Synergistic Interactions of Multiple Mutations on Catalysis during the Hydroxylation Reaction of *p*-Hydroxybenzoate Hydroxylase: Studies of the Lys297Met, Asn300Asp, and Tyr385Phe Mutants Reconstituted with 8-Cl-Flavin†

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ABSTRACT: The oxygen transfer to *p*-hydroxybenzoate catalyzed by *p*-hydroxybenzoate hydroxylase (PHBH) has been shown to occur via a C4a-hydroperoxide of the flavin. Two factors are likely to be important in facilitating the transfer of oxygen from the C4a-hydroperoxide to the substrate. (a) The positive electrostatic potential of the active site partially stabilizes the negative charge centered on the oxygen of the flavin-C4a-alkoxide leaving group during the transition state [Ortiz-Maldonado, M., Ballou, D. P., and Massey, V. (1999) *Biochemistry 38*, 8124−8137]. (b) The hydrogen-bonding network ionizes the substrate to promote its nucleophilic attack on the electrophilic C4a-hydroperoxide intermediate [Entsch, B., Palfey, B. A., Ballou, D. P., and Massey, V. (1991) *J. Biol. Chem. 266*, 17341−17349]. This ionization is also aided by the positive electrostatic potential of the active site [Moran, G. R., Entsch, B., Palfey, B. A., and Ballou, D. P. (1997) *Biochemistry 36*, 7548−7556]. Substituents on the flavin can specifically affect the stability of the alkoxide leaving-group, whereas changes to specific enzyme residues can affect the charge in the active site and the hydrogen-bonding network. We have used wild-type (WT) PHBH and several mutant forms, all with normal FAD and with 8-Cl-FAD substituted for FAD, to assess the relative contributions of the two effects. Lys297Met and Asn300Asp have decreased positive charge in the active site, and these variants engender ∼35-fold slower hydroxylation rates than the WT enzyme. Substitution of 8-Cl-FAD in these mutant forms gives ∼1.8-fold increases in hydroxylation rates, compared with a ≥4.8-fold increase for WT with this flavin. The hydroxylation catalyzed by Tyr385Phe, a mutant enzyme form with a disrupted hydrogen-bonding network that compromises the ionization of the substrate without changing the positive charge of the active site, is stimulated 1.5-fold by substituting the enzyme with 8-Cl-FAD. The substrate, tetrafluoro-*p*-hydroxybenzoate, is fully ionized in WT PHBH, but this phenolate is a poor nucleophile because of the electron-withdrawing effects of the fluorine substituents. With tetrafluoro-*p*-hydroxybenzoate as the substrate, substitution of FAD with 8-Cl-FAD in the WT enzyme stabilizes the leaving alkoxide and leads to a 2.3-fold increase in the hydroxylation rate compared to that with FAD. Either the use of substrates that do not communicate with the proton network or the mutation of amino acid residues that perturb this interaction may prevent a necessary conformational change that allows proper orientation between reactants during the hydroxylation reaction or permits the essential protonation of the initially formed nascent flavin-C4a-peroxide anion. Thus, both activation of substrate by the proton network and stabilization of the leaving alkoxide appear to be important for oxygen transfer catalyzed by PHBH. The full effect of the substituents on the flavin (4.8-fold) can only be realized when the optimal transition state can be achieved, and this optimal state is not fully realized with the mutant forms.

The reactivity of molecular oxygen with organic compounds is generally poor, even though the overall energetics are usually very favorable. This is largely due to the fact that the triplet ground state of oxygen must first react with a singlet organic compound in a one-electron step; moreover, the one-electron redox potential for oxygen is quite low (−0.33 V at pH >5), making this quite unfavorable (*1*). In reactions with oxygen, biological systems often use metalloproteins and flavoproteins, because they can readily undergo one-electron steps to generate reactive intermediates. External flavin monooxygenases carry out this reaction by catalyzing the reaction of the reduced flavin prosthetic group with dioxygen to form a reactive flavin-C4a-peroxide or hydroperoxide. Such intermediates can then react with specific substrates either nucleophilically or electrophilically to form hydroxylated products.

p-Hydroxybenzoate hydroxylase (PHBH,[1] EC 1.14.13.2) is an external flavoprotein monooxygenase that uses NADPH and oxygen to hydroxylate its natural substrate *p*-hydroxybenzoate (*p*OHB). This reaction is a major step in the

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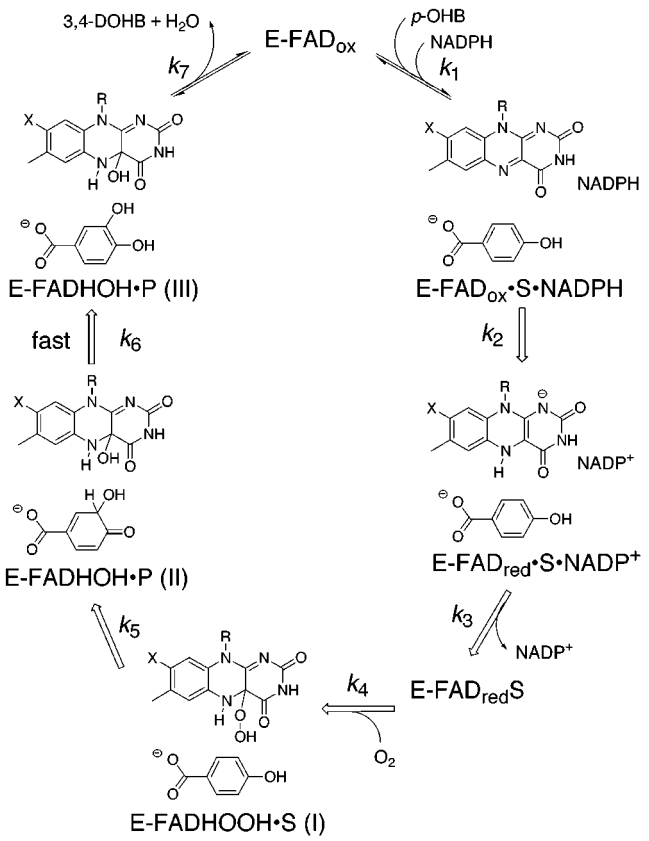
Scheme 1



