REVIEW

Monooxygenation of aromatic compounds by flavin-dependent monooxygenases

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Abstract: Many flavoenzymes catalyze hydroxylation of aromatic compounds especially phenolic compounds have been isolated and characterized. These enzymes can be classified as either single-component or two-component flavin-dependent hydroxylases (monooxygenases). The hydroxylation reactions catalyzed by the enzymes in this group are useful for modifying the biological properties of phenolic compounds. This review aims to provide an in-depth discussion of the current mechanistic understanding of representative flavin-dependent monooxygenases including 3-hydroxy-benzoate 4-hydroxylase (PHBH, a single-component hydroxylase), 3-hydroxyphenylacetate 4-hydroxylase (HPAH, a two-component hydroxylase), and other monooxygenases which catalyze reactions in addition to hydroxylation, including 2-methyl-3-hydroxypyridine-5-carboxylate oxygenase (MHPCO, a single-component enzyme that catalyzes aromatic-ring cleavage), and HadA monooxygenase (a two-component enzyme that catalyzes additional group elimination reaction). These enzymes have different unique structural features which dictate their reactivity toward various substrates and influence their ability to stabilize flavin intermediates such as C4a-hydroperoxyflavin. Understanding the key catalytic residues and the active site environments important for governing enzyme reactivity will undoubtedly facilitate future work in enzyme engineering or enzyme redesign for the development of biocatalytic methods for the synthesis of valuable compounds.

Abbreviations: 3,4,5-THCA, 3,4,5-trihydroxycinnamic acid; 3,4,5-THPA, 3,4,5-trihydroxyphenyl acetic acid; 3,4-DOHB, 3,4-dihydroxybenzoate; 5HN, 5-hydroxynicotinic acid; 5PAO, 5-pyridoxic acid oxygenase; AAMS, α-(*N*-acetylaminomethylene) succinic acid; C_1, reductase component of *p*-hydroxyphenylacetate 3-hydroxylase from *Acinetobacter baumannii*; C_2, oxygenase component of *p*-hydroxyphenylacetate 3-hydroxylase from *Acinetobacter baumannii*; DFT, density functional theory; DHPA, 3,4-dihydroxyphenylacetate; $FADH^-$, reduced FAD; $FMNH^-$, reduced FMN; HPA, 4-hydroxyphenylacetate; HPAH, 3-hydroxyphenylacetate 4-hydroxylase; MHPC, 2-methyl-3-hydroxypyridine-5-carboxylate; MHPCO, 2-methyl-3-hydroxypyridine-5-carboxylate oxygenase; NMHN, *N*-methyl-5-hydroxynicotinic acid; P2O, pyranose 2-oxidase; PHBH, 3-hydroxy-benzoate 4-hydroxylase; pOHB, 4-hydroxybenzoate; QM/MM, quantum mechanical and molecular mechanical; QSAR, quantitative structure and reactivity relationship.

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Introduction

Aromatic compounds in the phenolic acid family are some of the most abundant compounds found in nature. These compounds make up a significant part of lignocelluose, various natural products, and man-made chemicals, and have a wide spectrum of applications in chemical, agricultural, food, and pharmaceutical industries.[1–3] Many phenolic acids have useful biological activities such as antioxidant, anticancer, antimicrobial, antifungal, and anti-inflammatory activities, and are widely used in the pharmaceutical and food industries.[3] Hydroxylation of phenolic acid can generally increase the radical scavenging ability of the compound and may also increase other biological activities. For example, 3,4,5-trihydroxycinnamic acid (3,4,5-THCA), 3,4,5-trihydroxyphenylacetic acid (3,4,5-THPA), and gallic acid were reported to have greater antioxidant and anti-inflammatory effects than their corresponding non-hydroxylated phenolic acids which contain only the single hydroxyl group.[4–6] Therefore, the hydroxylation of phenolic acids, especially via enzymatic methods is important for enhancing their biological activities.[3,7]

Four enzyme types have been reported to catalyze aromatic hydroxylation, including the heme-containing cytochrome P450, non-heme iron, and pterin-dependent and flavin-dependent monooxygenases.[8] This review will only cover flavin-dependent systems and will aim to discuss in-depth, the current mechanistic understanding of representative flavin-dependent monooxygenases which catalyze only hydroxylation of aromatic compounds or hydroxylation plus additional reactions, and review the challenges facing their application. These flavin-dependent enzymes discussed can be divided into two types: the first type binds flavin (oxidized FAD) as a prosthetic group (single-component monooxygenase), while the second type does not bind tightly to oxidized flavin but rather, uses reduced flavin provided by a reductase as a substrate (two-component monooxygenase). Representatives of both types will be discussed. We discuss monooxygenases which catalyze merely hydroxylation including 3-hydroxy-benzoate 4-hydroxylase (PHBH, representative of single-component hydroxylases) and 3-hydroxyphenylacetate 4-hydroxylase (HPAH, representative of two-component hydroxylases), and also discuss monooxygenases catalyzing additional reactions in addition to hydroxylation including 2-methyl-3-hydroxypyridine-5-carboxylate oxygenase (MHPCO, a representative single-component enzyme that catalyzes aromatic-ring cleavage), and HadA monooxygenase (HadA, a representative two-component enzyme that catalyzes an additional group elimination reaction). Other flavin-dependent monooxygenases are beyond the scope of this review, and information regarding a wide variety of reactions catalyzed by flavin-dependent monooxygenases can be found in many excellent reviews.[9–18] In addition to mechanistic aspects, this review also discusses the potential applications of flavin-dependent monooxygenases and the challenges currently encountered in these areas.

Overall catalytic reactions of flavin-dependent monooxygenases

Single-component flavin-dependent monooxygenases.

A typical reaction cycle of single-component flavin-dependent monooxygenases catalyzing aromatic hydroxylation can be divided into two parts – reductive and oxidative half-reactions (Fig. 1). For the reductive half-reaction, an aromatic substrate binds to the enzyme to form an active enzyme:substrate complex which can be reduced by NAD(P)H much faster than in the absence of the aromatic substrate (Steps 1 and 2). Single-component monooxygenases use NAD(P)H directly as a reducing reagent to reduce their flavin cofactors, which to date, has only been reported to be FAD.[10,14,19] For the oxidative half-reaction, the reduced enzyme:substrate complex reacts with molecular oxygen to generate a C4a-hydroperoxyflavin intermediate (Step 3), which further pursues hydroxylation of the aromatic substrate, resulting in hydroxylated product and C4a-hydroxyflavin (Step 4). At the last step, the product is released from the active site and C4a-hydroxyflavin dehydrates to regenerate oxidized flavin (Step 6). In the absence of substrate, or in the presence of compounds that can bind to the enzyme but cannot be hydroxylated, C4a-hydroperoxyflavin only eliminates H_2O_2 to form the oxidized flavin (Step 7).

A reactive flavin species, C4a-hydroperoxyflavin, is a key intermediate of all flavin-dependent monooxygenases including sing-component and two-component types. This intermediate is the key reagent that inserts the hydroxyl group into aromatic substrates (C4a-hydroperoxyflavin acts as an electrophile) (Fig. 2). In other types of flavin-dependent monooxygenases which oxygenate different types of substrates such as Baeyer–Villiger monooxygenases, the reactive flavin species is instead, C4a-peroxyflavin (C4a-peroxyflavin acts as a nucleophile) (Fig. 2).[14,17] Monooxygenations by flavin-dependent monooxygenases are involved in a wide variety of biological processes such as in the

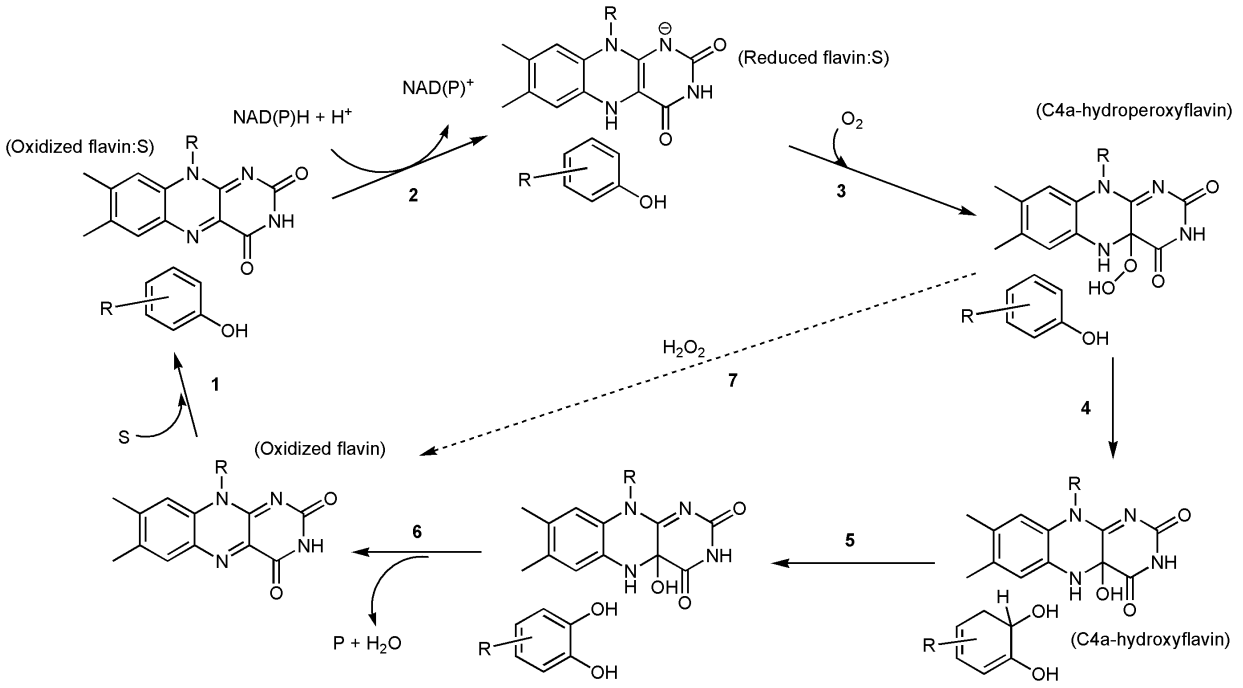


Figure 1. A typical catalytic cycle of phenolic hydroxylation catalyzed by single component flavin-dependent monooxygenases (hydroxylases).

