to be an attractive method for the improvement of the efficiency of substrate hydroxylation of HbpA and for the introduction of new activities.

Two-component flavin-dependent monooxygenases represent a newly recognized family of oxidative enzymes and their biocatalytic performance is unexplored. It is anticipated that studies from structural and functional genomics[106] will allow a more thorough characterization of this class of monooxygenases and provide future possibilities for synthetic applications.

BVMOs are extremely useful for the synthesis of esters and lactones and for asymmetric oxidations. HAPMO is the first described BVMO which is active with phenolic compounds. The scope of reactions of HAPMO has not yet been fully explored but it is clear that this enzyme converts a wide range of acetophenones into the corresponding phenyl acetates.

Flavoprotein oxidases such as VAO are capable of introducing side-chain modifications in *p*-substituted phenols. This is useful for the synthesis of flavors and fragrances and for the production of pure enantiomers. Studies from site-directed mutants have demonstrated that the stereoselectivity of VAO can be inverted by rational redesign.

Insight into the structure, mechanism and biochemical properties of oxidative flavoenzymes is of great help to select for biocatalysts with a high degree of chemo-, regio-, and stereoselectivity. In view of the need of expensive cofactors, it is expected that NAD(P)H-dependent flavoprotein monooxygenases will be mainly used in whole cell systems.[70,88,107–110] Structure-function relationship studies on PHBH and HAPMO have given

some insight why these enzymes are strictly dependent on NADPH. Such studies are not only useful for the redesign of the coenzyme specificity but will also teach us more about the evolutionary relationship of flavoenzymes.

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