

Flavins on the Move: Flavoprotein Hydroxylases and Epoxidases

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Introduction

Flavoprotein monooxygenases perform chemo-, regio- and/or enantioselective oxygenations of organic substrates under mild reaction conditions [1]. These properties along with effective preparation methods turn flavoprotein monooxygenases in focus of industrial biocatalysis. Here we describe two biocatalytically relevant subclasses of flavoprotein monooxygenases with a close evolutionary relation: class A represented by *p*-hydroxybenzoate hydroxylase (PHBH) and class E formed by styrene monooxygenases (SMOs).

PHBH family members perform highly regioselective hydroxylations on a wide variety of aromatic compounds. A rapid increase in available crystal structures and detailed mechanistic studies of such enzymes [2] are opening a new season of research in the field.

SMOs catalyze a number of stereoselective epoxidation and sulfoxidation reactions [3]. Mechanistic and structural studies expose distinct characteristics, which provide a promising source for future biocatalyst development [4]. Nearly all bacterial SMOs are two-component proteins comprising a reductase and a monooxygenase. Remarkably, in few cases, the reductase is fused to the monooxygenase [5]. Such a self-sufficient enzyme can also cooperate with a single monooxygenase, resulting in a novel type of two-component SMO [6].

Results & Discussion

Flavoprotein monooxygenases can be divided in six different subclasses based on structural features and oxygenation chemistry [1]. Table 1 gives an overview of the crystal structures of single-component flavoprotein aromatic hydroxylases (class A) and two-component styrene monooxygenases (SMO; class E). The aromatic hydroxylases use NAD(P)H for FAD reduction, while SMOs preferentially bind reduced FAD.

Table 1. Crystal structures of one-component flavoprotein hydroxylases and two-component epoxidases

PDB	Enzyme
1PBE	4-hydroxybenzoate 3-hydroxylase (PHBH)
1PNO 1FOH	phenol 2-monooxygenase (PHHY)
2DKH 2DKI	3-hydroxybenzoate 4-hydroxylase (3HB4H)
2QA1 2QA2	UW16 12-hydroxylase (PgaE/CabE)
2R0C 2R0G 2R0P	7-carboxy-K252c hydroxylase (RebC)
2VOU	2,6-dihydropyridine 3-hydroxylase (DHPH)
2RGJ	Phenazine-1-carboxylate hydroxylase (PhzS)
3IHG	Aklavinone 11-hydroxylase (RdmE)
3GMB 3GMC	2-methyl-3-hydropyridine-5-carboxylic acid oxygenase (MHPCO)
3IHM	Styrene monooxygenase (StyA)

Class A enzymes

PHBH catalyzes the conversion of 4-hydroxybenzoates into protocatechuates. PHBH catalysis is strictly controlled by the effector role of the aromatic substrate, resulting in a narrow substrate specificity [2]. Potential substrates such as 4-aminobenzoate and 2-hydroxy-4-aminobenzoate perfectly bind in the active site but are not converted because they fail to stimulate the reduction of the flavin by NAD(P)H [7]. PHBH hydroxylates fluorinated substrates predominantly at C3, but is less regioselective and efficient with chlorinated 4-hydroxybenzoates [8].

Phenol hydroxylase (PHHY) from *Trichosporon cutaneum* catalyzes the conversion of monophenols into catechols. The enzyme accepts hydroxyl-, amino-, halogen- or methyl derivatives of phenol [9]. PHHY shows significant uncoupling of substrate hydroxylation, and the regioselectivity of hydroxylation of fluorinated phenols is pH dependent [10]. Residues involved in the catalytic functions of PHHY are very different from those of PHBH [11].

3-Hydroxybenzoate 4-hydroxylase (3HB4H) from *Comamonas testosteroni* catalyzes the conversion of 3-hydroxybenzoates into protocatechuates. The enzyme is closely related to PHHY and residues in the active sites are well conserved. Attempts to switch the substrate specificity of 3HB4H from 3-hydroxybenzoate to phenol by directed evolution did not show the expected change. 3HB4H has been proposed to contain a hydrophobic tunnel, not present in PHBH or PHHY, for the transport of the substrate to the active site, and a hydrophilic channel for oxygen diffusion [13].

