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# New frontiers in flavin-dependent monooxygenases

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ABSTRACT

Flavin-dependent monooxygenases catalyze a wide variety of redox reactions in important biological processes and are responsible for the synthesis of highly complex natural products. Although much has been learned about FMO chemistry in the last ~80 years of research, several aspects of the reactions catalyzed by these enzymes remain unknown. In this review, we summarize recent advancements in the flavin-dependent monooxygenase field including aspects of flavin dynamics, formation and stabilization of reactive species, and the hydroxylation mechanism. Novel catalysis of flavin-dependent *N*-oxidases involving consecutive oxidations of amines to generate oximes or nitrones is presented and the biological relevance of the products is discussed. In addition, the activity of some FMOs have been shown to be essential for the virulence of several human pathogens. We also discuss the biomedical relevance of FMOs in antibiotic resistance and the efforts to identify inhibitors against some members of this important and growing family enzymes.

## 1. Introduction

Flavin-dependent monooxygenases (FMOs)[1] use oxygen and an external reductant, such as NADPH, to oxygenate a large number of compounds. The reaction involves the insertion of one oxygen atom into an organic substrate, while the other oxygen atom is reduced to water [1,2].

FMOs catalyze a wide variety of redox reactions in important biological processes, such as in the transformation of xenobiotic compounds, in the detoxification of drugs, in the biosynthesis of antibiotics and hormones, and in the hydroxylation of amino acids [3,4]. In addition, the activity of FMOs has been exploited for the production of intermediate compounds for industrial processes. For example, FMOs are known to perform epoxidation of substituted alkenes, which is essential in the formation of enantiopure organic compounds that are widely used in the pharmaceutical industry [5]. In addition, FMOs are responsible for the synthesis of highly complex natural products, some of which are potential new drugs for the treatment of cancers and bacterial and fungal infections [4,6,7]. This has led to an increase interest in the biological pathways responsible for the production of these compounds [8]. Biosynthetic gene clusters and characterization of enzymes in these pathways have revealed novel enzymatic functions, many of which are carried out by FMOs [8]. For instance, novel FMOs that perform double hydroxylation of aspartic acids in the biosynthesis of the antibiotic fosfazinomycin have been identified [9]. Similarly, the formation of oxime functional groups in the biosynthesis of caerulomycin A is also catalyzed by an FMO [10].

The catalytic cycle of FMOs can be simplified into two half-reactions: the reductive and oxidative half reactions (Fig. 1). These enzymes require a hydride equivalent from NAD(P)H to reduce the flavin and activate molecular oxygen. Oxygen activation involves a single electron transfer step that results in the formation of a flavin-semiquinone and a

*Abbreviations:* FMO, Flavin-dependent monooxygenase; Fl, flavin; FAD, flavin adenine dinucleotide; $Fl_{4a}$-OOH, C4a-hydroperoxiflavin; NMOs, *N*-monooxygenases; BVMOs, Baeyer-Villiger monooxygenases; NADPH, reduced nicotinamide adenine dinucleotide phosphate; $NADP^+$, oxidized nicotinamide adenine dinucleotide phosphate; PHBH, *p*-hydroxybenzoate hydroxylase; p-OHB, 4-hydroxybenzoate; APBS, Adaptive Poisson-Boltzmann Solver; PDB, Protein Data Bank; SKIEs, solvent kinetic isotope effects; QM/MM, quantum mechanics/molecular mechanics; DFT, density functional theory; HRC-ET hydroxyl, radical-coupled electron transfer mechanism; SidA, siderophore A; Ktzl, *Kutzneria* sp. 744; PvdA, pyoverdine A from *Pseudomonas aeruginosa*; FzmM, fosfazinomycin M; CreE, caerulomycin E; CrmH, caerulomycin H; 2AEPn, 2-aminoethylphosphonate; TetX, tetracycline destructase; RIFMO, rifampicin monooxygenase; RIF, rifampicin; KMO, kynurenine 3-monooxygenase; HTS, high throughput screening; SQLE, squalene epoxidase; SQS, squalene synthase; EpnF, eponemycin.

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[1] We note that many publications referred to flavin-containing monooxygenases as FMOs. In this review we used this acronym to refer to the family of flavin-dependent monooxygenases.

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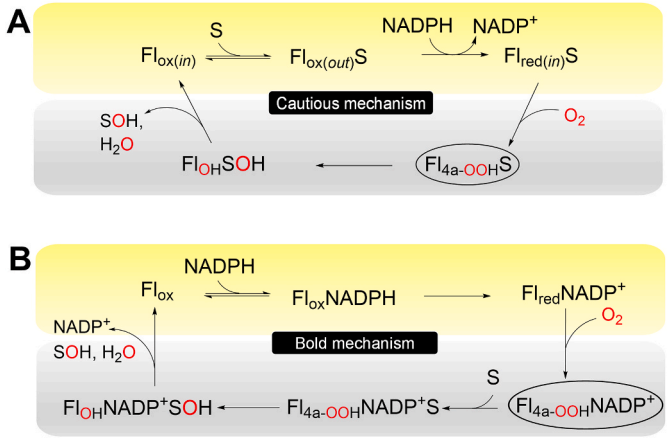


**Fig. 1.** Cautious and bold mechanisms of flavin monooxygenases. A) The cautious mechanism follows a ping-pong mechanism as the reaction with oxygen occurs in the presence of the substrate and the absence of $NADP^+$ (circled). B) The bold mechanism follows a sequential mechanism of substrate binding and the formation of $F_{4a}$-OOH (circled) occurs in the absence of substrate. Reductive-half reaction is shown in yellow and the oxidative-half reaction in gray. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

superoxide radical pair that combine to form C(4a)-hydroperoxyflavin ($Fl_{4a}$-OOH), which is responsible for substrate oxidation [11]. FMOs are unique in their ability to form and stabilize $Fl_{4a}$-OOH [12]. $Fl_{4a}$-OOH is tuned by the active site environment to catalyze the hydroxylation, epoxidation, or Baeyer–Villiger oxidation of a wide range of substrates [12].

FMOs are classified into eight distinct subclasses based on amino acid sequence and structural and biochemical properties. The eight subclasses (A-H) are divided into two groups based on mechanistic properties. Subclasses A, B, G, and H undergo reduction and oxidation by an electron donor within the same polypeptide chain and are known as single component monooxygenases. Subclasses C-F obtain the reduced flavin from a flavin oxidase enzyme. These are known as two-component monooxygenases [3,4].

This review aims to explore new frontiers in FMO catalysis and applications, with a focus on the biochemical, computational, and structural data of FMOs that hydroxylate N-atoms, also known as N-monooxygenases (NMOs). New insights into the mechanism of FMOs are described, including aspects of flavin conformational changes, formation and stabilization of $Fl_{4a}$-OOH, and the mechanism of hydroxylation. Furthermore, we also discuss recently characterized reactions catalyzed by novel NMOs. Additionally, relevant bio-medicinal applications of FMOs, such as drug resistance and FMOs as targets in drug discovery, are examined.

## 2. New insights into the mechanism of action of FMOs

FMOs catalyze a complex reaction that involves not only coordination of the flavin and several substrates but also reduction and oxidation of cofactors and substrates within the same active site. Although much has been learned in the last ~80 years of research on FMO chemistry, several aspects of the reactions catalyzed by these enzymes remain unknown [1,4]. Specific flavin motion and conformational changes have been outlined in the past but have only been attributed to one specific group of FMOs. A combination of experimental and computational methods has been used to investigate the molecular details related to flavin dynamics [13,14], formation and stabilization of reactive species [15–17], and hydroxylation mechanism of FMOs [18]. Here, we describe our current understanding of recent data that provides new insights into the mechanism of action of FMOs with a specific focus on recent discoveries from NMOs.

### 2.1. Flavin dynamics in FMOs

Flavins are well known for their remarkable chemical versatility. These cofactors are responsible for a variety of biological reactions that are derived from the chemical complexity of their catalytic center, the isoalloxazine ring system. The reactivity of the isoalloxazine ring is seen across an assortment of reactions catalyzed by flavoenzymes, including hydroxylation, hydration, desaturation, photochemistry, electron transfer, and oxygen activation [19–21]. It is well established that the protein matrix plays a major role in modulating the catalytic potential of the flavin cofactor, since free flavins are not capable of performing many of these reactions [22,23]. Understanding and predicting how flavin cofactor reactivity is modulated by the protein environment is still an active area of investigation [22].

Flavoenzymes have also been extensively studied to elucidate the mechanism of their conformational flexibility. It has been reported that conformational changes in the flavin cofactor itself are crucial to multiple steps in the catalytic cycle and these motions are accompanied by changes in protein conformation [24,25].

In FMOs, conformational changes of the flavin were first described in *p*-hydroxybenzoate hydroxylase (PHBH), followed by other related enzymes [24,26,27]. PHBH typifies the class A monooxygenases and has been extensively investigated by kinetic, spectroscopic, and crystallographic studies [25,28,29]. The mechanism of action of class A monooxygenases with oxygen has been described as the "cautious mechanism", which is characterized by the release of $NADP^+$ before the addition of oxygen (e.g., ping-pong kinetic mechanism) (Fig. 1A). The substrate to be hydroxylated (substrate henceforth) binds first and then the complex reacts with NAD(P)H. Substrate binding enhances the reaction with NAD(P)H by up to ~ $10^5$-fold [29]. The cautious mechanism lowers the risk of uncoupling (e.g., release of hydrogen peroxide without substrate hydroxylation), since the reaction of reduced flavin with oxygen to form the $Fl_{4a}$-OOH occurs only when the substrate is present and poised for hydroxylation [2,11].

