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Flavin-Based Catalysis

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7 Flavoprotein Monooxygenases and Halogenases

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7.1 Introduction

Flavoprotein monooxygenases (FPMOs) are widespread and catalyze a broad range of selective oxygenation reactions. To do so, they usually generate a covalent flavin–oxygen adduct that inserts one atom of molecular oxygen (O_2) into the substrate, while the other oxygen atom is reduced to water. During the last decades, FPMOs have become increasingly important as biocatalysts for the sustainable production of industrially relevant chemicals.
The first FPMO with exquisite versatility in terms of type of reaction, substrate scope, and attractive regiochemistry was reported in 1988 as a Baeyer–Villiger monooxygenase (BVMO), i.e. cyclohexanone monooxygenasefrom *Acinetobacter* sp. NCIMB 9871 (CHMO; EC 1.14.13.22) [1, 2]. By 2014, more than 130 different enzymes could be distributed into eight groups (A–H) on the basis of their structural and functional properties [3]. Currently, the FPMO family comprises approximately 250 members with distinct physiological functions. Among them are more than 60 enzymes for which the crystal structure has been solved.
In this review, we describe the biocatalytic scope of FPMOs. After briefly explaining the reaction mechanisms and classification of FPMOs, we present an overview of the oxygenation reactions of group A–H FPMOs. More information about specific mechanistic features and structural properties of FPMOs can be found in recent reviews [4–12].

7.1.1 Reaction Mechanisms of Flavoprotein Monooxygenases

FPMOs use different mechanistic strategies to convert their substrates. In the reductive part of the reaction, the flavin cofactor gets reduced by an external or internal electron donor, whereas in the oxidative part, the anionic-reduced flavin reacts with O_2 in a single-electron transfer mechanism before the substrate gets oxygenated [6, 7, 13]. The reaction of triplet-state O_2 with protein-bound reduced flavin is usually much faster than with free reduced flavin, yielding second-order rate constants up to 10^6 M^{-1} s^{-1} [13–15].
For a long time, it was assumed that FPMOs activate O_2 exclusively through formation of a flavin C4a-(hydro)peroxide [16]. Upon heterolytic splitting of the peroxide bond (for a discussion about homolytic splitting, see Ref. [17]), the distal oxygen atom of this flavin adduct is inserted into the substrate, leading to a mono-oxygenated product and flavin C4a-hydroxide (Scheme 7.1a). Elimination of water from the flavin C4a-hydroxide regenerates the oxidized flavin cofactor for a next reaction cycle. Depending on the type of enzyme–substrate complex, the flavin C4a-(hydro)peroxide can act as an electrophile or as a nucleophile, giving rise to a variety of oxygenation chemistries [6, 19]. Studies

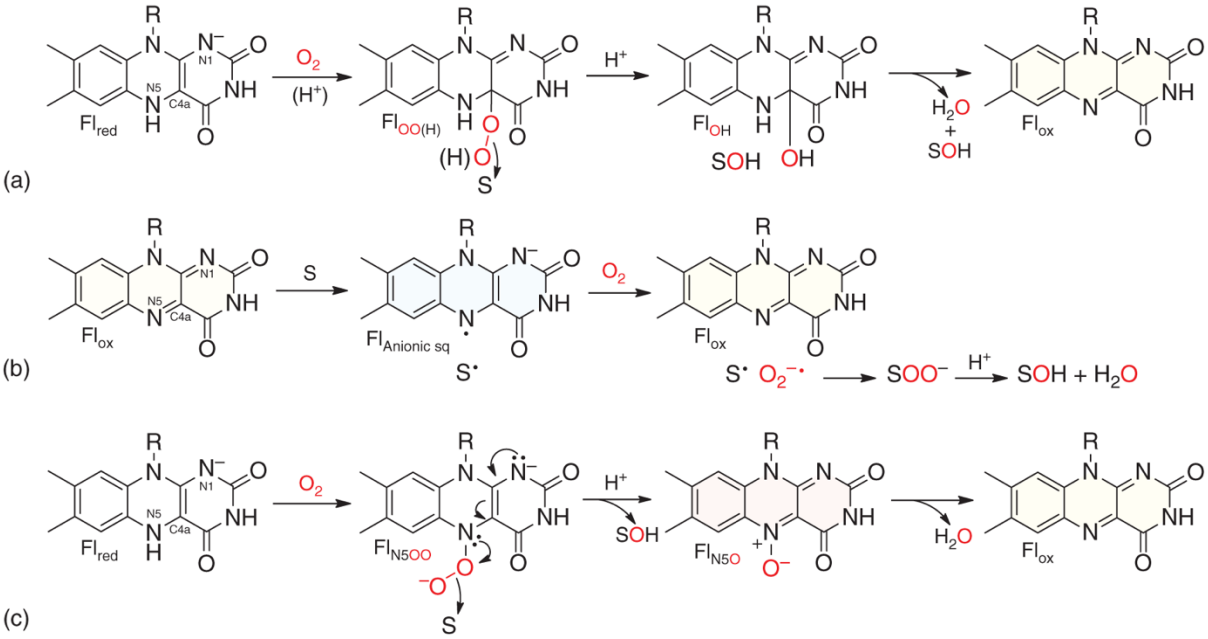
from transient kinetics have shown that the lifetimes of the flavin C4a-(hydro)peroxide and flavin C4a-hydroxide can vary dramatically [11, 16].

Internal FPMOs resemble flavin-dependent oxidases in hardly stabilizing the flavin C4a-(hydro)peroxide. They have been proposed to use enzyme-bound hydrogen peroxide for the monooxygenation of the intermediate product, generated during the reductive half-reaction [20, 21]. Nitronate monooxygenase (NIMO; EC 1.13.12.16; Figure 7.1) is a special internal FPMO [22]. NIMO is readily reduced by the substrate 3-nitropropionate (3-NPA), generating the 3-NPA radical and protein-bound anionic flavosemiquinone (Scheme 7.1b). Subsequent electron transfer from the anionic flavosemiquinone to O_2 generates superoxide anion, which reacts with the 3-NPA radical to yield 3-peroxo-3-nitropropanoate. This peroxygenated substrate then decays to the final products 3-oxopropanoate (malonic semialdehyde), nitrite, and water. The alternative would be that the 3-NPA radical reacts with O_2 to give a 3-peroxy-3-nitropropanoate radical, which subsequently receives an electron from the anionic flavosemiquinone yielding 3-peroxy-3-nitropropanoate [23].

Since 2013, several FPMOs that generate flavin N5–oxygen adducts during catalysis have been described [5, 24]. Among these enzymes, EncM was found to employ the flavin N5-oxide for substrate oxygenation [25], whereas RutA, DszA, and HcbA1 were recently proposed to use the transiently stable flavin N5-peroxide for substrate monooxygenation (Scheme 7.1c) [7, 18]. Based on the conservation of an oxygen reactivity consensus sequence, it was speculated that many more FPMOs oxygenate their substrates via flavin N5 oxyfunctionalization reactions.