PHBH, PHHY and 3HB4H perform *ortho*-hydroxylation reactions on related substrates. Their active sites are highly tuned for the conversion of a specific substrate. 3-Hydroxybenzoate 6-hydroxylase (3HB6H) catalyzes the *para*-hydroxylation of 3-hydroxybenzoate to 2,5-dihydroxybenzoate. Interestingly, 3HB4H and 3HB6H seem to have evolved mirror-image substrate binding sites for the conversion of the same substrate [14].

PgaE and CabE are flavoprotein polyketide hydroxylases [15] involved in gaudimycin C biosynthesis (Figure 1A), whereas aklavinone 11-hydroxylase (RdmE) is involved in the biosynthesis of rhodomycin (Figure 1B). RdmE stereospecifically recognizes the C9-R isomer. In contrast to other tailoring enzymes, RdmE does not accept substrates glycosylated at C7 [16].

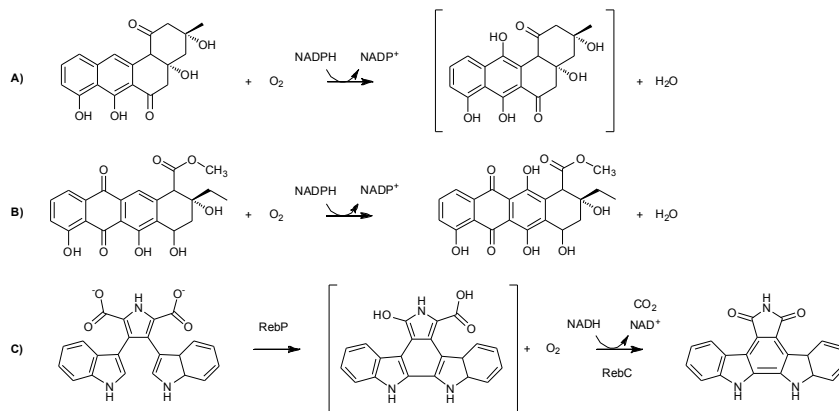


Figure 1. Reactions catalyzed by (A) PgaE/CabE (B) RdmE (C) RebC

RebC works in concert with RebP to produce the rebeccamycin precursor arciriaflavin A (Figure 1C). The RebC structure was solved with the putative substrate trapped in the active site [17]. The regioselectivity of RebC can be switched from rebeccamycin to staurosporine production through exchanging a pair of active-site

residues important in indolocarbazole ring contact [18]. RebC has been crystallized in the substrate free, substrate bound and cofactor reduced form [19]. The free enzyme shows a disordered helix that gets ordered upon substrate binding and reduction of the flavin. The reversible melting of this helix is supposed to guarantee substrate binding, entrapment and product release.

Single-component aromatic hydroxylases use NAD(P)H as electron donor for flavin reduction. Generally, the rate of flavin reduction is controlled by substrate binding. This, and the rapid dissociation of the oxidized pyridine nucleotide, discriminates the class A enzymes from Baeyer-Villiger monooxygenases. With the BVMOs (class B), the coenzyme remains bound throughout the catalysis, an essential requirement for the stabilization of the anionic peroxyflavin [20]. In the class A enzymes, the NAD(P)H reacts with the flavin outside the active site. Cofactor movement is one of the surprising features of flavoprotein aromatic hydroxylases. It was discovered in PHBH [8] and turns out to be a general mechanism. The aromatic substrate is not always necessary for flavin movement. An example is the substrate-free RebC where a loop-helix transition helps FAD to swing in after reduction [19].