A key feature of the caution mechanism is that the motion of the flavin cofactor has been shown to be essential. The structural basis for the mobility of the flavin isoalloxazine ring was identified in PHBH based on structural analysis of complexes with 4-hydroxybenzoate (*p*-OHB) [30] (Fig. 2) and the analog 2,4-dihydroxybenzoate [24,26]. The structure of the PHBH-*p*-OHB complex has both *p*-OHB and the isoalloxazine ring in the active site (the "in" conformation) (Fig. 2). In the structure of PHBH with the 2,4-dihydroxybenzoate, the isoalloxazine ring occupies a position that exposes the re-face to the solvent (the "out" conformation) (Fig. 2). Structural and mechanistic studies have shown that binding and deprotonation of *p*-OHB and binding of NADPH triggers flavin motion from the protein interior ("in" conformation) to an exposed position in contact with the solvent ("out" conformation) where reduction takes place [25]. The active site is electropositive and induces the electron rich reduced flavin to the "in" conformation (Fig. 2). Thus, the movement from the "out" to the "in" conformation is coupled to flavin reduction.

The conformational adaptability of the flavin cofactor in the catalytic cycle provides suitable environments for the different steps of the reaction, effectively separating the reduction and oxidative half-reactions catalyzed by PHBH. The net conformational change of the flavin movement in PHBH, and other related enzymes, with the C(2) ribityl chain functioning as the pivot point, is a "waving" movement of 7–8 Å (Fig. 3C) [27]. In terms of the protein environment, it is also suggested that the movement of Tyr222 (numbering in PHBH from *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*) away from the substrate aids in the displacement of the flavin [15]. This is supported by spectral and kinetic data collected on mutant enzymes PHBH Y222A and Y222V [31].

Class B monooxygenases were thought to utilize a different mechanism to prevent release of hydrogen peroxide. Members of this group are

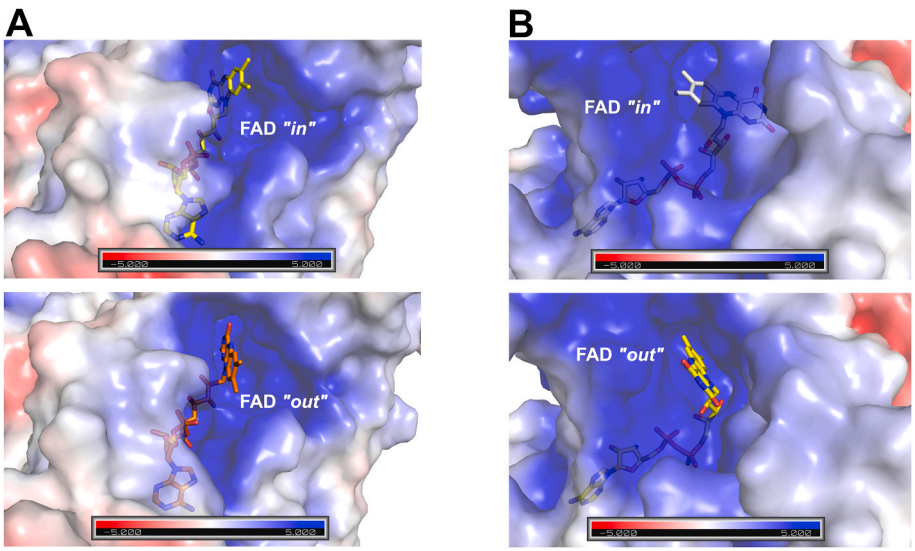


**Fig. 2.** Flavin mobility in class A and class B FMOs. (A) Surface electrostatic potential representation of *p*-hydroxybenzoate hydroxylase (PHBH) in complex with 4-hydroxybenzoate showing the "*in*" FAD (yellow) and 2,4-dihydroxybenzoate showing the "*out*" FAD (orange). 4-hydroxybenzoate and 2,4-dihydroxybenzoate were omitted for clarity. (B) Surface electrostatic potential representation of SidA. Reduced FAD is shown in the "*in*" (gray) and oxidized FAD is shown in the "*out*" (yellow) conformations in the active site. The color scale goes from red to blue, corresponding respectively to −5 and +5 $k_BT/e$. The surface electrostatic potential was calculated using the APBS plugin [32] for PyMOL to solve the linearized Poisson-Boltzmann equation at an ionic strength of 150 mM (NaCl), pH 7.0, a solvent radius of 1.4 Å, and its relative permittivity equal to 78. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

able to rapidly react with NAD(P)H. The reduced flavin then reacts with oxygen and the $Fl_{4a}$-OOH is formed in the absence of substrate. In this case, the $Fl_{4a}$-OOH is stabilized and ready for catalysis as it waits for the substrate to enter the active site. The half-life of $Fl_{4a}$-OOH has been calculated to be several minutes to hours [33]. Because these enzymes form the intermediate regardless of the substrate's presence, the mechanism has been referred to as the "bold mechanism" (Fig. 1B). It has been shown that the presence of $NADP^+$ is essential for the stabilization of $Fl_{4a}$-OOH. In fact, $NADP^+$ is the last product to be released.

The first Baeyer-Villiger monooxygenase (BVMO) structure, which is a class B FMO, was solved in 2004 [34] and the first structure of a flavin-containing monooxygenase[1] was solved in 2006 [35]. These seminal works established the structural domains of this family of enzymes and shed light onto the complexity of the mechanism of action. The structures did not have substrates bound and only the structure of the flavin-containing monooxygenase was solved in complex with $NADP^+$. $NADP^+$ was bound in an extended conformation and the nicotinamide ring was adjacent to the isoalloxazine ring; however, the $NADP^+$-C4-atom was ~5 Å away from the Fl-N5-atom and was not properly aligned for hydride transfer [36] (Fig. 4). Furthermore, the conformation of the nicotinamide ring was consistent with a hydride transfer from the pro*S* position, which is in contrast with extensive biochemical data that suggests that this occurs from the pro*R*-position (Fig. 4) [37–39]. The observed binding of $NADP^+$ led to different interpretations of its role in catalysis. One group suggested that hydride transfer occurred via the C2-position of the nicotinamide [35]. However, this possibility is not consistent with the mechanism of hydride transfer of NAD(P)H and kinetic isotopic data that shows that a hydride equivalent is transferred to the flavin from the C4-potion [38]. It was also suggested that the observed binding of $NADP^+$ was one that occurred after hydride transfer and might represent the conformation involved in the stabilization of the $Fl_{4a}$-OOH [40].

Several more structures of class B family members became available that support the so called "moonlighting" role of NAD(P)H in catalysis. Particularly informative were the structures of BVMO from *Thermobifida fusca* in different redox states [41] and the NMO ornithine hydroxylase, SidA, from *Aspergillus fumigatus* in complex with $NADP^+$ and ornithine in the oxidized (Fig. 3B), reduced, and re-oxidized states [36]. It was concluded that after flavin reduction, one of the key functions of $NADP^+$ was to interact with the Fl-N5-atom. When the flavin is in the oxidized form, the amine group of the nicotinamide functions as a hydrogen bond donor to the Fl-N5-atom. Upon reduction, the amide group flips ~180°, allowing the carbonyl oxygen to function as a hydrogen bond acceptor and the flavin-N5H as the hydrogen bond donor (Fig. 4C). Further structural, mechanistic, and biochemical studies were analyzed in terms of protein conformational changes that modulated binding of the nicotinamide ring in what has been referred to as the "cofactor sliding mechanism" [33,42–44].

Recently, a new crystal form of SidA revealed flavin conformational changes [14] (Fig.3D) that were not observed in previous SidA structures [36]. Along with SidA, flavin mobility was also reported in KtzI, an ornithine hydroxylase from the soil actinomycete Kutzneria sp. 744 [13,45]. SidA and Ktzl share high levels of sequence homology, with 34% identity.

The flavin conformational changes in class B NMOs are different from what was previously described in class A monooxygenases (Fig. 3). While the flavin motion in class A monooxygenases occurs nearly within the plane of the isoalloxazine ring, in these ornithine hydroxylase NMOs, the flavin motion involves rotations around two dihedral angles that result in a more pronounced movement of the isoalloxazine moiety with the ribityl tail not being disturbed [14] (Fig. 3).

Flavin mobility in NMOs is best understood as adapting either an "in" or an "out" conformation in which the movement of FAD is thought to aid in the release of $NADP^+$ from the enzyme active site after turnover in preparation for a new catalytic cycle [13]. In the "out" position, the isoalloxazine system moves away from the active site and becomes exposed to the solvent where the *si*-face of the oxidized FAD can be seen stacking with a conserved tyrosine (Tyr341 in SidA and Tyr 276 in KtzI). These Tyr are located in what has been called the Tyr-loop, which contains other residues that also undergo conformational changes (Fig. 5). After FAD reduction, the flavin cofactor moves back into the active site ("in" position) with the Tyr-loop rearranged by NAD(P)H binding.

Although the molecular nature of the flavin motion, as well as the Tyr loop, is similar in SidA and KtzI, the factors determining flavin dynamics differ. It is suggested that for SidA, both $NADP^+$ binding and flavin reduction drives FAD into the "in" conformation. The main difference between SidA and KtzI is found in the oxidized structures of both enzymes in the presence of $NADP^+$. While the KtzI-$NADP^+$ complex contains the oxidized FAD in the "out" position, the SidA-$NADP^+$ complex contains the oxidized FAD in the "in" position. However, in both structures, the Tyr-loop avoids steric clashes with $NADP^+$ by shifting away from the active site.

