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Review

# Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts

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## Abstract

During the last decades a large number of flavin-dependent monooxygenases have been isolated and studied. This has revealed that flavoprotein monooxygenases are able to catalyze a remarkable wide variety of oxidative reactions such as regioselective hydroxylations and enantioselective sulfoxidations. These oxidation reactions are often difficult, if not impossible, to be achieved using chemical approaches. Analysis of the available genome sequences has indicated that many more flavoprotein monooxygenases exist and await biocatalytic exploration. Based on the known biochemical properties of a number of flavoprotein monooxygenases and sequence and structural analyses, flavoprotein monooxygenases can be classified into six distinct flavoprotein monooxygenase subclasses. This review provides an inventory of known flavoprotein monooxygenases belonging to these different enzyme subclasses. Furthermore, the biocatalytic potential of a selected number of flavoprotein monooxygenases is highlighted.

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**Keywords:** Flavoprotein monooxygenase; Hydroxylation; Sulfoxidation; Epoxidation; Baeyer–Villiger oxidation; Biocatalysis

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## 1. Biocatalysis using monooxygenases

Efficient and specific insertion of one oxygen atom into an organic substrate is a reaction that is difficult to perform by chemical means. Although some catalysts have been developed that are able to catalyze specific oxygenations, the exquisite specificity of enzymes performing monooxygenations (monooxygenases) is still unequalled. This makes this class of enzymes (EC 1.13 and EC 1.14) of great interest for synthetic purposes. In the last few decades a multitude of monooxygenases have been discovered revealing a wide range of reactivities and selectivities. Nevertheless, only a small number have been explored for their synthetic value. This is partly due to a limited availability of enzymes, e.g. due to difficulties of enzyme expression or isolation. Another practical problem associated with

biocatalytic applications involving monooxygenases is the fact that most of these enzymes depend on expensive coenzymes for their activities. Recent developments have alleviated some of these practical issues by, e.g. employing improved expression systems, developing coenzyme regeneration systems or coenzyme replacements. Also, in recent years several enzymes have been discovered that can perform hydroxylations while being coenzyme-independent. As a consequence of these developments, effective practical biocatalytic applications with monooxygenases are in reach.

The efforts in discovery and characterization of monooxygenases have revealed that a number of different types of enzymes have evolved in nature to catalyze monooxygenation reactions. One of the best known examples is the family of cytochrome P450 monooxygenases. These heme-containing enzymes (EC 1.14.13,

EC 1.14.14 and EC 1.14.15) are relatively abundant, occur in many isoforms, and have been shown to catalyze a plethora of specific oxygenations. P450 genes are abundant in eukaryotic genomes, e.g. 57 P450 genes have been found in the human genome. Prokaryotic genomes have been shown to be less rich in P450 monooxygenases. At present, only ~300 P450 gene homologs can be identified in the presently sequenced bacterial genomes, corresponding to a frequency of just one P450 gene in each prokaryotic genome.

The general catalytic mechanism of P450 monooxygenases has been elucidated revealing that the catalyzed oxidation reaction is tightly coupled to substrate binding. Only upon substrate binding the heme cofactor can be reduced by a specific electron donor. The electrons are typically delivered by an associated flavin-containing reductase which in turn accepts electrons from NAD(P)H. A striking catalytic feature of P450 enzymes is their ability to hydroxylate unactivated carbon atoms. Regioselective hydroxylations catalyzed by P450 monooxygenases have been shown to be of value to modify sterols and steroids.

Except for P450 monooxygenases, other widespread monooxygenase classes are non-heme monooxygenases (EC 1.14.16), copper-dependent monooxygenases (EC 1.14.17 and EC 1.14.18), and flavin-dependent monooxygenases (EC 1.13.12 and EC 1.14.13). In recent years also a few new types of monooxygenases have been identified that do not contain any of the aforementioned cofactors. The polyketide monooxygenase ActVA-Orf6, involved in actinorhodin biosynthesis in *Streptomyces coelicolor* (Sciara et al., 2003), and YgiN, a quinol monooxygenase from *Escherichia coli* (Adams and Jia, 2005), oxidize multiringed aromatic substrates without the help of any cofactor. Aclacinomycin-10-hydroxylase, involved in anthracycline biosynthesis in *Streptomyces purpurascens*, is again another monooxygenase type as it depends on *S*-adenosyl-L-methionine as cofactor (Jansson et al., 2003; Jansson et al., 2005). The latter atypical monooxygenases appear to be relatively rare as only a few examples have been identified so far.

A major and perhaps most ubiquitous group of monooxygenases is formed by the flavin-dependent monooxygenases. Flavin-dependent monooxygenases have been shown to cover a wide range of different oxygenation reactions while being highly regio- and/or enantioselective. In this review an overview of

flavin-dependent monooxygenases is given with special emphasis on members that display interesting biocatalytic properties. To provide a clear and systematic overview, also a novel classification for all known flavo-protein monooxygenases is proposed.

## 2. Flavoprotein monooxygenases

The concerted reaction between O<sub>2</sub> and carbon in organic compounds is spin-forbidden. Nevertheless, a large number of enzymes have found a way to use molecular oxygen as a substrate to oxygenate an organic substrate. For such reactivity an enzyme has to be able to activate molecular oxygen. To create a species that transfers molecular oxygen, enzymes often use a transition metal which may or may not be bound to an organic cofactor, e.g. heme in P450 monooxygenase. However, flavin-dependent monooxygenases employ a purely organic cofactor for oxygenation reactions.

For reactivity with molecular oxygen a flavin cofactor has to be in the reduced form. This electron rich flavin intermediate is able to use molecular oxygen as a substrate (Massey, 1994). Upon a one-electron transfer from reduced flavin to oxygen, a complex is formed of superoxide and the flavin radical. A subsequent spin inversion results in formation of reduced oxygen (Ghisla and Massey, 1989). For most flavoprotein monooxygenases, a covalent adduct between the C<sub>(4a)</sub> of the flavin and molecular oxygen is formed and stabilized, yielding a reactive C<sub>(4a)</sub>-hydroperoxyflavin species (Fig. 1). Such a peroxyflavin is unstable and typically decays to form hydrogen peroxide and oxidized flavin. However, flavoprotein monooxygenases are able to stabilize this species in such a way that it can oxygenate a substrate (Entsch and van Berkel, 1995). Depending on the protonation state of the peroxyflavin, either a nucleophilic or electrophilic attack on the substrate is performed. As a result, a single atom of molecular oxygen is incorporated into the substrate, while the other oxygen atom is reduced to water (Fig. 1). Oxygenation reactions catalyzed by flavoprotein monooxygenases include amongst others hydroxylations, epoxidations, Baeyer–Villiger oxidations and sulfoxidations (Fig. 2). The specific type of oxygenation and selectivity depends on the shape and chemical nature of the active site of each specific monooxygenase. However, based on sequence and