Flavoprotein aromatic hydroxylases have evolved several strategies to permit substrate entrance into the active sites. In PHBH, the *open* conformation seems required to let the substrate enter directly from the FAD cleft [21]. In PHHY, phenol may enter from the same side, but no FAD *open* conformation was observed. Instead, cofactor movement in PHHY is coupled to a major conformational change of a helix that acts as a lid, closing the FAD cleft after substrate binding [22]. 3HB4H, PhzS and MHPCO display a tunnel probably needed to guide the substrate from the surface to the active site. Another adopted strategy is a loop-to-helix change observed in RebC and speculated in PhzS. Both hydroxylases act in concert with another enzyme, leaving the possibility that the loop-to-helix transition is part of a substrate channeling process. Substrate recognition, transport and product release are intriguing elements of flavoprotein hydroxylase catalysis, with ingenious diversity displayed among the family members.

Class E enzymes

Styrene monooxygenases (SMOs) convert styrene into styrene oxide. All characterized bacterial SMOs are flavin-dependent two-component proteins comprising a reductase (SMOB) and a monooxygenase (SMOA). Remarkably, in few cases the enzyme occurs as a fusion protein [5]. Bacterial SMOs show a high enantioselectivity and in almost all cases solely the (*S*)-enantiomer of styrene oxide is formed [5, 23-25]. Besides the natural substrate styrene also numerous derivatives are converted in more or less stereoselective manner (Figure 2).

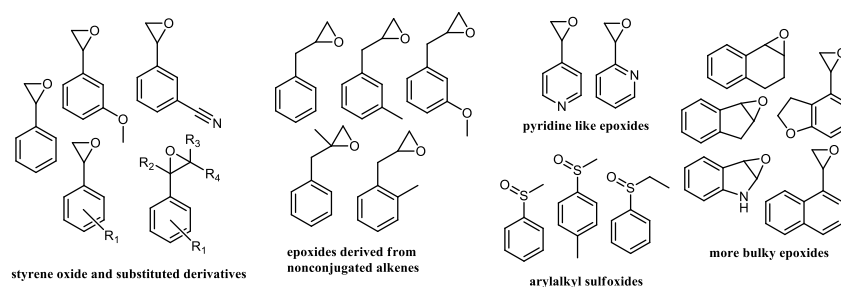


Figure 2. Epoxides and sulfoxides yielded from SMO-based biocatalysis. Yield and enantiopurity depends on the enzyme. R1 = -Cl, -Br, -F, -OH, or -CH₃; R2 and/or R3 = CH₃ or H; R4 = CH₂OH or CH₂Cl.

The 3D-structure of dimeric SMOA from *Pseudomonas* is quite homologous to that of PHBH [3]. Comparison of the apo-SMOA structure with wild-type and mutant structures of PHBH provides insight in identifying amino acid side chains that are thought to define the substrate and flavin-binding pockets of SMOA (Figure 3). The observed differences reflect fundamental differences in the mechanisms of these enzymes. PHBH binds tightly to both oxidized and reduced FAD and interacts directly with NADPH, whereas SMOA is completely reliant on an external source of reduced FAD.

The functional significance of active site amino acid side chains of SMOA has been investigated through evaluation of the relative catalytic activities of site directed mutants of the epoxidase and used to assist in evaluating computational models of SMOA [26]. Subsequent engineering efforts to improve or alter the catalytic activity of SMOA have met with some success. Error-prone mutagenesis proved successful in increasing the epoxidation activity of SMO for both styrene and indole [27]. Site directed mutagenesis guided by the results of docking substrate analogues led to the identification of mutant

enzyme forms with higher substrate specificity for α -methyl and α -ethyl styrenes [28]. These results suggest that there is a bright future for the production of SMO variants with enhanced catalytic activity and alternate substrate specificities.

