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Biochemical and Biophysical Research Communications 338 (2005) 590–598
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Review

Dynamics involved in catalysis by single-component and two-component flavin-dependent aromatic hydroxylases ☆,☆☆

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Received 13 September 2005
Available online 26 September 2005

Dedicated to Professors Osamu Hayaishi and the late Howard S. Mason in celebration of their important discovery, 50 years ago, of oxygenase enzymes.

Abstract

Flavoprotein monooxygenases are involved in a wide variety of biological processes including drug detoxification, biodegradation of aromatic compounds in the environment, biosynthesis of antibiotics and siderophores, and many others. The reactions use NAD(P)H and O_2 as co-substrates and insert one atom of oxygen into the substrate. The flavin-dependent monooxygenases utilize a general cycle in which NAD(P)H reduces the flavin, and the reduced flavin reacts with O_2 to form a C4a-(hydro)peroxyflavin intermediate, which is the oxygenating agent. This complicated catalytic process has diverse requirements that are difficult to be satisfied by a single site. Two general strategies have evolved to satisfy these requirements. *para*-Hydroxybenzoate hydroxylase, the paradigm for the single-component flavoprotein monooxygenases, is one of the most thoroughly studied of all enzymes. This enzyme undergoes significant protein and flavin dynamics during catalysis. There is an *open* conformation that gives access of substrate and product to solvent, and a *closed* or *in* conformation for the reaction with oxygen and the hydroxylation to occur. This *closed* form prevents solvent from destabilizing the hydroperoxyflavin intermediate. Finally, there is an *out* conformation achieved by movement of the isoalloxazine toward the solvent, which exposes its N5 for hydride delivery from NAD(P)H. The protein coordinates these dynamic events during catalysis. The second strategy uses a reductase to catalyze the reduction of the flavin and an oxygenase that uses the reduced flavin as a substrate to react with oxygen and hydroxylate the organic substrate. These two-component systems must be able to transfer reduced flavin from the reductase to the oxygenase and stabilize a C4a-peroxyflavin until a substrate binds to be hydroxylated, all before flavin oxidation and release of H_2O_2. Again, protein dynamics are important for catalytic success.

Keywords: Flavoprotein hydroxylase; Monooxygenase; Flavin hydroperoxide; Protein dynamics; Two-component flavin-dependent monooxygenase; Halogenase

The wide variety of flavoprotein monooxygenases

An important class of flavoproteins is the monooxygenase family, which uses NAD(P)H and O_2 as co-substrates. Single-component flavoprotein monooxygenases, such as *p*-hydroxybenzoate hydroxylase and phenol hydroxylase, play important roles in soil detoxification processes by hydroxylating a variety of aromatic and aliphatic compounds. One of the first studied enzymes of this class is salicylate hydroxylase, discovered by Hayaishi et al. [1]. Related flavoprotein monooxygenases in liver microsomes

☆ Financial support was received from the U.S. Public Health Service (Grant GM 647111 to D.P.B.) and the Australian Research Council (A 09906323 to B.E.).
☆☆ *Abbreviations:* FMO, flavin monooxygenase; PHBH, *p*-hydroxybenzoate hydroxylase; pOHB, *p*-hydroxybenzoate; HPAH, *p*-hydroxyphenylacetate hydroxylase; PAH, *p*-hydroxyphenylacetate; RebH, oxygenase responsible for halogenating tryptophan in the biosynthesis of rebeccamycin; PrnA, tryptophan-7-halogenase from *Pseudomonas fluorescens*; WT, wild type PHBH.
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doi:10.1016/j.bbrc.2005.09.081

(FMO) catalyze the oxygenation of a wide variety of nitrogen-, sulfur-, phosphorous-, selenium-, and other nucleophilic heteroatom-containing chemicals, including many drugs, in coordination with cytochromes P450 [2], and also in the regulation of the biosynthesis of auxin in plants [3]. Some other flavoprotein monooxygenases include human squalene monooxygenase (cholesterol biosynthesis) [4], ubiB, an oxygenase involved in ubiquinone biosynthesis [5], and ornithine-N^5-oxygenase [6], involved in biosynthesis of siderophores in microorganisms. In addition, there are flavoprotein monooxygenases that carry out Baeyer–Villiger oxygenations on aldehydes and ketones [7,8]. Recently, a number of two-component flavin-dependent monooxygenases have been described; these enzyme systems use a flavin reductase to produce reduced flavin that diffuses or is delivered to a monooxygenase component. Such two-component flavin-dependent enzyme systems are also involved in diverse biological reactions as mentioned above for single component enzymes.