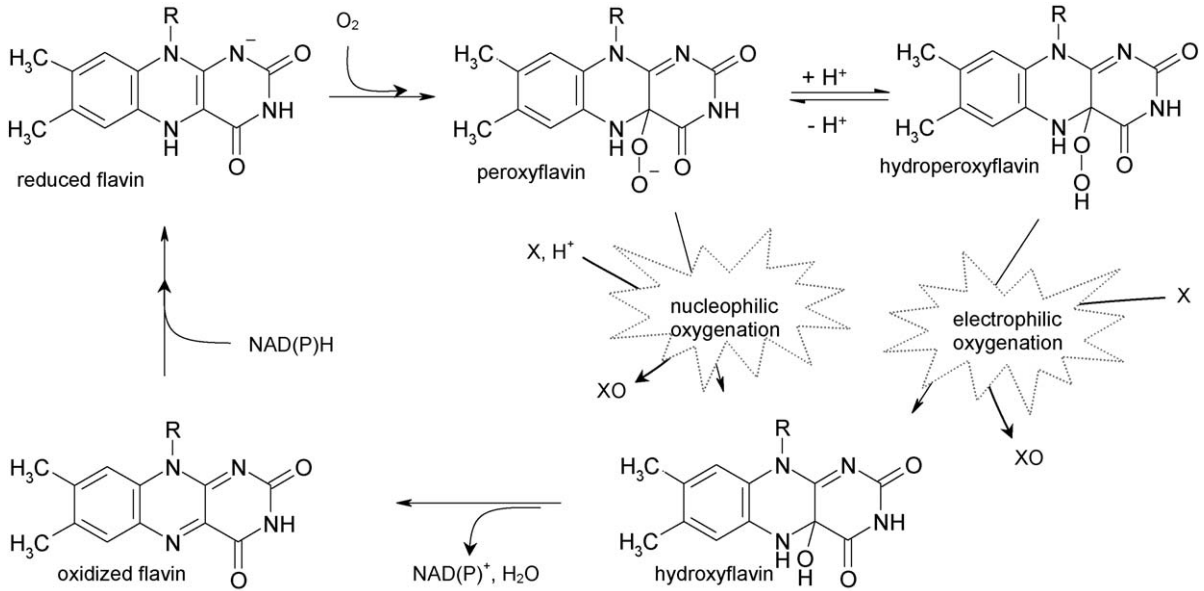


Fig. 1. General mechanism of oxygenation reactions catalyzed by external flavoprotein monooxygenases.

structural homology several flavoprotein monooxygenase subclasses can be discriminated revealing that each subclass appears to cover only a limited range of oxygenation reactions (see below). This suggests that the type of oxygenation catalyzed by a flavoprotein monooxygenase is dictated to some extent by its structural fold. Apparently, each fold favors catalysis of certain oxygenation reactions.

As mentioned above, a flavin can only react with molecular oxygen when it is in the reduced form. Most

monooxygenases equip the flavin cofactor with electrons by consuming reduced coenzymes, NADH or NADPH. These monooxygenases are called external flavoprotein monooxygenases (EC 1.14.13). However, some examples exist in which the flavin is reduced by the substrate itself. E.g. in the case of lactate monooxygenase (EC 1.13.12.4) lactate is oxidized into pyruvate, yielding reduced flavin (Sutton, 1957). In a subsequent step the reduced flavin reacts with molecular oxygen to oxidize the formed pyruvate into carbon dioxide and

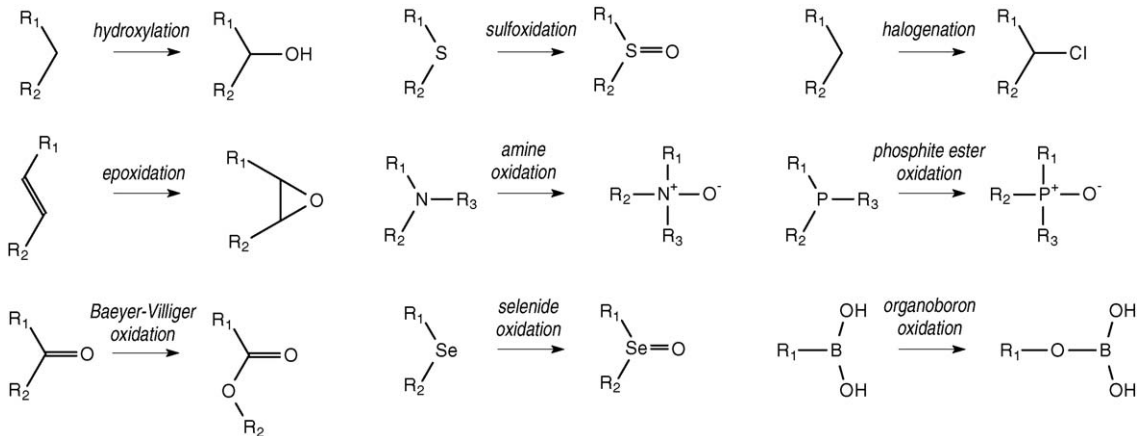


Fig. 2. Reactions catalyzed by flavoprotein monooxygenases.

acetate. These type of flavin-dependent monooxygenases are called internal monooxygenases (EC 1.13.12) and are extremely rare. Therefore, this review will only focus on the group of external monooxygenases.

Except from the external and internal monooxygenases, there is another group of flavin-dependent enzymes that can catalyze hydroxylation reactions. However, in these enzymes the flavin is not directly involved in the monooxygenation reaction but is needed to oxidize the substrate. The oxygen atom that is eventually incorporated into the oxidized (=activated) substrate is derived from water. Examples of such flavoenzymes are vanillyl-alcohol oxidase (EC 1.1.3.38) and *p*-cresol methyl hydroxylase (EC 1.17.99.1), which respectively, use molecular oxygen and cytochrome *c* for flavin reoxidation. Both these enzymes are able to hydroxylate a range of alkylphenols in an enantioselective manner (Reeve et al., 1990; Drijfhout et al., 1998; van den Heuvel et al., 2000).

### 3. Classification of flavoprotein monooxygenases

At present several hundreds of flavoenzymes have been characterized and described. Most of these flavoenzymes contain a non-covalently bound flavin as prosthetic group, but there are some that contain covalently bound FAD or FMN (Mewies et al., 1998). For example, the above-mentioned vanillyl-alcohol oxidase contains FAD linked to a histidine (de Jong et al., 1992) while *p*-cresol methyl hydroxylase contains FAD linked to a tyrosine (McIntire et al., 1985). For vanillyl-alcohol oxidase it was shown that such a covalent linkage is beneficial for catalysis as it enhances the oxidative power of the flavin cofactor (Fraaije et al., 1999). So far, all internal and external flavoprotein monooxygenases have been shown to bind the flavin cofactor, FAD or FMN, in a non-covalent manner (Fig. 3).

Classification of flavoenzymes has been done using different criteria, such as the type of chemical reaction that is catalyzed, the nature of the reducing and oxidizing substrates, homology in sequence or in topology of 3D structures (Massey, 2000). In this review we have classified the group of external flavoprotein monooxygenases according to the last two criteria: sequence

and (if available) structural data. An overview of the resulting classification is given in Table 1. External flavoprotein monooxygenases described to date can be grouped in six subclasses: A–F. Discrimination is based on sequence similarity and specific structural features which are reflected in sequence similarity and the presence of specific protein sequence motifs. The typifying characteristics and a description of some known members belonging to each subclass are indicated in the next paragraphs.

#### 3.1. Class A flavoprotein monooxygenases

##### 3.1.1. General characteristics of class A flavoprotein monooxygenases

- Encoded by a single gene;
- contain a tightly bound FAD cofactor;
- depend on NADH or NADPH as coenzyme;
- NADP<sup>+</sup> is released immediately upon flavin reduction;
- structurally composed of one dinucleotide binding domain (Rossmann fold) binding FAD.