A new study on the kinetic and structural characterization of the SidA variant M101A provides another piece of evidence that supports the role of active site dynamics in controlling $NADP^+$ release in class B monooxygenases. Met101 was previously shown to change conformation upon reduction and contact the pyrimidine ring of FAD in the "in"

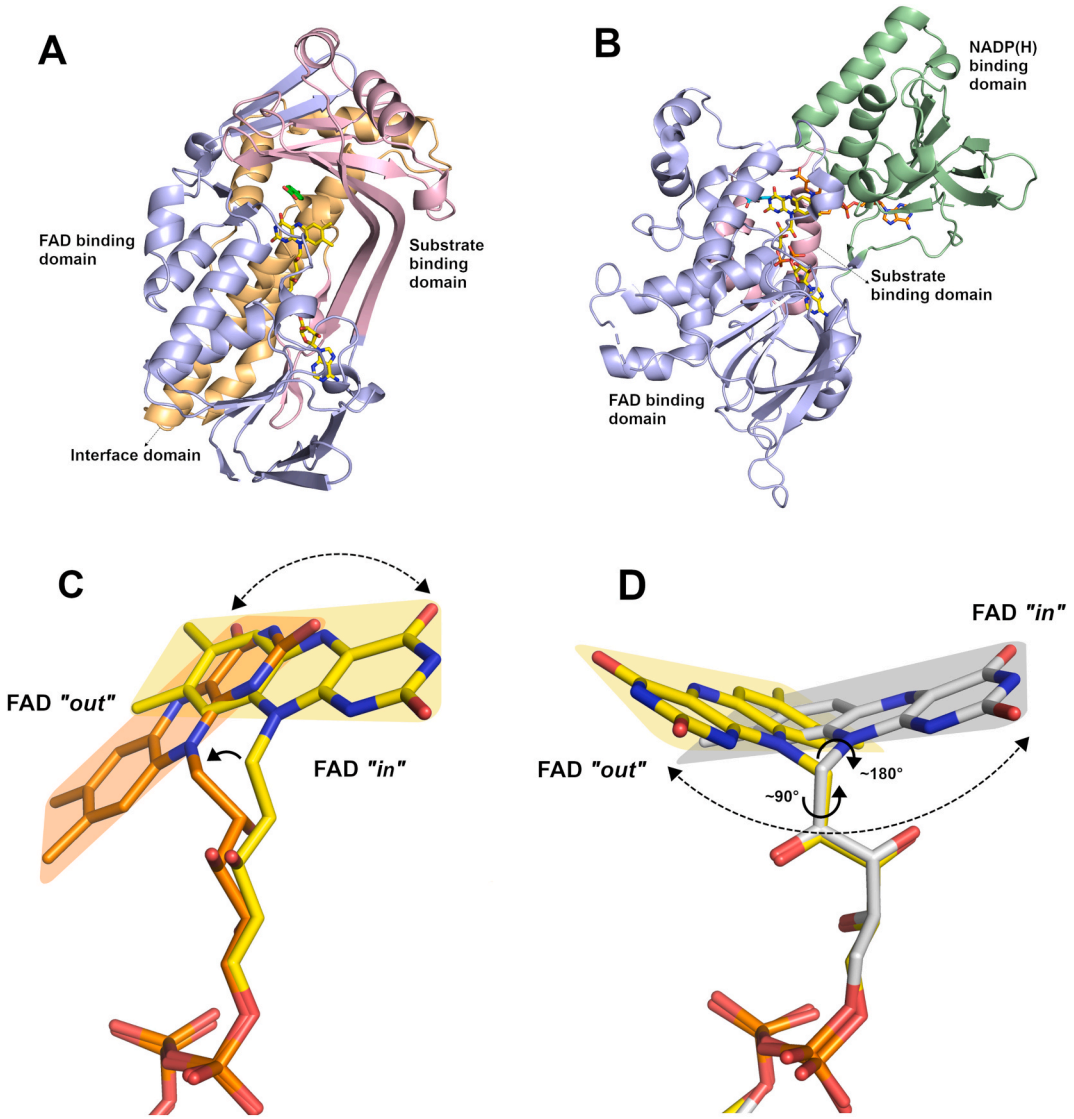


**Fig. 3.** Structures of PHBH and SidA. (A) Cartoon representation of the overall structure of class A FMO PHBH monomer (PDB ID: 1PBE) colored by domains (FAD binding domain in light blue, substrate binding domain in light pink and interface domain in orange). Stick representation of the FAD and 4-hydroxybenzoate, carbon atoms are colored in yellow and green, respectively. (B) Cartoon representation of the overall structure of class B FMO SidA monomer (PDB ID: 4B63) colored by domains (FAD binding domain in light blue, substrate binding domain in light pink and NADP(H) domain in green). Stick representation of the FAD, NADPH and ornithine, carbon atoms are colored in yellow, orange, and cyan, respectively. (C) Flavin mobility in class A FMOs. Overlaying the FAD "*in*" (yellow) (PDB ID: 1PBE) and "*out*" (orange) (PDB ID: 1DOD) states for class A monooxygenases showing the motion of the flavin along the isoalloxazine plane, resembling a "waving" motion. (D) Flavin mobility in class B FMOs. The "*in*" (gray) (PDB ID: 6X0J) and "*out*" (yellow) (PDB ID: 6X0H) conformation in class B NMOs occurs via a rotation around two dihedral angles, ~90° around the C1′– C2' bond and ~180° around the N10 – C1' bond. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

position [36](Fig. 5C). Steady-state and pre-steady-state kinetic experiments performed using the SidA M101A variant show that the mutation slows down events occurring after the flavin reduction step. Furthermore, the crystal structure of M101A in complex with $NADP^+$ displays the FAD in the "out" position, in contrast to what is observed for the wild-type SidA. It is proposed that M101 is involved in regulation of flavin motion and mutation to Ala reduced the flavin motion and makes $NADP^+$ release the rate limiting step [46].

The exact mechanism of how mobile flavins play a role in the mechanisms of NMO catalyzed reactions remains unclear. However, it is becoming evident that flavin motion is not only present in class A monooxygenases. In addition, new information from various enzyme complexes and conformational changes could be exploited in NMOs since these enzymes are potential drug targets. Applications of NMOs as potential drug targets will be discussed in Section 2.3.

### 2.2. Formation and stabilization of the $Fl_{4a}$-OOH

The activation of molecular oxygen has been extensively studied and significant progress has been made over the years [47]. In this section, important advancements in our understanding of $Fl_{4a}$-OOH formation and stabilization in FMOs will be discussed. The activation of oxygen involves a single electron transfer from the reduced flavin forming a flavin semiquinone and superoxide anion radical pair that recombines to constitute a $Fl_{4a}$-$OO^-$, which protonates forming $Fl_{4a}$-OOH [12,47].

For BVMO reactions, $Fl_{4a}$-$OO^-$ is required for catalysis and it is believed that the presence of a conserved arginine residue in the active site provides an environment that stabilizes the unprotonated form of the intermediate [48]. FMOs that catalyze hydroxylation reactions require $Fl_{4a}$-OOH and the mechanism of protonation depends on the enzyme class. The active site of NMO and flavin-containing

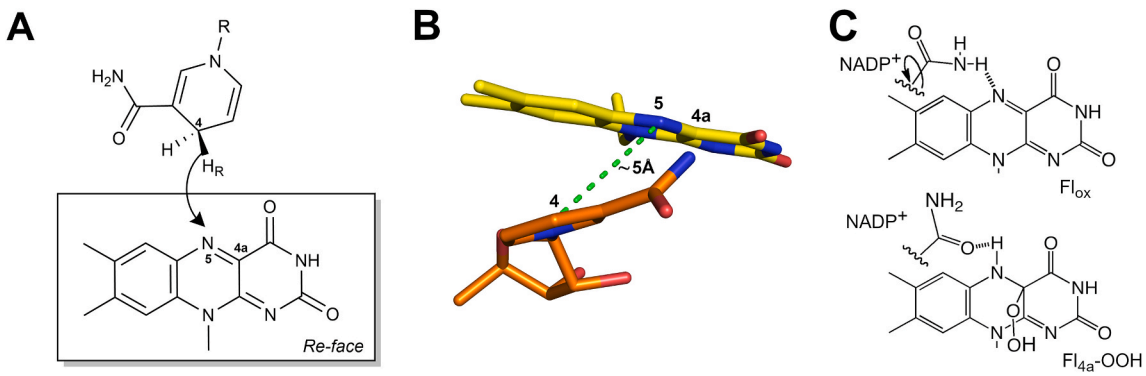


**Fig. 4.** Representation of the binding conformation of $NADP^+$ in class B monooxygenases. A) Hydride transfer of the pro*R*-position of the NADPH to the flavin-N5 atom supported by extensive biochemical studies. B) $NADP^+$ binding in class B monooxygenases is not optimal for hydride transfer. The coordinates for SidA (PDB ID: 4B63) were used in this figure. C) Predicted hydrogen bonding patterns for the complex with $NADP^+$ and $Fl_{ox}$ or $Fl_{4a}$-OOH.

monooxygenases lack residues adjacent to the flavin-C4a-atom that can facilitate protonation of the $Fl_{4a}$-$OO^-$. However, the 2′-OH group of the $NADP^+$ ribose is ~5 Å from the C4a-position. Solvent kinetic isotope effects (SKIEs) and density functional theory (DFT) have shown that the 2′-OH is involved in rapid proton transfer (Fig. 6) [15]. Structural studies showed that there is a water mediated hydrogen bond network that links the 2′-OH with bulk solvent, facilitating this process. Rapid reaction kinetics and pH studies have shown that in the two component monooxygenase *p*-hydroxyphenylacetate-3-hydroxylase (HPA) (class D), protonation of the $Fl_{4a}$-$OO^-$ is catalyzed by a His residue (His396 in HPA from *Acinetobacter baumannii*) that is ~4.6 Å from the flavin-C4a position [16]. SKIEs suggest that proton transfer is not rate limiting in the formation of the $Fl_{4a}$-OOH. DFT studies on pyranose oxidase, which also stabilizes the $Fl_{4a}$-OOH, suggest that protonation of the superoxide anion before covalent attachment to the flavin C4a-positon might occur to neutralize the negative charge [17]. It might also be possible that protonation of superoxide anions by $NADP^+$-2′-OH occurs before formation of $Fl_{4a}$-OOH. Regardless of the timing of the protonation step, members of class B FMOs take advantage of the presence of $NADP^+$ in the active site to form $Fl_{4a}$-OOH, while class D FMOs recruit active site residues to act as proton donors. Similar studies have not been performed with the class A monooxygenases, but it is reasonable to assume that protonation will occur from conserved active site residues.