Chemical issues

The overall reaction catalyzed by flavoprotein monooxygenases involves three general chemical processes. (A) Reduction of the flavin by NAD(P)H, (B) reaction of the reduced flavin with O_2 to provide a C4a-flavin (hydro)peroxide, which is the oxygenating reagent (it is the peroxide for electrophilic substrates and the hydroperoxide for nucleophilic substrates), and (C) binding, orienting, and activating the substrate for its oxygenation by the C4a-(hydro)peroxide. Each of these three processes has unique requirements and it is not surprising that more than one catalytic active site would be required. Two general strategies have evolved to deal with this complex chemical problem. First, in the case of the single-component flavin monooxygenases, for which *p*-hydroxybenzoate hydroxylase (PHBH) is the model [9–15], it has been found that the isoalloxazine ring of the flavin moves several angstroms and the protein undergoes significant rearrangements in the course of catalysis, so that effectively, there are multiple active sites.

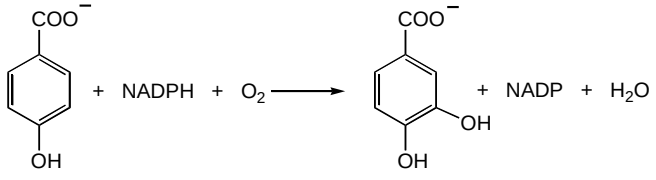
The second approach uses two components to separate the catalytic tasks. This group of two-component hydroxylases has been described mostly in the last decade, and functions by using an oxidoreductase to generate reduced flavin, and another enzyme, the oxygenase, to receive the reduced flavin, and react with O_2 and hydroxylate the substrate. Very little detailed mechanistic information is available on these systems. The two-component systems have no structural or sequence similarities to the one-component enzymes, and thus, these two groups probably evolved independently to carry out identical chemistry. The mechanism of transferring the labile reduced flavin from the reductase to the oxygenase in the two-component systems is not well understood, although recent investigations suggest that for most the reduced flavin diffuses to the oxygenase component quickly before it reacts with oxygen, so that appropriate hydroxylating species can be formed without production of H_2O_2 [16–18].

Reduction of the flavin by NAD(P)H in preparation for its reaction with O_2 occurs by hydride transfer stereospecifically, usually with the pro-*R* hydrogen of the reduced pyridine nucleotide transferring to the N5 of the flavin isoalloxazine [19] in reactions with deuterium isotope effects of 6–10 [20]. Reduction of the flavin is often a critical control point for catalysis by flavoprotein monooxygenases. Thus, for most of the known single-component flavoprotein aromatic hydroxylases, reduction is quite ineffective in the absence of substrate, and this control prevents the wasteful use of NAD(P)H that would produce reactive oxygen species such as H_2O_2. In contrast, controlling the rate of reduction does not regulate the Baeyer–Villiger oxygenases or the mammalian flavin monooxygenases [21–23]. NADPH reduces these enzymes at the same rate in the presence or absence of substrates and the NADP product remains tightly bound. The reduced enzyme-bound flavin then reacts with oxygen to form a C4a-(hydro)peroxyflavin that is quite stable in the absence of substrates; the bound NADP stabilizes the intermediate, which decays slowly over several minutes [21–23]. When substrate is present, reaction with the C4a-(hydro)peroxyflavin occurs quickly to yield oxygenated product [7,8]. Thus, turnover to produce H_2O_2 and oxidized flavin is avoided by a completely different mechanism than with the single-component aromatic hydroxylases.