Class A monooxygenases can be NADPH or NADH dependent. The C<sub>(4a)</sub>-hydroperoxyflavin is the oxygenating flavin species, that performs an electrophilic attack on the aromatic ring (Fig. 1). Typical substrates are aromatic compounds that contain an activating hydroxyl or amino group. This is an important difference with the P450 enzymes that can also hydroxylate non-activated aliphatic or aromatic compounds. Members of the class A flavoprotein monooxygenases usually are involved in the microbial degradation of aromatic compounds by *ortho*- or *para* hydroxylation of the aromatic ring (Moonen et al., 2002) and display a narrow substrate specificity. The prototype enzyme of this subclass is 4-hydroxybenzoate 3-monooxygenase (EC 1.14.13.2) from *Pseudomonas* that has been studied comprehensively (Entsch and van Berkel, 1995; Entsch et al., 2005). Other members are, e.g. 2-hydroxybiphenyl 3-monooxygenase (EC 1.14.13.44) from *Pseudomonas azelaica* (Suske et al., 1997), phenol 2-monooxygenase (EC 1.14.13.7) from *Trichosporon cutaneum* (Neujahr and Gaal, 1973), and salicylate 1-monooxygenase (EC 1.14.13.1) from *Pseudomonas putida*, the latter being the first characterized enzyme of this subclass (White-Stevens and Kamin, 1972). Monooxygenases

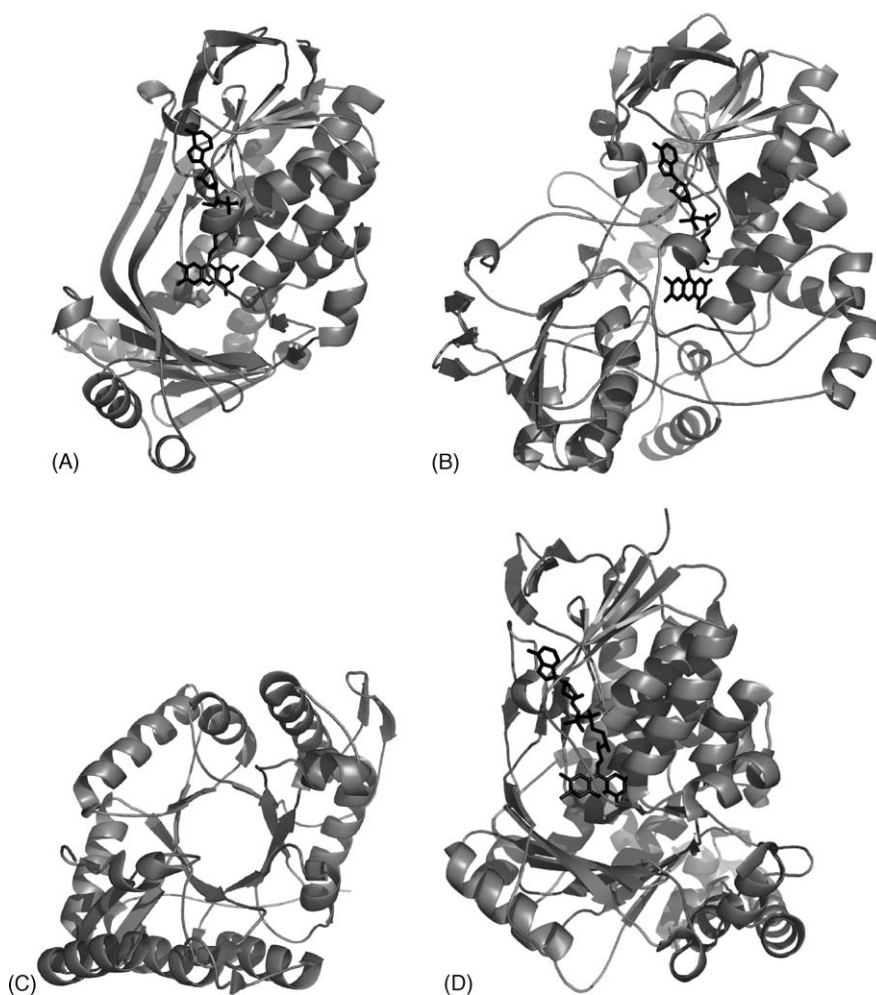


Fig. 3. Structures of several prototype flavoprotein monooxygenases. (A) Structure of a class A flavoprotein monooxygenase: 4-hydroxybenzoate 3-monooxygenase from *Pseudomonas fluorescens*. The FAD cofactor is shown in sticks. (B) Structure of a class B flavoprotein monooxygenase: phenylacetone monooxygenase from *Thermobifida fusca*. The FAD cofactor is shown in sticks. (C) Structure of a class C flavoprotein monooxygenase: alkanesulfonate monooxygenase from *Escherichia coli*. The FMN coenzyme was not bound in the crystalline enzyme preventing exact localization of the active site. (D) Structure of a class F flavoprotein monooxygenase: tryptophan 7-halogenase from *Pseudomonas fluorescens*. The FAD coenzyme is shown in sticks.

belonging to this subclass have also been implicated with the biosynthesis of ubiquinone (2-polyprenyl-6-methoxyphenol 4-monooxygenase; Gin et al., 2003) and modification of aromatic polyketides (e.g. monooxygenases involved in biosynthesis or modification of oxytetracycline (Yang et al., 2004), rifampin (Andersen et al., 1997), mithramycin (Prado et al., 1999), chromomycin (Menendez et al., 2004), auricin (Novakova et al., 2005), and griseorhodin (Li and Piel, 2002)).

Except for hydroxylations, also enzymes belonging to this subclass that catalyze epoxidations have been described. A well-known example is squalene monooxygenase (EC 1.14.99.7), a key enzyme in the committed pathway for cholesterol biosynthesis in eukaryotic cells. Squalene monooxygenase is bound to the endoplasmic reticulum where it catalyzes the epoxidation of squalene across a C–C double bond to yield 2,3-oxidosqualene. The enzyme is difficult to purify and rather unstable. The soluble truncated recombinant

Table 1  
Classification of external flavoprotein monooxygenases

Sub-class	Prototype	Reactions <sup>a</sup>	Subunits	Cofactor	Coenzyme	Genome frequency <sup>b</sup>	Structure (PDB codes); fold
A	4-OH-benzoate hydroxylase	Hydroxylation epoxidation	$\alpha$	FAD	NAD(P)H	570/309	2 (1PHH, IFOH); 1 FAD/NAD(P)-binding domain
B	Cyclohexanone monooxygenase	Baeyer–Villiger; N-oxidation	$\alpha$	FAD	NADPH	214/309	1 (1W4X); 2 FAD/NAD(P)-binding domains, 1 helical domain
C	Luciferase	<i>Light emission</i> ; S-oxidation; Baeyer–Villiger	$\alpha + \beta$	–	FMN NAD(P)H	432/309	2 (1BRL, 1M41); TIM barrel
D	4-OH-phenylacetate hydroxylase	Hydroxylation	$\alpha + \beta$	–	FAD NAD(P)H	43/309	0; Acyl-CoA dehydrogenase
E	Styrene monooxygenase	Epoxidation	$\alpha + \beta$	–	FAD NAD(P)H	21/309	0; 1 FAD/NAD(P)-binding domain
F	Tryptophan 7-halogenase	Halogenation	$\alpha + \beta$	–	FAD NAD(P)H	102/309	1 (2APG); 1 FAD/NAD(P)-binding domain, 1 helical domain

<sup>a</sup> The most commonly found in vivo oxidation activities are given.