7.1.2 Classification of Flavoprotein Monooxygenases

FPMOs use a noncovalently bound reduced flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) cofactor for activating O_2. Next to their protein structural properties (Figure 7.1) and oxygenation chemistries, the way of flavin reduction is an important criterion for the classification of FPMOs [3, 26].



Scheme 7.1 Reactions of FPMOs with O_2. (a) Reaction of reduced flavin (Fl_{red}) and O_2 leads to flavin C4a-(hydro)peroxide ($Fl_{OO(H)}$), which reacts with a substrate (S) to form product (SOH) and flavin C4a-hydroxide (Fl_{OH}). Fl_{OH} decays upon release of water to oxidized flavin (Fl_{ox}). (b) Reaction of NIMO with S leads to anionic flavosemiquinone ($Fl^{\bullet -}$) and a substrate radical ($S^{\bullet}$). $Fl^{\bullet -}$ then reacts with O_2 to Fl_{ox} and superoxide anion ($O_2^{\bullet -}$). $O_2^{\bullet -}$ then reacts with $S^{\bullet}$ to give a peroxygenated substrate (SOO^-), which decays to the final products 3-oxopropanoate (SOH), nitrite, and water. An alternative reaction of $S^{\bullet}$ with O_2 is described in the main text. (c) Reaction of Fl_{red} and O_2 leads to flavin N5-peroxide (Fl_{N5OO}), which reacts with the substrate to form product and flavin N5-oxide (FlN5O).Source: Saleem-Batcha et al. [7] and Matthews et al. [18].

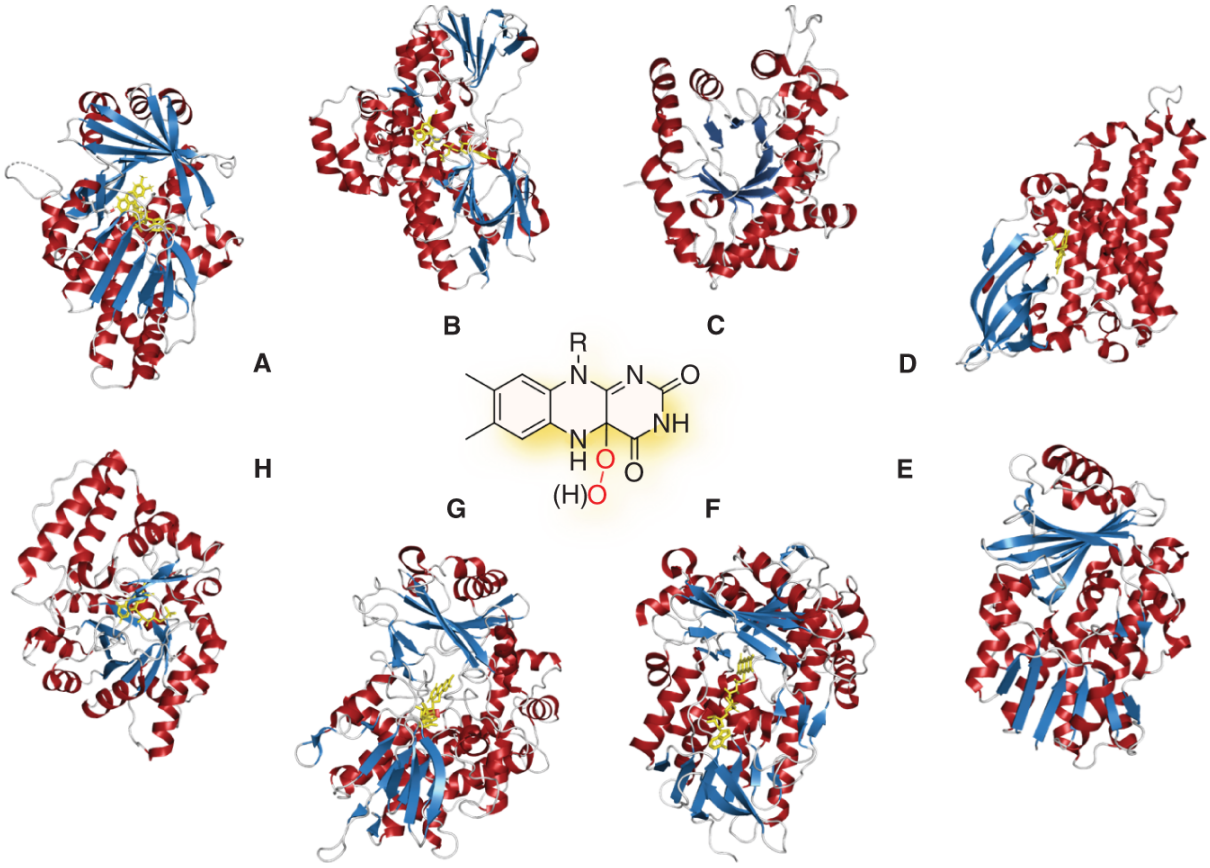


Figure 7.1 Crystal structures of FPMOs groups A to H according to mode of flavin reduction: external (A, B), two-component (C–F), internal (G, H). Protein Data Bank (PDB) number used in brackets. (**A**) TropB (6NES); (**B**) CHMO (4RG3); (**C**) bacterial luciferase (1LUC); (**D**) XiaF (5LVW); (**E**) StyA (3IHM); (**F**) RebH (2OAL); (**G**) tryptophan 2-monooxygenase (4IV9); (**H**) NIMO (6BKA).

Groups A and B constitute the largest groups of FPMOs, making up more than half of the currently known FPMOs. They rely on NAD(P)H as external electron donor and combine flavin reduction and substrate monooxygenation in a single polypeptide chain. Group A and B members contain similar three-layer ββα sandwich domains for binding the FAD cofactor, but differ in the mode of NAD(P)H recognition and kinetic mechanism. They can be discriminated on the basis of specific amino acid sequence fingerprints that map different patches of the FAD-binding domain [27–29], the absence (group A) or presence

(group B) of an internal NAD(P)H domain, and the way they have undergone domain fusion events [30]. With group A enzymes, substrate binding can stimulate the rate of flavin reduction, and the oxidized $NAD(P)^+$ is immediately released [16, 31]. Group B enzymes bind the substrate after flavin reduction and keep the $NAD(P)^+$ bound during the oxidative half-reaction [6, 16].

Group C–F enzymes comprise two-component FPMOs that use an NAD(P)H-dependent flavin reductase for delivery of reduced FMN or FAD to the monooxygenase component. Flavin transfer either occurs via free diffusion or through coupled channeling. Group C enzymes are FMN-dependent and have a triosephosphate isomerase (TIM)-barrel fold [32]. The flavin N5-(per)oxide-generating enzymes RutA, DszA, and HcbA1 belong to this group [18]. Group D members are FMN- or FAD-dependent and have an acyl-CoA dehydrogenase fold [11]. Group E and F enzymes are evolutionarily related to group A members [30, 33], but differ in oxygenation chemistry by catalyzing predominantly epoxidation (group E) or halogenation (group F) reactions [34].