degradation of many aromatic compounds by soil microbes. The activation of oxygen by PHBH has been shown to occur via formation of an electrophilic flavin-C4a-hydroperoxide intermediate, which is attacked by *p*OHB (Scheme 1) (*2*−*5*). In addition to activating oxygen in this reaction, the enzyme must also activate the substrate for optimal catalytic activity. A hydrogen-bonding network, comprising Tyr201, Tyr385, His72, and one or two water molecules, facilitates the deprotonation of *p*OHB to form the phenolate upon binding to the oxidized enzyme (*6*) (Figure 1). When *p*OHB binds to the oxidized enzyme, the pK_a of the 4-hydroxyl of *p*OHB is shifted from pH 9.3 to 7.4 (*6*). Changing any of these residues disrupts the proton network and makes ionization and activation of substrate much more difficult. In addition, several other mutant forms of PHBH, such as Lys297Met and Asn300Asp, are also impaired in their ability to activate the substrate (see Figure 1 for the arrangement of residues Lys297 and Asn300 in the active site). In these cases, the positive electrostatic environment is diminished, presumably disfavoring formation of the phenolate. Although Lys297Met and Asn300Asp mutant forms of PHBH do not promote the ionization of *p*OHB [the pK_a of the 4-hydroxyl proton for *p*OHB bound to either of these altered enzymes is >9.0 (*7*, *8*)], these mutant enzymes fully hydroxylate *p*OHB, but at much lower rates than the WT enzyme. Decreased reactivity of the FADHOOH in the mutant enzymes might also be due to either a decreased stability of the transition state for monooxygen transfer or the formation of an early (reactant-like) transition state necessitated by the lack of *p*OHB activation.

Monooxygen transfer in WT PHBH appears to be directed mainly by the type of substrate used during catalysis. Substrates that can be activated by the enzyme are hydroxylated rapidly, whereas substrates that cannot be activated are hydroxylated slowly or not at all. For most monooxygen transfer reactions that occur via electrophilic aromatic substitution, the transfer of oxygen is directed by the HOMO electron density of the substrate (*9*). The HOMO of *p*OHB is centered at the C(3) position, the site of hydroxylation (*10*). However, monooxygen transfer in PHBH is directed not only by the HOMO electron density of the substrate but also by the ability of the reactive flavin C4a-hydroperoxide intermediate to be a strong electrophile. Previously, we have determined Hammett and Brønsted parameters for PHBH that had been reconstituted separately with nine 8-substituted flavins (*11*). Those results show that the kinetic rate constant for the hydroxylation reaction of *p*OHB is dependent on the electrophilicity of the FADHOOH intermediate and/or the stability (acidity) of the $FADHO^-$ leaving group.

To investigate the relative importance of substrate activation and the electrophilicity of FADHOOH during the monooxygen transfer step, five PHBH forms (WT, Ser212Ala, Lys297Met, Asn300Asp, and Tyr385Phe) were reconstituted with 8-Cl-FAD and the reaction kinetics were compared with those of normal FAD enzyme forms in the presence of *p*OHB or with the substrate F_4-*p*OHB, which is a nucleophilically challenged substrate.

EXPERIMENTAL PROCEDURES

Materials. Substituted 8-Cl-riboflavin was obtained from J. P. Lamboy (University of Maryland, College Park, MD). The conversion of riboflavin into the FAD analogue was achieved using FAD synthetase and flavokinase isolated from *Brevibacterium ammoniagenes* as reported in ref *12*. The extinction coefficients used for the free 8-substituted FAD analogues were as follows: ϵ_{448} (Cl) = 10 600 M^{-1} cm^{-1} and ϵ_{450} (CH_3) = 11 300 M^{-1} cm^{-1}. F_5-Benzoic acid was used as purchased from Sigma; the xylene mixture was obtained from Mallinckrodt, while other chemicals were as described in ref *11*. The concentrations of the following solutions were estimated spectrophotometrically using an ϵ_{340} of 6220 M^{-1} cm^{-1} for NADPH, an ϵ_{258} of 13 600 M^{-1} cm^{-1} for 2,4-DOHB (in 1 N HCl), and an ϵ_{282} of 16 300 M^{-1} cm^{-1} for *p*OHB (in 1 N NaOH).