<sup>b</sup> At September 12, 2005 the 309 annotated bacterial genomes available at the NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>) were screened for the presence of monooxygenase homologs using the prototype protein sequence and the BLAST tool. The derived numbers only indicate a lower limit for the abundance of the respective monooxygenases as this analysis is blind to distant homologs.

form of human squalene monooxygenase is dependent upon NADPH-cytochrome P450 reductase for reducing equivalents, requires Triton X-100 for activity and easily loses FAD (Laden et al., 2000).

Another class A flavoprotein epoxidase concerns zeaxanthin epoxidase (EC 1.14.13.90). This plant enzyme is located on the chloroplast stromal side of the thylakoid membrane where it catalyzes the conversion of zeaxanthin to antheraxanthin and violaxanthin, an important reaction in the xanthophyll cycle that protects the photosynthetic system against damage by excess light. Zeaxanthin epoxidase has a specificity for carotenoids with a 3-hydroxy- $\beta$ -cyclohexenyl ring and requires besides from NADPH and FAD, also ferredoxin-like reductives and specific lipids for activity (Bugos et al., 1998; Hieber et al., 2000).

Recently, a class A flavoprotein monooxygenase has been discovered that is catalyzing a Baeyer–Villiger oxidation (Prado et al., 1999; Gibson et al., 2005). This bacterial enzyme (MtmOIV) is involved in the biosynthesis of mithramycin, an anticancer drug and calcium-lowering agent. As the monooxygenase has only been discovered recently, little is known concerning its catalytic potential and mode of action. It will be interesting to elucidate how this class A monooxygenase has evolved to catalyze a Baeyer–Villiger oxidations as it has been proposed that a flavin-catalyzed Baeyer–Villiger reaction requires a peroxyflavin intermediate (see Section 3.2) while class A flavoprotein monooxygenases typically form a hydroperoxyflavin instead. Crystallization of this monooxygenase has been reported recently (Wang et al., 2005). Elucidation of the crystal structure will shed more light on this mechanistic issue.

The 3-D structure has been solved for two representatives of the class A flavoprotein monooxygenases: 4-hydroxybenzoate 3-monooxygenase (Wierenga et al., 1979; Schreuder et al., 1989) and phenol 2-monooxygenase (Enroth et al., 1998). Both enzymes are NADPH dependent and their sequences contain two sequence motifs for the FAD binding region. The N-terminal GxGxxG sequence is indicative for the  $\beta\alpha\beta$ -fold (or Rossmann fold) that binds the ADP moiety of FAD (Wierenga et al., 1986). The amino acids of the second motif, GD, are in contact with the riboflavin moiety of FAD (Eggink et al., 1990). Interestingly, there is no distinct domain that binds the coenzyme NADPH. This is in line with the fact that NADPH only forms a transient complex to reduce the flavin



cofactor. The formed NADP<sup>+</sup> is rapidly released. Nevertheless, recognition of NADPH is very specific which has triggered several groups to study the structural basis of coenzyme recognition by 4-hydroxybenzoate 3-monooxygenase. Eppink et al. (1997) have described an additional fingerprint sequence for this subclass of monooxygenases. This fingerprint, containing a highly conserved DG motif, is involved in binding the pyrophosphate moieties of both FAD and NADPH. Furthermore, recent mutagenesis and crystallographic studies have revealed a plausible binding mode for NADPH in PHBH (Eppink et al., 1999; Wang et al., 2002).

### 3.1.2. EC members

EC 1.14.13.1 salicylate 1-monooxygenase; EC 1.14.13.2 (and EC 1.14.13.33) 4-hydroxybenzoate 3-monooxygenase; EC 1.14.13.3 4-hydroxyphenylacetate 3-monooxygenase; EC 1.14.13.4 melilotate hydroxylase; EC 1.14.13.5 imidazoleacetate 4-monooxygenase; EC 1.14.13.6 orcinol hydroxylase; EC 1.14.13.7 phenol hydroxylase; EC 1.14.13.9 kynurenine 3-monooxygenase; EC 1.14.13.10 2,6-dihydropyridine 3-monooxygenase; EC 1.14.13.18 4-hydroxyphenylacetate 1-monooxygenase; EC 1.14.13.19 taxifolin 8-monooxygenase; EC 1.14.13.20 2,4-dichlorophenol 6-monooxygenase; EC 1.14.13.23 3-hydroxybenzoate 4-monooxygenase; EC 1.14.13.24 3-hydroxybenzoate 6-monooxygenase; EC 1.14.13.27 4-aminobenzoate 1-monooxygenase; EC 1.14.13.35 anthranilate 3-monooxygenase; EC 1.14.13.38 anhydrotetracycline monooxygenase; EC 1.14.13.40 anthraniloyl-CoA monooxygenase; EC 1.14.13.44 2-hydroxybiphenyl 3-monooxygenase; EC 1.14.13.50 pentachlorophenol monooxygenase; EC 1.14.13.58 benzoyl-CoA 3-monooxygenase; EC 1.14.13.63 3-hydroxyphenylacetate 6-monooxygenase; EC 1.14.13.64 4-hydroxybenzoate 1-monooxygenase; EC 1.14.13.90 zeaxanthin epoxidase; EC 1.14.12.4 2-methyl-3-hydroxypyridine-5-carboxylic acid oxygenase.

### 3.1.3. Other members

4-Methyl-5-nitrocatechol monooxygenase, 4-aminobenzoate 3-monooxygenase, 2-octaprenyl-6-methoxyphenol 4-monooxygenase, squalene monooxygenase, rifampin monooxygenase and oxytetracy-

cline monooxygenase (rendering microbes antibiotic-resistant), a number of monooxygenases involved in polyketide biosynthesis routes (including MtmOIV).

## 3.2. Class B flavoprotein monooxygenases

### 3.2.1. General characteristics of class B flavoprotein monooxygenases

- Encoded by a single gene;
- contain a tightly bound FAD cofactor;
- depend on NADPH as coenzyme;
- keep the coenzyme NADPH/NADP<sup>+</sup> bound during catalysis;
- composed of two dinucleotide binding domains (Rossmann fold) binding FAD and NADPH, respectively.

This subclass is also referred to as multifunctional flavin-containing monooxygenases as representatives of this subclass are able to oxidize both carbon atoms and other (hetero)atoms. This subclass comprises three sequence-related flavoprotein monooxygenase subfamilies (Fraaije et al., 2002):

- flavin-containing monooxygenases (FMOs);
- microbial N-hydroxylating monooxygenases (NMOs);
- Baeyer–Villiger monooxygenases (Type I BVMOs).

All members of these three monooxygenase families are single-component FAD-containing enzymes and specific for NADPH. The protein sequences of these monooxygenases contain two Rossmann-fold motifs indicative for two binding domains for FAD and NADPH. In 2004 the first crystal structure for a member of this monooxygenase subclass was solved (see below; Malito et al., 2004).