Groups G and H (EC 1.13.12.x) comprise internal FPMOs. These single-component enzymes reduce the flavin cofactor through substrate oxidation and contain either a ββα sandwich domain for binding FAD (group G) or a TIM-barrel domain for binding FMN (group H) [3].

The flavin N5-oxide-stabilizing EncM does not belong to any of the abovementioned FPMO groups. EncM belongs structurally to the vanillyl alcohol oxidase (VAO)/*p*-cresol methyl hydroxylase (PCMH) flavoprotein family [35], and binds its FAD cofactor in a covalent mode. Using the flavin N5-oxide for initial substrate attack, EncM catalyzes the dual oxidation of an acyl carrier protein-bound dihydrooctaketide into desmethyl-5-deoxyenterocin in a coenzyme-independent oxygenation–dehydrogenation type of reaction [24]. EncM, tentatively classified as the first member of group I, can be regarded as an "inverted internal oxygenase" since it catalyzes oxygenation first (enabled by the stable N5-oxide) before substrate dehydrogenation [25].

7.1.3 Oxygenation Reactions

FPMOs catalyze a remarkable diversity of chemoselective oxygenation reactions (Table 7.1). Using the FPMO classification, we observe that group A–D enzymes show the highest diversity in oxygenation chemistries and that, in several cases, a certain type of reaction is not reserved to a specific protein fold. Table 7.1 also shows that the three-layer ββα sandwich fold (Class, Architecture, Topography, and Homology (CATH) code 3.50.50.60) is the most common protein scaffold for hosting the flavin cofactor of FPMOs.

7.2 Types of Reactions Catalyzed by Group A–H Enzymes

7.2.1 Group A Reactions

Group A represents the largest group of FPMOs. Currently, approximately 90 group A members with a distinct physiological function have been purified. Most of these enzymes catalyze hydroxylation reactions of activated aromatic compounds, including anilines, phenols, phenolic acids, alkylphenols, pyridines, indoles, phenazines, phytoalexins, polyketides, and benzoquinones. The electrophilic aromatic substitution character of these hydroxylation reactions determines that the electron-donating substituents of the substrates act as *ortho*/*para*-directors and that the flavin C4a-hydroperoxide acts as an electrophile. The high regioselectivity and narrow substrate specificity explain the wide

variety in substrate-binding poses and poor sequence conservation of the substrate-binding pockets [33]. By the same token, certain group A enzymes react with the same physiological substrate but form another product, e.g. 3-hydroxybenzoate 4-hydroxylase (3HB4H; EC 1.14.13.23) and 3-hydroxybenzoate 6-hydroxylase (3HB6H; EC 1.14.13.24). The aromatic hydroxylase 2-hydroxybiphenyl 3-monooxygenase (HbpA; EC 1.14.13.44) has been studied extensively for applied biocatalysis (Scheme 7.2a) [36–38].

Table 7.1 Oxygenation chemistries of FPMOs.

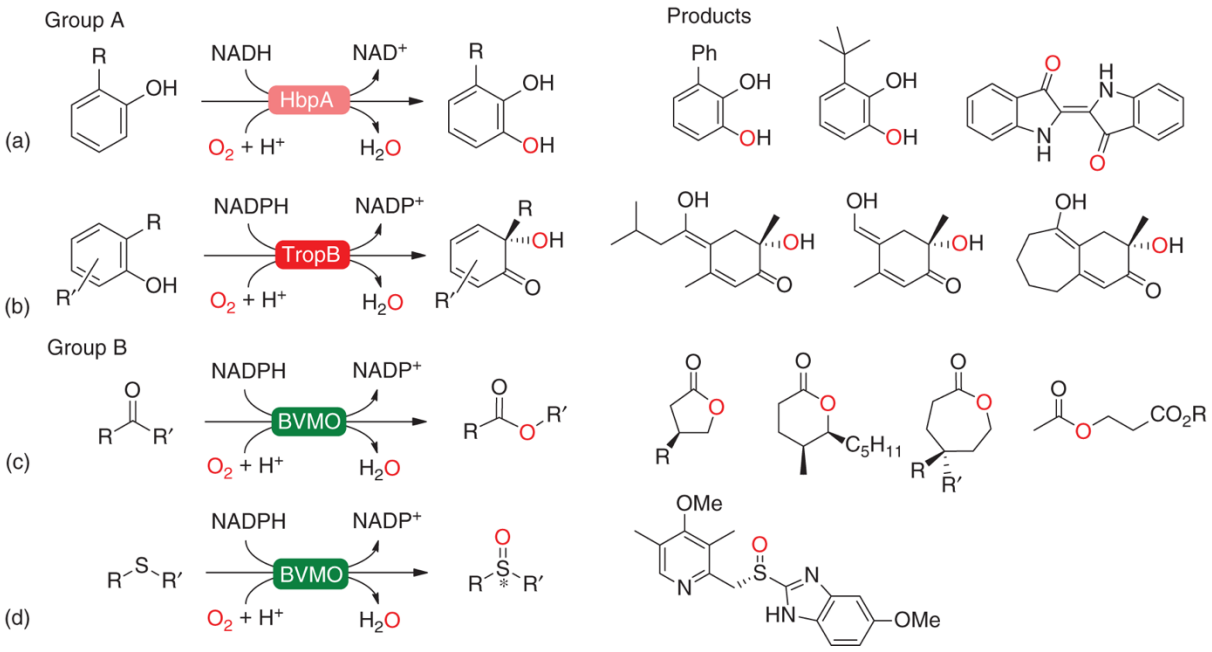
Type of reaction	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Hydroxylation	*							
Oxidative decarboxylation							*	*
Oxidative dehalogenation				*				
Oxidative deamination							*	
Oxidative dearomatization	*							
Baeyer–Villiger oxidation		*						
Oxidative ring opening								
N-Hydroxylation								
Sulfoxidation		*						
Epoxidation					*			
Heteroatom oxygenation								
Indole oxidation								
Oxidative halogenation						*		
Oxidative cyclization								
Light emission								
Oxidative desulfonation			*					
Oxidative dealkylation								
Oxidative decarboxymethylation								
Oxidative biaryl homocoupling								
Oxidative denitration				*				

Group A–H enzymes are colored according to type of flavin-binding domain. Yellow: ββα sandwich fold (FAD); blue: TIM-barrel fold (FMN); green: acyl-CoA dehydrogenase fold (FAD/FMN). Reactions indicated with an asterisk are depicted in Schemes 7.2 and 7.3.