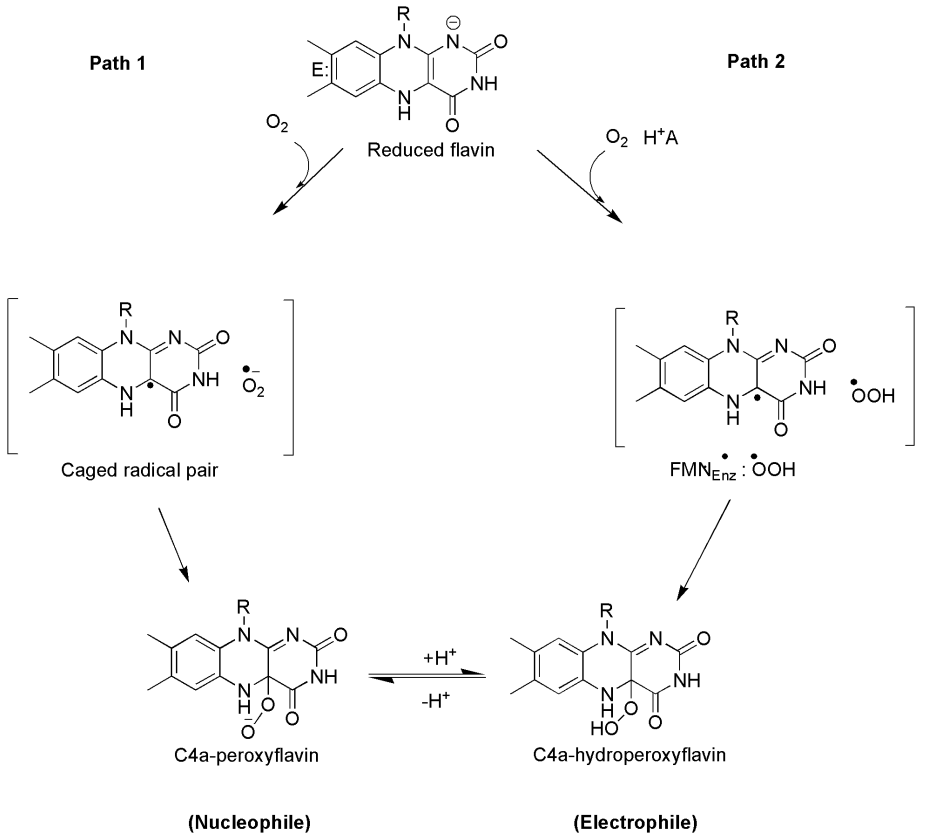
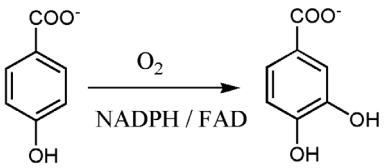
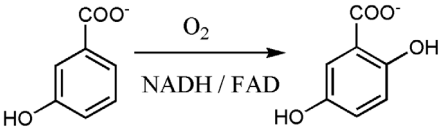
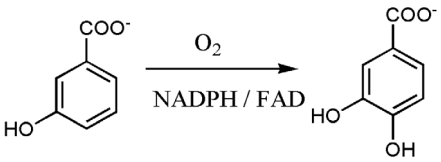
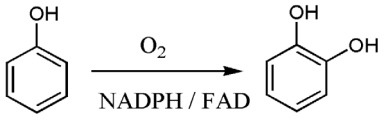
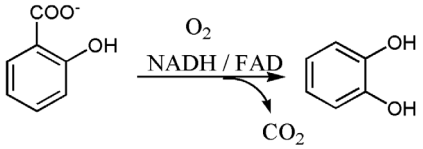
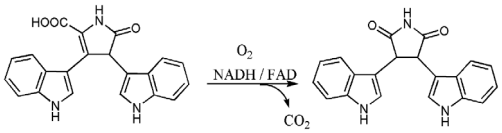
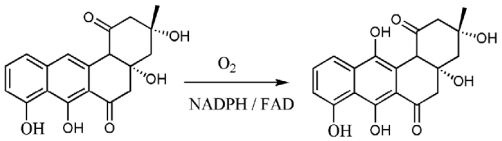
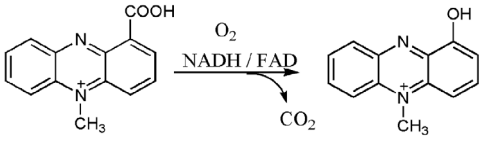
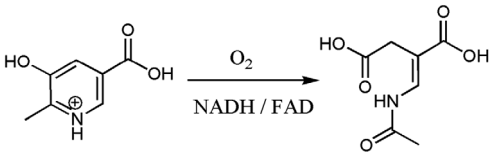
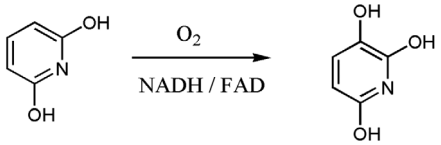
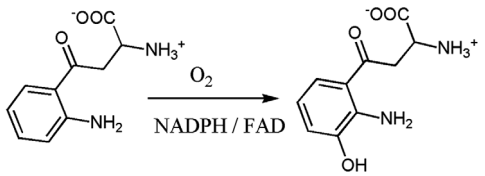


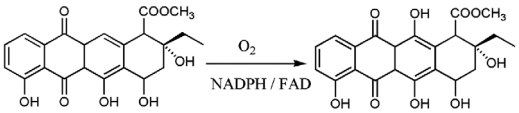
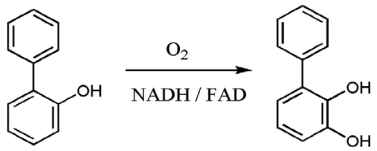
Figure 2. The reaction of reduced flavin with dioxygen to form C4a-(hydro)peroxyflavin. The first step of the reaction is an electron transfer from reduced flavin to oxygen to generate a radical pair of flavin semiquionone and oxygen superoxide. Path 1, a model that has been traditionally proposed for reactions of flavin-dependent monooxygenases, only involves electron transfer in the first step and combination of the radical pair to result in C4a-peroxyflavin which can be further protonated in the following step. Using combined methods of theoretical chemistry and transient kinetic investigation of pyranose 2-oxidase (P2O) and C_2 monooxygenase, the electron transfer step was found to be synchronous with a proton transfer (proton-coupled electron transfer) as in Path 2 to result in C4a-hydroperoxyflavin.

Table I. *Single Component Flavin-Dependent Monooxygenase Catalyzed Hydroxylation of Aromatic Compounds*

Enzyme	Reaction	Crystal structure	References
4-Hydroxybenzoate 3-hydroxylase (PHBH) from *Pseudomonas fluorescens* catalyzes the conversion of *p*-hydroxybenzoate to 3,4-dihydroxybenzoate.		1PBE	22
3-Hydroxybenzoate-6-hydroxylase (3HB6H) from *Rhodococcus jostii* RHA1catalyzes the para-hydroxylation of 3-hydroxybenzoate to 2,5-dihydroxybenzoate		5HYM	23
3-Hydroxybenzoate 4-hydroxylase (MHBH) from *Comamonas testosteroni* KH122-3s catalyzes the conversion of 3-hydroxybenzoate to 3,4-dihydroxybenzoate		2DKH	24
Phenol hydroxylase (PHHY) from *Trichosporon cutaneum* catalyzes the ortho-hydroxylation of phenol to catechol		1FOH	25
Salicylate 1-hydroxylase (SALH) from *Pseudomonas putida* S-1 catalyzes the hydroxylation of salicylate to catechol with the release of CO_2 by decarboxylation with a 1:1:1 stoichiometry		5EVY	26
RebC catalyzes hydroxylation in the biosynthesis of rebeccamycin in *Lechevalieria aerocolonigenes*		2R0P	27
PgaE catalyzes hydroxylation in angucycline biosynthesis in *Streptomyces*		2QA1	28
PhzS catalyzes hydroxylative decarboxylation in the biosynthesis of pyocyanin in *Pseudomonas aeruginosa*		2RGJ	29
MHPCO catalyzes hydroxylation and ring-opening of 2-methyl-3-hydroxypyridine-5-carboxylic acid to form α-(N-acetylaminomethylene) succinic acid in *Mesorhizobium loti*		3GMC	30
2,6-Dihydroxypyridine-3-hydroxylase catalyzes hydroxylation of 2,6-dihydroxypyridine to 2,3,6-trihydroxypyridine in the nicotine degradation pathway in *Arthrobacter nicotinovorans*		2VOU	31
Kynurenine 3-monooxygenase catalyzes the hydroxylation of L-kynurenine (L-Kyn) to 3-hydroxykynurenine (3OHKyn) in the pathway for tryptophan catabolism		5Y7A	32

(Continues)

Table I. *Continued*

Enzyme	Reaction	Crystal structure	References
Aklavinone-11-hydroxylases catalyzes the hydroxylation of Aklavinone to ε-rhodomycinone in the biosynthesis of anthracyclines in *Streptomyces purpurascens*		3IHG	33
2-Hydroxybiphenyl 3-monooxygenase (HbpA) catalyzes the *ortho*-hydroxylation of 2-hydroxybiphenyl to 2,3-dihydroxybiphenyl in the degradation of the fungicide 2-hydroxybiphenyl		5BRT	34

biodegradation of aromatic compounds in the environment, in the detoxification of drugs, and in the biosynthesis of antibiotics, hormones and vitamins.[10,14] Many of these enzymes also catalyze reactions with high regio- and/or stereo-specificity, making them attractive for biocatalytic applications.[3,7,10,13,14,20,21]

As single-component flavin-dependent monooxygenases catalyze flavin reduction and substrate oxygenation in the same active site, the flavin cofactor remains bound to the protein throughout all steps of the catalytic cycle. Table I summarizes the biochemical and catalytic features of known single-component monooxygenases. It is interesting to note that all of the

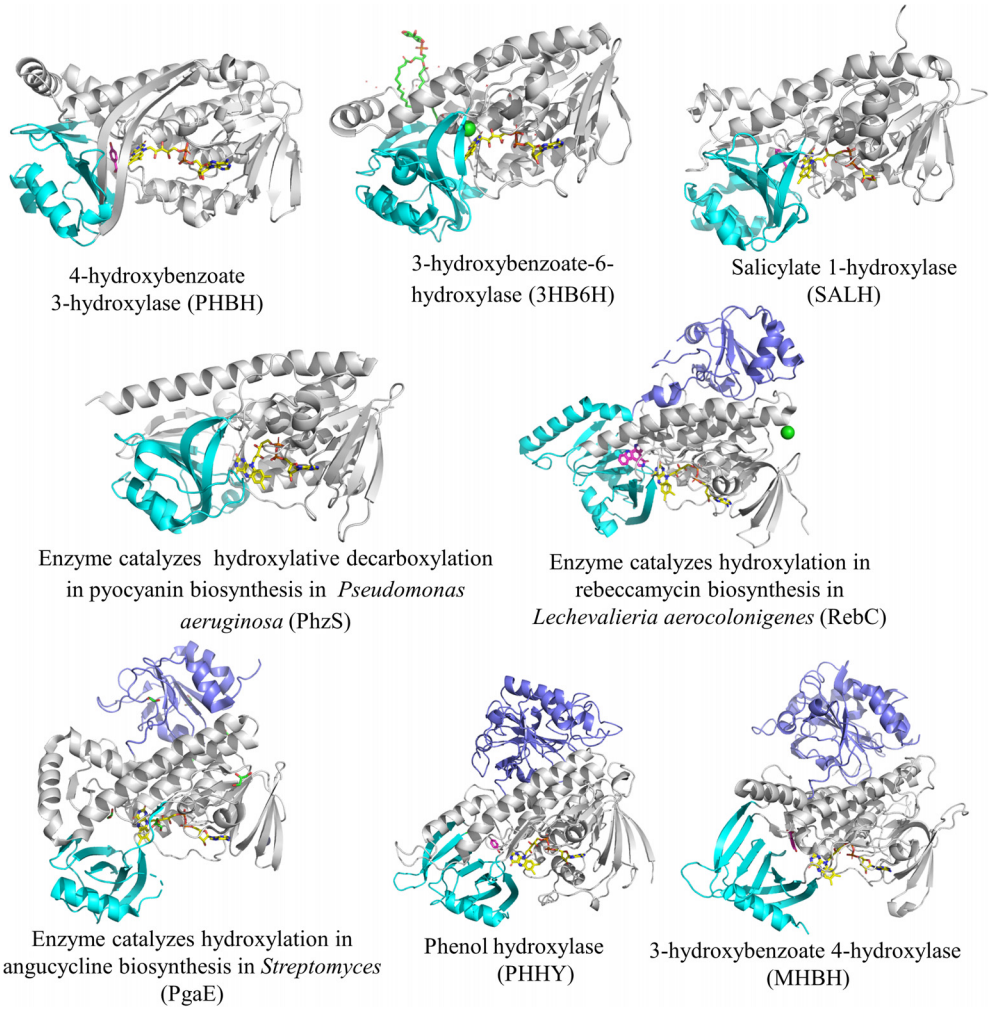
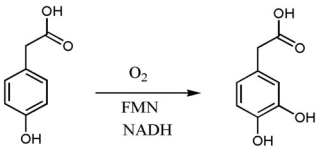
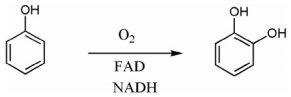
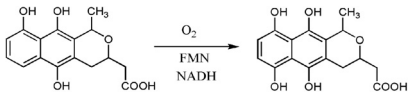
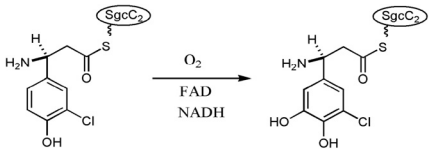
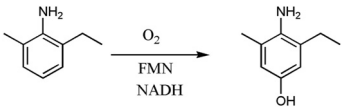
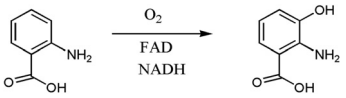
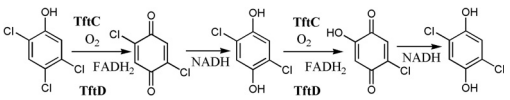
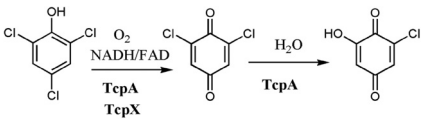