FMOs (EC 1.14.13.8) were originally identified in liver microsomes and named ‘mixed-function oxidases’. The name of these enzymes was later changed to flavin-containing monooxygenase (FMO; see for a recent review, Ziegler, 2002). FMOs are found in all mammals and other eukaryotic organisms. In mammals and human several isoforms exist, each having a different substrate specificity and tissue localization. Six FMO genes and five FMO pseudogenes have been identified in the human genome. FMO3 is the most important isoform in the liver (Furnes et al., 2003; Hernandez et al., 2004). FMOs play an important role

in detoxification of drugs and other xenobiotics in the human body thereby complementing the activities of the cytochrome P450 system. The membrane-associated enzymes catalyze the monooxygenation of carbon-bound reactive heteroatoms: nitrogen, sulfur, phosphorus, selenium, or iodine. In contrast to mammalian FMOs, yeast FMO does not oxidize nitrogen-containing compounds but is only active with biological thiols (Suh et al., 1996). It was shown that the yeast enzyme is required for proper folding of disulfide-containing proteins by generating an oxidizing environment in the endoplasmic reticulum (Suh et al., 1999). In plants, an FMO was found to be involved in the biosynthesis of auxin, a crucial hormone in plant development (Zhao et al., 2001). Genome analysis has revealed that FMO gene homologs are frequently encountered in plant genomes, e.g. 29 gene homologs are present in the genome of *Arabidopsis thaliana*. Recently, the first bacterial FMO gene was cloned (Choi et al., 2003). Overexpression and partial characterization revealed that this enzyme is able to oxidize a number of amines. Also indole is readily oxidized by the enzyme as large amounts of indigo were formed by the recombinant *E. coli* cells expressing the bacterial FMO. Another bacterial FMO homolog has been suggested to catalyze a Baeyer–Villiger oxidation to form the polyketide pederin (Piel, 2002). In contrast to eukaryotic genomes, searching the 309 presently available bacterial genomes using a FMO specific sequence motif (Fraaije et al., 2002) reveals that bacterial FMOs are relatively rare as only a few representatives can be identified.

Microbial NMOs, e.g. lysine N(6)-hydroxylase (EC 1.14.13.59), catalyze the N-hydroxylation of long-chain primary amines. They play a role in the biosynthesis of various bacterial and fungal siderophores (low molecular weight iron chelators). Cloning and sequencing of several NMO genes has revealed that they share sequence homology with FMOs (Stehr et al., 1998). As for FMOs, NMOs specifically need NADPH to perform catalysis. Limited biochemical data are available for these enzymes, which is partly caused by their low affinity for FAD hindering mechanistic studies (Stehr et al., 1999).

Several class B BVMOs, previously classified as Type I BVMOs, have been cloned and characterized (Fraaije et al., 2002; Kamerbeek et al., 2003). These enzymes catalyze an atypical oxygenation reac-

tion: the Baeyer–Villiger oxidation of a ketone (or aldehyde) to an ester or lactone. The prototype of BVMOs is cyclohexanone monooxygenase (EC 1.14.13.22) from *Acinetobacter* sp. NCIB 9871. This enzyme was originally isolated and characterized in 1976 (Donoghue et al., 1976). Extensive research has shown that cyclohexanone monooxygenase carries out Baeyer–Villiger oxidations on a wide variety of cyclic ketones with exquisite chemo-, regio-, and enantioselectivities (see for recent reviews, Stewart, 1998; Mihovilovic et al., 2002). The cyclohexanone monooxygenase gene was cloned in 1988 (Chen et al., 1988) and only 11 years later the next BVMO, steroid monooxygenase (EC 1.14.13.54) from *Rhodococcus rhodochrous*, was cloned (Morii et al., 1999). From then on more sequences became steadily available including the genes encoding 4-hydroxyacetophenone monooxygenase (EC 1.14.13.84; Kamerbeek et al., 2001), cyclododecanone monooxygenase (Kostichka et al., 2001), cyclopentanone monooxygenase (EC 1.14.13.16; Iwaki et al., 2002) and ethionamide monooxygenase (Fraaije et al., 2004). Some BVMO sequences have been patented that are linked to detoxification of fungal toxins (e.g. US patent 6822140). A BVMO acting on the monocyclic monoterpene ketones 1-hydroxy-2-oxolimonene, dihydrocarvone and menthone, was purified to homogeneity from *Rhodococcus erythropolis* DCL14 (van der Werf, 2000). Also several cyclohexanone monooxygenase homologs have been identified and exploited for biocatalytic studies (Kyte et al., 2004). By studying the catalytic properties of a number of these cyclohexanone monooxygenase homologs it was found that clustering of sequences by sequence similarity coincides with stereopreference (Mihovilovic et al., 2005).

Sequence similarity analysis using a large set of sequences has shown that class B BVMOs can be identified using a specific protein sequence motif. This has facilitated genome mining as BVMO-encoding genes can be readily annotated using the sequence motif. By this approach, a BVMO was identified in the genome of the thermophilic soil bacterium *Thermobifida fusca*. This monooxygenase has been overexpressed and characterized revealing activity towards aromatic compounds while also aliphatic ketones are converted. As the enzyme is very efficient in oxidizing phenylacetone it has been named phenylacetone monooxygenase (EC 1.14.13.92; Fraaije et al., 2005). Phenylacetone

monooxygenase represents the first class B monooxygenase for which the crystal structure has been determined (Malito et al., 2004). The structure is composed of two Rossmann fold domains binding the FAD cofactor and the NADPH coenzyme. As a result the structure resembles to some extent other known flavoprotein structures. The dinucleotide binding domains are flanked by two helical domains which appear to be unique for this type of monooxygenases. Close to the reactive part of the flavin cofactor a conserved arginine residue is located which is predicted to assist in formation and stabilization of the oxygenating peroxyflavin intermediate. Replacing this residue has been shown to eliminate oxygenating activity confirming the crucial catalytic role of this arginine (Kamerbeek et al., 2004). The availability of the phenylacetone monooxygenase structure will facilitate enzyme redesign studies. An example of structure inspired mutagenesis of phenylacetone monooxygenase has been described recently (see Section 4.5; Bocola et al., 2005).

### 3.2.2. EC members

EC 1.14.13.8 dimethylaniline monooxygenase (eukaryotic FMO); EC 1.14.13.16 cyclopentanone monooxygenase; EC 1.14.13.22 cyclohexanone monooxygenase; EC 1.14.13.54 ketosteroid monooxygenase; EC 1.4.13.59 lysine N6-monooxygenase, EC 1.14.13.66 2-hydroxycyclohexanone 2-monooxygenase; EC 1.14.13.84 4-hydroxyacetophenone monooxygenase; EC 1.14.13.92 phenylacetone monooxygenase.

### 3.2.3. Other members

Indigo forming bacterial FMO, monocyclic monoterpene ketone monooxygenase, L-ornithine N5-monooxygenase, cyclododecanone monooxygenase, and ethionamide monooxygenase.

## 3.3. Multi-component flavoprotein monooxygenases

In addition to the single polypeptide flavoprotein monooxygenases discussed in the previous two paragraphs, also multi-component monooxygenases have been discovered that use flavin as a coenzyme rather than a cofactor. The variety in this group of monooxygenases is quite large concerning coenzyme use, coenzyme specificity and sequence homology (Chaiyen et al., 2001). Generally, these enzymes consist of two

different polypeptide chains that have different functions: a reductase component carrying out the flavin reduction and an oxygenase component oxidizing the substrate by molecular oxygen. The reducing equivalents needed for the oxygenation are transferred from NAD(P)H to a flavin that is bound to the reductase component. Subsequently, the reduced flavin is transferred to the oxygenase component. Upon reaction with molecular oxygen, the peroxygenated flavin is formed and the monooxygenation reaction takes place. While only a few structures are known of the monooxygenase components of these multi-component enzyme systems, sequence information indicates that this group can be dissected into four clearly distinct subclasses: C–F (Table 1). Each of these subclasses appears to have evolved to catalyze specific type of reactions and is discussed below.