Synthesis of F_4-pOHB. F_4-*p*OHB was synthesized using the procedure reported in ref *13*. Pentafluorobenzoic acid (1 g) was dissolved in 20 mL of a 10% KOH solution. The solution was equilibrated under argon pressure and refluxed for 10 h. Once the solution was cooled, concentrated HCl was added until the pH was ≤1. Product, F_4-*p*OHB, was extracted three times with 25 mL of ether. The ether phase was treated with anhydrous Na_2SO_4 (10 g) for 30 min and

[1] Abbreviations: PHBH, *p*-hydroxybenzoate hydroxylase from *Pseudomonas aeruginosa*; Lys297Met PHBH, *p*-hydroxybenzoate hydroxylase with Lys297 replaced with Met; Asn300Asp PHBH, *p*-hydroxybenzoate hydroxylase with Asn300 replaced with Asp; Tyr385Phe PHBH, *p*-hydroxybenzoate hydroxylase with Tyr385 replaced with Phe; Ser212Ala PHBH, *p*-hydroxybenzoate hydroxylase with Ser212 replaced with Ala; WT, wild-type; *p*OHB, *p*-hydroxybenzoate; 2,4-DOHB, 2,4-dihydroxybenzoate; 3,4-DOHB, 3,4-dihydroxybenzoate; F_4-*p*OHB, tetrafluoro-*p*-hydroxybenzoate; FAD, flavin adenine dinucleotide; ApoPHBH, *p*-hydroxybenzoate hydroxylase from which FAD has been removed; $FADH^-$, anionic reduced flavin adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide monophosphate; P_i, phosphate.

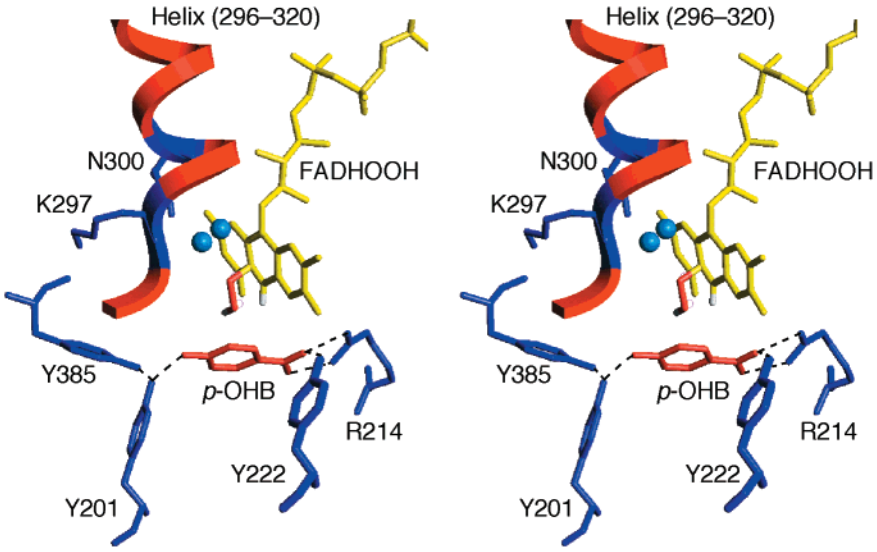


FIGURE 1: Stereoview of the active site environment of PHBH. The active site of PHBH contains positively charged residues (i.e., Lys297 and Arg214), a positive dipole from the α-helix comprising residues 296−320, two residues (Tyr201 and Tyr385) that are part of the proton network that participates in the ionization of *p*OHB, and two water molecules close to the isoalloxazine ring of the FAD. The FAD is presented as the FADHOOH intermediate, without geometric optimization, to show the suitable conformation for aromatic substitution.

then filtered. The treated ether phase was dried under vacuum using a rotary evaporator, and the white solid product was crystallized twice using a xylene mixture (73% yield). The crystals were dried under vacuum, and the melting point was determined to be 152−154 °C. The concentration of F_4-*p*OHB was estimated spectrophotometrically using an ϵ_{260} of 11 600 M^{-1} cm^{-1} (in 1 N NaOH).

Ser212Ala, Lys297Met, Asn300Asp, and Tyr385Phe PHBH Purification. PHBH and the Ser212Ala, Lys297Met, Asn-300Asp, and Tyr385Phe mutant forms were expressed and purified from extracts of *Escherichia coli* JM105/pIE-130 using published procedures (*6*, *7*, *14*); 10 L of cell culture provides ∼500 mg of purified enzyme. The concentration of purified PHBH forms, which have A_{280}/A_{450} ratios of <9.0, were estimated using an ϵ_{450} of 10 300 M^{-1} cm^{-1} for Ser212Ala PHBH (*15*), an ϵ_{450} of 10 400 M^{-1} cm^{-1} for Lys297Met PHBH (*8*), an ϵ_{450} of 9800 M^{-1} cm^{-1} for Asn300Asp PHBH (*7*), and an ϵ_{450} of 10 300 M^{-1} cm^{-1} for Tyr385Phe PHBH (*6*). ApoPHBH mutants were prepared by the procedure reported by Müller and van Berkel (*16*), but with the modifications reported in ref *11*, implemented to increase the apoenzyme yield. The purified apoenzyme forms had no detectable activity in assay mixtures lacking FAD and no measurable absorbance at wavelengths above 310 nm.

Apoenzyme forms were reconstituted with 8-Cl-FAD as described in ref *11*. 8-Cl-FAD binds tightly to the mutant forms of PHBH, and no detectable flavin dissociates from the enzyme during gel filtration or ultrafiltration. The determination of the extinction coefficients for the oxidized enzymes, the enzymatic hydroxylation rate constants, and the dissociation constants for *p*OHB and for 2,4-DOHB have been described in detail in ref *6*. The redox potentials of PHBH variants reconstituted with 8-Cl-FAD, in the presence or absence of *p*OHB, were measured as described by Massey (*17*). Indigo trisulfonate was used as the reference dye [E_m(pH 7) = −81 mV].

Stopped-Flow Instrumentation. Rapid reaction studies were carried out with either a Kinetic Instruments, Inc., or a Hi-Tech Scientific SF-61 stopped-flow spectrophotometer as described by Palfey et al. (*18*). All reactions and measurements, unless noted otherwise, were carried out at 4 °C in 50 mM KP_i buffer (pH 6.5).