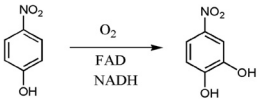
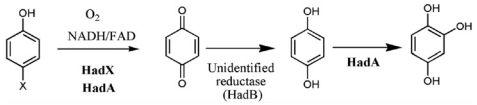
Figure 3. X-ray structures of single component flavin-dependent monooxygenases catalyze hydroxylation of aromatic compounds.

Table II. *Two-Component Flavin-Dependent Monooxygenases Catalyze Hydroxylation of Aromatic Compounds*

Function	Reaction	Reductase component	Oxygenase component	References
Enzymes catalyze the hydroxylation of p-hydroxyphenylacetate (HPA) yielding 3,4-dihydroxyphenyl acetate (DHPA) in *Escherichia coli*,[35] *Pseudomonas aeruginosa,*[36] *Pseudomonas putida,*[37] *Klebsiella pneumonia,*[38] *Sulfolobus tokodaii,*[39] *Thermus thermophiles,*[40] and *Acinetobacter baumannii*[41]		HpaA (17 kDa) HpaC (19.4 kDa) MhaB, (6 kDa) HpaH (19 kDa) HpaC (18 kDa) HpaC (16 kDa) C_1 (35.5 kDa)	HpaB (59 kDa) HpaA (58. kDa) MhaA (63 kDa) HpaA (59 kDa) HpaB - HpaB (54 kDa) C_2 (47 kDa)	35 36 37 38 39 40 41
Enzymes catalyze hydroxylation in the first step in the degradation of phenol in *Bacillus thermoglucosidasius A7*		PheA2 (18 kDa)	PheA1 (57 kDa)	42
Enzymes catalyze hydroxylation involved in the antibiotic actinorhodin biosynthesis in *Streptomyces coelicolor*		ActVB (18 kDa)	ActVA (39.7 kDa)	43 44
Enzymes catalyze hydroxylation of a carrier protein-tethered substrate during the biosynthesis of the Enediyne antitumor antibiotic C-1027 in *Streptomyces globisporus*		SgcE6 (19.5 kDa)	SgcC (57.9 kDa)	45
Enzymes catalyze hydroxylation of 2-methyl-6-ethylaniline (MEA) in the downstream catabolic pathway of chloroacetanilide herbicides in *Sphingomonads*		MeaY (21 kDa)	MeaX (46 kDa)	46
Enzymes catalyze hydroxylation to convert anthranilate to 3-HAA in the initial reaction of the degradation pathway in *Geobacillus thermodenitrificans* NG80-2		GTNG_3158 -	GTNG_3160 -	47
Enzymes catalyze the oxidation of 2,4,5-trichlorophenoxyacetic acid (2,4,5-TCP) to 2,5-dichloro-*p*-benzoquinone and then to 2,5-dichloro-*p*-hydroquinone (2,5-DiCHQ). TftD then oxidizes the latter to 5-chloro-2-hydroxy-*p*-benzoquinone in the degradation of 2,4,5-TCP in *Burkholderia cepacia AC1100*		TftC (22 kDa)	TftD (58 kDa)	48
Enzymes catalyze reactions to convert 2,4,6-trichlorophenol to 6-chlorohydroxyquinone in the degradation of 2,4,6-trichlorophenol in *Cupriavidus necator* JMP134		TcpX (21 kDa)	TcpA (60.7 kDa)	49,50

(Continues)

Table II. *Continued*

Function	Reaction	Reductase component	Oxygenase component	References
Enzymes catalyze hydroxylation of 4-nitrophenol to 4-nitrocatechol in the 4-NP degradation pathway in *Rhodococcus sp. strain* PN1		NphA2 (20 kDa)	NphA1 (53 kDa)	51
Enzymes catalyzes hydroxylation plus group elimination (halide) at position 4 of phenolic compounds with a para-substituent in *R. pickettii*		HadX (~19 kDa)	HadA (~59 kDa)	52,53

single-component flavin-dependent monooxygenases use FAD as a cofactor, while FAD and FMN are both used in two-component monooxygenases. This cofactor specificity must be due to the difference in structural motifs that interact with flavins. The overall folding of single-component monooxygenases generally consists of three domains (Fig. 3): the FAD binding domain (gray), substrate binding domain (cyan), and dimer interface domain (purple). The FAD binding domain has a typical Rossmann fold which recognizes the ADP part of FAD, thus making FAD the only type of flavin typically found as a cofactor in single-component monooxygenases.

Two-component flavin-dependent monooxygenases. Unlike single-component monooxygenases, two-component flavin-dependent monooxygenases require two proteins to carry out their reactions. The reductase is usually smaller than the oxygenase, ranging in size from 6 to 22 kDa for the reductase, and from 39 to 63 kDa for the monooxygenase (Table II). The reductase component (C_1) of *p*-hydroxyphenylacetate 3-hydroxylase (HPAH) from *Acinetobacter baumannii* is significantly larger (35 kDa) than those of other reductases[41,54] because C_1 has an extra C-terminal domain which serves as the binding site for the aromatic substrate which subsequently induces a conformational change in the enzyme to increase the rate of flavin reduction and release of reduced flavin.[15,55,56]

The flavin reduction in these enzymes takes place in the reductase component. These reductases typically bind FAD or FMN as cofactors or can be isolated in apoenzyme form.[15,41,57,58] They can be reduced directly by NAD(P)H without requiring the binding of aromatic compounds (Fig. 4, Step 1). The exception can be found in several reductases such

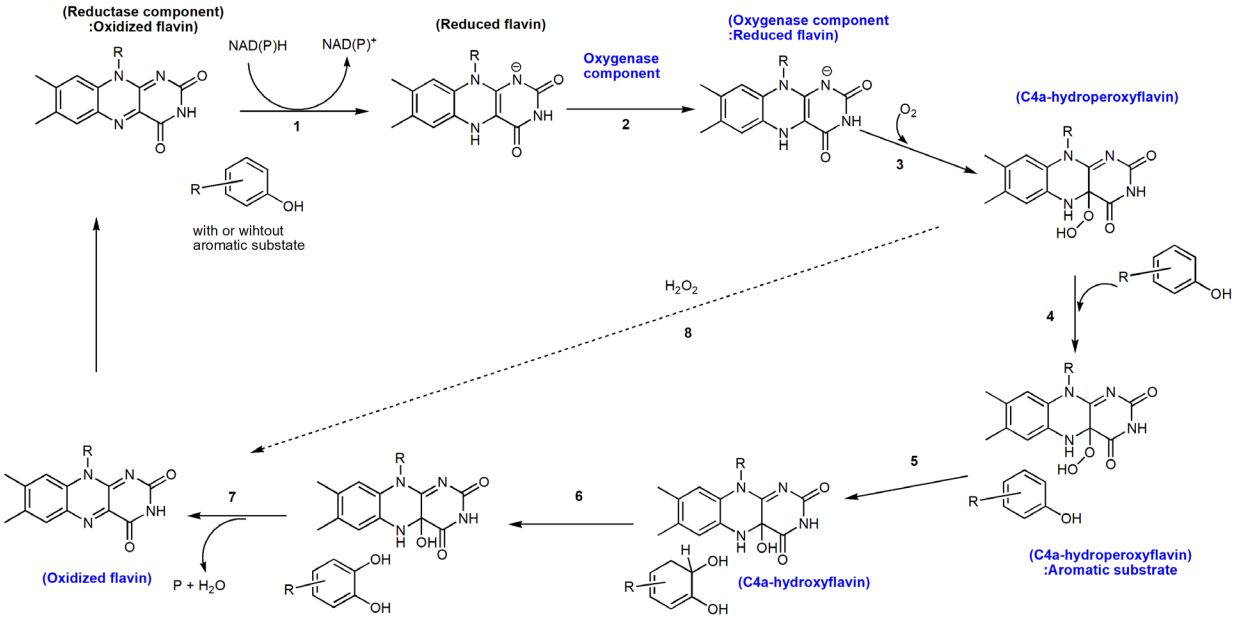


Figure 4. A typical catalytic cycle of phenolic hydroxylation catalyzed by two component flavin-dependent monooxygenases (hydroxylases).

as C_1 in which aromatic compounds can bind and stimulate the flavin reduction (see more discussion later).

For the oxygenation reaction of two-component enzymes, the enzyme-bound reduced flavin reacts with oxygen to form C4a-hydroperoxyflavin (Fig. 4, Step 3). All types of reduced flavins including FAD, FMN, and riboflavin can be used as substrates by two-component enzymes, depending on their specific recognition features.[15,54,57] The variety of flavin usage observed in two-component monooxygenases is due to the flavin binding feature because only the isoalloxazine part is buried inside the active sites, while ribityl phosphate of FMN or ADP part of FAD generally protrudes outside[59,60] (Fig. 5).