## 3.4. Class C flavoprotein monooxygenases

### 3.4.1. General characteristics of class C flavoprotein monooxygenases

- Encoded by multiple genes encoding one or two monooxygenase components and a reductase component;
- use reduced FMN as a coenzyme (generated by the reductase);
- the reductase can use NADPH and/or NADH as coenzyme;
- the structural core of the monooxygenase subunit(s) displays a TIM-barrel fold.

A number of monooxygenases have been described that belong to this subclass of flavoproteins monooxygenases on the basis of sequence homology. The most extensively studied representatives are bacterial luciferases (EC 1.14.14.3). Bacterial luciferases are able to emit light upon oxidation of long-chain aliphatic aldehydes. The luciferases are composed of a heterodimeric oxygenase component and require a reductase for delivery of reduced FMN. The two subunits of the heterodimer are homologous in sequence and the elucidated crystal structure of the oxygenase component from *Vibrio harveyi* has revealed that they also have a similar structure, both exhibiting a TIM-barrel fold (Fisher et al., 1995; Baldwin et al., 1995). Nevertheless, only one of the subunits contains an active site.

Also several BVMOs belonging to this subclass of flavoprotein monooxygenase have been described (Taylor and Trudgill, 1986). The best-described examples are from *Pseudomonas putida* ATCC 17453, a strain that is able to grow on camphor (Trudgill, 1984). To degrade this compound, the bacterium recruits three BVMOs (one of class B and two of class C). Initial degradation is catalyzed by the class C (Type II) BVMOs. The organism uses 2,5-diketocamphane 1,2-monooxygenase (EC 1.14.15.2) or 3,6-diketocamphane 1,6-monooxygenase (EC 1.14.15.2) for ring expansion of (+)- or (–)-camphor, respectively. It was shown that the 2,5-diketocamphane 1,2-monooxygenase is built up by an oxygenase component and an flavin reductase component (Taylor and Trudgill, 1986). The oxygenase component consists of two subunits of equal size and strongly binds one FMN molecule closely resembling the above-mentioned luciferases. In fact, bacterial luciferases have also been classified as Type II BVMOs (Villa and Willetts, 1997) based on the similar oligomeric structure and the fact that luciferases are also able to catalyze a reaction similar to a Baeyer–Villiger oxidation (Eckstein et al., 1993). Consistent with this, it was shown that the luciferases from *Vibrio fischeri* and *Photobacterium phosphoreum* perform Baeyer–Villiger reactions on different ketones (Villa and Willetts, 1997). Nevertheless, the Type II BVMOs have never been reported to emit light during catalysis.

Except for luciferases and Type II BVMOs, also some other monooxygenases have been described that fit into this subclass of monooxygenases. *E. coli* harbors an alkanesulfonate monooxygenase (EC 1.14.14.5) by which it is able to utilize alkanesulfonates as sulfur sources for growth. The enzyme depends on reduced FMN for activity and is able to convert a wide range of alkanesulfonates. The crystal structure of alkanesulfonate monooxygenase has been elucidated revealing a homotetrameric assembly of subunits displaying a TIM-barrel fold (Eichhorn et al., 2002). Kinetic studies have suggested that efficient catalysis requires formation of a complex between the monooxygenase, FMN and the reductase (Eichhorn et al., 1999). Other class C monooxygenases have been found to be involved in desulfurization pathways oxidizing dibenzothiophene into the corresponding sulfone. The initial degradation of nitrilotriacetate, a widely applied chelator, is also catalyzed by a class C flavoprotein monooxy-

genase (Uetz et al., 1992; Knobel et al., 1996; Xu et al., 1997).

#### 3.4.2. EC members

EC 1.14.14.3 luciferase; EC 1.14.15.2 2,5-diketocamphane 1,2-monooxygenase and 3,6-diketocamphane 1,6-monooxygenase.

#### 3.4.3. Other members

Alkanesulfonate monooxygenase, nitrilotriacetate monooxygenase, dibenzothiophene monooxygenase, dibenzothiophene sulfone monooxygenase.

### 3.5. Class D flavoprotein monooxygenases

#### 3.5.1. General characteristics of class D flavoprotein monooxygenases

- Encoded by two genes encoding a monooxygenase and a reductase;
- use reduced FAD as a coenzyme (generated by the reductase);
- the reductase can use NADPH and/or NADH as coenzyme;
- no structure available, sequence homology suggests a structural resemblance with the acyl-CoA dehydrogenase fold (mainly  $\alpha$ -helical).

The prototype enzyme for this subclass of monooxygenases is 4-hydroxyphenylacetate 3-monooxygenase (EC 1.14.13.3) which has been identified in a number of bacteria. The best studied 4-hydroxyphenylacetate 3-monooxygenases come from *E. coli* W (Galan et al., 2000a; Prieto and Garcia, 1994) and *Acinetobacter baumannii* (Chaiyen et al., 2001; Thotsaporn et al., 2004; Sucharitakul et al., 2005). Similar to class A flavoprotein monooxygenases, members of class D typically are active on aromatic substrates, e.g. 4-hydroxyphenylacetate, phenol (Duffner et al., 2000; Kirchner et al., 2003; van den Heuvel et al., 2004) and 4-nitrophenol (Kadiyala and Spain, 1998). Furthermore, representatives of this subclass seem to be restricted to only one type of oxygenation: (regioselective) hydroxylation. With 4-nitrophenol monooxygenase, the enzyme catalyzes sequential *ortho*- and *para* hydroxylations of which the latter reaction is accompanied with nitrite release. With 2,4,5-trichlorophenol monooxygenase (Xun, 1996; Gisi and Xun, 2003), the *para*- and *ortho* hydroxylation steps both are accompanied by elimination of chloride.

With 2,4,6-trichlorophenol monooxygenase (Xun and Webster, 2004), the enzyme catalyzes sequential dechlorinations by oxidative and hydrolytic reactions. Class D monooxygenases have also been shown to be involved in hydroxylation of 4-chlorophenol (Nordin et al., 2004), indole (Choi et al., 2004; Lim et al., 2005) and polyketides (Brunke et al., 2001).

Although no structure is available for any of these monooxygenases, a structural model of 4-hydroxyphenylacetate-3-monooxygenase can be built using the recently elucidated crystal structure of 4-hydroxybutyryl-CoA dehydratase: a sequence related flavoprotein (Martins et al., 2004). The structure of 4-hydroxybutyryl-CoA dehydratase is similar to known structures of flavin-containing acyl-CoA dehydrogenases. This indicates that the acyl-CoA dehydrogenase fold allows catalytic promiscuity as several kinds of reactions can be catalysed by members of this flavoprotein superfamily: hydroxylations, dehydratations and oxidations.

### 3.5.2. EC members

EC 1.14.13.3 4-hydroxyphenylacetate monooxygenase; EC 1.14.13.7 phenol monooxygenase.

### 3.5.3. Other members

4-nitrophenol 2,4-monooxygenase, 4-chlorophenol monooxygenase, 2,4,5-trichlorophenol 4,2-monooxygenase, 2,4,6-trichlorophenol 4,6-monooxygenase, phenazine monooxygenase, naphthocyclinone monooxygenase, indole monooxygenase.