After formation, $Fl_{4a}$-OOH stabilization varies depending on the enzyme class. Class B monooxygenases rely on interactions with $NADP^+$ to provide the high $Fl_{4a}$-OOH stability observed in these enzymes. As briefly mentioned in the previous sections, hydrogen bonding between the nicotinamide carbonyl oxygen and the Fl-N5-H atom is essential to prevent the expulsion of hydrogen peroxide from the $Fl_{4a}$-OOH. The important stabilizing role of this interaction is supported by computational analyses and was probed using $NADP^+$ analogs [18,33,41,49]. Additional stabilization is obtained from hydrogen bonding with the O-atom at the 2′-ribose (Fig. 6). Two-component monooxygenases utilize a similar approach, where hydrogen bonding with the Fl-N5-H and the $Fl_{4a}$-OOH distal oxygen occur; however, this happens with conserved active site residues (Fig. 6) [50].

### 2.3. Hydroxylation mechanism

A combination of experimental and computational methods has been used to investigate the hydroxylation mechanism and substrate selectively in FMOs at the molecular level. $Fl_{4a}$-OOH is generally considered an "$OH^+$" donor, functioning as an electrophile to be attacked by a nucleophile (Fig. 7). Experimental data was analyzed in favor of the nucleophilic mechanism (Fig. 7) from studies of C8-modified FAD with PHBH. It was shown that electron withdrawing groups accelerated the rate constant for hydroxylation and it was concluded that electrophilic aromatic substitution best explained the data [52]. Computational studies have been performed in support of this mechanism and, in general, the nucleophilic mechanism has been accepted as the mechanism of action of FMOs [11,53,54].

The availability of more structures, including complexes with substrates, as well as advances in computational biomolecule modeling allowed further evaluation of the hydroxylation mechanism of FMOs. QM/MM (quantum mechanics/molecular mechanics) and DFT studies suggested a homolytic cleavage of the O-OH bond and transfer of a hydroxyl radical as an alternative mechanism for PHBH [55]. The calculations highlighted the stabilization of the reacting OH group of the $Fl_{4a}$-OOH intermediate by Pro293. In one study, the backbone of Pro293 (numbering in PHBH from *Pseudomonas fluorescens*) was proposed to interact with the reacting OH through hydrogen bonding, which lowered the energy of the transition state [56]. This is in agreement with the observation that the PHBH P293S mutant had a lower rate of hydroxylation [57]. Another study pinpointed this interaction with the Pro293 amide oxygen, although a nucleophilic mechanism was applied in this calculation [53].

Biochemical evidence in support of a homolytic bond cleavage has been available since the late 80's from reports of the detection of hydroxyl radicals generated by pulse radiolysis [58,59]. Further support of this hydroxyl radical mechanism in NMOs has come from DFT studies with SidA. SidA is one of the few FMOs for which structures with several ligands are available. In addition to the structures with $NADP^+$ and ornithine, the structure with lysine is also available. Lysine is only one carbon longer than ornithine; however, with this amino acid, turnover results in ~90% $H_2O_2$ release and only ~10% hydroxylated-lysine. In contrast, in the presence of ornithine, the hydroxylation is ~95% efficient. DFT studies with SidA in complex with ornithine or lysine were consistent with homolytic cleavage of the $Fl_{4a}$-OOH and hydroxylation by hydroxyl radical transfer. The studies also suggested that ornithine binds with the N5-atom in the deprotonated form, which was later supported by pH studies [60]. The hydroxyl radical mechanism provides a logical explanation for the substrate selectivity of SidA. It shows that due to the extended binding position of lysine in the active site, the hydrogen bonding interactions in the transition state are not optimized for lysine hydroxylation (Fig. 8). This results in an increase in the activation energy of 8 kcal/mol (Fig. 8) [18]. The fact that substrate selectivity in SidA can be explained by transition state stabilization of the hydroxyl radical provides further support for this mechanism.

A more recent study also used DFT simulations to investigate the mechanism of substrate hydroxylation in the fungal enzyme TropB, a class A flavin-dependent hydroxylase [61]. It was established that in the reaction catalyzed by TropB, a hydroxyl radical-coupled electron transfer mechanism (HRC-ET) is favored by ~ 7 kcal/mol compared to the electrophilic aromatic substitution. TropB, as well as PHBH, is involved in the hydroxylation of aromatic compounds and these enzymes have similar structures. These computational studies suggest that

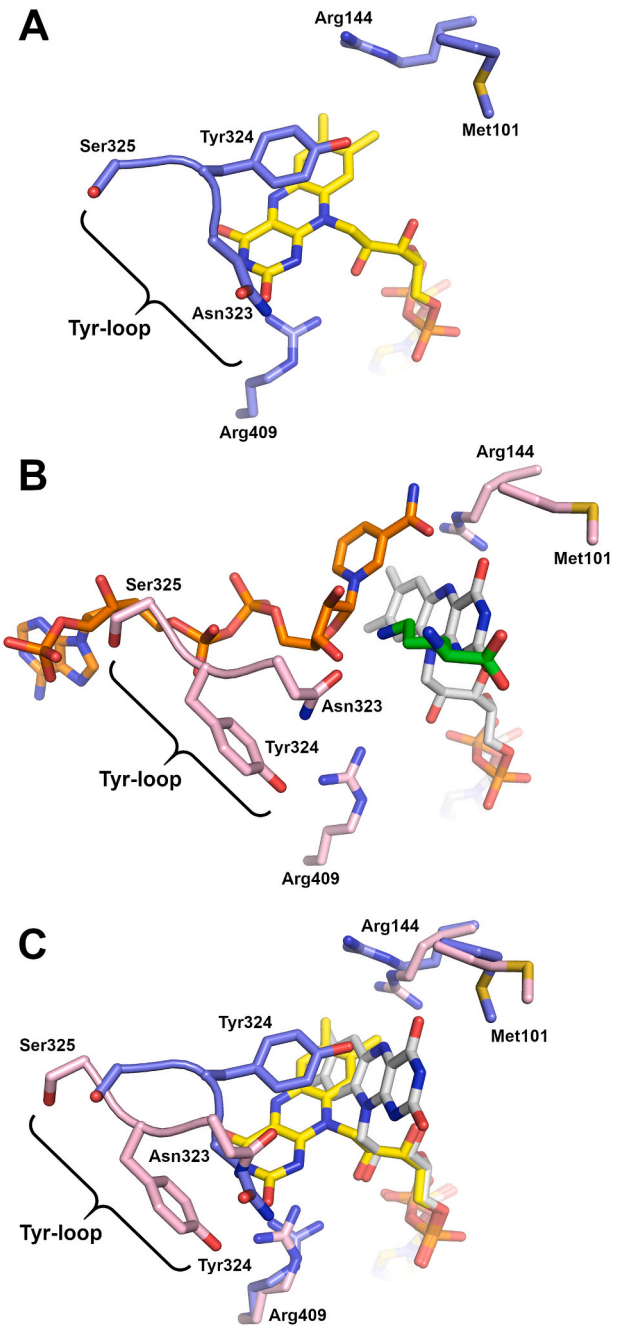


**Fig. 5.** Conformational changes in the SidA active site. (A) Ligand free form (PDB ID 6X0H) with the oxidized flavin stabilized in the "out" conformation (yellow) by Tyr324. Side chains around the flavin are shown in light blue. (B) NADPH-reduced SidA (PDB ID 6X0J) in complex with $NADP^+$ (orange) and ornithine (green). The reduced "in" FAD is colored in gray and the side chains around the flavin are shown in light pink. (C) The overlay of panes A and B, NADPH, and L-Ornithine were omitted for clarity. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the mechanism of hydroxylation by FMOs requires re-evaluation. Computational data is now available that support a hydroxyl radical mechanism for class A and B monooxygenases and might represent a general mechanism of action for FMOs.

## 3. Novel catalysis and applications of FMOs

Bacterial genome sequencing projects and metagenomic studies have greatly accelerated the identification of biosynthetic gene clusters of natural products with significant health benefits, such as anti-cancer, antibiotic, and anti-inflammatory activities, among others [62–64]. Some of these natural products have unique functional groups that are important for the structure or chemical properties of these compounds and many novel FMOs are being identified in these biological pathways.