Aromatic substrates can be present or absent when the enzyme-bound reduced flavin reacts with oxygen, depending on requirement of each enzyme. Although following the oxygenation step, the reaction of two-component enzymes generally occurs similar to the reaction of single-component enzymes, there are several distinctive features that are different between these two enzyme types. These include differences in the stability of C4a-hydroperoxyflavin, the order of substrate binding, and the mode of reduced flavin transfer (Fig. 4, Step 2). Transfer of the reduced flavin is an essential part in the reaction of two-component monooxygenases, but is not required for the single-component monooxygenases (discussed more later in Section reaction mechanism of 4-hydroxyphenylacetate 3-hydroxylase).

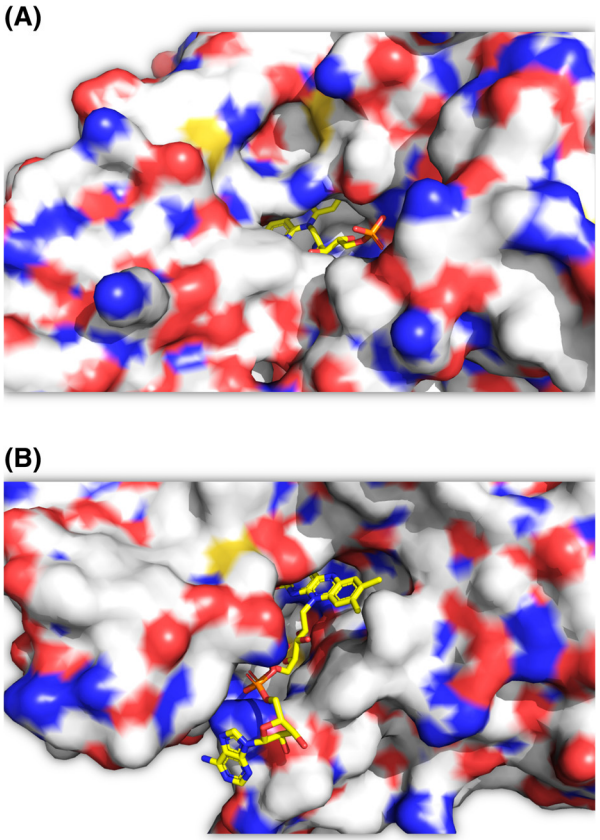


Figure 5. X-ray structures of (A) C_2 (pdb id: 2jbs) and (B) HpaB (pdb id: 2yyi), the isoalloxazine part is buried inside the active sites, while the ribityl phosphate of FMN or ADP part of FAD protrudes outside.

Flavoenzymes catalyzing phenolic acid hydroxylation

Flavin-dependent monooxygenases catalyzing hydroxylation of phenolic compounds are enzymes that are most extensively studied among the class. Here, the reactions of PHBH and HPAH are used as representative reactions of single-component and two-component enzymes, respectively.

Reaction mechanism of 4-hydroxybenzoate 3-hydroxylase (PHBH) (single-component monooxygenase).

PHBH represents one of the most extensively investigated single-component hydroxylases. It catalyzes the hydroxylation of 4-hydroxybenzoate (pOHB) to generate 3,4-dihydroxybenzoate (3,4-DOHB) using NADPH and O_2 as co-substrates [Fig. 6(A)].[9,19,61,62] The enzyme is classified as a member of Class A flavoenzyme monooxygenases.[10] The reaction catalyzed by PHBH can be divided into two parts – the reductive half-reaction and the oxidative half-reaction, as described in Figure 1. The enzyme conformational change is important for controlling various steps in both half-reactions.

During the catalytic cycle, the isoalloxazine ring of the FAD moiety can change in position due to a change in the enzyme conformation to enhance catalysis. The first crystal structure of the enzyme in the form of a PHBH-FAD-pOHB complex shows that FAD is buried inside the active site.[22] This enzyme form is designated as the "*in*" conformation, and this enzyme conformation does not facilitate the reduction of FAD by NADPH. However, in the presence of NADPH, the binding of pOHB allows the flavin ring to move to the "*out*" position. This *out* conformation was observed in the wild-type enzyme in complex with 2,4-dihydroxybenzoate.[63] In this form, NADPH can bind to the *re*-side of the isoalloxazine ring, and promote the flavin reduction, which is the transfer of a proR hydride of NADPH to the N5 of FAD.[64] In comparison to the "*in*" conformation, the flavin ring in the "*out*" conformation is rotated around the ribityl side chain, resulting in movement of the N5 position of the isoalloxazine ring by about 7 Å (Fig. 7) closer to the protein surface where NADPH can bind closer to FAD.

The crystal structure of PHBH with bound NADPH could only be observed with the structure of the Arg220Gln variant.[65] However, the position of the flavin ring is different from what was previously designated as the "*in*" or "*out*" conformations (Fig. 7). In this protein complex, the isolloxazine ring swings out slightly from the active site to the protein surface in order to create space for the substrate and product

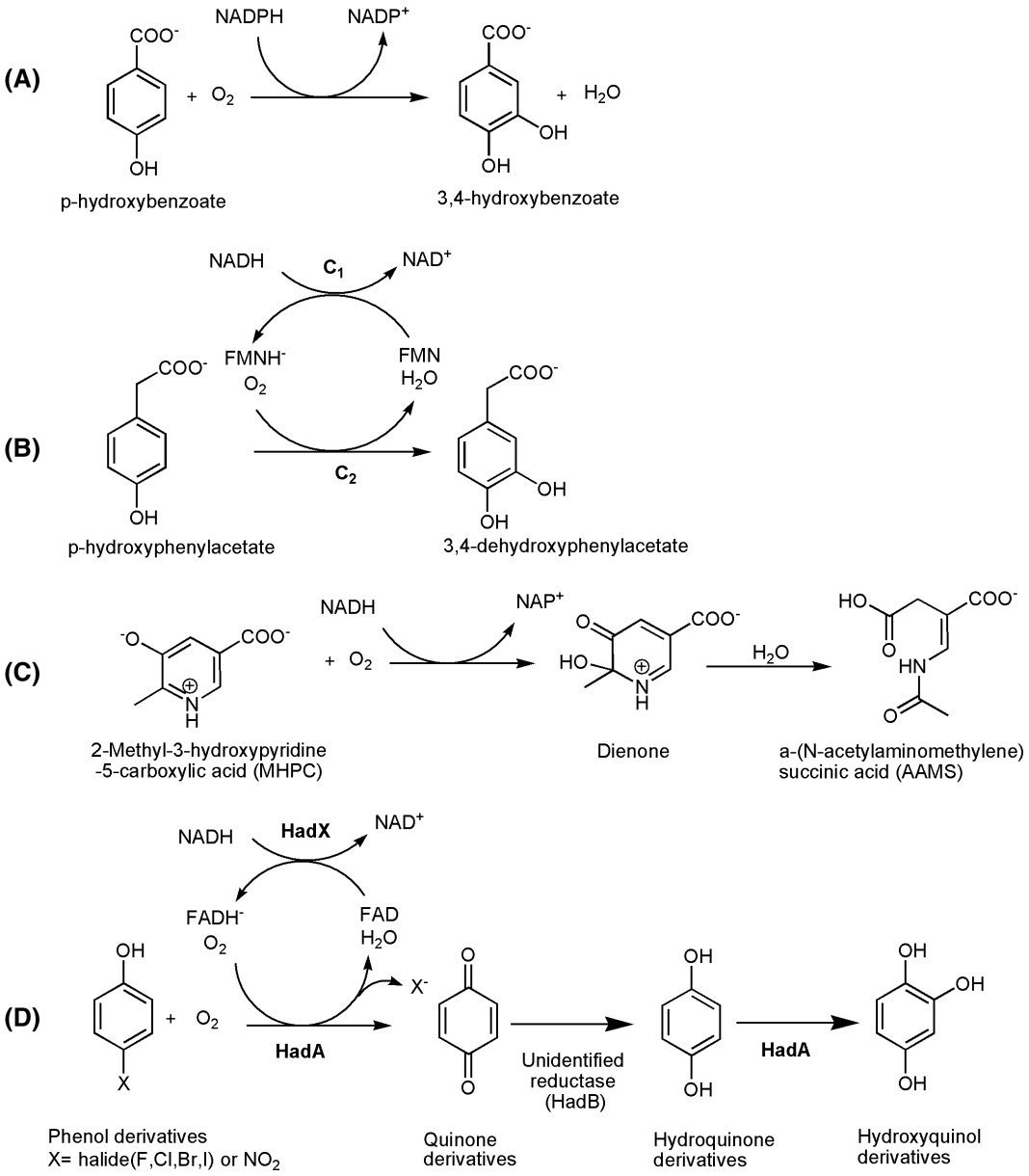


Figure 6. Reactions catalyzed by flavin-dependent monooxygenases in this review. (A) 3-Hydroxy-benzoate 4-hydroxylase (PHBH), (B) 3-hydroxyphenylacetate 4-hydroxylase (HPAH), (C) 2-methyl-3-hydroxypyridine-5-carboxylate oxygenase (MHPCO), and (D) HadA monooxygenase.

to access the active site. NADPH binds in an elongated conformation along the cleft on the surface of PHBH. However, with this binding form, the nicotinamide ring is too far from FAD to allow flavin reduction. Thus, this conformation cannot facilitate the flavin reduction and was assigned as the "*open*" conformation. At this stage, the phenolic substrate (pOHB) can directly form hydrogen bonds with surface amino acids.[65,66] From the structure of the PHBH:pOHB complex, the substrate phenolic oxygen participates in a hydrogen bond network that extends about 13 Å and connects the backbone carbonyl of Pro293, Tyr201, Tyr385, a water molecule, and His72 (Fig. 8).[22] These hydrogen-bond networks can lower the pK_a of the phenolic hydroxyl group by about 2 units.[61] The deprotonated form of the substrate can trigger movement of the flavin ring to the "*out*" position in which NADPH can reduce FAD.[67] Pre-steady-state kinetic data also support the importance of pOHB binding in promoting formation of the active enzyme conformation. The flavin reduction in the presence of aromatic substrate is 10^5 time faster than that of the reaction without aromatic substrate.[61] After reduction, $FADH^-$ rotates back into the "*in*" conformation and may be stabilized by the positive electrostatic environment of the active site.[68] Comparison of the structure of PHBH with other structures of enzymes in the same class such as phenol hydroxylase[25] does not show similar H-bond networks. Thus, the controlled reduction of flavin by the deprotonation of the substrate hydroxyl group may be unique to PHBH.

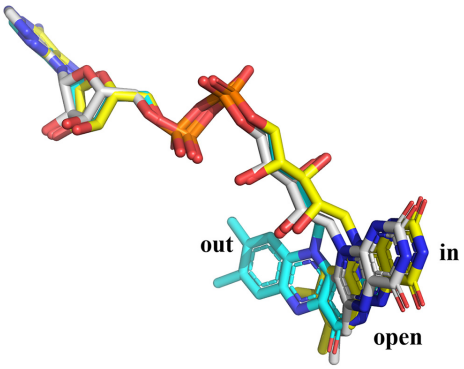


Figure 7. The "*out*", "*open*," and "*in*" conformations of the flavin ring were aligned and compared using pdb id: 1dod, 1k0j, and 1pbe, respectively.