## 3.6. Class E flavoprotein monooxygenases

### 3.6.1. General characteristics of class E flavoprotein monooxygenases

- Encoded by two genes encoding a monooxygenase and a reductase;
- use reduced FAD as a coenzyme (generated by the reductase);
- the reductase can use NADPH and/or NADH as coenzyme;
- no structure is available, sequence analysis indicates the presence of one dinucleotide binding domain (Rossmann fold) thereby suggesting an evolutionary link with the Class A flavoprotein monooxygenases

Another subclass of two-component monooxygenases is represented by styrene monooxygenase from

*Pseudomonas* sp. VLB120 (Otto et al., 2002). So far, a very limited number of representatives of this flavoprotein monooxygenase subclass are known. In fact, only some other styrene monooxygenases from pseudomonads with similar characteristics have been reported (Hartmans et al., 1990; Di Gennaro et al., 1999; Santos et al., 2000; O'Leary et al., 2002; Kantz et al., 2005). This low abundance is in agreement with genome analysis. Inspection of the available bacterial genomes for the presence of additional class E flavoprotein monooxygenases resulted in the identification of only a few homologs (Table 1). This shows that class E flavoprotein monooxygenases are relatively rare. Nevertheless, we recently discovered a new member of this flavoprotein monooxygenase subclass by metagenome screening (M.W. Fraaije, personal communication).

All described styrene monooxygenases are highly enantioselective in oxidizing styrene and some of its derivatives. As enantiopure epoxides are interesting building blocks in the fine-chemical industry, effort has been put into developing efficient biocatalytic systems based on this biocatalyst to produce styrene oxides (see Section 4.1). Furthermore, some mechanistic details have emerged in the last few years revealing that (1) reduced FAD does not have to be actively delivered by the reductase to the monooxygenase subunit, and (2) the monooxygenase subunit is well able to form and stabilize the peroxyflavin after binding reduced FAD and reacting with molecular oxygen (Otto et al., 2002). A recent kinetic study has suggested that the reductase component can stimulate catalysis of the monooxygenase subunit. This phenomenon is rationalized by the hypothesis that FAD can remain bound to the monooxygenase component via the ADP moiety of FAD while the isoalloxazine part of the flavin binds to the active site of the reductase subunit (Kantz et al., 2005). The validity of this mechanism awaits the elucidation of the first styrene monooxygenase structure.

### 3.6.2. Known member

Styrene monooxygenase.

## 3.7. Class F flavoprotein monooxygenases

### 3.7.1. General characteristics of class F flavoprotein monooxygenases

- Encoded by two genes encoding a monooxygenase and a reductase;

- use reduced FAD as a coenzyme (generated by the reductase);
- the reductase can use NADPH and/or NADH as coenzyme;
- two domain structure: one FAD binding domain (Rossmann fold) and a helical domain.

Many halogenation reactions occurring in nature are catalyzed by flavoenzymes. Members of this halogenase family have been identified in the biosynthetic pathways of antibiotics, antitumor agents and other halometabolites (van Pee and Unversucht, 2003). One of the best studied flavin-dependent halogenases is tryptophan 7-halogenase (Keller et al., 2000; Yeh et al., 2005). While the reaction catalyzed by this enzyme does not result in formation of an oxygenated product, the proposed catalytic mechanism is similar to that of a flavoprotein monooxygenase. As for the other two-component flavoprotein monooxygenases, the monooxygenase component of the enzyme needs reduced flavin (FAD), produced by a reductase, and molecular oxygen for catalysis. A recent study has shown that a rhodium organometallic complex can be used for reduced FAD regeneration with formate as the electron donor (Unversucht et al., 2005). This shows that direct interaction with the reductase component is not a prerequisite for catalysis.

Very recently, the structure of tryptophan 7-halogenase has been determined revealing some structural resemblance with the structure of 4-hydroxybenzoate 3-monooxygenase (Dong et al., 2005). The tryptophan 7-halogenase structure is a dimer with each subunit forming a single domain. Besides from the conserved FAD binding module, the halogenase subunit contains a helical substrate binding module. The structure is compatible with a 'monooxygenase' type of catalytic mechanism. Inspection of the FAD-bound halogenase structure suggests that the reduced coenzyme will react with molecular oxygen to form the hydroperoxyflavin intermediate. Based on the location of the bound ligands/substrates, Cl<sup>-</sup> and tryptophan, it is suggested that a chloride ion will perform a nucleophilic attack on the hydroperoxyflavin resulting in the formation of HOCl. This highly reactive molecule will travel through a 10 Å tunnel to reach and halogenate the bound tryptophan molecule. As a consequence of this mechanism the regioselectivity of halogenation is dictated by the way

the substrate is bound with respect to the end of the tunnel. Apparently, these halogenases have combined two chemical reactions in one protein scaffold in which first the reduced flavin coenzyme is used to form reactive HOCl. The latter compound is subsequently shuttled to another active site to react with the organic substrate. As a consequence, the halide ion can be regarded as substrate for the 'monooxygenase' half-reaction of these halogenases. The availability of the halogenase structure will facilitate future structure-inspired studies, e.g. it will be possible to modify selectively the tryptophan binding site by which regioselectivity and substrate specificity can be tuned.

### 3.7.2. Some known members

Tryptophan 7-halogenase, tryptophan 5-halogenase, and 'pyrrolyl' halogenase (Dorrestein et al., 2005).

## 4. Biocatalysis with flavin-dependent monooxygenases

Regio- and/or enantioselective insertion of oxygen is not straightforward to be accomplished when using chemical techniques. Therefore, flavin-dependent monooxygenases represent promising biocatalytic tools (Schmid et al., 2001a). This section will discuss some of the monooxygenases that have been shown to be of value for biocatalytic processes. While many more monooxygenases have been explored for their biocatalytic properties, the monooxygenase examples illustrated below provide a good view on the catalytic capability of flavin-dependent monooxygenases.

### 4.1. Styrene monooxygenase: a selective epoxidation catalyst

Enantiopure styrene oxides are important building blocks for the pharmaceutical industry. A promising example of an enantioselective monooxygenase is styrene monooxygenase from *Pseudomonas* sp. VLB120 (class E monooxygenase). This enzyme catalyzes the conversion of styrene into (*S*)-styrene oxide at an enantiomeric excess larger than 99% (Panke et al., 1998). A recombinant *E. coli* expressing StyA and StyB was developed (Panke et al., 2000) and this whole-cell biocatalyst showed a broad substrate spectrum that gives access to various chiral aryloxides (Schmid et al.,

2001b). The system was successfully scaled-up and it was possible to produce almost 400 g (*S*)-styrene oxide at pilot-scale (30 L fed-batch bioconversion, two-liquid phase system; Panke et al., 2002). Introduction of an apolar organic solvent reduces toxic effects of substrate and/or product and the use of whole cells instead of an isolated biocatalyst has the advantage of in vivo coenzyme regeneration. The same research group that developed this whole cell biocatalyst also designed a small-scale cell-free system. The only enzymatic component of this system is the monooxygenase (StyA). The reductase component (StyB), NAD and artificial formate dehydrogenase NADH regenerating system could be replaced by the organometallic complex pentamethyl-cyclopentadienyl rhodium bipyridine  $[\text{Cp}^*\text{Rh}(\text{bpy})(\text{H}_2\text{O})]^{2+}$ . This redox-catalyst receives electrons from the oxidation of formate to carbon dioxide and is able to regenerate reduced FAD directly. The epoxidation rate of this chemoenzymatic system was  $\sim 70\%$  of a fully enzymatic reaction (Hollmann et al., 2003). The flavin coenzyme can also be reduced by direct electrochemical reduction using an electrode. In this way, the need for expensive NAD(P)H coenzyme is circumvented facilitating biocatalytic applications.