Reductive Half-Reaction. Transient kinetic experiments of the reductive half-reaction were initiated by mixing anaerobic oxidized enzyme with an anaerobic buffered solution containing various concentrations of NADPH. Rate constants were calculated from exponential fits of absorbance traces recorded at appropriate wavelengths using the programs KISS (Kinetic Instruments) and Program A, which use the Marquardt algorithm (*19*).

Oxidative Half-Reaction. Transient kinetic studies of the oxidative half-reaction for the enzymes reconstituted with 8-Cl-FAD were initiated by mixing reduced enzyme with a buffered solution containing O_2 (final concentrations of 0.062−0.98 mM) as described previously (*6*, *7*). Anaerobic enzyme (∼30 μM) in a tonometer (with substrate present) was reduced slowly by using an NADH generating system consisting of glucose-6-phosphate and glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (*11*). The oxygenation of F_4-*p*OHB by PHBH with FAD or reconstituted with 8-Cl-FAD was also initiated by mixing reduced enzyme with an oxygenated buffered solution by an asymmetric (1:10) mixing technique available in the Hi-Tech Scientific SF-61 stopped-flow spectrophotometer. This made it possible to use a greater final oxygen concentration (1.7 mM). In this case, anaerobic enzyme (∼110 μM) in a tonometer (with 40 mM F_4-*p*OHB present) was reduced by adding 1.1 equiv of NADPH. After mixing, the concentration of PHBH was 10 μM.

Formation and decay of transient intermediates were detected from absorbance traces recorded at appropriate isosbestic wavelengths and by fluorescence emission traces (515 nm cutoff filter) resulting from excitation at 400 nm or otherwise stated. Rate constants were calculated from exponential fits by the procedures reported previously (*18*), and spectra for the chemical intermediates were calculated from analysis of reaction traces of the reaction of reduced, substrate-saturated enzyme with 0.62 mM oxygen recorded

Table 1: Selected Properties of WT, Lys297Met, Asn300Asp, and Tyr385Phe Forms of PHBH

PHBH	flavin	$E^{\circ\prime}$ (mV)[a] without *p*OHB	$E^{\circ\prime}$ (mV)[a] with *p*OHB	K_d (μM) *p*OHB[c]	K_d (μM) 2,4-DOHB[c]	K_d (μM) NADPH[d]	$\%_{hydrox}$ of *p*OHB[b]	pK_a of *p*OHB[k]
WT	FAD	−163[e]	−165[e]	9.5[f]	22[f]	220	100	7.4
	8-Cl-FAD	−83[g]	−81[g]	30[g]	43	400	100[g]	7.6
Lys297Met	FAD[h]	−176	−176	7.5	49	373	92	>9.0
	8-Cl-FAD	−100	−97	29	85	429	97	nd[l]
Asn300Asp	FAD[i]	−180	−205	7.8	104	990	100	>9.0
	8-Cl-FAD	−102	−136	14	300	610	94	nd[l]
Tyr385Phe	FAD[e]	−163	−154	7.9	10	240	75	>9.0
	8-Cl-FAD	−94	−82	17	30	365	74[j]	nd[l]

[a] Redox potentials were determined in 50 mM KP_i buffer (pH 7.0) at 25 °C using the method of Massey (*17*). Individual measurements for the given enzyme form varied ≤4 mV. [b] The hydroxylation stoichiometry was determined by quantifying NADPH consumed in the presence of 50 μM *p*OHB in 100 mM Tris-sulfate buffer (pH 8.0) at 25 °C. Measurements for the individual enzyme forms varied by ≤2%, unless otherwise stated. [c] Dissociation constants for *p*OHB and 2,4-DOHB were determined from titrations in 50 mM KP_i buffer (pH 6.5) at 4 °C. Individual measurements for the given enzyme form varied ≤2 μM. [d] Dissociation constants for NADPH were calculated from the kinetics of reduction of the enzyme complexed with *p*OHB. Individual measurements for the given enzyme form varied ≤5%. [e] From ref *6*. [f] From ref *2*. [g] From ref *11*. [h] From ref *8*. [i] From ref *7*. [j] The hydroxylation stoichiometry was determined by product analysis as described in ref *6*. [k] pK_a values were determined by titrating the enzyme with *p*OHB at different pH values as described in ref *6*. [l] Not determined.

at multiple wavelengths. The SpecFit program, which fits data using a global least-squares method by factor analysis and Marquardt minimization (developed by R. A. Binstead and A. D. Züberbuhler, Spectrum Software Associates), was used to calculate spectra from the collected data.

RESULTS

Properties of the Mutant Forms of Enzymes with 8-Cl-FAD. The FAD prosthetic group in altered forms of PHBH can be removed by the same procedure used for the preparation of WT apoPHBH (*11*). Reconstitution with 8-Cl-FAD is complete and occurs as facilely as reconstitution of WT apoPHBH. The redox potential of the WT and mutant enzymes reconstituted with 8-Cl-FAD increased as expected due to the electron-withdrawing effect of the chloro group at the 8-position of the flavin (Table 1). As observed with the WT enzyme, the redox potential of the flavin analogue bound to the Lys297Met mutant enzyme is not affected by binding *p*OHB to the enzyme (Table 1 and Figure 2). However, the redox potential of Asn300Asp PHBH, reconstituted with either FAD or 8-Cl-FAD, decreases upon *p*OHB binding (Table 1). On the other hand, the redox potential of Tyr385Phe PHBH with either flavin analogue increases upon substrate binding (Table 1).