As our knowledge of FMO chemistry and catalysis expands, new applications for FMOs become possible. FMO activity is applied in nature to initiate the catabolism of xenobiotics [65]. This activity has also been recruited by bacteria for the degradation or inactivation of clinically used antibiotics. FMO activity has also been shown to be essential for the virulence of several human pathogens, which has led to several drug discovery programs targeting FMOs. Here, we focus on selected cases of novel FMOs involved in natural product biosynthesis and FMOs identified in antibiotic resistant mechanisms and as potential drug targets.

### 3.1. Double hydroxylation reactions by novel NMOs

SidA and PvdA have been intensively studied due to their essential function in siderophore biosynthesis. These NMOs catalyze the single hydroxylation of amine-containing substrates, such as lysine and ornithine, to hydroxylamine products. However, several recently identified NMOs are capable of catalyzing consecutive oxidations of amines to generate oximes or nitrones (Fig. 9). CrmH from *Streptomyces caeruleus* was the first NMO found to generate oximes [10]. The catalytic mechanism of CrmH is believed to involve the double hydroxylation of the substrate, caerulomycin N, to form dihydroxylamine, which then dehydrates and tautomerizes to form the oxime-containing caerulomycin A. Caerulomycin A has a unique 2,2′-bipyridine skeleton and shows attractive potential bioactivities such as antifungal, antiamoebic, and antitumor [62] properties. In *Streptomyces* species, PcxL and Hpx catalyze similar oxidation reactions of 2-aminoethylphosphonate (2AEPn) and (R)-1H2AEPn, respectively, to corresponding aldoximes, which are further oxidized to nitrosylated phosphonic acid products through multiple steps [66]. Oxime-containing small molecules are reported to have interesting biological properties such as antibacterial, antifungal, insecticidal, antitumor, immunosuppressive, and acetylcholinesterase reactivation activities [66].

OxaD from *Penicillium oxalicum* F30 generates nitrone alkaloid roquefortine L [67]. In the proposed mechanism, oxidation of roquefortine C to the hydroxylamine product is further oxidized to the nitrone in roquefortine L. Roquefortine is an example of a biosynthetically related prenylated indole alkaloid. Potent antibacterial, anticancer, antiparasitic, and insecticidal activities [63,64] have been reported for this class of compounds.

The NMOs, FzmM and its analog CreE convert aspartic acid to nitrosuccinate via double hydroxylation. Nitrosuccinate is subsequently converted to nitrous acid and fumarate by FzmL or CreD [9,68,69]. Nitrous acid is the N-donor for the formation of the N-N bond in the biosynthetic pathways of fosfazinomycin and cremeomycin. The structures of these enzymes are currently unavailable, and their amino acid sequences show low conservation to other FMOs. FzmM and CreE are distantly related to PcxL and HpxL, which were previously discussed.

Detailed mechanistic and structural studies of these interesting newly identified NMOs have the promise of shedding new light onto FMO chemistry. Determining the kinetic mechanism and solving the three-dimensional structure of these enzymes will allow the elucidation of the chemical mechanisms that will be important for engineering novel molecular scaffolds with improved biological activities.

### 3.2. Role of FMOs in antibiotic resistance

Antibiotic resistance poses increasing risks to patients by limiting the function of currently used antibiotics for the treatment of a wide range of infections, including life-threatening pneumonia [70]. Due to the broad

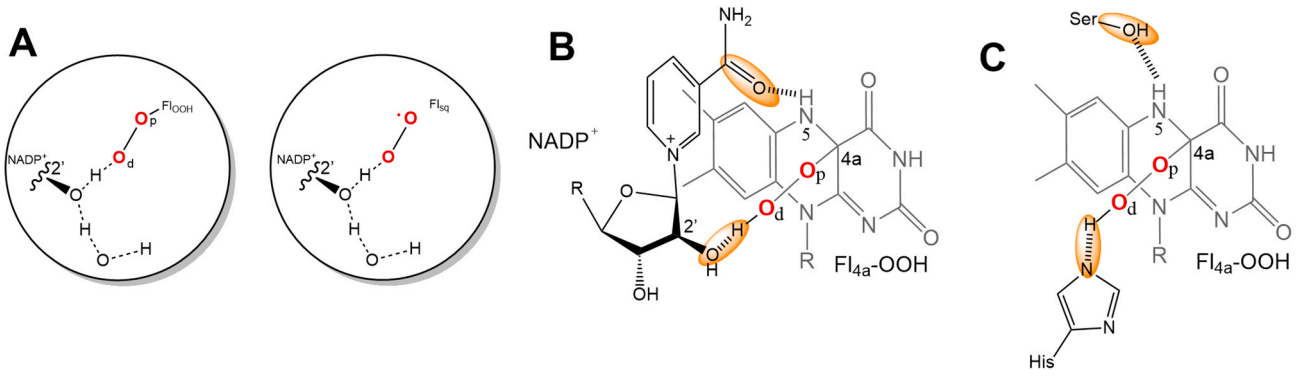


**Fig. 6.** Protonation and stabilization mechanism in FMOs. A) Protonation mechanism in class B monooxygenases. Protonation of the distal oxygen of $Fl_{4a}$-$OO^-$ is – facilitated by the ribose 2′-OH of $NADP^+$ (right) or protonation might occur directly to the superoxide anion (left). In two component monooxygenases, the proton donor is a His residue. B) Hydrogen bonding interactions involved in $Fl_{4a}$-OOH stabilization in class B monooxygenases or (C) two component monooxygenases (Ser171 and His396 in HPA) [51].

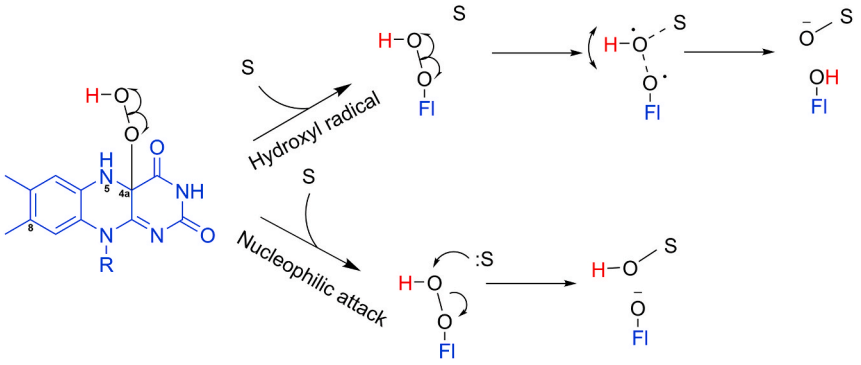


**Fig. 7.** Proposed hydroxylation mechanism of substrates in FMOs.

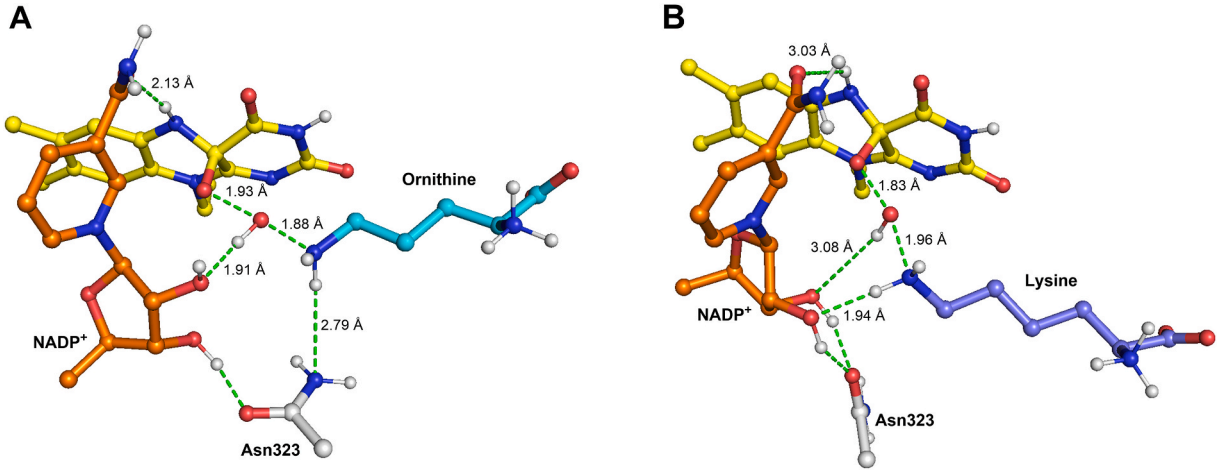


**Fig. 8.** Hydrogen bonding interaction in the transition state for the hydroxylation of (A) ornithine (shown in cyan), and (B) lysine (shown in purple) in the SidA active site obtained by DFT calculations [18]. NADPH is shown in orange and the Asn323 side chain is shown in gray. The transition state for hydroxyl radical transfer with ornithine is stabilized by a strong hydrogen bond with the $NADP^+$-2′OH. The transition state for lysine is instead stabilized by a weak hydrogen bond with the $NADP^+$-3′OH. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

substrate scope of FMOs, the active sites of microbial FMOs can accommodate antibiotics for oxidation reactions, leading to deactivation and, ultimately, resistance to certain antibiotics. Several microbial FMOs have been discovered and characterized for their functions in resistance to tetracyclines, rifampicin, sulfonamides, and imipenem antibiotics.