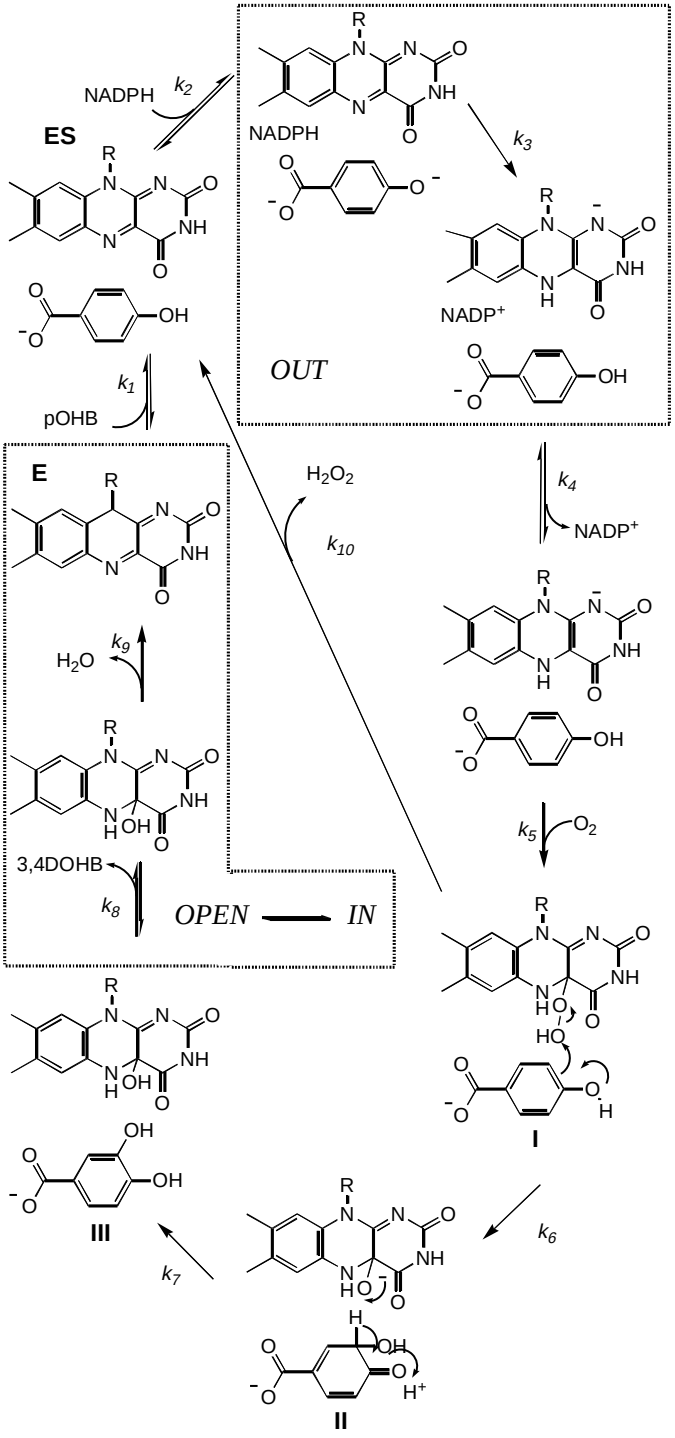
Aromatic substrate single-component flavoprotein hydroxylases

p-Hydroxybenzoate hydroxylase (PHBH)

PHBH is one of the most thoroughly studied enzymes and is the paradigm for the mechanisms of aromatic hydroxylases. PHBH catalyzes the reaction shown in Scheme 1. The chemistry involving the flavin in the individual reactions of this enzyme is illustrated in Scheme 2. Each of the flavin forms shown in Scheme 2 has a unique absorbance and/or fluorescence signature and can be identified. For example, Fig. 1 shows spectra derived from stopped-flow spectrophotometric studies of various species involved in the reaction of reduced PHBH with oxygen in the presence of the alternative substrate, 2,4-dihydroxybenzoate [24]. In the PHBH mechanism, dynamics involving the protein and flavin are crucial in catalysis [13]. X-ray structures and kinetic analysis of wild type and mutant forms of PHBH [10,25–28] have revealed three conformations that



Scheme 1.



Scheme 2.

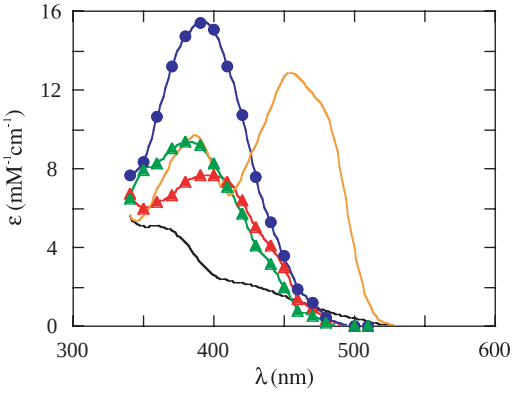


Fig. 1. Spectra of reduced (black), C4a-hydroperoxyflavin (red), C4a-hydroxyflavin (green), C4a-hydroxyflavin plus product dienone (blue), and oxidized flavin (orange). These are intermediates observed by stopped-flow spectrophotometry using 2,4-dihydroxybenzoate as substrate [24].

are important in catalysis: one in which the isoalloxazine is *in*, one in which it is *out*, and another in which the protein is in an *open* conformation. The *in* (or *closed*) conformation isolates the flavin from solvent and prevents solvent-induced breakdown of the flavin hydroperoxide to H_2O_2 and oxidized flavin (k_{10}). The *out* conformation permits access of the isoalloxazine to NADPH for reduction by hydride transfer to the N5 of the flavin [10,26–29]. The *open* structure permits import and egress of substrates and products, and was first recognized in the structure of Arg220Gln PHBH, a variant in which the *open* conformation is stabilized [30]. In addition to revealing the *open* structure, other structures of this variant showed for the first time where NADPH binds, and how, after its binding, additional dynamics of the protein are necessary to place the nicotinamide into proper juxtaposition to the FAD to carry out the stereospecific hydride transfer [30]. Scheme 2 indicates the various conformations used for the chemical steps in catalysis. The boxed regions represent the isoalloxazine position either as an equilibrium between *open* and *in*, or as *out*, as indicated. Species not boxed have *in* (*closed*) conformations. Fig. 2 displays crystal structures of PHBH in the three conformations, and the wire framing denotes the solvent access in each conformation. It is quite clear that the *open* conformation permits room for the substrate and product to access the active site, *out* permits access of solvent to the N5 of the isoalloxazine, and *in* protects both faces of the isoalloxazine from solvent. Residues shown to be critical to catalysis, and to dynamic processes, are labeled.