The dissociation constants of the 8-Cl-FAD-reconstituted mutant enzymes for *p*OHB and 2,4-DOHB are similar to those for the WT enzyme reconstituted with the flavin analogue (Table 1). The spectral perturbations upon ligand binding to the mutant enzymes with 8-Cl-FAD are also similar to those observed for the mutants with FAD and for the WT enzyme either with FAD (*20*, *21*) or with 8-Cl-FAD (*11*) (Figure 3). The pK_a of the 4-hydroxyl proton in *p*OHB is decreased from 9.3 to ∼7.4 on binding to native PHBH (*6*), which helps to activate the substrate for the hydroxylation step. A similar effect is observed with the WT enzyme reconstituted with 8-Cl-FAD; the pK_a for bound *p*OHB is decreased from 9.3 to 7.6 (*11*). This similar decrease of the pK_a of the bound substrate, along with the data showing similar dissociation constants, indicates that the chloro substituent on the flavin has only small effects on the interaction of the enzyme with the substrate. The Lys297Met, Asn300Asp, and Tyr385Phe enzyme forms do not decrease the pK_a of the bound 4-hydroxyl proton of *p*OHB (*6*−*8*).

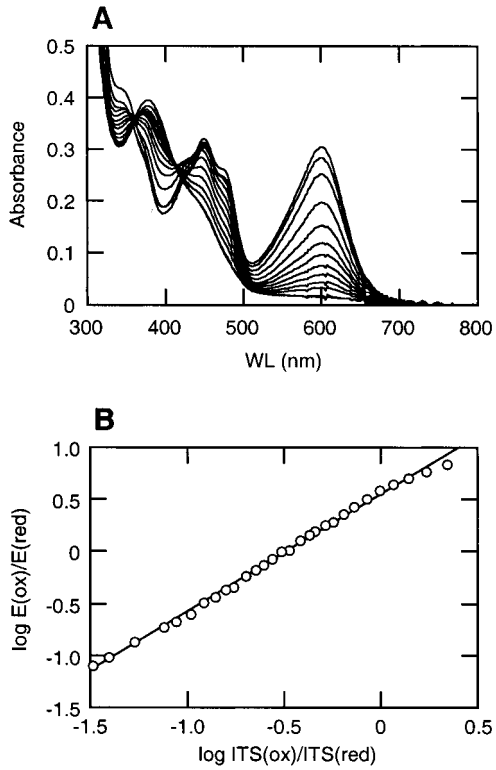


FIGURE 2: Determination of the redox potential of Lys297Met PHBH reconstituted with 8-Cl-FAD. (A) The anaerobic enzyme (30 μM) with *p*OHB (0.5 mM) and indigo-5,5′,7-trisulfonate (ITS) (∼15 μM) in 100 mM KP_i (pH 7.0) at 25 °C was slowly reduced using xanthine (200 μM), xanthine oxidase (∼40 nM final concentration), and benzyl viologen (2 μM). (B) The reduction of ITS was monitored by the decrease in absorbance at 600 nm, and the reduction of the enzyme was monitored by the decrease in absorbance at 488 nm. The slope of the plot of log E_{ox}/E_{red} vs log ITS_{ox}/ITS_{red} is 1.2.

Lys297Met and Asn300Asp mutant forms have decreased positive electrostatic potential at the active site, thereby disfavoring deprotonation of the substrate. Tyr385Phe is part of the proton transfer network required for effecting deprotonation of the substrate. Thus, substitution of native flavin with 8-Cl-FAD in the mutant enzymes is expected to mainly affect the reactivity of the flavin-C4a-hydroperoxide because significant activation of the substrate does not occur in these mutant forms. Although Lys297Met and Asn300Asp do not

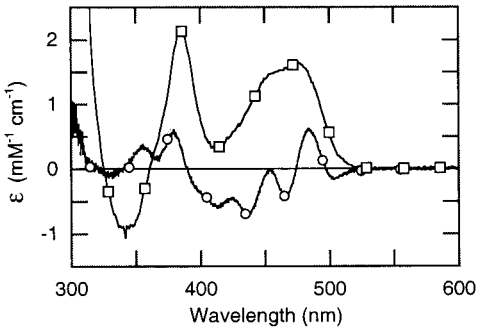


FIGURE 3: Difference spectra produced in titrations of Lys297Met PHBH reconstituted with 8-Cl-FAD upon ligand binding. Experiments were carried out in 50 mM KP_i (pH 6.5) containing 1 mM EDTA at 4 °C. Curves were generated by subtracting the spectrum of the *free* enzyme from the spectrum of the enzyme after addition of saturating ligand: *p*OHB (○) and 2,4-DOHB (□). Difference spectra were similar to those obtained with Lys297Met PHBH and the WT enzyme with normal flavin under the same conditions.

facilitate the ionization of *p*OHB, these mutant forms of PHBH with either FAD or 8-Cl-FAD were nevertheless essentially fully coupled; 94−100% of the enzyme-bound *p*OHB was converted to the product, 3,4-DOHB (Table 1). The Tyr385Phe mutation, on the other hand, yields only 75% hydroxylation, either with FAD or with 8-Cl-FAD bound to the altered enzyme. The remaining oxidation of NADPH leads to formation of H_2O_2. The characteristic intermediates previously reported for these mutant enzyme forms with FAD, both in the reduction of the flavin by NADPH and in the reaction of the reduced flavin with oxygen (*6*−*8*), were also observed with enzymes reconstituted with 8-Cl-FAD. Thus, the general kinetic mechanism of these enzymes was the same as for WT, except for Tyr385Phe, which permits the flavin-C4a-hydroperoxide to partially release H_2O_2.