During the oxidative half-reaction, the isoalloxazin ring of the reduced enzyme complex with pOHB bound is in the "*in*" conformation in which it is buried and inaccessible to solvent. The pOHB hydroxyl group forms H-bond networks with amino acid residues and is positioned close to the C4a position of the isoalloxazine ring. The active site of PHBH can facilitate the rapid reaction of oxygen with reduced flavin to form a C4a-hydroperoxyflavin intermediate with a rate constant of 2.6×10^5 M^{-1} s^{-1}.[69] This reaction is about 10^3 time faster than the reaction of free reduced flavin with oxygen.[69,70] In the presence of pOBH, C4a-hydroperoxyflavin hydroxylates the aromatic substrate *via* the electrophilic aromatic substitution mechanism and produces C4a-hydroxyflavin and a non-aromatic dienone product. The non-aromatic dienone rapidly tautomerizes to generate the aromatic product (Fig. 1, Step 5).

After the hydroxylation, C4a-hydroxyflavin dehydrates to eliminate water in order to regenerate the oxidized flavin. This step occurs synchronously with the release of aromatic product (3,4-DOHB). At high concentrations of pOHB, the substrate inhibits the dehydration step of C4a-hydroxyflavin, forming a trapped C4a-hydroxyflavin:product complex which can slow down the overall turnover.[71] The rate of product release is pH-dependent and consistent with a pK_a value of 7.1, suggesting that H-bond networks that control substrate deprotonation with pK_a of 7.1 are also involved with the process of product release. It was suggested that deprotonation of the *para*-hydroxyl group of 3,4-DOHB can result in the product dianionic form which can interact with Pro293 to induce the enzyme conformational change to promote positioning of the flavin in the "*out*" position, accelerating the dehydration process of C4a-hydroxyflavin.[22,65,72]

Reaction mechanism of 4-hydroxyphenylacetate 3-hydroxylase (two-component monooxygenase). *p*-Hydroxyphenylacetate 3-hydroxylase (HPAH) is one of the most extensively studied two-component flavin-dependent monooxygenases in which the reaction mechanisms can be used as a model for understanding the reactions of other enzymes in this class. HPAH catalyzes the hydroxylation of 4-hydroxyphenylacetate (HPA) to yield 3,4-dihydroxyphenylacetate (DHPA) [Fig. 6(B)]. Although the net reactions of HPAH and PHBH are similar, as they both catalyze hydroxylation of phenolic acids, the modes of operation by these two enzymes are remarkably different. This is mainly due to the nature of one being a single-component *versus* the other being a two-component enzyme in which flavin reduction and oxygenation occur in a single or in separate active sites, respectively.

Many HPAHs from several bacteria species have been isolated and characterized as shown in Table II. Among these, the reaction mechanism of HPAH from *Acinetobacter baumannii* is the most extensively investigated. This enzyme consists of a reductase component (C_1) and an oxygenase component (C_2).[41,54] The enzyme is part of the HPA degradation pathway in bacteria.[73] C_1 is a flavoprotein that binds FMN as a cofactor and catalyzes the reduction of

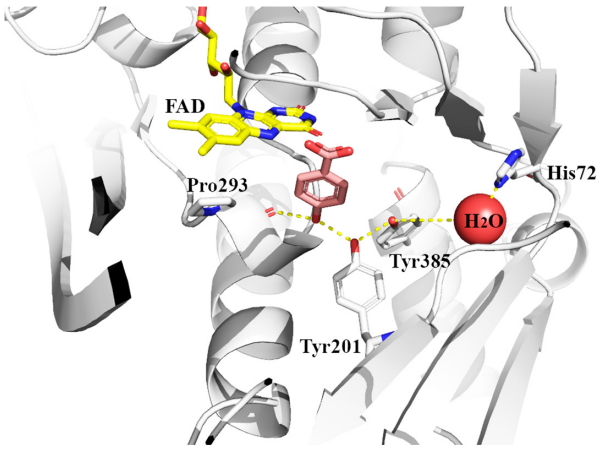


Figure 8. The hydrogen bond network along Pro293, Try201, Try385, a water molecule, and His72 residues (pdb id: 1pbe) in PHBH.

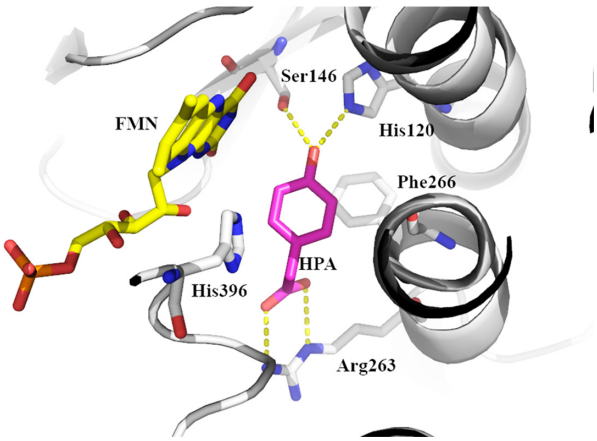


Figure 9. Important amino acid residues in the active site of C_2 (pdb id: 2jbt).

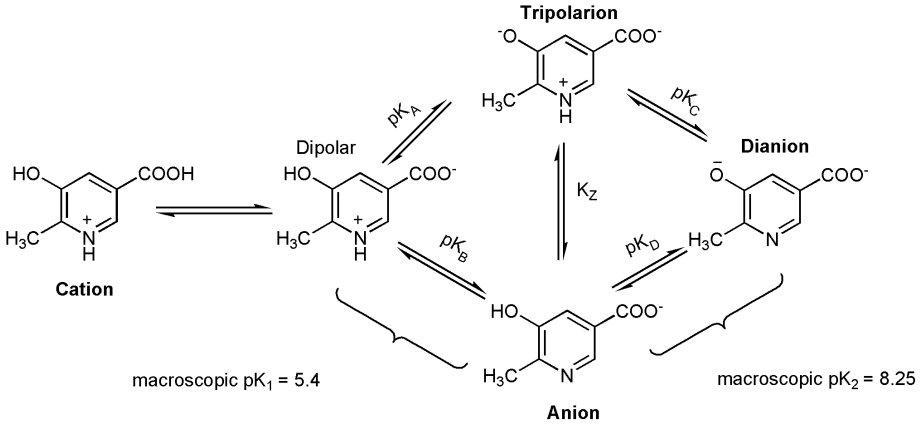


Figure 10. Protonation status of MHPC at various pHs.

oxidized FMN to generate reduced FMN using NADH as a reductant. Recently, the structure of C_1 has been reported.[74] C_2 catalyzes the hydroxylation of HPA using oxygen and $FMNH^-$, $FADH^-$ or reduced riboflavin as co-substrates.[54,70]

For two-component flavin-dependent monooxygenases, the reduced flavin generated by a reductase has to be transferred to a corresponding monooxygenase. The mode of reduced flavin transfer can occur via two modes – by free diffusion or through protein–protein interactions. Evidence from pre-steady-state kinetic experiments of several enzymatic systems suggests that the reduced FMN can be transferred from the reductase to the oxygenase efficiently without a requirement for protein–protein interaction.[15,52,56,75,76] Investigation of the reduced flavin transfer mechanism from C_1 to C_2 indicates that the reduced flavin can be transferred *via* free diffusion and the rate-limiting step of the process is the release of $FMNH^-$ from C_1.[15,76] In HPAH, the substrate HPA acts as an effector to stimulate the reduction of FMN by changing the enzyme into a more active conformation.[56,74,76] The rate constant of the FMN reduction increases by about 30 times in the presence of HPA. Recently, the X-ray structure of C_1 has been reported. Studies using small angle X-ray diffraction analysis indicate that the presence of HPA induces an overall shape expansion of the enzyme.[74] Presumably, this conformational change occurs to facilitate formation of a more active conformation of C_1 in which it is able to interact more effectively with NADH. Interestingly, this effector enhancing NADH reduction of flavin is not present in all reductases involved with two-component monooxygenase systems. Besides C_1, experimental evidence of this property has only been observed for HPAH from *Pseudomonas putida*.[77] However, based on the sequence analysis, several reductases such as the reductase of the nitrilotriacetate monooxygenase system have been proposed to have the ability to bind with their effectors and achieve rate enhancement of flavin reduction.[54,73,74] Investigation on the control mechanism of the rate of flavin reduction indicates that the C-terminal domain of C_1 acts as an autoinhibitory domain in which the inhibition of NADH reduction is only alleviated upon HPA binding.[56] In a recent report, the residues Tyr207 and Phe216 were identified based on site-directed mutagenesis to be involved in HPA binding.[74]

For the oxygenation which occurs on C_2, $FMNH^-$ is the first substrate to bind to the enzyme. The binding of $FMNH^-$ to C_2 occurs very rapidly with a second order rate constant of at least 10^7 M^{-1} s^{-1} and a K_d value for the binding of ~1.2 μM.[70] The C_2 crystal structure shows that the isoalloxazine ring is buried inside the protein and is inaccessible to outside solvent.[59] This binding arrangement is commonly found in other two-component flavin-dependent monooxygenases.[60,78,79] The complex of C_2:$FMNH^-$ reacts with O_2 very quickly to form the C4a-hydroperoxyflavin intermediate with a rate constant of 1.1×10^6 M^{-1} s^{-1}. Molecular dynamics simulations indicate that the oxygen entrance into the active site of C_2 is controlled by the gate-keeping residue Phe266 (Fig. 9)[80] in which the side chain of Phe266 is

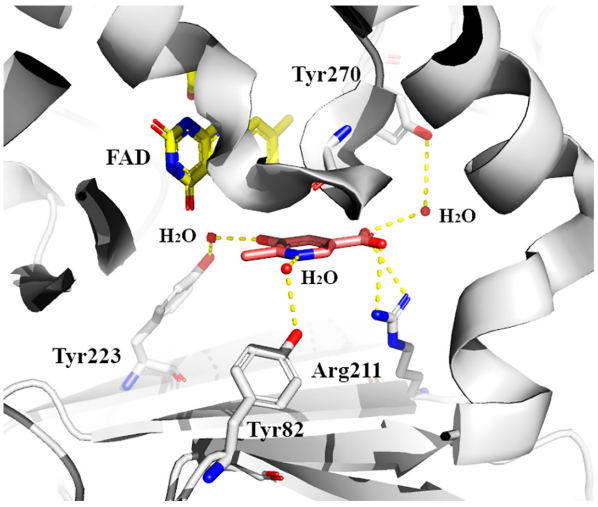


Figure 11. Important amino acid residues in the active site of MHPCO (pdb id: 3gmc).

required to move away to allow for oxygen diffusion to the C4a-position of flavin.