The catalytic process begins at state E, a dynamic equilibrium between the *open* and *in* conformations. The *open* conformation permits binding of *p*-hydroxybenzoate (pOHB) to form ES (k_1), and the binding of substrate shifts the equilibrium to the *closed* position. These steps occur so fast under physiological conditions that they cannot be measured by stopped-flow methods, implying that the *open*-to-*closed* transition is also very fast. The thermodynamic balance of this equilibrium has been shown to be critical to catalysis. For example, the Ala45Gly variant does not bind substrate rapidly because, as shown by enzymological and X-ray structural work [31], the smaller volume of the glycyl residue strongly stabilizes the *in* conformation inhibiting attainment of the *open* conformation necessary for initial substrate binding. By contrast, the related variant, Ala45Val, favors the *open* structure because the additional bulk of the valine side chain prevents movement to the *in* conformation. This variant permits very fast substrate binding, but the binding is much weaker

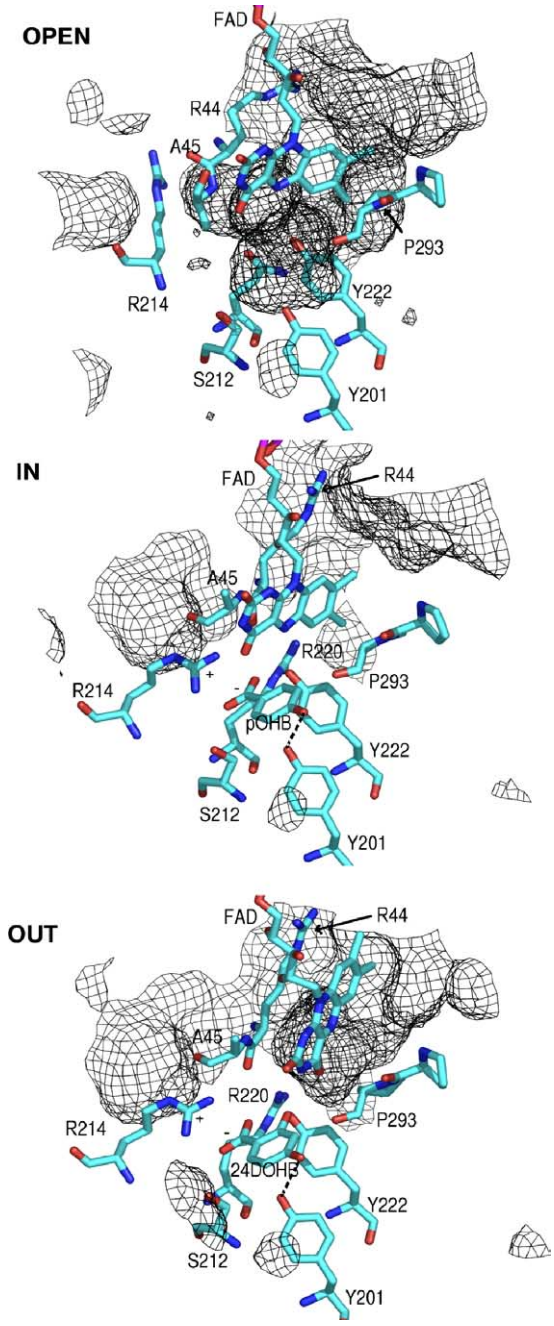


Fig. 2. Conformations of PHBH important for catalysis. Top, *open* conformation illustrated by Arg220Gln PHBH with no ligand bound (Gatti) (PDB ID, 1K0L); middle, *in* conformation illustrated by wild type PHBH with *p*-hydroxybenzoate bound (PDB ID 1PBE); bottom, *out* conformation illustrated by wild type PHBH with 2,4-dihydroxybenzoate bound (PDB ID 1DOD). The surface shown as black mesh illustrates the portion of the active site of PHBH that is accessible to solvent.

than with WT, because the *in* structure, which is required to accommodate the full complement of protein–substrate interactions, is disfavored. Thus, the binding of substrate is at least a two-step process, with initial binding followed by rearrangement to the *in* conformation.