Reductive Half-Reaction of the Mutant Enzymes with 8-Cl-FAD in the Presence of pOHB. Oxidized enzyme forms substituted with 8-Cl-FAD (15 μM) and complexed with *p*OHB (0.5 mM) were mixed with NADPH under anaerobic conditions in a stopped-flow spectrophotometer. Traces at 450 nm for the Asn300Asp and Tyr385Phe enzyme forms showed a decrease in absorbance in a single phase, whereas the Lys297Met PHBH form showed a lag followed by a decrease in absorbance (data not shown). The observed lag at 450 nm during the reduction of Lys297Met PHBH reconstituted with 8-Cl-FAD by NADPH is due the release of $NADP^+$ being faster than the hydride transfer step (see below). Similar kinetic behavior was observed with this mutant containing FAD (*8*). The observed rate for the reduction step (k_2) of the mutant enzymes substituted with 8-Cl-FAD was hyperbolically dependent on NADPH concentration with dissociation constants for NADPH similar to those for enzyme containing FAD (Table 1). The rate constant k_2 for the mutants of the form E·8-Cl-FAD are in each case about 1.7-fold greater than those for E·FAD forms (Table 2).

Transient absorbance was also observed at 690 nm during the reduction reaction of Lys297Met PHBH with 8-Cl-FAD; this is due to a charge-transfer complex between $NADP^+$ and reduced flavin. The kinetic traces for the WT enzyme could be fit to two phases with the first characterized by the same rate constant observed at 450 nm. By contrast, with Lys297Met PHBH with 8-Cl-FAD, the second phase was

Table 2: Reduction Rate Constants[a] of WT, Lys297Met, Asn300Asp, and Tyr385Phe PHBH Forms in the Presence of *p*OHB

PHBH	flavin	k_2 (s^{-1})	k_3 (s^{-1})
WT	FAD[b]	50	25
	8-Cl-FAD	80	54
Lys297Met	FAD[c]	13.6	25
	8-Cl-FAD	23.6	84.5
Asn300Asp	FAD[d]	0.15	nd[f]
	8-Cl-FAD	0.29	nd[f]
Tyr385Phe	FAD[e]	0.50	nd[f]
	8-Cl-FAD	0.75	nd[f]

[a] The estimated uncertainty for the rate constants is ≤5%. [b] From ref *2*. [c] From ref *8*. [d] From ref *7*. [e] From ref *6*. [f] Not detected. See Scheme 1 for identification of rate constants.

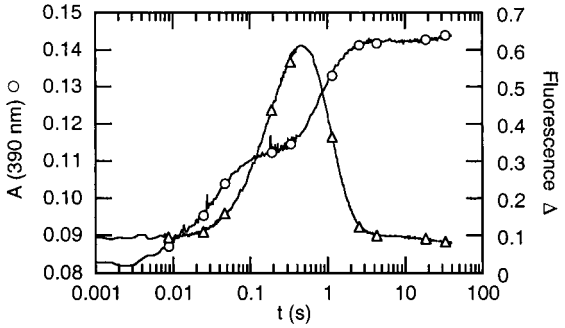


FIGURE 4: Kinetic traces from the oxidative half-reaction of 8-Cl-FAD Lys297Met PHBH in the presence of *p*OHB. The reduced enzyme (15 μM) with bound *p*OHB (0.5 mM) in 50 mM KP_i (pH 6.5) was reacted with 0.62 mM oxygen at 4 °C in the same buffer solution using a stopped-flow spectrophotometer. Absorbance (○) at 390 nm and fluorescence (△) with a λ_{ex} of 400 nm and a λ_{em} of >510 nm are shown.

dependent on NADPH concentration and corresponds to the reduction rate observed at 450 nm, while the first phase (84.5 s^{-1}) was independent of NADPH concentration. Although the decrease in absorbance at 690 nm is due to the dissociation of $NADP^+$ from the reduced enzyme, the rate observed is that of reduction of the flavin. This is because the dissociation rate is greater than the reduction rate (Table 2). This is also true for the reaction of Lys297Met PHBH with FAD (*8*) (Table 2). In contrast to Lys297Met, the absorbance due to the complex of reduced Asn300Asp and Tyr385Phe enzyme forms and $NADP^+$ (k_3) was not detected, either with FAD (*6*, *7*) or with 8-Cl-FAD. Thus, for these enzymes, $k_3 \gg k_2$ (Scheme 1).

Oxidative Half-Reaction of Lys297Met and Asn300Asp PHBH Forms Reconstituted with 8-Cl-FAD in the Presence of pOHB. The reactions of oxygen with mutagenically altered forms of PHBH containing reduced 8-Cl-FAD were measured in the presence of *p*OHB. Three phases could be detected in the reaction of Lys297Met and Asn300Asp enzyme forms reconstituted with 8-Cl-FAD (Figure 4), indicating the existence of two chemical intermediates. Similarly, native PHBH exhibits three phases (k_4, k_5, and k_7 in Scheme 1) (*2*). The first phase is complete at ∼70 ms, the second phase at 400 ms, and the last phase at 4 s (Figure 4). In all cases, the most rapid phase exhibited a first-order dependence on oxygen concentration (k_4) (Table 3) and generated the 8-Cl-flavin-C4a-hydroperoxide intermediate (8-Cl-FADHOOH) as indicated by the similarity of the calculated spectra of the 8-Cl-FADHOOH species to those of the 8-Cl-FADHOH intermediates formed after the hydroxylation