#### 4.2. Hydroxybiphenyl 3-monooxygenase: an example of monooxygenase engineering

Held et al. (1998, 1999) developed a recombinant *E. coli* strain expressing hydroxybiphenyl 3-monooxygenase (class A monooxygenase) for the conversion of 2-substituted phenols into 3-substituted catechols, which are difficult to synthesize chemically. During production of 3-phenylcatechol, in situ product removal prevented toxic effects on the whole-cell biocatalyst. Isolated hydroxybiphenyl 3-monooxygenase has been used in organic-aqueous reaction media in combination with enzymatic coenzyme regeneration (Lutz et al., 2002). Directed evolution has been employed to improve and broaden the substrate specificity and efficiency of this monooxygenase (Meyer et al., 2002a,b).

#### 4.3. Monooxygenases involved in desulfurization

Combustion of sulfur-containing fuels such as diesel leads to formation of sulfur oxides, which are air pollu-

tants. Until 70% of the sulfur content of diesel oil is presented as dibenzothiophenes. The conventional chemical hydrodesulphurization process is not adequate for complete removal of these compounds (Abbad-Andaloussi et al., 2003). Therefore, biocatalytic processes using flavoprotein monooxygenases may find application in the desulfurization of fossil fuels (Gray et al., 2003). Different approaches have been used to engineer strains with improved desulfurization activity exploiting the catalytic potential of dibenzothiophene monooxygenases (class C monooxygenases). Overexpression of a flavin reductase in *E. coli* and *P. putida* enhanced the overall rate of desulfurization as compared to the use of wild-type strains (Galan et al., 2000b; Reichmuth et al., 2000). To circumvent catabolite repression of the monooxygenase gene cluster by sulfonate, cysteines or methionines, these genes have been placed under control of the *tac* promoter in various *Pseudomonas* strains (Gallardo et al., 1997). Furthermore, activities towards (highly) alkylated DBTs have been improved using a chemostat approach (Arensdorf et al., 2002) and gene shuffling (Coco et al., 2001).

#### 4.4. Cyclohexanone monooxygenase: a versatile monooxygenase

From all the flavoprotein monooxygenases, BVMOs have been studied most extensively for their biotechnological applications. BVMOs typically can deal with a large number of different substrates while exhibiting an exquisite enantio- and/or regioselectivity. The most extensively studied BVMO is cyclohexanone monooxygenase (class B monooxygenase). Currently, more than 100 substrates have been described for this bacterial enzyme (Mihovilovic et al., 2002). Nowadays, also several cyclohexanone monooxygenase homologs have been discovered and overexpressed enabling comparative biocatalytic studies. This has revealed that, while sequence identity among these homologs can be high, the regioselectivity can vary widely (Mihovilovic et al., 2005). Reetz et al. (2004a,b) have also shown by directed evolution that enantioselectivity of cyclohexanone monooxygenase can be significantly improved by altering only a single amino acid.

A nice example of the applicability of a cyclohexanone monooxygenase was recently given by

Hilker et al. (2005). They demonstrated that *E. coli* cells expressing cyclohexanone monooxygenase could be used to produce industrially relevant lactones at kilogram scale. A high productivity for the asymmetric Baeyer–Villiger oxidation of racemic bicyclo[3.2.0]hept-2-en-6-one was obtained combining a resin-based in situ substrate feeding and product removal (SFPR) methodology, a glycerol feed control, and an improved oxygenation device. Both regioisomeric lactones [(–)-(1*S*,5*R*)-2 and (–)-(1*R*,5*S*)-3] were obtained in nearly enantiopure form (*ee* > 98%) and good yield. This novel technology opens the way to further (industrial) upscaling of highly valuable (asymmetric) monooxygenase reactions.

#### 4.5. Phenylacetone monooxygenase: a new and thermostable oxygenating biocatalyst

Phenylacetone monooxygenase (class B monooxygenase) was recently discovered by genome mining. The phenylacetone monooxygenase gene was identified in the genome of the thermophilic bacterium *Thermobifida fusca* and as a result it displays an interesting feature: it is relatively thermostable (Fraaije et al., 2005). Biocatalytic conversions using previously discovered BVMOs often are hampered by the instability of the respective monooxygenases. Except for being thermostable, phenylacetone monooxygenase also appears to be tolerant towards solvents (de Gonzalo et al., 2006). In fact, the use of solvent can greatly increase the enantioselectivity of the enzyme when oxidizing sulfides. The substrate range for this BVMO seems to be tuned towards aromatic ketones. However, also aromatic sulfides and sulfoxides, aliphatic ketones, and organoboron compounds are oxidized by the enzyme (Fraaije et al., 2005; de Gonzalo et al., 2005a). The enzyme also displays excellent enantioselective behaviour with a number of sulfides and ketones. Furthermore, it has been shown that, as for styrene monooxygenase (see above), an organometallic complex can be used to replace the coenzyme NADPH (de Gonzalo et al., 2005b). By sequence alignment with cyclohexanone monooxygenase and inspection of the phenylacetone monooxygenase structure, several deletion mutants of phenylacetone monooxygenase have been prepared. As predicted, these enzyme mutants are able to convert relatively bulky substrates when compared with the wild

type enzyme (Bocola et al., 2005). This shows that by knowing the enzyme structure in atomic detail, it will be possible to create novel enzyme variants with properties that are fine-tuned for biocatalytic purposes.

## 5. Concluding remarks

The inventory of known flavoprotein monooxygenases described in this review shows that several protein scaffolds have been equipped with an oxygenating capacity. Apparently, monooxygenases have evolved at several occasions during evolution. The central reaction in these enzymes is always the same: formation of a peroxyflavin intermediate by reaction of reduced flavin with molecular oxygen. Depending on the type of monooxygenase, the flavin is reduced (1) in the monooxygenase moiety itself, representing a tightly bound flavin cofactor (subclasses A and B flavoprotein monooxygenases), alternatively (2) the flavin acts as a coenzyme being reduced by a auxiliary flavin reductase component after which the reduced flavin is bound by the monooxygenase component (subclasses C–F). The final oxygenation reaction catalyzed by each flavoprotein monooxygenase depends on the microenvironment of the (peroxy)flavin bound to the monooxygenase subunit, e.g. the protonation state of the peroxy function can be modulated by amino acids in the vicinity of the C<sub>(4a)</sub> of the flavin thereby regulating the nucleophilic or electrophilic character of the peroxyflavin. Furthermore, the microenvironment of the active site will determine which substrate will be able to approach and bind in an appropriate position. As a result each monooxygenase exhibits unique catalytic properties. Each monooxygenase subclass appears to be biased towards specific oxygenation reactions (Table 1), e.g. the class A monooxygenases primarily catalyze hydroxylations and epoxidations while the class F representatives only catalyze halogenations. This suggests that each fold favors a certain type of oxidative activity.

This review illustrates that flavoprotein monooxygenases are very flexible with respect to the type of oxidations reactions that are catalyzed and the range of substrate molecules that are accepted. It is expected that future research will even extend the range of reactions that can be catalyzed by flavoprotein monooxygenases as these type of enzymes are frequently encountered in



microbial genomes. Furthermore, recently developed enzyme redesign methods allows fine tuning of selected flavoprotein monooxygenases. Successful applications of monooxygenases will depend on methodologies that will facilitate efficient catalysis by, e.g. ensuring effective coenzyme recycling, oxygen transfer and product recovery. By combining the enzyme discovery and redesign efforts with process engineering, more applications that involve flavoprotein monooxygenases as biocatalysts will emerge.

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