The presence of substrate increases the rate of reduction of the flavin by NADPH by $>10^5$-fold [32]. This increased rate is due to the effects of substrate on the dynamics of *in*-to-*out* transformations (almost no change in redox potential of the flavin occurs on binding substrate). When the substrate is in place, the binding of NADPH (k_2) triggers the transition to the *out* conformation and reduction ensues (k_3) [33,34]. NADPH can bind before pOHB, but the *out* conformation is not achieved until substrate is bound. This phenomenon constitutes an elegant method to avoid production of reactive oxygen species in the absence of substrate.

Detailed kinetic and spectral studies of the His72Asn variant showed that a proton network is involved in controlling the rapid movement of the isoalloxazine from *in* to *out* to permit its reduction [33,34]. This proton network, which is illustrated in Fig. 3, permits rapid deprotonation of the substrate phenolic group, and the resulting phenolate interacts with the protein to initiate the conformational change that moves the isoalloxazine to the *out* position. Mutation of any of the residues of the proton network impairs catalysis. His72 is on the surface of the protein and provides the contact between the proton network and solvent. Therefore, the disrupted proton network of the His72Asn variant cannot efficiently deprotonate the substrate, so the isoalloxazine does not move to the *out* position rapidly. Studies using the Pro293Ser variant showed that the carbonyl of Pro293 is the actual sensor of the ionized substrate, and repulsion of this carbonyl by the substrate phenolate promotes the movement of the isoalloxazine to the *out* position as shown in Scheme 3 [34]. Pro293 is part of a highly conserved rigid peptide that cradles the isoalloxazine on its *re* side.

After reduction and loss of the NADP product (k_4), the anionic isoalloxazine moves back to the *in* position where it can react efficiently with O_2 (k_5) to form C4a-hydroperoxy-FAD (**I**). The movement back to the *in* position is driven

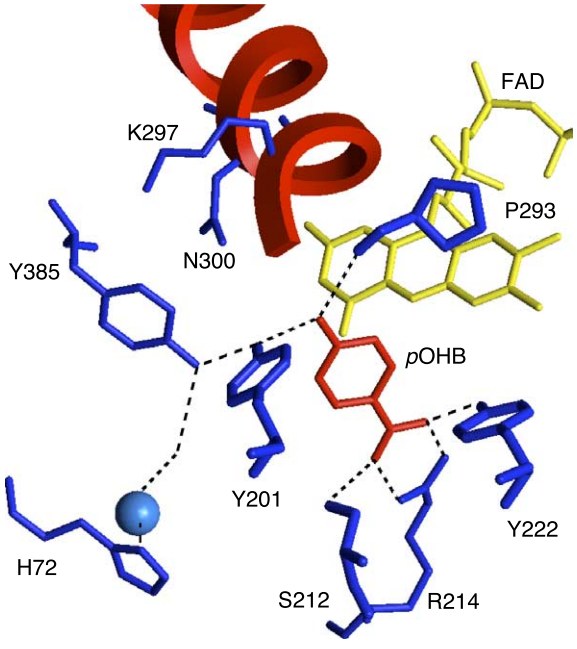
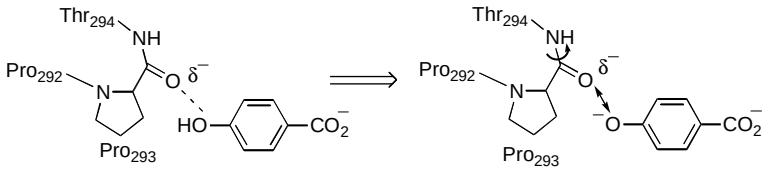


Fig. 3. X-ray structure of PHBH in the *in* position showing the hydrogen bond network. Figure constructed from PDB ID, 1K0L.



Scheme 3.

by the positive electrostatic field of the *in* active site, which attracts the $FADH^-$ anion [11]. This conformational rearrangement is the rate-determining step in optimum catalysis by the WT enzyme. The *in* conformation sequesters the flavin from solvent and positions it optimally to hydroxylate the substrate. Because the positive electrostatic field can only support one negative charge, the substrate, which has a higher pK_a than the reduced flavin, becomes protonated via the proton network.