Table 3: Oxidative Half-Reaction Rate Constants of WT, Lys297Met, Asn300Asp, and Tyr385Phe Forms of PHBH in the Presence of *p*OHB

PHBH	EFlH$^-$·S + O_2	k_4 (M^{-1} s^{-1})[a] →	EFlHOOH·S	k_5 (s^{-1})[a] →	EFlHOH·P	k_7 (s^{-1})[a] →	E_{ox}·S
WT	FAD[b]	2.8×10^5		47 ± 2		14	
	8-Cl-FAD[c]	4.1×10^4		225 ± 10[d]		40	
Lys297Met	FAD[e]	2.4×10^5		1.80 ± 0.09		0.9	
	8-Cl-FAD	3.9×10^4		2.7 ± 0.1		1.96	
Asn300Asp	FAD[b]	3.9×10^5		1.10 ± 0.06		0.12	
	8-Cl-FAD	5.7×10^4		2.3 ± 0.1		0.27	
Tyr385Phe	FAD[f]	4.2×10^5		2.00 ± 0.09		>20	
	8-Cl-FAD[g]	6.9×10^4		3.0 ± 0.2		>30	

[a] Values of rate constants were the average of at least four determinations. Rate constants varied from one another by ≤5%. [b] From ref *7*. [c] From ref *11*. [d] Value taken from simulation experiments of the oxidative half-reaction of 8-Cl-FAD PHBH (*11*). [e] From ref *8*. [f] Twenty-five percent of the total enzyme formed an incompetent FADHOOH ($k_{4a} = 1.7 \times 10^5$ M^{-1} s^{-1}). [g] Twenty-five percent of the total ezyme formed an incompetent 8-Cl-FADHOOH ($k_{4a} = 2.4 \times 10^4$ M^{-1} s^{-1}). See Scheme 1 for definition of rate constants.

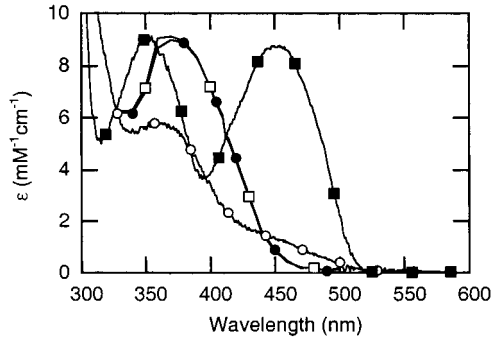


FIGURE 5: Spectra of the chemical species detected during the oxidative half-reaction of 8-Cl-FAD Asn300Asp PHBH in the presence of *p*OHB*:* (○) the reduced 8-Cl-FAD enzyme·*p*OHB complex, (●) 8-Cl-FAD-C4a-hydroperoxide, (□) 8-Cl-FAD-C4a-hydroxide, and (■) the oxidized 8-Cl-FAD·*p*OHB complex. Reaction conditions are the same as those described in the legend of Figure 4.

of *p*OHB (Figure 5). This intermediate was observed previously for these enzymes with FAD (*7*, *8*). Because of the similarity of the spectra of these C4a-hydroperoxy and C4a-hydroxy intermediates (Scheme 1), an accurate determination of the rate constant for the hydroxylation of *p*OHB by the mutant enzymes with 8-Cl-FAD was not possible by monitoring the reaction in the UV−visible absorbance region. However, the rate constants for hydroxylation by the altered enzyme forms could be more accurately determined by assessing the formation (k_5) and decay (k_7) of fluorescence resulting from excitation of the 8-Cl-FADHOH species at 400 nm. 8-Cl-FADHOH is much more fluorescent than 8-Cl-FADHOOH, at least when bound to the enzyme. The 8-Cl-FADHOOH intermediate of each mutant hydroxylates *p*OHB ~1.8-fold more rapidly than does the normal FADHOOH species (k_5, Table 3). However, the rate constant for hydroxylation of *p*OHB by WT PHBH reconstituted with 8-Cl-FAD is at least 4.8-fold larger than that for the WT enzyme with FAD (*11*) (Table 3). The rates of decay of 8-Cl-FADHOH (k_7) for these mutant forms are ~2.3-fold greater than for the mutant enzymes with FAD (Table 3).

Oxidative Half-Reaction of Tyr385Phe PHBH Reconstituted with 8-Cl-FAD in the Presence of pOHB. Hydroxylation of *p*OHB by Tyr385Phe PHBH with native FAD and enzyme reconstituted with 8-Cl-FAD is 75% coupled toward making the hydroxylated product. The reaction exhibits three phases at wavelengths between 320 and 500 nm. The first phase is complete at ~20 ms, the second phase at 180 ms, and the last phase at 2 s (Figure 6). The first two phases