An important hydroxylation reaction catalyzed by several group A enzymes concerns the *ipso*-attack [46]. In such reaction, the entering hydroxy group attaches to a position in the aromatic ring already carrying a substituent, and this substituent can either be expelled or migrate in a subsequent step. A classic example concerns salicylate 1-hydroxylase (SalH; EC 1.14.13.1) catalyzing oxidative decarboxylation [47].

Related to the *ipso*-attack is the oxidative dearomatization reaction catalyzed by group A members TropB (Figure 7.1), SorbC, and AzaH [48–50]. This site- and stereoselective reaction is particularly interesting for applied biocatalysis by providing access to a wide

range of substituted *ortho*-quinol enantiomers (Scheme 7.2b), including oxidative cyclization products [51–57].
Several group A enzymes catalyze Baeyer–Villiger oxidations. These FPMOs are involved in the microbial biosynthesis of complex natural products and are referred to as atypical or type O BVMOs [12]. Type O BVMOs are mostly multifunctional enzymes that possess both BVMO and hydroxylase activity.
Multiple group A enzymes catalyze epoxidation reactions. Examples include FPMOs from *Streptomyces* species, which catalyze sequential enantioselective epoxidations during the biosynthesis of polyether antibiotics [58], and several recently characterized diastereoselective FPMOs involved in fungal indole alkaloid biosynthesis [59, 60].



Scheme 7.2 Selected group A and B FPMO-catalyzed reactions with gram-scale potential. (a) HbpA-catalyzed aromatic hydroxylation leading to catechols, and to hydroxylated indole, which oxidizes to indigo; (b) TropB-catalyzed oxidative dearomatization; (c) BVMO-catalyzed Baeyer–Villiger oxidation leading to chiral lactones and esters; (d) BVMO-catalyzed asymmetric sulfoxidation leading to the pharmaceutical drug esomeprazole. Source: (a) Meyer et al. [36]; Lutz et al. [37]; Bregman-Cohen et al. [38]. (d) Modified from Bong et al. [39].

7.2.2 Group B Reactions

Group B FPMOs comprise several sequence-related subgroups, including BVMOs, flavin-containing monooxygenases (FMOs), and *N*-hydroxylating monooxygenases (NMOs) [26]. Among these subgroups, the microbial NADPH-dependent BVMOs (also referred to as type I BVMOs; EC 1.14.13.x) have received the most attention because of their excellent enantioselectivity [61–63], broad substrate scope, and ease of recombinant production.
Type I BVMOs catalyze the conversion of ketones to chiral esters or lactones (Scheme 7.2c). Nucleophilic attack by the flavin C4a-peroxide on the carbonyl group of the ketone substrate results in the formation of a tetrahedral Criegee intermediate, which undergoes a rearrangement involving migration of one of the substituents of the

carbonyl carbon and subsequent heterolytic cleavage of the peroxidic bond, yielding the ester and acid products (see Ref. [63] for a historical account). Type I BVMOs are also active with aldehydes, yielding the corresponding acids or formate derivatives [64–66], and can perform enantioselective sulfoxidations, heteroatom oxidations, and epoxidations [1, 65].

Since the first crystal structure of a type I BVMO (i.e. phenylacetone monooxygenase (PAMO); EC 1.14.13.92) was elucidated in 2004 [67], the number of studies about the production, engineering, and biocatalytic properties of BVMOs has exploded [65, 68–71]. Next to the discovery and characterization of new members [72–74], close attention was paid to improving the substrate scope and thermal stability of BVMOs [10, 75–81]. A remarkable improvement in sulfoxidation performance was achieved for the CHMO-mediated synthesis of the gastric acid inhibitor esomeprazole from pyrmethazole (Scheme 7.2d) [39]. With 41 mutations, the enzyme obtained improved activity and thermo- and solvent stability, became resistant to oxygen supply, and formed minor amounts of sulfone byproduct, resulting in 99.6% conversion and 99.9% of the (*S*)-isomer.

FMOs (EC 1.14.13.8) were first discovered in mammals and humans, where they are involved in the detoxification of a vast spectrum of nitrogen- and sulfur-containing compounds [82]. Later, FMOs were also found to be present in prokaryotes [83] and yeasts [84], and especially abundant in marine heterotrophic bacteria, where they convert trimethylamine into the osmolyte trimethylamine-*N*-oxide [85]. In humans, this dietary risk factor is produced by the hepatic isoform FMO3 [86]. FMO5 is the only human isoform that shows BVMO activity [87, 88], possibly because of a Glu-His switch [89].

FMO from *Methylophaga* sp. strain SK1 was explored for its synthetic capacity and found to produce high amounts of indigo from indole [90]. Besides converting a series of indole derivatives into the corresponding indigoid dyes, this bacterial FMO showed moderate-to-good enantioselectivity in sulfoxidation reactions [91]. The FMO from *Stenotrophomonas maltophilia* was one of the first FMOs that showed a relaxed coenzyme specificity and BVMO activity [92]. In addition to type I BVMOs, the actinobacterium *Rhodococcus jostii* RHA1 appeared to contain several FMOs with NAD(P)H specificity and BVMO activity. Based on these properties and distinctive phylogeny, these FMOs were classified as type II FMOs [29, 93].

A screening with a set of microbial group B enzymes showed that BVMOs and type II FMOs have overlapping substrate profiles with mammalian FMOs [94]. FMO from *Nitrincola lacisaponesis* was the first characterized FMO from an alkaliphile. This enzyme showed a good thermal stability and organic solvent tolerance, and was active with a range of established BVMO substrates [95].

FMOs are also present in plants, insects, and nematodes. Plant FMOs have been divided into three clades [96], with clade I containing a pipecolate *N*-hydroxylase, clade II containing the YUCCA enzymes (EC 1.14.13.168), and clade III containing FMOs leading to organosulfur compounds with health beneficial properties. Despite their interesting properties, plant FMOs have not yet been extensively studied for their biocatalytic potential [97].

NMOs are microbial group B enzymes that are involved in the biosynthesis of secondary metabolites, such as siderophores and antimicrobial agents [98]. NMOs catalyze the *N*-hydroxylation of soft nucleophiles, such as ornithine and lysine, using mechanisms similar to FMOs [16]. The structural properties and catalytic mechanism of NMOs, as well as their phylogeny and involvement in siderophore biosynthesis, have recently been summarized [99]. NMOs play a crucial role in the biosynthesis of

diazo-containing antibiotics [100]. One route starts with the NMO-mediated double hydroxylation of aspartate affording nitrosuccinate [101]. In another route, NMO converts lysine to *N*6-hydroxylysine, allowing the subsequent formation of hydrazinoacetate [102]. The NMO-catalyzed *N*5-hydroxylation of ornithine is the first step in the production of piperazate [103, 104], an important nonproteinogenic building block for many natural products [105]. Recent bioinformatics studies suggest that certain actinobacteria employ an NMO-fused chimeric enzyme for the synthesis of piperazate [106], strengthening the idea that NMOs might be useful in enzymatic cascade reactions for the synthesis of valuable diazo compounds [99].