The anionic reduced isoalloxazine reacts with O_2 (k_5) to initially form the C4a-flavin peroxide, but the nascent peroxide has a higher pK_a and rapidly abstracts (possibly indirectly) a proton from the phenolic substrate, forming both the more electrophilic C4a-hydroperoxide and the more nucleophilic substrate phenolate. This primes the electrophilic attack of the hydroperoxide on the phenolate to bring about hydroxylation (k_6), yielding the dienone form of the product and the C4a-flavin alkoxide [35]. Formation of the phenolate to activate the substrate for electrophilic attack is catalyzed by the same proton network that is used in the reduction of the flavin [36]. The nature of the transition state for the oxygenation step was probed by determining the rate of the oxygenation step using a series of FADs modified by substitution in the 8-position [35]. This position is accessible to solvent, and X-ray structures indicate that it does not have any obvious interactions with the protein. The inductive effects of the substituents (CN, Cl, CH_3, H, SCH_3, OCH_3, NH_2, S^-, $N(CH_3)_2$, and OH) modified the stability (pK_a) of the leaving group in the hydroxylation, the C4a-flavin alkoxide. Electron-withdrawing groups lowered the pK_a of the leaving group and increased the rate of oxygenation, whereas electron-donating groups had the opposite effect. A linear Hammett relationship was consistent with a transition state nearly midway between the reactants and products; the Hammett plot suggested that the leaving group, $FADHO^-$, bore a -0.42 charge. The positive electrostatic field surrounding the isoalloxazine in the *in* position was shown to be important for stabilizing this charge; thus, a charge-change variant, Lys297Met, which decreased the net positive electrostatic field surrounding the isoalloxazine in the *in* conformation, decreased the hydroxylation rate [11], while the Glu49Gln variant, which increased the net positive charge, resulted in greater rates of hydroxylation [37].

Rearomatization of the dienone (k_7) yields the product, 3,4-dihydroxybenzoate, and the C4a-hydroxyflavin. With *p*-hydroxybenzoate, rearomatization is very fast compared to the subsequent step and cannot be observed, while with 2,4-dihydroxybenzoate, this step is readily followed by stopped-flow spectrophotometry [24]. At this point the product is ionized as the phenolate, which probably favors the *open* conformation so that product can dissociate, analogously to the earlier substrate binding.

Overall, it is not surprising that catalysis of such complicated reactions as these requires significant protein (and flavin) conformational changes, together with proton exchanges. Although the thermodynamics of the overall hydroxylation reactions are favorable, the protein is required to orchestrate the conformational dynamics so that an orderly sequence of chemical events proceeds. With PHBH it can be seen that once initiated, the series of mechanistic steps proceeds like a "domino effect" to bring about efficient catalysis. Although many enzymes must also utilize critical dynamic events such as described above, in few cases does one have an intrinsic probe such as a flavin to enable experimental monitoring of dynamics.

Two-component flavin-dependent hydroxylases

Reductases of the two-component flavin-dependent monooxygenase system

The flavin reductases of the two-component flavin-dependent monooxygenase systems and their modes of regulation are diverse. Flavin reductases are known to have several roles in Nature. In addition to reducing flavins for the hydroxylases, they are also involved in reducing iron complexes in preparation for iron uptake and metabolism in prokaryotes, in reducing the iron center in ribonucleotide reductase [38], in reducing methemoglobin in red blood cells [39], and in the bioluminescence (luciferase) reactions of bacteria [40]. In fact, the luciferase systems are the first two-component flavin-dependent oxygenases to be studied. All of the known flavin reductases are small proteins with subunit masses of 17–32 kDa and most of them are dimeric. Some can only reduce FAD, some FMN, while others can reduce FAD, FMN, or riboflavin.

Described below are three different types of flavin reductases involved in two-component flavin dependent hydroxylase systems that were chosen to illustrate some of the different properties of the known flavin reductases.

HPAH reductase from Pseudomonas aeruginosa

This is the simplest type of flavin reductase, and in sequence and function is quite similar to the analogous reductase for HPAH from *Escherichia coli* [16,41]. It is a dimer of 19 kDa units. This reductase was cloned and expressed in

E. coli as a 4-His-tag fused to the C-terminus, resulting in a stable enzyme when expressed in *E. coli* [42]. This HPAH reductase is specific for NADH and does not bind the substrate, *p*-hydroxyphenyl acetate (HPA). It binds both FAD and NADH quite tightly, with K_d values of approximately 2 μM. Thus, some fraction of the enzyme can be isolated with FAD bound. NADH reduces FAD at a rate of $\sim 5\ s^{-1}$ at 4 °C, and then $FADH^-$, which binds much less tightly, is released from the enzyme. The reductase is also capable of reducing FMN and riboflavin, although the efficiency for the latter two is lower than for FAD [42].

HPAH reductase from Acinetobacter baumannii

This reductase is similar in sequence to that from *Pseudomonas aeruginosa*, but contains an additional 14-kDa domain at the C-terminus, resulting in a total molecular mass of 32 kDa [43]. The reductase binds FMN very tightly (K_d ~0.006 μM) so that the enzyme is isolated with bound FMN when cloned and expressed in *E. coli*. It does not bind or reduce FAD or riboflavin. The unique feature of this reductase is that the extra domain enables the enzyme to bind the substrate HPA, and this binding increases its rate of reducing FMN by several hundred-fold (to $\sim 300\ s^{-1}$ and a K_d ~25 μM at 4 °C). This regulation of catalysis by HPA is analogous to the control of catalysis in PHBH discussed above. In addition to regulating reduction of FMN, binding of HPA also increases the rate of release of $FMNH^-$ from the reductase, and this facilitates the transfer of reduced flavin to the oxygenase (J. Sucharitakul, personal communication). Thus, binding of HPA to the reductase causes important dynamic rearrangements of the reductase to facilitate both reduction of FMN and release of $FMNH^-$.