In the absence of HPA, the C4a-hydroperoxyflavin intermediate is quite stable because it slowly eliminates H_2O_2 with a rate constant of 0.003 s^{-1}. Similar rate constants for reduced flavin reacting with oxygen and for the elimination of hydrogen peroxide were also observed in HPAH from *Pseudomonas aeruginosa*.[36] This property is also unique to two-component monooxygenases because similar stability of C4a-hydroperoxyflavin in the absence of substrate can also be found in other two-component monoxygenases such as bacterial luciferase,[81] flavin-dependent dehalogenase,[53] styrene monooxygenase,[82] and FMN-dependent monooxygenase ActVA-ActVB from *Streptomyces coelicolor*.[83] Investigations using site-directed mutagenesis and pre-steady-state kinetics suggest that a cavity located in front of the reduced FMN are perfectly fit for housing the peroxide moiety of the C4a-hydroperoxyflavin intermediate[13,59] which can be stabilized by an H-bond between the Oγ atom of Ser171 and the flavin N(5)-H atom.[84] Interruption of this H-bond in a Ser171Ala variant increases the rate of H_2O_2 elimination by about 1400-fold compared with that of the wild-type enzyme.[84]

The His396 side chain located at the active site of C_2 (Fig. 9) is important for C4a-hydroperoxyflavin formation since when this residue was mutated to alanine, valine, and asparagine, the rate constant for the formation of intermediate decreased from 1.1×10^6 M^{-1} s^{-1} to $3.3–3.8 \times 10^3$ M^{-1} s^{-1}.[84] The data imply that His396 may act as an instantaneous proton provider for the formation of C4a-hydroperoxyflavin. This residue is also important for maintaining the protonation of C4a-hydroperoxyflavin by acting as a hydrogen bond acceptor.[85] Investigation using solvent kinetic isotope effects indicates that the proton transfer from His396 occurs rapidly before the formation of C4a-hydroperoxyflavin and it is not the rate-limiting step of the overall process of C4a-hydroperoxyflavin formation.[85] The information correlates well with results from density functional calculations in that the formation of C4a-hydroperoxyflavin occurs via a proton-coupled electron transfer mechanism in which molecular oxygen accepts an electron from reduced FMN simultaneously with a proton transfer from a protonated histidine (His396). The C_2 active site is evolved to optimize this proton-coupled electron transfer process so well because the energy associated with the C4a-hydroperoxyflavin formation is nearly barrierless.[86]

Analysis by rapid-quench flow experiments indicates that the C4a-hydroperoxyflavin intermediate hydroxylates HPA to generate DHPA with a hydroxylation rate constant of 15–17 s^{-1}.[87] The hydroxylation rate constant is pH-independent over the range of pH 6.2–9.9. Site-directed mutagenesis, pH-dependent, and pre-steady-state kinetics studies suggest that His120 (Fig. 9) may be positively charged throughout this pH range to facilitate formation of the deprotonated HPA, which is more prone to the oxygenation reaction via an electrophilic aromatic substitution mechanism.[88] Site-directed mutagenesis studies also show that Ser146 (Fig. 9) is important for the proper binding of substrate for efficient hydroxylation, as the rate of hydroxylation in the Ser146Ala variant was six-fold slower than the wild-type enzyme.

Flavin-dependent monooxygenases that catalyze additional reactions after hydroxylation

Some of flavin-dependent monooxygenases that can incorporate a hydroxyl group into aromatic or phenolic substrates can also further catalyze additional reactions. Here, we discuss two enzymatic systems in which one catalyzes breakage of an aromatic ring and another one catalyzes a group elimination reaction in addition to the initial hydroxylation reaction.

Flavin-dependent monooxygenase that catalyzes oxidative aromatic ring-cleavage reactions (single-component monooxygenase). Flavin-dependent monooxygenases that catalyze aromatic ring-cleavage reactions include 2-methyl-3-hydroxypyridine-5-carboxylic acid oxygenase (MHPCO)[89] and 5-pyridoxic acid oxygenase (5PAO).[90] These two flavoenzymes can be classified as members of Class A single-component flavoprotein monooxygenases that mainly catalyze hydroxylation of aromatic substrates.[10] MHPCO was successful overexpressed in *Escherichia coli* and its three-dimensional structure is known. Much information regarding the MHPCO reaction and kinetic mechanisms is available. This review will highlight the current understanding of the MHPCO reaction as a representative flavoenzyme that can catalyze aromatic ring-cleavage.

MHPCO is a single-component flavoenzyme monooxygenase that catalyzes the ring-opening reaction of a pyridine compound derivative, MHPC, to form an aliphatic product, α-(N-acetylaminomethylene) succinic acid (AAMS) [Fig. 6(C)]. This reaction is part of the vitamin B6 degradation pathway.[91–93] Oxygen isotope labeling experiments showed that only one atom of molecular oxygen was incorporated into the AAMS product, while another atom of oxygen was from H_2O.[94] Similar to other flavoenzyme monooxygenases, the reaction of MHPCO can be divided into two parts – reductive and oxidative half-reactions – similar to the reaction of PHBH.[95]

During the reductive half-reaction, investigations using a substrate analog indicated that the tripolar ionic form of MHPC (Fig. 10) is the form that binds to the oxidized enzyme to form an active MHPCO–MHPC complex.[96] The nitrogen atom of the substrate presumably remains protonated throughout the catalytic reaction. Site-directed mutagenesis studies

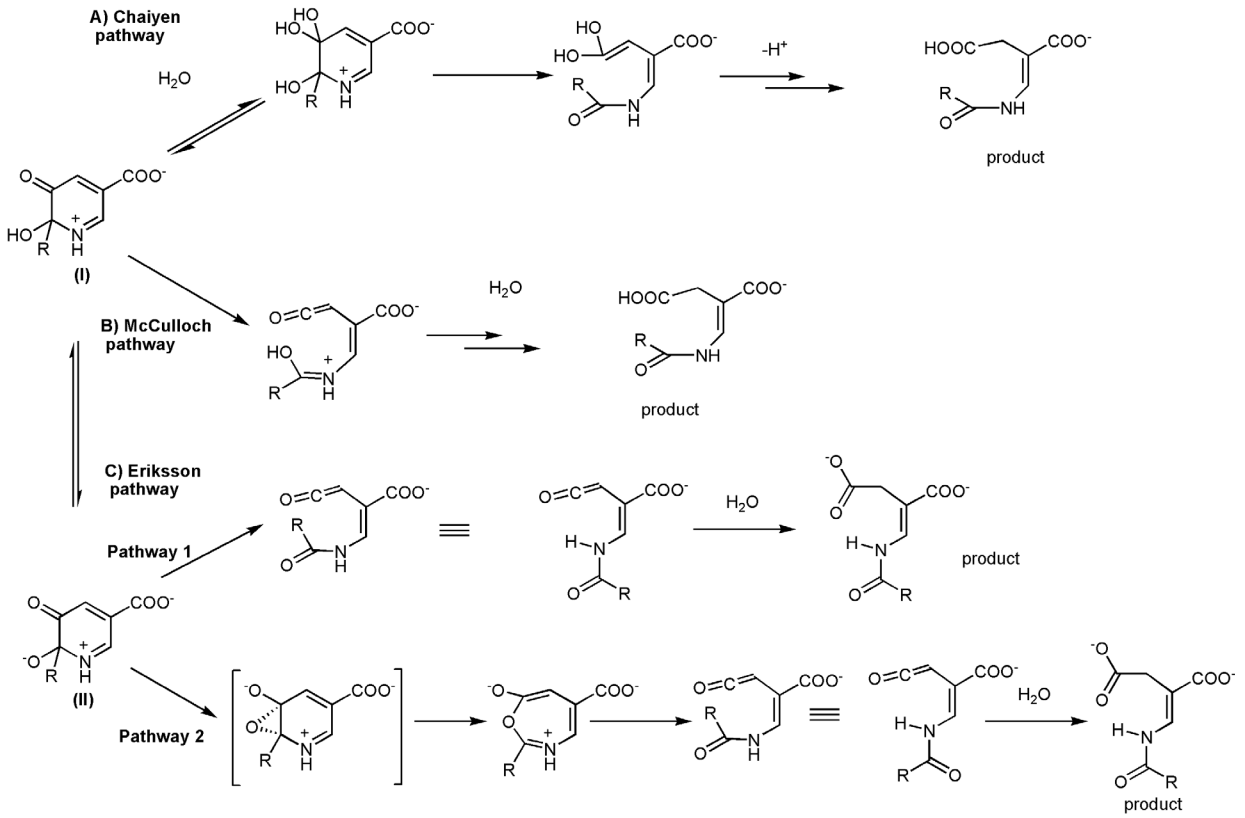


Figure 12. Proposed reaction mechanisms by Chaiyen et al., McCulloch et al., and Eriksson et al.

indicate that the protonation status of MHPC is probably stabilized by Tyr82 which can form an H-bond with H_2O and the nitrogen atom of the substrate as shown in Figure 11.[30,97] Tyr82 is crucial for the selective binding of the tripolar ionic form of the substrate. Upon changing Tyr82 to histidine and phenylalanine, the enzyme variants lost their selectivity for binding the tripolar ionic form of the substrate.[97]

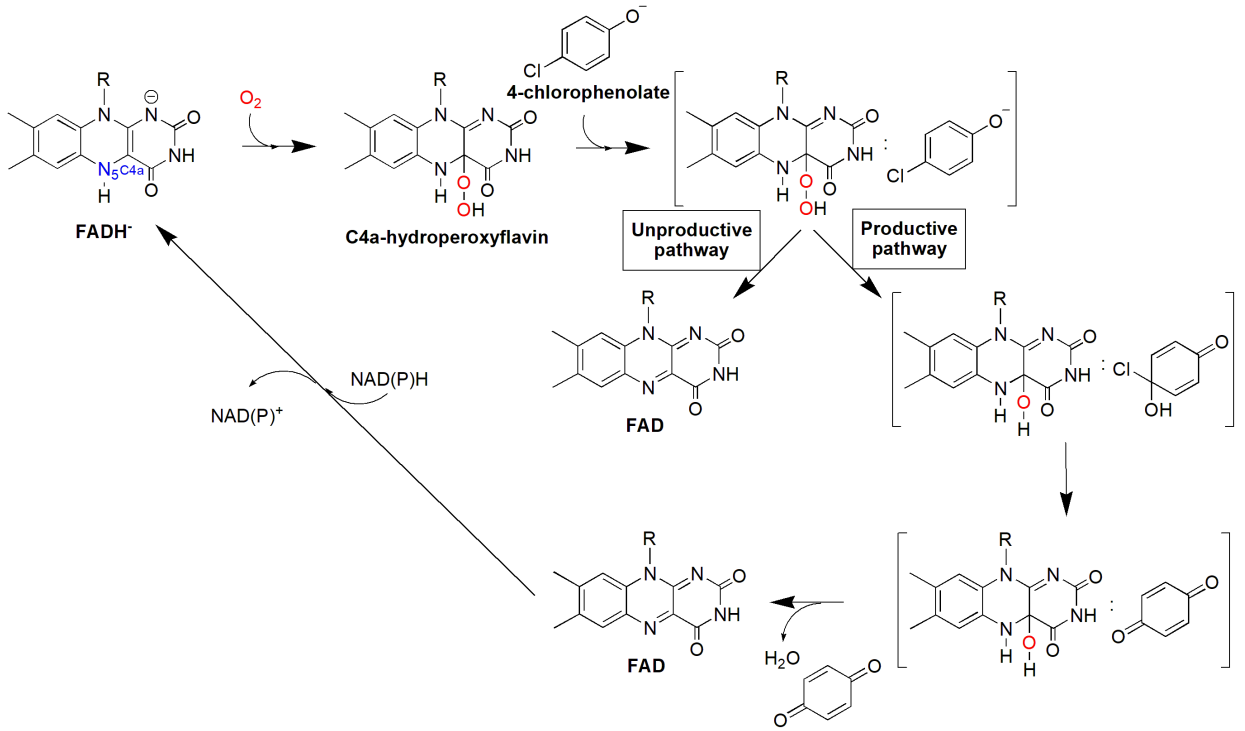


Figure 13. Kinetic mechanisms of the HadA reaction with 4-chlorophenol.