The enzyme tetracycline destructase, TetX, was the first FMO discovered that conferred antibiotic resistance against multiple tetracyclines, including tigecycline. Initially discovered from Bacteroides, this class A FMO was found to hydroxylate the C11a of tigecycline to produce 11a-hydroxytigecycline (Fig. 10A). The crystal structure of tetracycline-bound TetX revealed the short distance between tetracycline C11a and the flavin C4a (~5.9 Å) [71]. A strongly hydrophobic pocket was assigned as the putative oxygen binding site. Together with a

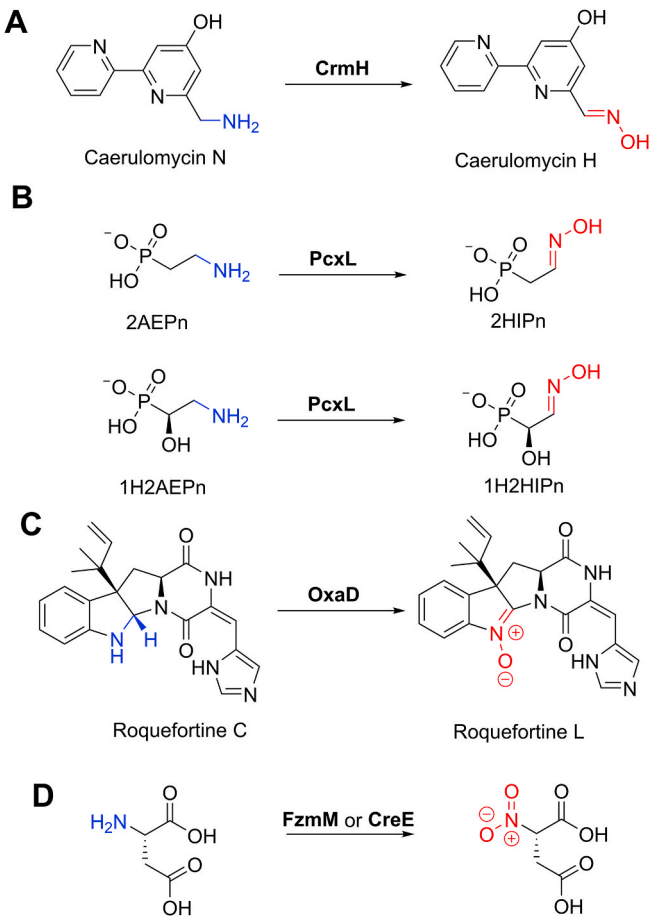


**Fig. 9.** Reactions catalyzed by (A) CrmH, (B) HpxL and PcxL, (C) OxaD, and (D) FzmM and CreE.

crystal structure of TetX2, a TetX ortholog, it was determined that TetX is a class A FMO [72]. Wild type TetX (Mab TetX) from *Mycobacterium abscessus* was recently found to also contribute to the high tetracycline resistance [73]. Moreover, genes encoding TetX3 and TetX4 in numerous Enterobacteriaceae and Acinetobacter have been isolated from animals and humans, which could cause compromised infection treatment by tetracyclines [74].

Rifampicin monooxygenase (RIFMO), another class A FMO, is encoded by the rox gene in *Nocardia farcinica* and was initially suggested to convert the frontline anti-tuberculosis drug rifampicin (RIF) to 2′-N-hydroxy-4-oxo-rifampicin (RIF-2′-N-OH) [75]. However, evidence of RIF-2′-N-OH existence was only supported by mass spectroscopy. Closer scrutiny of the crystal structure of the RIFMO-RIF complex by NMR analysis found that the oxidation product was RIF-OH (Fig. 10B), formed through hydroxylation of C2 followed by hydrolytic cleavage of the ansa-bridge [76,77].

A novel Baeyer-Villiger monooxygenase (Ar-BVMO, class B FMO), isolated from *Acinetobacter radioresistens*, was found to have the activity of oxidizing the carbonyl moiety of the β-lactam ring (Fig. 11A). Asymmetric oxygen insertion yielded two products [78]. The crystal structure of SaFMO, a BVMO from methicillin- and vancomycin-resistant *Staphylococcus aureus* strain MU50, has been reported [79]. Although a molecular docking study revealed the accessibility of the SaFMO active site to vancomycin and/or methicillin, the function and substrate specificity remains to be explored.

The biosynthesis of 1,2,4-trihydroxybenzene involves the sequential action of two monooxygenase components, SadA and SadB. The reductase component that provides the reduced FMN is known as SadC (Fig. 11B). SadA converts sulfamethoxazole to 4-aminophenol, which is subsequently converted by SadB to 1,2,4-trihydroxybenzene [80]. These products do not show antibiotic activity, so the presence of SadA results in a reduction of the bacteriostatic effect of sulfonamides. A different two component FMO system, encoded by the monooxygenase gene sulX and the reductase gene sulR (Fig. 11C), was found to catalyze similar oxidation steps to SadA/SadC [81]. In this system, the sulfonamide monooxygenase was a very close analog of SadA (95.5% identity) [81]. These monooxygenases belong to the class C FMOs.

### 3.3. Drug discovery program targeting FMOs

Only a few FMOs have been investigated as potential drug targets. Among these, kynurenine 3-monooxygenase (KMO), a class A FMO, is the most studied due to its role in tryptophan catabolism [82]. Catalyzing the hydroxylation of L-kynurenine, KMO generates the neurotoxin 3-hydroxykynurenine and causes neurodegeneration and/or neuroinflamation [82]. Targeting KMO is an attractive treatment approach for illnesses such as Alzheimer's disease [83] and acute pancreatitis [84,85]. KMO inhibitor development has been accomplished through fluorescence-based high throughput screening (HTS) and rational drug design (Fig. 12A) [86,87]. However, structural analogs of L-kynurenine should be studied with caution to inhibit KMO since they uncouple the oxidation process and generate high levels of cytotoxic hydrogen peroxide [87,88].

Inhibition of FMOs involved in the cholesterol synthesis pathway can also be beneficial. Human squalene epoxidase (SQLE) inhibition causes accumulation of toxic squalene and presents itself as a novel target for neuroendocrine cancers [89]. NB-598 was found to specifically inhibit SQLE ($IC_{50}$ = 63 nM) [90] and suppress de novo cholesterol biosynthesis in small cell lung cancer cells ($IC_{50}$ = 76 nM) [91]. A high resolution crystal structure of human SQLE has also been reported revealing the inhibitor binding site [90]. The key residue of human SQLE, Tyr195, was found to form hydrogen bonds with the Gln168 side chain in the presence and absence of hydrophilic substrate. However, upon binding with inhibitors, Tyr195 changes its residue orientation, which favors its interaction with the amine groups of the inhibitors. This binding mode could inspire design of next-generation inhibitors. In the cholesterol synthesis pathway, another potential FMO target studied was squalene synthase (SQS), which has been investigated as an antifungal target against *Aspergillus flavus* [92]. Celastrol was found to be an inhibitor of *Af*SQS, with an $IC_{50}$ of 0.83 μM.

Our group developed a colorimetric high-throughput screening platform and identified celastrol (Fig. 12B) as an inhibitor of *A. fumigatus* growth (MIC = 2 μM) [93]. The screening was performed against SidA and its higher $IC_{50}$ value ($IC_{50}$ = 11 μM) suggested that it might be a multi-target inhibitor [92]. Similar drug discovery strategies might apply to other N-hydroxylating monooxygenases involved in siderophore synthesis, such as PvdA and NbtG [44,94]. More recently, the "out" conformation adopted by the FAD in the active site of SidA has been exploited as a potential new binding pocket for drug discovery. A fragment-mapping approach was used for SidA to identify new ligand binding spots [14].

Since TetX has been proven to be the deactivator of tetracycline drugs, administration of TetX inhibitor in combination with tetracyclines should restore the activity of tetracyclines [95]. Anhydrotetracycline, a biosynthetic precursor of tetracycline, has been shown to prevent tetracycline degradation by TetX, thus, proving the concept of such a co-administration strategy [95,96]. In fact, it was established that anhydrotetracycline binds to the active site of TetX and locks FAD in the unproductive "out" conformation. This mechanism exists in a relevant clinical application of flavin mobility in monooxygenases.