The above overview shows that group B FPMOs catalyze a wide range of oxygenation reactions and that the borders between the different subgroups are fading. In this context, it is important to note that FMOs can also catalyze halogenation reactions, illustrated by the discovery of a 4-hydroxybenzoate decarboxylative brominase (Bmp5). Bmp5 catalyzes two consecutive steps in the biosynthesis of polybrominated diphenyl ethers in the marine organism *Pseudoalteromonas luteoviolacea* [107]. In the first step, Bmp5 catalyzes the NADPH- and O_2-dependent conversion of 4-hydroxybenzoate to 3-bromo-4-hydroxybenzoate, while in the second step this monobrominated intermediate is converted via oxidative decarboxylative bromination to 2,4-dibromophenol. Bmp5 can also brominate monosubstituted 4-hydroxybenzoates [108]. Unlike the two-component flavin-dependent halogenases (*vide infra*), Bmp5 is active with iodide, but not with chloride, raising questions about its precise reaction mechanism.

7.2.3 Group C Reactions

Assisted by a reductase partner that provides $FMNH_2$, group C FPMOs catalyze a motley collection of oxygenation reactions. Besides the unique light emission reaction catalyzed by bacterial luciferase (EC 1.14.14.3), group C members perform hydroxylation, Baeyer–Villiger oxidation, oxidative ring opening, oxidative desulfonation, oxidative dealkylation, and oxidative decarboxymethylation reactions (Table 7.1).

The crystal structure of luciferase from *Vibrio harveyi* [109] paved the way for structure–function relationship studies of FPMOs with a luciferase-like $(\beta/\alpha)_8$ domain (Figure 7.1; CATH code 3.20.20.30). The bioluminescence reaction of bacterial luciferase can be applied in prokaryotic and eukaryotic reporter gene systems for the detection of a wide range of specific analytes [110, 111].

LadA monooxygenases from thermophilic *Geobacillus* species (EC 1.14.14.28) catalyze the hydroxylation of long-chain alkanes to the corresponding primary alcohols [112]. These enzymes are not only of interest for the treatment of environmental oil pollution, but also for the synthesis of complex molecules. Random mutagenesis and structural analysis of LadA from *Geobacillus thermodenitrificans* revealed that most mutations improving catalysis were connected to residues located outside the active site [113].

2,5-Diketocamphane 1,2-monooxygenase (2,5-DKCMO; EC 1.14.14.108) is a group C enzyme involved in the degradation of camphor in *Pseudomonas putida* ATCC 17453 [114]. 2,5-DKCMO catalyzes the Baeyer–Villiger oxidation of (+)-bornane-2,5-dione to 5-oxo-1,2-campholide. This chiral ketolactone spontaneously hydrolyzes to (1*R*)-2,2,3-trimethyl-5-oxocyclopent-3-enyl acetate. 2,5-DKCMO and the enantiocomplementary 3,6-diketocamphane 1,6-monooxygenase (3,6-DKCMO; EC 1.14.14.155) are generally referred to as type II BVMOs [115]. The DKCMOs catalyze different regio- and enantiospecific oxidations of cyclic and aliphatic ketones thereby

generating chiral lactone products of value as synthons in chemoenzymatic synthesis [114].

Several group C enzymes catalyze carbon–sulfur cleavage reactions that are useful for acquisition of sulfur or for bioremediation purposes. Dimethylsulfide monooxygenase (DmoA; EC 1.14.13.131), dimethylsulfone monooxygenase (SfnG; EC 1.14.14.35), and methanesulfonate monooxygenase (MsuD; EC 1.14.14.34) catalyze specific oxidative demethylation steps during sulfur assimilation in soil bacteria. Alkanesulfonate monooxygenase (SsuD) catalyzes an oxidative desulfonation/dealkylation reaction yielding C1–C14 primary aldehydes (Scheme 7.3a) [8]. Dibenzothiophenesulfone monooxygenase (DszA; 1.14.14.22) catalyzes an oxidative ring-opening reaction. Recently, it was proposed that this reaction initially involves covalent adduct formation between the anionic flavin N5-peroxide and the substrate and that, after subsequent splitting of the carbon–sulfur bond, the oxygen–oxygen bond of the flavin–substrate adduct is cleaved, affording the monooxygenated product 2-(2-hydroxyphenyl)-benzene sulfonate and flavin N5-oxide [18].

Nitrilotriacetate monooxygenase (NmoA; EC 1.14.14.10) and EDTA monooxygenase (EmoA; EC 1.14.14.33) catalyze oxidative decarboxymethylation reactions, thereby assisting in the microbial degradation of the extensively used chelating agents. EmoA sequentially removes two carboxymethyl groups from ethylenediaminetetraacetate (EDTA) and has a broader substrate range than NmoA.