MHPCO can also bind to the substrate analogues 5-hydroxynicotinic acid (5HN) and N-methyl-5-hydroxynicotinic acid (NMHN); the K_d values for binding of MHPC, 5HN, and NMHN are 9.2 μM, 5.2 μM, and 55 μM, respectively.[96] Similar to PHBH, the binding of MHPC or substrate analogues (5-HN and NMHN) to MHPCO can increase the reduction rate of FAD by NADH. In the presence of MHPC, the flavin reduction rate is about 1600 time faster than the reaction without MHPC.[96] Based on X-ray structures, site-directed mutagenesis and steady state kinetics, the data suggest that Tyr270 (Fig. 11), which can form an H-bond with a carboxylic group of the substrate through a water molecule, is crucial for maintaining the optimal conformation for reduction of FAD by NADH.[98]

During the oxidative half-reaction, the complex of reduced enzyme with MHPC reacts with molecular oxygen to form the C4a-hydroperoxyflavin intermediate with a bi-molecular rate constant of 5.5×10^4 M^{-1} s^{-1}.[94] This reactive intermediate can oxygenate an aromatic substrate via an electrophilic aromatic substitution mechanism and generates the hydroxylated product and C4a-hydroxyflavin. 5HN and NMNH can be used as efficient substrates with hydroxylation efficiencies of 92% and 70%, respectively, compared with the efficiency of MHPC of 91%.[94,96,99] 5HN is a useful substrate for investigating the reaction mechanism of MHPCO because the compound serves as a good substrate for MHPCO,[96] and unlike MHPC, 5HN is commercially available. Comparison of the crystal structures of the MHPCO–5HN and MHPCO–MHPC complexes indicates that the binding mode of 5HN is almost the same as that of MHPC.[98] Moreover, the rate constants of the MHPCO reaction using 5HN as a substrate are in the same range or slightly higher than the reaction using MHPC.[94,96]

After hydroxylation, the process by which the hydroxylated product undergoes a ring opening reaction is still unclear and remains a mechanistic challenge for this enzyme. Three mechanisms have been proposed as shown in Figure 12. Mechanisms **A** and **B** were proposed by Chaiyen et al. and McMulloch et al., respectively. In both of these reactions, the ring-cleavage reaction starts from the protonated hydroxy-MHPC (**I**) (Fig. 12). In Chaiyen's pathway,[94–96] the protonated hydroxy-MHPC is attacked by water at the C3-keto moiety group and is followed by rearrangements to yield AAMS as the product. In McMulloch's pathway,[30] the protonated hydroxy-MHPC undergoes an electrocyclic ring-opening to form a ketene intermediate prior to attack by a water molecule. Recently, the Eriksson group performed density functional theory (DFT) calculations on a small model containing only C4a-hydroperoxyflavin and substrate (using 5HN as substrate).[100] The results suggest that both proposed mechanisms previously mentioned are less likely to occur since the activation energy for both of them are high ($\Delta\Delta G^{\#}_{gas}$ = 38.5 kcal mol^{-1} and 41.9 kcal mol^{-1} for the Chaiyen pathway and McCulloch pathway, respectively). However, these values are not consistent with rate constants measured experimentally. The DFT calculations also suggest that the ring-opening process must occur in the protein active site because in aqueous solution, the free hydroxylated 5HN can undergo re-aromatization to form the hydroxylated aromatic product spontaneously.[100] Based on DFT calculations, after hydroxylation, the proton of the protonated hydroxy-MHPC (**I**) is proposed to be transferred to C4a-hydroxyflavin intermediate. The deprotonated hydroxy-MHPC (**II**) can proceed with the ring-cleavage reaction with a much lower activation energy. Two pathways were proposed for the ring-cleavage process, starting from the deprotonated hydroxy-MHPC (**II**) (Fig. 12, pathway C). The energies associated with the two pathways are reasonably low ($\Delta\Delta G^{\#}_{gas}$ = 14.1 kcal mol^{-1} and 11.1 kcal mol^{-1}), allowing the reaction to proceed.[100,101] However, no experimental support is available to support results from the DFT calculations.

Flavin-dependent dehalogenase that catalyzes additional dehalogenation or denitration of aromatic compounds (two-component monooxygenase). Halogenated compound waste is found wide spread in the biosphere as a consequence of their heavy usage as solvents, pharmaceuticals[102] and agrochemicals such as pesticides, herbicides, insecticides, and fungicides. The toxic nature of halogenated compounds and their potential long-term persistence in the environment raises a serious threat to the environment, and to the health of humans and animals. Oxidative dehalogenation involves the replacement of a halogen with a hydroxyl group from molecular oxygen or hydrogen peroxide which typically can be catalyzed by flavin-dependent oxygenases, cytochrome P450 oxidases, and dehaloperoxidases.[17,103–105]

Several aerobic microbes possess dehalogenases with the promising ability to degrade halogenated compounds to much less toxic compounds such as benzoquinone.[52,53,106] Degradation of chlorophenols can be carried out by various enzymatic systems such as *tft* clusters from *Burkholderia cepacia* AC1100,[48] *tcp* clusters from *Ralstonia eutropha* JMP134,[107] *had* clusters from *Ralstonia pickettii* DTP0602,[53,108] and *pcp* clusters from *Sphingobium chlorophenolicum*.[109] Many enzymes that catalyze oxidative dehalogenation can use a broad range of substrates.[110,111] A well-studied single-component dechlorinase is PcpB from *S. chlorophenolicum*. This enzyme has FAD as a prosthetic group and uses NADPH and molecular oxygen as co-substrates for the conversion of pentachlorophenol to 2,3,5,6-tetrachlorobenzoquinone. The quinone product can be chemically reduced to 2,3,5,6-tetrachloro-*p*-hydoquinone.[109,112]

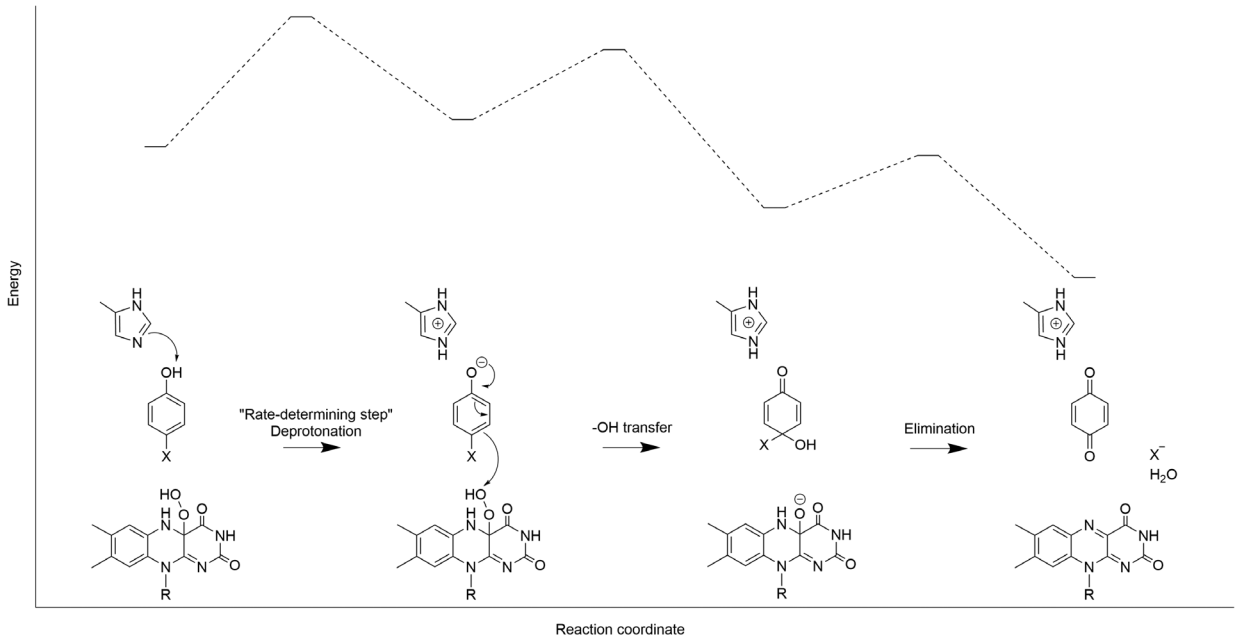


Figure 14. The proposed model and energy diagram for oxidative dehalogenation and denitration of HadA.

Many oxidative dechlorination reactions are also catalyzed by two-component flavin-dependent enzymes. Of these enzymes, the reaction mechanism of HadA from *R. pickettii* is the most extensively studied [Fig. 6 (D)].[52,53,106] Transient kinetics based on stopped-flow and rapid-quench flow experiments have shown that HadA binds to reduced flavin as the first substrate to form a binary complex which can further react with oxygen to generate C4a-hydroperoxyflavin (Fig. 13).[53] Previous investigations of TftD also detected a species which was speculated to be a C4a-flavin adduct (C4a-hydroperoxyflavin or C4a-peroxyflavin), but transient kinetic experiments have never been carried out with the TftD system.[113] Studies of PcpB also demonstrated the existence of C4a-flavin adducts.[109,112] 4-Chlorophenol binds to the HadA enzyme after the reactive intermediate C4a-hydroperoxyflavin forms.[53] The ternary complex then bifurcates into the coupling path of hydroxylation plus Cl^- elimination and the uncoupling path of H_2O_2 elimination (Fig. 13).[53]

Recently, studies using various substrates and density functional analysis to investigate the reaction mechanism of HadA indicate that HadA has dual activities of oxidative dehalogenation (hydroxylation plus halide elimination) and denitration (hydroxylation plus nitro elimination). Quantitative structure and reactivity relationship (QSAR) analysis was performed in order to correlate the rate constants associated with the individual steps of the HadA reactions with various physio-chemical parameters. This analysis indicated that the deprotonation of the phenolic substrate is the step with the highest activation energy amongst all of the steps involved in product formation because the deprotonation of substrates controls the overall reaction of product formation. Phenolic substrates with a lower pK_a give higher rate constants of hydroxylation than substrates with higher pK_a values, indicating that substrate deprotonation has a higher energy barrier than the other steps such as –OH transfer, and thus controls the hydroxylation step (Fig. 14). These results contradict previous TftD computational calculations. Results from quantum mechanical and molecular mechanical (QM/MM) calculations of TftD showed that the –OH

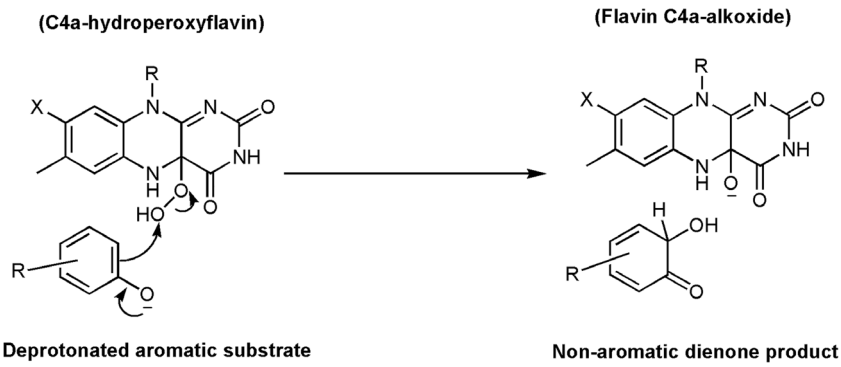


Figure 15. Electrophilic aromatic substitution mechanism.

transfer has the highest energy barrier and did not give a high energy barrier of substrate deprotonation, thus explaining why the proton and –OH transfer is a concerted process.[114]

The substrate deprotonation mechanism of HadA and other two-component monooxygenases are likely different from that of single-component monooxygenases. The substrate deprotonation mechanism of PHBH is mediated through an H-bond network on the enzyme surface through the active site.[9] The deprotonation of pOHB is important for the –OH transfer from C4a-hydroperoxyflavin to the substrate via an electrophilic aromatic substitution mechanism which is the key step that controls the hydroxylation.[115–118] QM/MM studies of phenol hydroxylase suggest that binding of the phenol substrate to Asp54 facilitates substrate deprotonation to the phenolate form.[119] For MHPCO, the hydroxypyridine binds to the enzyme in the deprotonated form[95] and the enzyme active site environment ensures substrate deprotonation.[97] Whereas for HadA, TftD, and TcpA, an active site His was proposed to be the key catalytic base for initiation of proton abstraction.[48,120] However, it still remains unclear how the environment of the HadA active site can promote additional group elimination in addition to hydroxylation.