A more recently discovered BVMO, MymA, is involved in mycolic acid synthesis and serves as a target of isoniazid [97]. A thioalkylbenzoxazole compound, discovered by GSK scientists through screening of intracellular *Mycobacterium tuberculosis* growth was found to have anti-tuberculosis activity. Since S-oxidation is known to activate

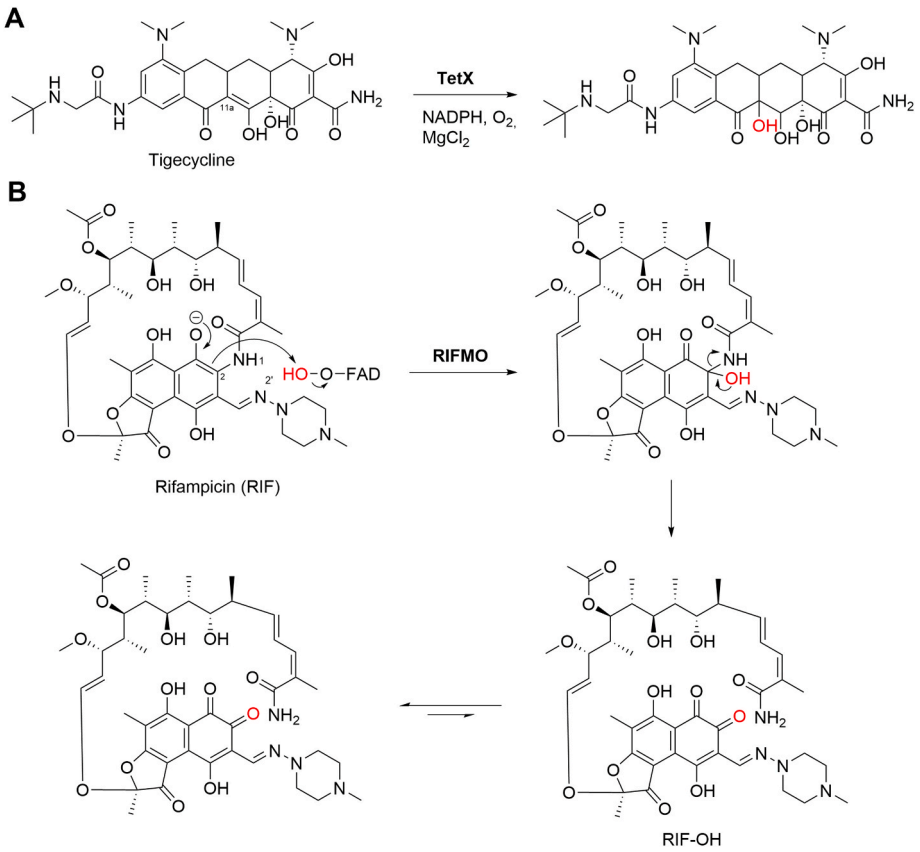


**Fig. 10.** (A) TetX-catalyzed hydroxylation of tigecycline. (B) Proposed mechanism of RIFMO-catalyzed hydroxylation of rifampicin.

anti-tuberculosis drugs, this compound was tested against a panel of *ethA* and *mymA* mutant strains. The increased resistance of *mymA* mutants suggested that this compound was bioactivated by MymA, presumably to the corresponding sulfoxide and sulfone (Fig. 13) [98]. However, the catalytic mechanism remains to be elucidated.

### 3.4. FMO in synthesis of drug candidates

EpnF is a flavin-dependent enzyme from *Streptomyces hygroscopicus* that was found to catalyze a key step in the biosynthesis of epoxyketone pharmacophore. The reaction is characterized as a cascade of decarboxylation-dehydrogenation-monooxygenation reactions (Fig. 14). TMC-86 A is a natural α,β-epoxyketone that covalently modifies the essential threonine residue of the proteasome β-subunit.

It is well established that epoxyketone proteasome inhibitors constitute a class of therapeutic agents for cancer treatment [99]. In fact, carfilzomib, a synthetic tetrapeptide epoxyketone proteasome inhibitor, was approved for multiple myeloma treatment. Epoxyketones have also shown antiparasitic activities [100]. In the context of natural product-based drug discovery, synthetic substrates have been incubated with EpnF to afford analogs of TMC-86A when searching for candidates with improved activity [101].

## 4. Conclusions

The flavoenzymology field has advanced our understanding of the reactions catalyzed by flavoenzymes in vast detail. The combination of biochemical, structural, and computational tools allowed the elucidation of the mechanisms of C-H and O-H bond cleavage by oxidases [102,103]. The study of FMOs has also significantly benefited from multidisciplinary approaches to probe the various steps in this multi-substrate/redox reaction. Trapping and characterization of intermediates in *crystallo* have been particularly important. For many years, the characterization of the "waving" flavin in class A FMOs provided a logical explanation of the kinetic mechanism, where substrate binding is required to trigger flavin motion for reaction with NADPH. Similarly, the structure of FMOs in complex with $NADP^+$ highlighted potential interactions that provided clues to the mechanism of $Fl_{4a}$-OOH stabilization in class B FMOs. Further biochemical studies, in particular stopped-flow spectroscopy combined with kinetic isotope effects, were used to study the various steps in the oxidative half-reactions, particularly the protonation of $Fl_{4a}$-$OO^-$ and $H_2O_2$ or $H_2O$ elimination. The availability of three-dimensional structures in complex with substrates and in different redox states provided valuable information that has been used in computational studies to probe the hydroxylation mechanism.

Altogether, the field of FMOs was believed to be represented by class A monooxygenases utilizing the cautious mechanism with the "waving" flavin allowing the reduction to occur in a different site than hydroxylation. On the other hand, class B monooxygenases were thought to follow the bold mechanism, performing the reduction and oxidation reaction in the same active site, with NADP(H) reducing the flavin and stabilizing the $Fl_{4a}$-OOH. An updated version of the NMOs' mechanism with emphasis on the data from SidA is shown in Fig. 15. Flavin motion in NMOs is proposed to be modulated by the Tyr-loop rearrangements. In the "out" position, the flavin isoalloxazine ring can be seen stacking with a conserved Tyr (Fig. 15A). This type of interaction was previously characterized in ferredoxin-$NADP^+$ oxidases. In this flavoenzyme, a C-terminus conserved tyrosine residue stacks with the flavin isoalloxazine system. The motion of the phenol ring of this tyrosine residue was proposed to be responsible for the decrease in the binding energy of the charge transfer complex between NADPH-$Fl_{ox}$ or $NADP^+$-$Fl_{red}$, that otherwise would form a tight dead-end complex [104–106]. It is possible that in NMOs, similar to what has been described for class A FMOs, reaction with NADPH occurs with the flavin in the "out" position

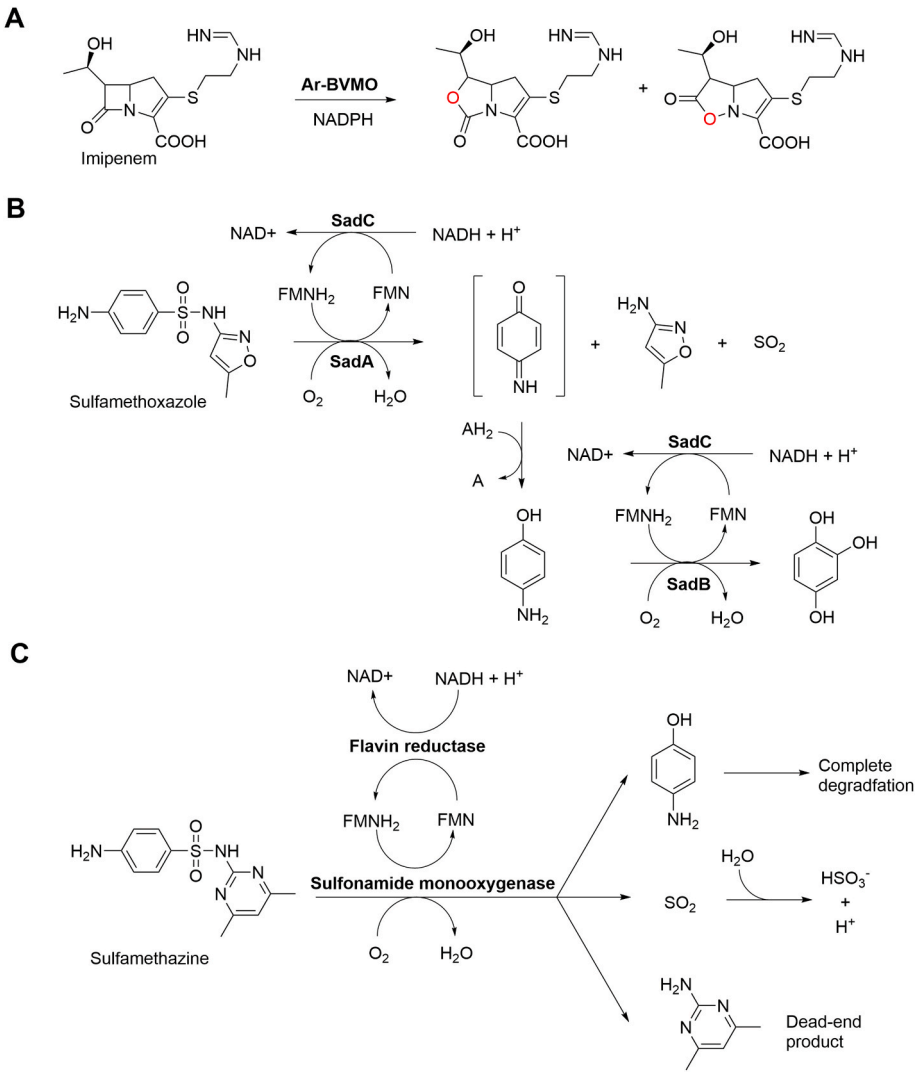


**Fig. 11.** (A) Ar-BVMO-catalyzed oxidation of imipenem. (B) Proposed mechanism of sulfamethoxazole degradation catalyzed by SadA/SadC and SadB/SadC. (C) Proposed mechanism of sulfamethazine degradation catalyzed by a two-component FMO system encoded by sulX/sulR.