7.2.4 Group D Reactions

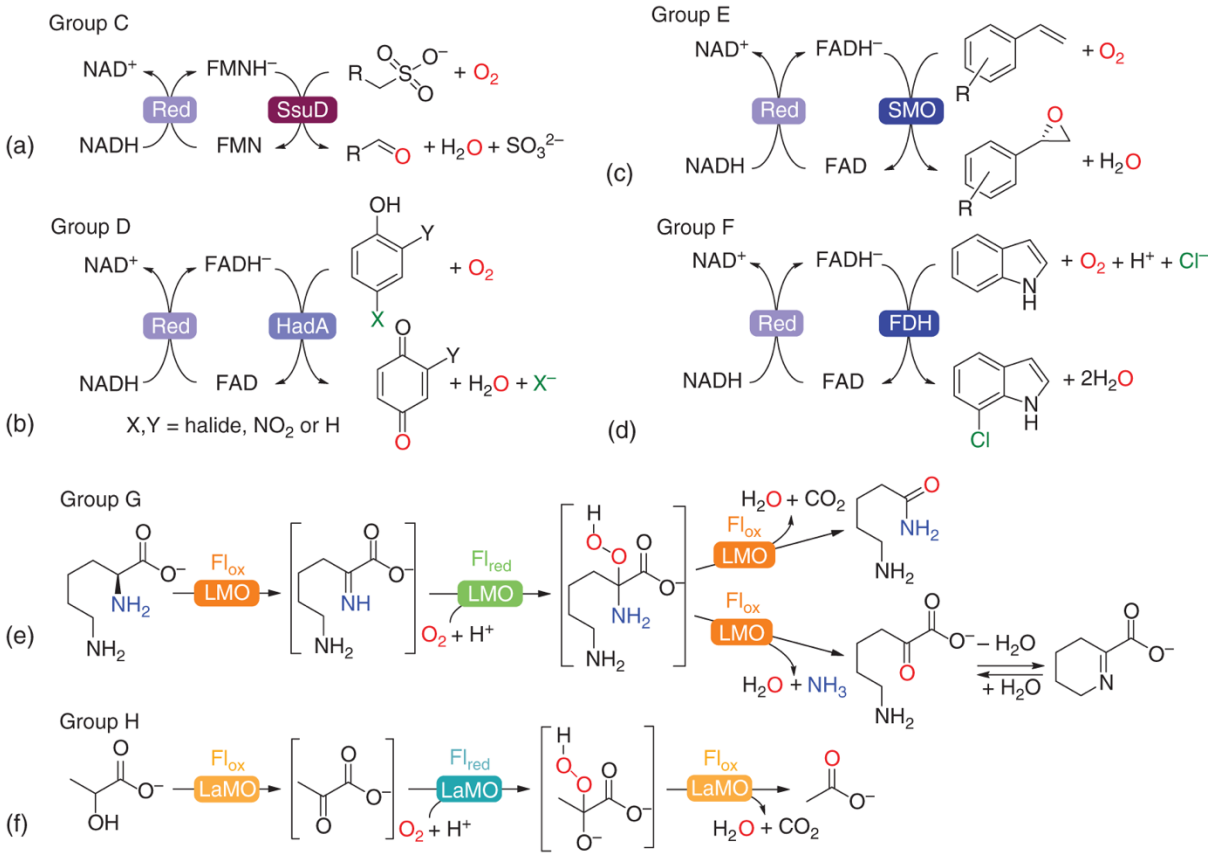
Group D FPMOs contain an acyl-CoA dehydrogenase fold (Figure 7.1; Table 7.1). They are phylogenetically divided over FAD- and FAD/FMN-dependent clades [34]. Today, approximately 40 different group D members have been described. Most of these enzymes, spread over both clades, catalyze hydroxylation reactions and are involved in the microbial degradation of (halo)phenols [11, 116].

FAD-dependent 4-hydroxyphenylacetate 3-hydroxylase (C2-HpaH; EC 1.14.14.9) is the prototype aromatic hydroxylase of group D. The reaction mechanism and structure–function relationship of C2-HpaH has been studied in great detail, and its mode of oxygen and substrate activation has been compared with that of single-component flavoprotein aromatic hydroxylases [117, 118].

C2-HpaH has also been engineered for biocatalytic applications. Using site-directed mutagenesis, it was possible to create enzyme variants that hydroxylated alternative substrates like 4-hydroxycinnamate, 4-aminophenylacetate, tyramine, and octapamine yielding valuable antioxidants and catecholamine synthons [118].

Genome mining was used to search for monooxygenases that can convert the nutritional supplement (*S*)-equol to commercially unavailable hydroxyequols [119]. It was found that one of the TtHpaH homologs from *Rhodococcus opacus* efficiently converted both (*R*)- and (*S*)-equols to the corresponding 3′-hydroxyequols with a slight preference for the (*S*)-enantiomer, and that one of the TtHpaH homologs from *Photorhabdus luminescens* regioselectively hydroxylated the (*R*)- and (*S*)-equols to the corresponding 6-hydroxyequols with clear preference for the (*S*)-enantiomer.

3-Hydroxy-9,10-seconandrost-1,3,5(10)-triene 9,17-dione monooxygenase (HsaA; EC 1.14.14.12), involved in the catabolism of cholesterol in *Mycobacterium tuberculosis*, is a group D member that is active with a steroid substrate.



Scheme 7.3 Selected group C to H FPMO-catalyzed reactions. Two-component FPMOs C-F: (a) SsuD-catalyzed oxidative desulfonation to primary aldehydes; (b) HadA-catalyzed oxidative dehalogenation and denitration; (c) SMO-catalyzed asymmetric epoxidation; (d) FDH-catalyzed oxidative halogenation of indole. Internal FPMOs G-H: (e) LMO-catalyzed oxidative decarboxylation or deamination of lysine; (f) LaMO-catalyzed oxidative decarboxylation of L-lactate to acetate. Source: (a) Based on Robbins and Ellis [8]. (b) Modified from Pimviriyakul et al. [40]. (c) Di Gennaro et al. [41] and Panke et al. [42]. (e) Matsui et al. [43] and Trisrivirat et al. [44]. (f) Modified from Edson [45].

Several group D members are bifunctional enzymes. 4-Nitrophenol 4-monooxygenase (PnpA; EC 1.14.13.167) from *R. opacus* catalyzes the oxidative denitration of 4-nitrophenol to hydroquinone, which is then further converted to 1,2,4-trihydroxybenzene [120]. 4-Chlorophenol monooxygenase (HadA) from *Ralstonia pickettii* has been engineered to catalyze sequential oxidative dehalogenation and denitration reactions (Scheme 7.3b) [40]. Dibenzothiophene monooxygenase (DszC; EC 1.14.14.21) from *Rhodococcus erythropolis* catalyzes the sequential sulfoxidation of dibenzothiophene (DBT) to DBT-sulfoxide and DBT-sulfone, respectively. In this way, the enzyme initiates the microbial desulfurization of crude oil [121].

Another interesting reaction catalyzed by a group D member concerns the oxidative desulfonation of the widely used antibiotic sulfamethoxazole. This reaction is catalyzed by sulfonamide monooxygenase (SadA), which initiates the catabolism of various sulfonamides in *Microbacterium* species through an *ipso*-hydroxylation mechanism [122]. In the next step, another FMN-dependent group D member, 4-aminophenol monooxygenase(SadB), converts the released 4-aminophenol

by sequential oxidative deamination and hydroxylation to 1,2,4-trihydroxybenzene [122].
Several group D enzymes catalyze indole oxidations, such as 2-methylindolylpyruvate 3-hydroxylase (TsrE) from *Streptomyces laurentii*. This enzyme, which initiates an unusual indole-ring expansion mechanism in the biosynthesis of thiostrepton, was found to hydroxylate the synthetic substrate mimic 2-methyl-3-propylindole, triggering a double-bond shift within the pyrrole ring, yielding the (3*S*)-isomer of the imine product with an enantiomeric excess (ee) value of up to 96% [123].
Indole 3-acetate monooxygenase (IacA; EC 1.14.13.235) catalyzes the initial step in the degradation of the plant hormone auxin in *P. putida* yielding 2-hydroxyindole-3-acetate as most likely product [124]. IacA is also capable of oxidizing indole to indoxyl, which spontaneously dimerizes to the blue pigment indigo. Indosespene 3-hydroxylase (XiaF, Figure 7.1) catalyzes the conversion of indosespene to the carbazole moiety of the indolosesquiterpenoid xiamycin in *Streptomyces* endophytes [125]. This cryptic cyclization reaction is believed to involve the initial 3-hydroxylation of the indole moiety of indosespene. XiaF also oxygenates indole to indoxyl, which spontaneously oxidizes to yield indigo and indirubin.
Group D enzymes also catalyze *N*-hydroxylations. One example is isobutylamine *N*-monooxygenase (IBAH; EC 1.14.14.30) from *Streptomyces viridifaciens*, which catalyzes the oxidation of isobutylamine to isobutylhydroxylamine, a key step in the biosynthesis of the azoxy antibiotic valanimycin [126]. Other *N*-hydroxylations are catalyzed by dTDP-L-evernosamine *N*-hydroxylase (RubN8; EC 1.14.13.187) from *Micromonospora carbonacea* [127], dTDP-L-epi-vancosamine *N*-hydroxylase (DnmZ) from *Streptomyces peucetius* [128], and dTDP-3-amino-2,3,6-trideoxy-4-keto-3-methyl-D-glucose *N*-hydroxylase (KijD3) from *Actinomadura kijaniata*[129]. The first two enzymes mediate the double oxidation of their dinucleotide aminosugar substrates to the corresponding nitrososugars, whereas KijD3 produces a nitrosugar [130].