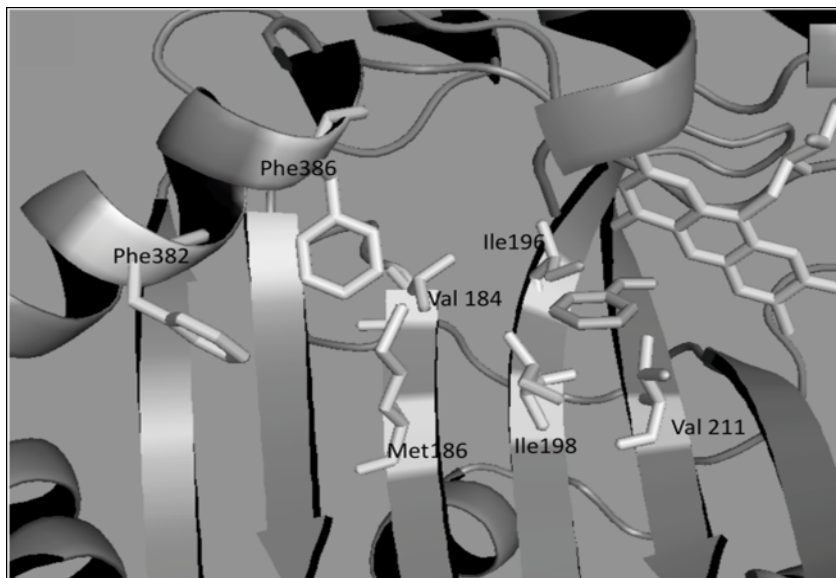


Figure 3. Projection of oxidized FAD and bound substrate locations observed in PHBH onto the active site of SMOA. Hydrophobic side chains lining the proposed styrene-binding pocket of SMOA are shown.

The reduced flavin of SMOA binds 8000-fold more tightly than does oxidized flavin. In turnover, reduced FAD supplied by SMOB, binds firmly as a substrate to apo-SMOA but then reacts catalytically with molecular oxygen as a coenzyme to form tightly-bound flavin-oxygen intermediates associated with the synthesis of styrene oxide. FAD then dissociates from SMOA as a product of the epoxidation reaction (Figure 4). This result demonstrates the versatility of the flavin hydroperoxide and emphasizes the important role of the SMOA active site in carefully orientating and poising styrene and flavin-hydroperoxide to allow the exquisite, enantioselective delivery of oxygen observed in the SMOA-catalyzed epoxidation of styrene [29].

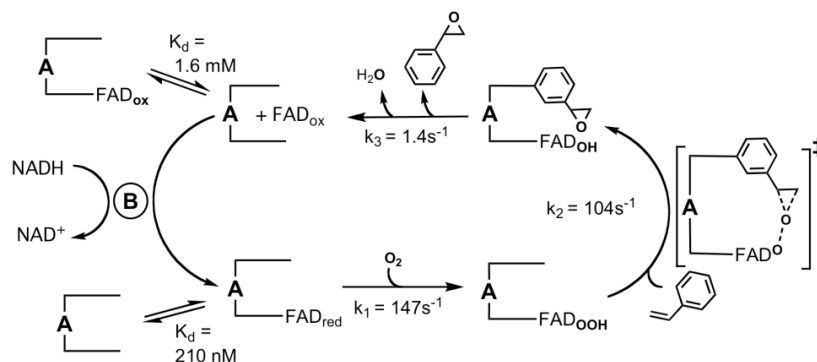


Figure 4. Styrene-epoxidation and FAD-recycling reactions of SMO. The FAD C4a-hydroperoxide intermediate catalyzes the stereoselective addition of oxygen to styrene to yield the styrene oxide and FAD C4a-hydroxyflavin product complex. Subsequent release of styrene oxide and elimination of water yields a weakly interacting SMOA-oxidized flavin complex. Oxidized FAD is recycled in the NADH-dependent flavin reduction reaction of SMOB. Given rate constants are derived from rapid reaction studies at 25°C [29].