Reductase of the phenol hydroxylase system from Bacillus thermoglucosidasius A7

This reductase is similar in size to the *P. aeruginosa* HPAH reductase [44]. Curiously, this reductase is thought to bind FAD very tightly and use it as a cofactor; thus, it is a flavoprotein that reduces flavins. It reduces FMN and riboflavin about as efficiently as it does FAD. An X-ray structure of this enzyme is available [44], and it shows that NADH binds in a hairpin conformation with its adenine stacked on the pyridine.

Oxygenases of two-component flavin-dependent monooxygenases

Three oxygenases of the two-component flavin-dependent systems are described below: the HPAH oxygenases from *P. aeruginosa* and *Acinetobacter baumannii*, and the oxygenase involved in the halogenation of tryptophan in the biosynthetic path for rebeccamycin. These systems demonstrate some of the variety of mechanisms employed by the two-component systems. The hydroxylases use the same basic chemistry discussed above for PHBH (Scheme 2). $FADH^-$ binds to the oxygenase and reacts with oxygen to form a flavin-C4a-hydroperoxide, which then hydroxylates the substrate to yield product and the flavin-C4a-hydroxide. Water is eliminated from the flavin hydroxide to complete the chemistry of catalysis. In contrast to the single-component flavoprotein hydroxylases, after product dissociates, the FAD must then be released from the oxygenase and passed back to the reductase to be reduced for the next cycle of catalysis. Although the chemistry is similar, the regulation of the overall reaction and the dynamics involved are very different from those found with PHBH as described above.

HPAH oxygenase from P. aeruginosa

As mentioned above, the reductase in this HPAH system is not regulated by the substrate, HPA. Instead, catalysis is regulated at the level of the oxygenase. This oxygenase is only functional with FAD, not with FMN or riboflavin, although its reductase catalyzes the reduction of all three flavins. The oxygenase MW is ~60 kDa and it has sequence similarity to the *E. coli* HPAH [16,41,45]. It binds reduced FAD rapidly and tightly, and then reacts with oxygen with a second-order rate constant of $\sim 3.5 \times 10^5\ M^{-1}\ s^{-1}$ to form a stable C4a-flavin hydroperoxide [42]. In the absence of HPA, the half-life of the hydroperoxide is ~80 s at 4 °C. This contrasts with PHBH, which forms a hydroperoxide that is so unstable in the absence of its substrate that it cannot be observed by stopped-flow techniques under most conditions. The C4a-flavin hydroperoxide of HPAH has a spectrum like that of PHBH (Fig. 1), except that the molar extinction at 390 nm is greater (~13 vs. ~9 $mM^{-1}\ cm^{-1}$). When present, HPA binds rapidly to the enzyme–C4a-flavin hydroperoxide complex, and hydroxylation ensues at about 10^3-fold faster than the natural decay of the peroxide. The resulting C4a-hydroxyFAD is highly fluorescent, like the analogous species (**III** in Scheme 2) with PHBH. This fluorescence is lost as water is eliminated from the intermediate to form FAD still bound to the enzyme.

The oxidized FAD is released from the oxygenase at a much slower rate than other steps in the reaction; this bound FAD can be seen by its resolved and blue-shifted spectrum and its lack of fluorescence compared to FAD in solution [42]. When the bound FAD is released, a less-resolved spectrum and a large increased fluorescence result, typical of FAD in solution. The release of FAD clearly involves substantial conformational changes in the protein. Thus, because the release is energetically favorable (but slow), addition of oxidized FAD cannot reverse the process to reproduce the bound spectrum observed during catalysis. The release of FAD is the rate-limiting step in catalysis, so that during turnover the FAD is largely in the oxidized state [42].

HPAH oxygenase from A. baumannii

(Data from J. Sukaritakul, personal communication) The overall HPAH system from *A. baumannii* is significant-

ly different from that from *P. aeruginosa*. Although its reductase will only catalyze the reduction of FMN, the oxygenase functions nearly as well with FMN or with FAD and somewhat less efficiently with riboflavin. The oxygenase (MW ~50 kDa) reacts very rapidly with O_2 with second-order kinetics ($1.7 \times 10^6\ M^{-1}\ s^{-1}$) [46]. The resulting C4a-flavin hydroperoxide is stable in the absence of HPA, like that with the *P. aeruginosa* HPAH, thereby providing regulation similar to that of the *P. aeruginosa* enzyme, in addition to that at the reductive step. Curiously, when HPA is bound, the reaction with oxygen is considerably slower ($4.8 \times 10^4\ M^{-1}\ s^{-1}$) than in its absence, suggesting that the normal order of reaction is to form the C4a-flavin hydroperoxide and then to bind HPA. This is not required, however, because the slower rate with O_2 is nevertheless fast enough not to limit catalysis.