7.2.5 Group E Reactions

Group E FPMOs catalyze enantioselective epoxidation and sulfoxidation reactions. Styrene monooxygenase (SMO), composed of epoxidase StyA (EC 1.14.14.11; Figure 7.1) and flavin reductase StyB (EC 1.5.1.36), is the most extensively studied member of this group. After its discovery in 1990 [131], SMO soon received attention as a (recombinant) biocatalyst for the production of optically pure epoxides (Scheme 7.3c) [41, 42]. In the beginning of this century, by using whole *Escherichia coli* cells harboring a plasmid containing the *sty*AB genes of *Pseudomonas taiwanensis* VLB120, Witholt and coworkers could produce gram-scale amounts of (*S*)-styrene oxide and related chiral oxiranes in a two-liquid phase fed-batch bioreactor [132, 133].
Purification of recombinant proteins allowed a more thorough characterization of the catalytic and structural properties, the mode of flavin transfer [134], and also facilitated the development of nonenzymatic systems for the regeneration of the reduced FAD cofactor of StyA [135, 136].
During the last decade, many new SMOs were discovered, allowing a subdivision in different phylogenetic groups [34, 137]. Interesting findings concerned the discovery of a natural fusion between StyA and StyB in *R. opacus* 1cp (StyA2B) [138, 139], and the identification of a group of indole monooxygenases (IMOs; IndAB) that prefer the conversion of indole to indole 2,3-epoxide [140]. IMOs can be discriminated from SMOs

on the basis of specific active site-sequence motifs [141], and based on this finding, StyA2B can now be considered as an IndA2B enzyme [137, 142].
Heine et al. constructed artificial fusions of StyA and StyB from *Pseudomonas fluorescens* ST for the synthesis of Tyrian purple and other indigoid dyes [140, 143]. Corrado et al. used a similar flexible linker to join StyA and StyB from *P. taiwanensis* VLB120, and coexpressed this self-sufficient SMO with formate dehydrogenase for the efficient production of (*S*)-styrene oxides [144]. The chimeric SMO was then integrated in a multienzyme cascade [145] for the regio- and stereoselective aminohydroxylation of *trans*-β-methylstyrene to obtain either enantiopure (1*R*,2*R*)- or (1*S*,1*R*)-phenylpropanolamine diastereomers [146].
SMOs have a broad substrate scope. They are most active with a wide range of aromatic and aliphatic alkenes, but also oxidize aromatic sulfides, sulfanes, and benzothiophenes [147–149]. SMOs usually produce (*S*)-epoxides and (*R*)-sulfoxides. IMOs, on the other hand, prefer (*S*)-sulfoxidations. Recently, Wu and coworkers identified an SMO from the genome of *Streptomyces* sp. NRRL S-31 that showed complementary stereoselectivity toward alkenes and only formed the (*R*)-epoxides [150].
Besides SMOs and IMOs, there are some other FAD-dependent epoxidases that have been classified as group E FPMOs [3]. Zeaxanthin epoxidase (ZEP; EC 1.14.13.90) and squalene epoxidase (SQLE; EC 1.14.14.17) are involved in xanthophyll and cholesterol biosynthesis, respectively [151]. They have group A-like flavin-binding characteristics, but being membrane-associated, they receive their reducing equivalents from electron-transfer protein complexes. A crystal structure of the catalytic domain of SQLE was recently obtained [152, 153], which might stimulate future biocatalytic applications.

7.2.6 Group F Reactions

FPMOs of group F comprise two-component FAD-dependent halogenases (FDHs) that typically catalyze bromination or chlorination steps during the biosynthesis of natural products [154–156]. FDHs can be discriminated from related FPMOs by a specific WxWxIP active-site sequence motif [157]. *In vivo*, FDHs receive $FADH_2$ from a flavin reductase, but *in vitro* they can also use (photo)chemically reduced FAD [158–160].
Tryptophan 7-halogenase from *P. fluorescens* (PrnA; EC 1.14.19.9) was the first FDH obtained in pure form [161]. The elucidation of the crystal structure of PrnA provided support for the proposal that oxidation of chloride anion by flavin C4a-hydroperoxide in the enzyme active site results in the formation of hypochlorite [162]. This halenium ion is then guided through a short tunnel to the substrate-binding site where it reacts in a regioselective way with tryptophan to produce 7-chlorotryptophan. Subsequent mutagenesis and crystallography studies on tryptophan 7-halogenases PrnA and RebH (Figure 7.1), tryptophan 5-halogenase PyrH (EC 1.14.19.58), and Trp 6-halogenases ThaI and BorH (EC 1.14.19.59), confirmed the vital importance of the catalytic lysine and its positioning relative to tryptophan for the regioselective halogenation reaction [163].
KtzR (EC 1.14.19.60) is a tryptophan halogenase involved in the assembly of the antifungal secondary metabolite kutzneride, and selectively converts 7-chlorotryptophan, originally produced by KtzQ, to 6,7-dichlorotryptophan [164]. Another unusual tryptophan halogenase, obtained from the marine sponge *Theonella swinhoei* WA, catalyzes the 6-chlorination of 5-hydroxytryptophan, also being quite active with serotonin and 5-methoxyindole [165].
FDHs are also capable of catalyzing sequential halogenations. The first example of such reaction was reported for PltA from *P. fluorescens*, which catalyzes the dichlorination of

pyrrolyl-S-PltL during the biosynthesis of the antifungal natural product pyoluteorin [166]. Another example is represented by MalA and MalA′ from *Malbranchea aurantiaca*. Both FDH isoforms catalyze the chlorination and bromination of premalbrancheamides during late-stage fungal alkaloid biosynthesis [167]. ClmS from *Streptomyces venezuelae* is one of the few FDHs known that halogenates an alkyl substrate, forming the dichloroacetyl group of chloramphenicol [168].