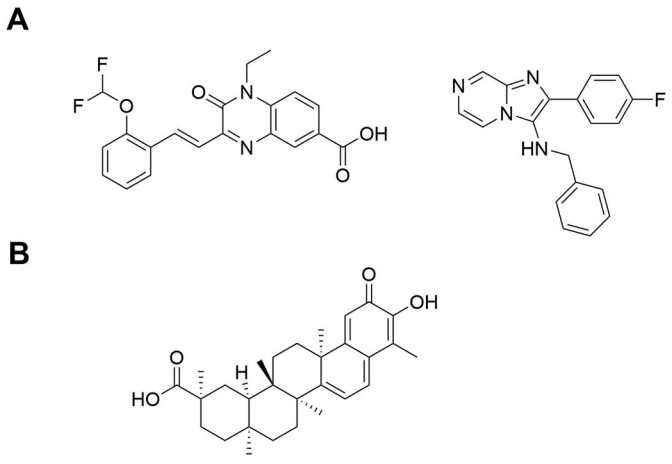


**Fig. 12.** (A) Structures of screen hits for KMO. (B) Structure of SidA inhibitor celastrol.

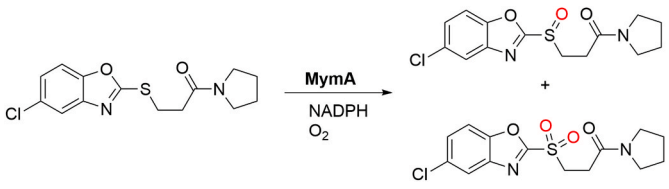


**Fig. 13.** Activation of anti-tuberculosis agent by FMO MymA.

(Fig. 15B). In this context, the conserved tyrosine residue would be responsible for modulating the interaction between the nicotinamide and the isoalloxazine ring to prevent the formation of a tight stable complex. It is also possible that the tyrosine residue is involved in the positioning of the nicotinamide for pro*R* hydride transfer in the orientation required for stabilization of the $Fl_{4a}$-OOH (Fig. 15C). Solving the structure of the complex between NADPH and class B FMOs remains a key piece of the puzzle that is still missing. Molecular dynamic studies that can identify the conformational motions involved in flavin dynamics and NADP(H) entrance and exit of the active site should be a major focus in the FMO field. Although, a Tyr-loop is not generally

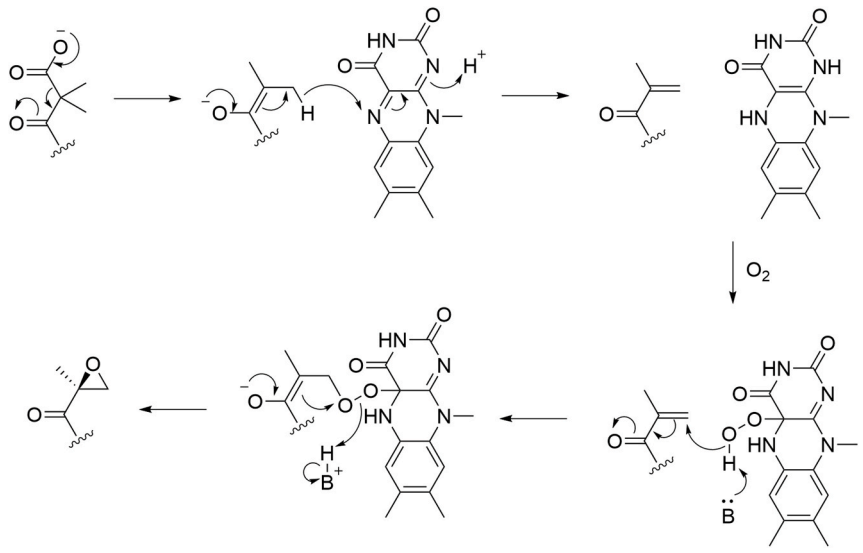

**Fig. 14.** Proposed catalytic cascade of EpnF.

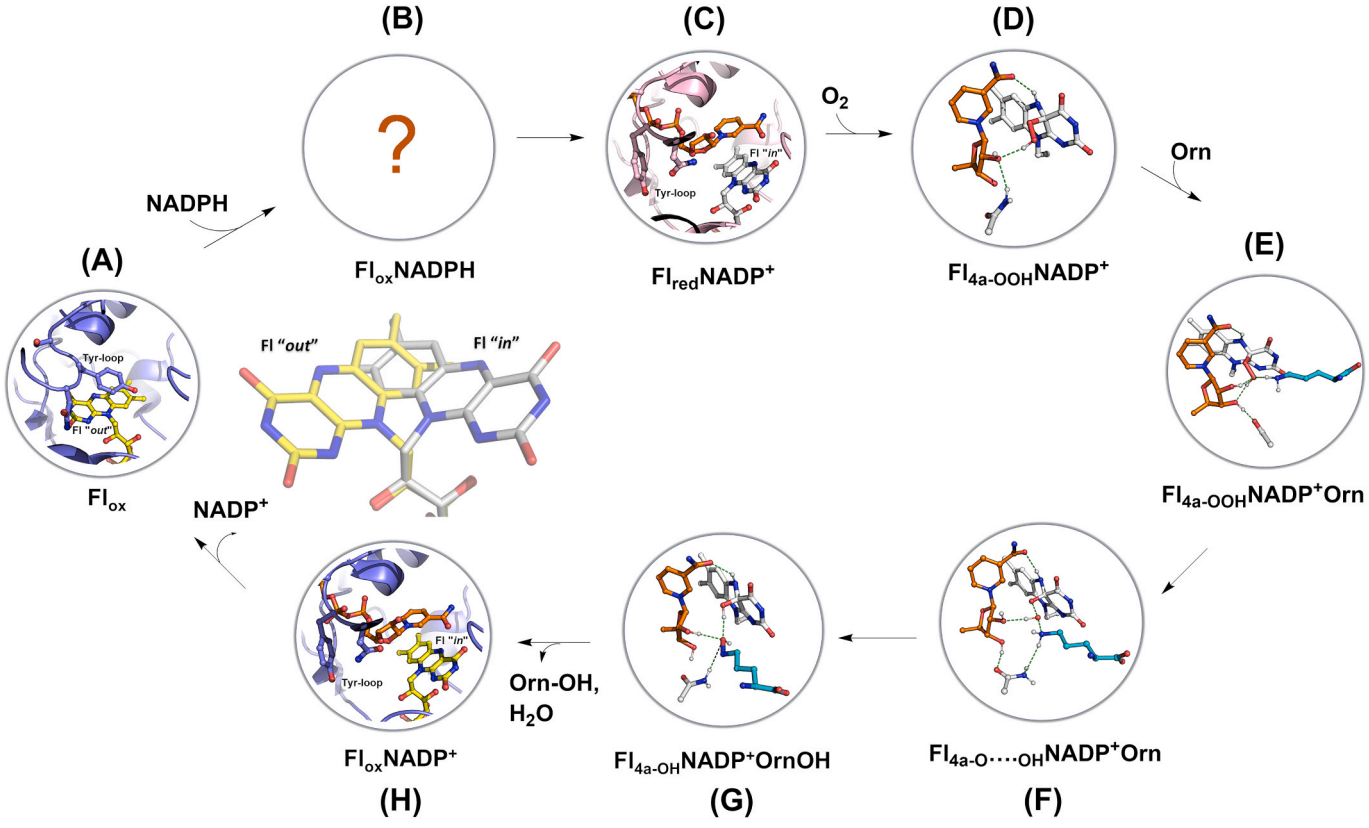


**Fig. 15.** Proposed mechanism for NMOs from biochemical, structural, and computational studies on SidA. $NADP^+$ and ornithine are shown in orange and cyan, respectively. (A) SidA ligand free form (PDB ID 6X0H) with the oxidized flavin. In this complex, the flavin is in the "out" position and interacts with Tyr-loop. (B). Unknown molecular details on the complex formed between the oxidized flavin and NADPH (C). The NADPH-reduced SidA (PDB ID 4B65) in complex with $NADP^+$. (D) $Fl_{4a}$-OOH stabilization by hydrogen bonding with $NADP^+$. Protonation of the distal oxygen by the 2′OH is not shown. (E) Complex between bond in $Fl_{4a}$-OOH and ornithine. (F) Hydrogen bonding interaction in the transition state for the hydroxylation of ornithine through homolytic cleavage of the O-OH. (G) Proposed complex of $Fl_{4a}$-OH, $NADP^+$, and hydroxyornithine. After $H_2O$ elimination and hydroxyornithine release, the complex of oxidized enzyme with $NADP^+$ is generated (PDB ID 6X0I) (H) and release of $NADP^+$ completes the catalytic cycle. The predicted conformation changes of the flavin to the "out" position, to facilitate $NADP^+$ release (H–A) and to the "in" position upon NADPH binding/reduction (A–C) are also shown. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

conserved in all members of class B monooxygenase, flavin motion could also take place in BVMO and flavin-containing monooxygenases by interactions with yet unidentified residues.

Stabilization of the $Fl_{4a}$-OOH is dependent on hydrogen bonding with $NADP^+$. Ribose hydroxyl groups are involved in protonation in addition to stabilization (Fig. 15D). The precise timing of the protonation is not clearly understood as protonation of the superoxide has been proposed. The interaction is also conserved in two component FMOs but occurs with active site residues as $NADP^+$ is not present. The mechanism of hydroxylation of substrates in FMOs has been probed by computational studies. Homolytic cleavage and hydroxyl radical transfer have reemerged as possible mechanisms for class A and B FMOs (Fig. 15E). Further support for these mechanisms will come from more computational studies as well as from mechanistic studies with alternative substrates and enzymes variants.

Ultimately, the biochemical and structural characterization of new FMOs involved in the functionalization of N-groups will provide the basis for the engineering of new pharmacological agents. Similarly, inactivation of FMOs can be targeted for the identification of small molecules for the treatment of microbial infections and other diseases. The unique conformational states that FMOs undergo during catalysis can be exploited in the development of new and selective inhibitors against these enzymes.

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## Notes

The authors declare no competing financial interest.

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