RebH, an oxygenase of the two-component flavin-dependent enzyme system that chlorinates tryptophan

RebH catalyzes the 7-chlorination of tryptophan. Similar systems carry out chlorinations of electron-rich aromatic rings in the biosynthesis of many compounds in microorganisms. Very little is known about the mechanisms, but RebH has sequence homology with the two-component oxygenases, and the reaction requires an $FADH^-$ (generated by a reductase, RebF), tryptophan, oxygen, and Cl^- for catalysis [47]. Thus, it is anticipated that RebH binds $FADH^-$ and reacts with O_2 to form a C4a-flavin hydroperoxide.

Three mechanisms have been proposed to account for the chlorination of tryptophan. Yeh et al. [47] suggest that Cl^- would attack the proximal oxygen of the flavin C4a-hydroperoxide to form FAD–O–Cl, which chlorinates Trp. A second proposal is that the flavin C4a-hydroperoxide first would form a tryptophan epoxide, which would be attacked by chloride to form the halohydrin, and selective elimination of H_2O would yield product. A third alternative is that Cl^- attacks the distal oxygen of the hydroperoxide to give HOCl plus the flavin C4a-hydroxide. HOCl would then chlorinate tryptophan. This requires that RebH orient the nascent HOCl and tryptophan so that stereoselectivity would be achieved. A crystal structure of a similar two-component FAD-dependent Trp-7-halogenase, PrnA from *Pseudomonas fluorescens* [48], indicates that the flavin and tryptophan are bound with a separation of about 12 Å. There is a tunnel between the flavin and the tryptophan, as well as side chains that could potentially orient the putative HOCl appropriately for the stereospecific chlorination. There is almost no mechanistic information available for these halogenases, but research in this area is likely to be very interesting, especially with a crystal structure available.

Some general principles with two-component flavin-dependent oxygenases

Concentrations of oxygenase are generally greater than that of available flavin in the cell [45]. Therefore, most of the flavin present is bound to the oxygenase, either as the C4a-flavin hydroperoxide or as oxidized flavin. Sequestering the flavin prevents reductases from rapidly producing free reduced flavin anion that could react with O_2 to generate reactive oxygen species. The formation of a stable flavin hydroperoxide prevents the futile reduction and oxidation of flavin to form H_2O_2 and is an important means of regulation analogous to that used by the Baeyer–Villiger [30] and mammalian FMO oxygenases described above, but very different from regulation with PHBH, which is controlled at the level of reduction of the flavin.

Earlier in vitro experiments with two-component systems have usually used excesses of free flavin to be sure that there is sufficient flavin for optimal catalysis by the reductase. Not surprisingly, this condition gives rise to considerable formation of H_2O_2 that is largely derived from the excess free reduced flavin reacting with oxygen. In vivo, coupling to hydroxylation is likely to be considerably tighter because there is virtually no free flavin present in cells. We have found that coupling is more efficient when flavin is provided to a two-component system at a lower concentration than that of the oxygenase involved [42].

This description of the function of two-component aromatic hydroxylases raises important questions about oxygenase dynamics. One-component systems like PHBH have evolved to position the substrate against the flavin in a solvent-free environment before reaction with oxygen. In contrast, the HPAH systems described above, which are able to stabilize the C4a-flavin hydroperoxide intermediate, have evolved to bind HPA after reaction with oxygen. This is remarkable, because substrate binding must require solvent access, and any solvent access is likely to destroy the flavin hydroperoxide. Clearly, the dynamics to satisfy this requirement must be complex. A similar problem exists for the Baeyer–Villiger oxygenases and the mammalian flavin monooxygenases, although these enzymes do not catalyze difficult aromatic hydroxylations. Moreover, in both of these systems NADP remains bound, and this is critical to the stabilization of the flavin peroxides. Without NADP bound, the flavin peroxides are very unstable. With HPAH, after hydroxylation, water is lost from the flavin-C4a-hydroxide considerably faster than the FAD dissociates from the enzyme. Therefore, the flavin hydroxide-product complex must have a conformation that permits some solvent access to eliminate water, but not easily permit FAD to dissociate. A larger conformational change that is rate-determining in the overall catalysis is required to release the oxidized flavin (see above). Perhaps either the $FADH^-$ or the flavin hydroperoxide can promote the conformational change necessary for stabilizing the hydroperoxide, whereas FAD cannot. Thus, release of oxidized FAD occurs, but is rate-determining. It is clear that there is much to be learned about the two-component flavin-dependent oxygenases, especially with regard to dynamics in catalysis.

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