Currently, about 40 different FDHs have been (partially) characterized. Most of them catalyze halogenations of free indoles (Scheme 7.3d), pyrroles, and phenols, whereas others are active with carrier-protein-bound substrates in nonribosomal peptide synthetase or polyketide synthase assembly lines [155, 156]. FDHs have received increasing interest as biocatalysts because of their capacity to selectively install halogen atoms in aromatic and aliphatic compounds of varying complexity under mild and green conditions [169]. Next to engineering the regioselectivity of halogenation and the discovery of novel family members, efforts have been spent toward increasing the catalytic efficiency, broadening the substrate scope, and improving the thermal and operational stability of FDHs [156, 170]. Employing genome mining with a previously unappreciated sequence motif, Goss and coworkers discovered the first FDH (VirX1) that exhibits a clear preference for iodination and acts on a diverse set of organic substrates [171].

Group F halogenases can also catalyze biaryl homocoupling reactions [172]. A pair of FDHs (Mpy10 and Mpy11) from *Streptomyces* sp. CNQ-418 was shown to be responsible for the chiral *N*,*C*-biaryl coupling of two molecules of monodeoxypyoluteorin, which resulted in densely halogenated marinopyrroles possessing potent antibiotic activity.

Recently, Lewis and coworkers employed family-wide activity profiling to obtain sequence-function information on FDHs [173]. Using a high-throughput mass-spectrometry-based screen of more than 100 putative FDH sequences, they identified halogenases with novel substrate scope and complementary regioselectivity, and used these enzymes for preparative scale C–H functionalization.

7.2.7 Group G and H Reactions

Group G and H members represent the internal FPMOs. These enzymes do not need an external electron donor, in principle an advantage for applied biocatalysis.

Group G is made up of amino acid monooxygenases with a monoamine oxidase fold. They catalyze oxygenative decarboxylation reactions of arginine (AMO; EC 1.13.12.1), lysine (LMO; EC 1.13.12.2), tryptophan (TMO; EC 1.13.12.3; Figure 7.1), and phenylalanine (PAO; EC 1.13.12.9), to give the corresponding amides. Depending on the amino acid used, group G enzymes can also function as oxidases, thereby converting the intermediate imino acids through oxidative deamination to α-keto acids. LMO from *Pseudomonas* sp. AIU813 catalyzes both the decarboxylation and deamination of L-lysine (Scheme 7.3e) [43]. Results from rapid kinetics and mass-spectrometry product analysis suggested that this LMO uses *in situ*–generated enzyme-bound hydrogen peroxide to decarboxylate the imino lysine to 5-aminovaleramide (Scheme 7.3e) [44]. These results merit further investigation, not only from a mechanistic point of view, but also to explore whether group G reactions can be optimized for the production of interesting platform chemicals.

Group H comprises internal FPMOs with a $(\beta/\alpha)_8$ TIM-barrel fold. In Section 7.1.1, we already described the proposed reaction mechanism of NIMO. Here, we can add that the

widespread NIMOs seem to have a restricted substrate scope [174], and that the crystal structures of NIMO from *Pseudomonas aeruginosa* PAO1 [175] and *Cyberlindnera saturnus* [176] are highly conserved.
Lactate monooxygenase (LaMO; EC 1.13.12.4) catalyzes the oxidation of L-lactate to acetate via the oxidative decarboxylation of the initially formed pyruvate (Scheme 7.3f) [45]. LaMO shares many properties with flavin-dependent α-hydroxyacid oxidases [177–179], but the structural requirements for its monooxygenase activity remained unclear for a long time. Based on the crystal structure of LaMO from *Mycobacterium smegmatis*, it was recently proposed that a large and compact lid is responsible for the slow release of pyruvate from the reoxidized enzyme, facilitating its reaction with enzyme-bound hydrogen peroxide [180]. In conclusion, the reactions of internal FPMOs have fascinated scientists for already more than 60 years.

7.3 Further Considerations for Biocatalyst Applications

Several aspects need consideration when selecting FPMOs for biocatalytic applications. Aspects that relate to the biophysical and biochemical properties of the FPMOs will shortly be discussed here. For reaction engineering aspects, we recommend a recent review that also addresses the critical issues regarding oxygen supply [181].
Functional expression of the FPMO of interest is a first step toward an attractive biocatalytic system. In case of poor expression or inclusion body formation, a solubility tag can be used to improve the enzyme yield [81]. Saturating the FPMO with flavin cofactor might be required to achieve optimal enzyme activity [182].
Because many FPMOs need an external electron donor such as NAD(P)H, cofactor regeneration is a critical point. Therefore, one should consider if the FPMO-driven reaction might best be performed with whole cells or isolated enzyme [181, 183, 184]. Related to the electron donor supply is the coupling efficiency of the FPMO. Efficient coupling of the two half-reactions, i.e. flavin reduction and substrate oxygenation, is strongly dependent on the rate of substrate oxygenation and the rate of spontaneous decomposition of the flavin oxygenation species. Besides producing potential harmful hydrogen peroxide, this results in the unproductive consumption of expensive NAD(P)H and thus poor atom efficiency. The addition of catalase might be useful to decompose the hydrogen peroxide and improve the overall reaction stoichiometry [181]. Organic solvent tolerance in case of hydrophobic substrates should also be considered, such as in the case of SMO and IMO [185].
Several systems have been developed for NAD(P) coenzyme regeneration [186], with the most common ones being the coupled-enzyme (addition of another NAD(P)-dependent biocatalyst and its substrate) and coupled-substrate (addition of a sacrificial substrate) methods. Because of the quasi-irreversible nature of the reactions catalyzed by FPMO, the coupled-substrate method cannot be applied. Chemical, electrochemical, and photochemical regeneration of NAD(P)H has been demonstrated to be efficient but possible enzyme inhibition, NAD(P)H loss to secondary reactions, and photosensitivity of the FAD/FMN cofactor, have to be taken into account. An elegant way to achieve coenzyme recycling is to include the FPMO in a redox neutral cascade in which a second enzyme regenerates the nicotinamide coenzyme while producing either a precursor or the final product of the cascade [187, 188]. In recent years, synthetic nicotinamide coenzyme biomimetics (NCBs), appealing for their cost efficiency [189–191], have been coupled to various FPMOs to replace NAD(P)H as electron donor. NCBs have been found active with group A hydroxylases [192], group C luciferases [110], group E SMOs [136],

and group F halogenases [160]. Because of their catalytic mechanism and coenzyme recognition motif, group B BVMOs do not accept NCBs as coenzyme [192].

7.4 Conclusions and Outlook

FPMOs have come a long way from their initial discovery to their current use in applied biocatalysis. A wide range of group A–H monooxygenases are available. Current efforts demonstrate an increasing stream of newly discovered FPMOs that can perform novel oxygenation chemistries and FPMOs that are improved for industrial applications.

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