Current mechanistic understanding and challenging aspects of flavin-dependent monooxygenases that hydroxylate aromatic compounds

Hydroxylation mechanism. Studies of flavin-dependent monooxygenases have been instrumental in understanding the mechanisms of oxygen activation in many flavoenzymes.[13,17,86,121,122] There is no specific binding site for oxygen in flavoenzymes that use oxygen as a substrate. The combined approaches of molecular dynamics simulations, site-directed mutagenesis and transient kinetics indicate that oxygen diffuses through specific pathways in these enzymes to enter the area close to the C4a-position of the flavin of C_2,[80] alditol oxidase from *Streptomyces coelicolor* A3,[80] aryl alcohol oxidase,[123] etc. The reaction of reduced enzyme with molecular oxygen was proposed to occur via a single electron transfer from reduced flavin to molecular oxygen to form a radical pair of flavin semiquinone and superoxide anion before the radical pair collapses to form a C4a-peroxyflavin intermediate prior to its protonation to form C4a-hydroperoxyflavin (Fig. 2, Path 1).[11,124–126] Recently, DFT calculations of the oxygenase component (C_2) of *p*-hydroxyphenylacetate 3-hydroxylase (HPAH) indicate that the formation of C4a-hydroperoxyflavin occurs via a proton-coupled electron transfer mechanism[86] in which the single electron transfer process to generate the radical pair is tightly coupled with proton transfer (Fig. 2, Path 2). This mechanism is also consistent with the mechanism of C4a-hydroperoxyflavin formation in pyranose 2-oxidase (P2O).[122] P2O is an oxidase that can form C4a-hydroperoxyflavin as an intermediate before elimination of the typical H_2O_2 product for flavoenzyme oxidases. Although all of the flavin-depdendent monooxygenases can form C4a-flavin oxygen adducts as intermediates, different monooxygeases facilitate different protonation states of the adduct (either to form C4a-hydroperoxyflavin or C4a-peroxyflavin), depending on its requirement to act electrophile or nucleophile.[10,14,17] It still remains unclear at this stage how the environment inside these proteins can control different protonation states so that the enzymes can be fine-tuned to perform the expected tasks.

Based on the currently available information, the mechanisms of –OH group transfer from C4a-hydroperoxyflavin to aromatic substrates in flavin-dependent monooxygenases are mostly consistent with electrophilic aromatic substitution in which the –OH moiety of C4a-hydroperoxyflavin acts as an electrophile (Fig. 15). In PHBH and MHPCO, the QSAR correlation using flavin analogues with different substituents at the 8-postion indicates that the rate constants of hydroxylation increase with more electron withdrawing substituents due to the stabilization of the flavin C4a-alkoxide leaving group after hydroxylation (Fig. 15). These data suggest that an OH-moiety of C4a-hydroperoxyflavin is transferred as an electrophile to aromatic substrate.[118,127] The QSAR analysis for the correlation of k_{cat} values with the E_{LUMO}–E_{HOMO} energy of –OH transfer in a series of fluorinated *p*-hydroxybenzoate in PHBH[128] and halogenated phenol for phenol hydroxylase[119] indicate that this energy gap controls the hydroxylation because the k_{cat} values of PHBH and phenol hydroxylase are higher when the molecular orbital energy gaps of C4a-hydroperoxyflavin and the substrates are lower. The results suggest that the –OH transfer is the rate-limiting step for PHBH and phenol hydroxylase. In the HadA reaction discussed previously, the –OH transfer is not the rate-limiting step in the reaction, but rather, the deprotonation process is the step with the highest energy.[106] Therefore, within the same –OH incorporation via electrophilic aromatic substitution mechanism, the energy landscape that controls these reaction steps are varied. It would be interesting to learn more about the energy landscapes associated with other flavin-dependent monooxygenases. Although most of the mechanisms are consistent with the electrophilic aromatic substitution, it remains unclear for the mode of substrate activation to facilitate this –OH group transfer. As the deprotonated form of the substrate would promote the incorporation of +OH into the aromatic ring, the active site features that facilitate the process are undoubtedly important for this catalysis. In the reaction catalyzed by PHBH, it was clearly demonstrated that protein and FAD conformational changes coupled with interactions through H-bond networks control the process of substrate deprotonation. Studies using QM/MM methods for

PHBH,[129] phenol hydroxylase[119] salicylate hydroxylase[130] suggest that the deprotonation of the aromatic substrate increases the rate of hydroxylation by decreasing the energy barrier. In PHBH, the hydrogen bond network between Pro293, Tyr201, Tyr385, a water molecule, and His72 (Fig. 8) promotes formation of the deprotonated from of the hydroxyl group of pOHB by decreasing the pK_a of pOHB by about 2 units.[22] Interruption of this H-bond network by site-directed mutagenesis resulted in a decrease in the hydroxylation rate constant.[131,132] In other systems, although candidate residues for interacting with a substrate hydroxyl group have been proposed (previously discussed), detailed explanation regarding their control of substrate deprotonation are less clear and remain to be investigated.

Mechanisms related to additional reactions. A major challenge regarding the reaction mechanism of MHPCO is to understand how the enzyme active site facilitates additional ring-cleavage after hydroxylation. It is not clear how the active site promotes attack of the dienone hydroxylated MHPC by water and why this water addition does not occur in other aromatic hydroxylases. The ring-breaking mechanisms proposed by DFT calculations remain to be investigated to for consistency with experimental results. An understanding of the active site architecture that is crucial for this C–C bond breaking will be valuable for future applications in biocatalysis or enzyme redesign to construct an organic-biomimetic catalyst capable of converting aromatic to aliphatic compounds without involvement of metal.

Although the hydroxylation of HadA likely proceeds via an electrophilic aromatic substitution mechanism in which the substrate deprotonation controls the overall process, it is still unclear why the active site of HadA promotes group elimination in addition to hydroxylation. The next step for investigating the HadA reaction requires an X-ray structure of the HadA:substrate complex so that the residues involved in the group elimination reaction can be identified and further analyzed by site-directed mutagenesis. An understanding of the group elimination reaction catalyzed by HadA should be valuable for biocatalysis applications, as it would allow the incorporation of the group elimination activity of HadA in other flavin-dependent hydroxylases through enzyme engineering, allowing these enzymes to perform tasks that are useful for detoxification or for the synthesis of valuable chemicals.

Potential use in biocatalysis. Regio- and stereospecific oxygen insertions by oxygenase enzymes are useful for industrial applications for production of pharmaceutical ingredients and fine chemicals.[133–136] Expansion of enzymatic specificity is generally accomplished by enzyme engineering through a random, semi-random, or rational approach.[137,138] Various aromatic hydroxylases that are useful in biocatalysis have been documented.[21] The HPAH system can efficiently synthesize antioxidants 3,4,5-THPA and 3,4,5-THCA from HPA and *p*-coumaric acid, respectively. Rational engineering of HPAH resulted in the production of the Tyr398Ser variant that was shown to be more effective than the wild-type enzyme for preparation of 3,4,5-THCA.[139,140] HPAH possesses several properties that are suitable for biocatalytic applications, including the ability to obtain a high yield of protein overexpression during production, stability over a pH range of 6–10[87] and high thermostability.[139] Engineering of HPAH to use an aniline instead of a phenolic compound was achieved in the Ser146Ala variant which can convert 4-aminophenylacetic acid to 3-hydroxy-4-aminophenylacetic acid.[140] This reaction should be useful for the synthesis of many pharmaceutical compounds.[141] Recently, the reaction of HPAH has been demonstrated to be applicable to conversion of agricultural waste such as phenolic compounds from palm oil mill effluent to antioxidants such as THCA.[142]

Dehalogenation and denitration by the HadA reaction should be useful for biodetoxification of halogenated phenols and nitrophenols accumulated in environment. Potential applications in this aspect should be further explored by engineering cells to contain clusters of enzymes capable of degrading these toxic compounds.[105,143,144] In addition, low-cost detoxification via biodegradation also offers a means for converting toxic waste into value-added products, and have the potential to contribute to waste biorefining and the development of a circular economy. The resulting quinone and hydroquinone resulting from the HadA reaction can be further metabolized by other enzymes in the *had* operon to yield common metabolites in cells. Some of these metabolites such as di-acid and hydroxyl-acid can be used as commodity chemicals such as precursors in bioplastics.[7]

In order to successfully apply these monooxygenases in industrial applications, further developments regarding all engineering aspects need to be achieved. Comprehensive activity profiling and enzyme engineering are necessary for understanding the value of each enzyme in biocatalysis. This can be achieved by rational design, directed-evolution or by the use of ancestral reconstruction and characterization of evolutionary pathways between distinct functional families.[145–147] Enzyme engineering is necessary to overcome protein stability issues in some hydroxylases such as HadA. In order to accomplish economically viable biocatalysis, process optimization to design efficient scale-up is necessary for industrial implementation. Recent improvements in enzyme immobilization methods should also be helpful in providing enzyme reusability, recyclability, and operational stability, thus leading to reduced cost of

production. The techniques employed for hydroxylase immobilization in the past included carrier bonding, cross-linking, and entrapment.[148–150]

Conclusions

Many flavoenzymes catalyze hydroxylation of phenolic compounds and hydroxylation plus additional reactions have been isolated and characterization in both single component and two component flavin-dependent hydroxylase. Despite some common features in the overall catalytic reactions, single-component and two-component aromatic hydroxylase are different in most catalytic respects. MHPCO and HadA are examples of one-component and two-component monooxygenases that can catalyze additional reactions beside hydroxylation. It is not known how the key active site features in these enzymes facilitate these additional reactions. Many outstanding questions regarding the mechanistic details of these monooxygenases remain to be investigated. Further developments in engineering aspects are necessary for the successful implementation of these enzymes in large-scale or industrial applications.

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