The dimeric NADH-specific flavin reductase SMOB serves as the primary supply line for reduced FAD required in the SMOA-catalyzed epoxidation reaction [23]. SMOB is closely related to PheA2, the flavin reductase of the two-component phenol hydroxylase PheA [30,31]. In steady-state turnover, SMOB catalyzes the rapid and indiscriminate reduction of oxidized FAD, FMN, and riboflavin [23]. This apparent lack of specificity is interesting, considering that the flavin-catalyzed epoxidation reaction of SMOA is FAD-specific. When FAD is in excess over the enzyme active site concentration and SMOA and SMOB are present in equimolar concentrations, the rate at which SMOB catalyzes the reduction of the FAD pool greatly exceeds the rate at which SMOA can employ it in styrene epoxidation. This uncoupling of flavin reduction and styrene epoxidation is inefficient and results in the production of reactive oxygen species, mainly in the form of hydrogen peroxide. Rates of NADH consumption by SMOB and styrene oxide synthesis by SMOA can be matched if SMOA is present in excess over SMOB or at equal SMOA and SMOB concentrations if the FAD concentration is less than or equal to the concentration of available active sites. Under the latter conditions FAD species, occurring during catalysis (Figure 4), are predominantly bound to the SMO components [32].

Although efficient coupling of the NADH-consumption and styrene-epoxidation reactions is possible over a range of protein- and flavin-

concentrations, there may be an additional need for SMOA-SMOB complex formation to explain the full range of reaction coupling efficiency [32]. This possibility that a protein-protein complex may form during catalysis is an area of current research.

Recently, we characterized the first representative of a new type of SMO in which the reductase component is fused to the monooxygenase component [5]. The fusion protein StyA2B from *Rhodococcus opacus* 1CP is the first self-sufficient styrene monooxygenase reported. A low epoxidizing activity of the fused monooxygenase as well as the occurrence of a second single monooxygenase gene (*styA1*) encoded directly upstream to *styA2B* raised questions on a functional dependence of both proteins. Indeed, it could be demonstrated that StyA1 and StyA2B together are a more efficient SMO [6]. StyA1 accepts reduced FAD also from other reductases yielding a similar monooxygenase activity, demonstrating that this component is the main epoxidase in this SMO-type and that StyA2B is mainly acting as a FAD reductase. The reductase subunit of StyA2B is sufficient to deliver reducing equivalents to both monooxygenases (A1 and A2). Furthermore, a kind of protein cross-talk or transient complex formation between both proteins during which reduced FAD is channeled from reductase to monooxygenases is supposed since highest epoxidation activity and efficiency is observed at an equimolar ratio of both components [6]. Based on genome mining only few representatives of this fusion-type SMO were identified yet. Most of them are found among the Gram-positive Actinobacteria [6]. Considering the gene/protein organization and subunit characteristics, two groups of subclass E flavoprotein monooxygenases can be designated: E1 comprising StyA/StyB typically from *Pseudomonas*-species and E2 represented by StyA1/StyA2B from *Rhodococcus opacus* 1CP.

Conclusions

The structural and mechanistic studies on flavoprotein aromatic hydroxylases have revealed that these enzymes use a number of strategies to perform efficient catalysis. Tightly controlled FAD reduction avoids the unproductive consumption of NAD(P)H, and active sites are optimized for the regioselective incorporation of a hydroxyl group.

So far, relatively few hydroxylases have been described that consist of a reductase and a monooxygenase component [33-36]. Nevertheless,

these enzymes are of main interest for synthetic applications because they might adapt to new substrates more quickly than the hydroxylases that must coordinate the reductase and oxygenase functions in a single polypeptide chain [36]. It is anticipated that many more two-component monooxygenase systems will be uncovered from the metagenome, either in free form or fused as in StyA2B.

The nature and possible advantages of the self-sufficient one-component SMO (StyA2B) needs further investigation. Elucidation of the structure and mechanism of this protein might help to design highly active and selective one-component SMOs for biotechnological purposes.

The progress in chemical, electrochemical, and light-driven cofactor regeneration show the general applicability for flavoprotein monooxygenases in biocatalysis [37]. The broad substrate range converted by these enzymes provides access to many valuable building blocks for chemical syntheses. The recently published structures and insights in mechanisms allow further approaches in protein engineering in order to alter active sites for higher activities or acceptance of alternative substrates.

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