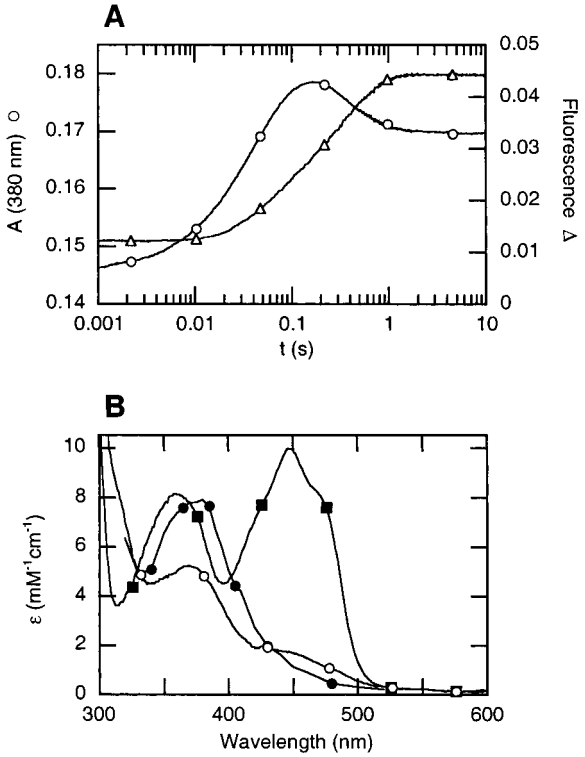
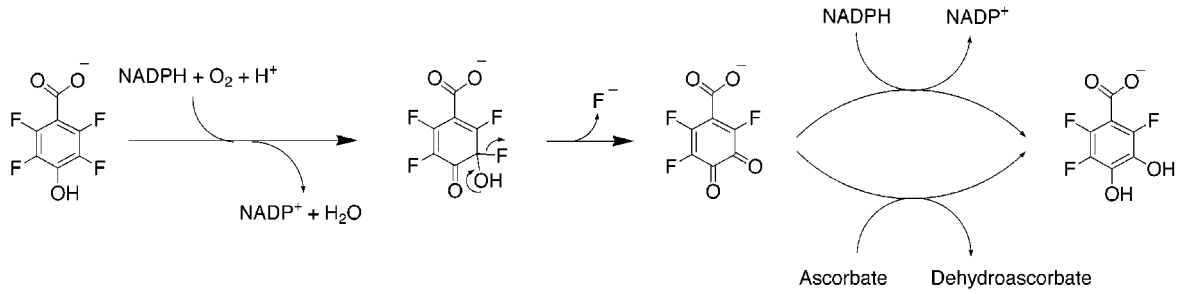


FIGURE 6: Kinetic traces and spectra of species calculated from the oxidative half-reaction of 8-Cl-FAD Tyr385Phe PHBH in the presence of *p*OHB. The reduced enzyme (20 μM) with bound *p*OHB (0.5 mM) in 50 mM KP_i (pH 6.5) was reacted with 0.62 mM oxygen at 4 °C in the same buffer solution using a stopped-flow spectrophotometer. (A) Absorbance (○) at 380 nm and fluorescence (△) with a λ_{ex} of 380 nm and a λ_{em} of >530 nm are shown. (B) (○) The reduced 8-Cl-FAD enzyme·*p*OHB complex, (●) 8-Cl-flavin-C4a-hydroperoxide, and (■) the oxidized 8-Cl-FAD·*p*OHB complex.

exhibit a first-order dependence on oxygen concentration (k_4 and k_{4a}, Table 3) and generate the flavin-C4a-hydroperoxide intermediate (as indicated by the spectrum of the intermediate in Figure 6B). The phase with the largest second-order rate constant (75% of the total fraction) forms a competent 8-Cl-FADHOOH that hydroxylates the aromatic substrate. The second phase (25% of the total fraction) forms an incompetent 8-Cl-FADHOOH, accounting for the decreased level of hydroxylation coupling. The third phase in the oxidation reaction is independent of oxygen concentration and indicates the hydroxylation step. The first phase appeared as a lag in the traces of fluorescence (Figure 6A), which, as above, is primarily due to the formation of the flavin-C4a-hydroper-

Scheme 2



oxide intermediate. Because the rate constant for decay of the flavin-C4a-hydroxide intermediate is much larger than the rate constant for its formation (at least 10-fold larger), the FADHOH intermediate cannot be observed in Tyr385Phe substituted with 8-Cl-FAD by monitoring either the UV−visible or fluorescence properties (Figure 6). This is also true when FAD is used in this mutant (*6*). The calculated spectrum of the flavin-C4a-hydroperoxide is shown in Figure 6B. The similarities to the analogous reaction with FAD suggest that the overall mechanism of the Tyr385Phe enzyme was not altered by substituting the enzyme with 8-Cl-FAD.

Oxidative Half-Reaction of Ser212Ala PHBH Reconstituted with 8-Cl-FAD in the Presence of pOHB. Hydroxylation of *p*OHB by Ser212Ala PHBH with FAD was efficient. and the rate constants for the oxidative steps were very similar to those observed for WT PHBH under the same conditions (*15)*. Native PHBH exhibits three phases (k_4, k_5, and k_7 in Scheme 1) (*2*). With Ser212Ala, a slow fourth phase was also prominent in the hydroxylation reaction. This slow phase was attributed to the formation of the dead-end complex between the flavin-C4a-hydroxide and *p*OHB, which is present at a high concentration in the reaction mixture (6 mM) because of the high K_d for binding of the substrate to the reduced enzyme (*15*). The reaction of oxygen with this form of PHBH containing reduced 8-Cl-FAD was assessed by absorbance measurements in the presence of *p*OHB. Three phases could be detected (not shown), indicating the presence of one chemical intermediate in addition to the dead-end complex between the flavin-C4a-hydroxide and *p*OHB. The first phase is complete at ~48 ms, the second phase at 260 ms, and the last phase at 16 s (not shown). The most rapid phase exhibited a first-order dependence on oxygen concentration ($k_4 = 5.4 \times 10^4$ M^{-1} s^{-1}) and generated the 8-Cl-flavin-C4a-hydroxide intermediate (8-Cl-FADHOH). This precluded the exact determination of the rate constant for hydroxylation (k_5), as found similarly with WT PHBH reconstituted with 8-Cl-FAD under the same conditions (*11*). The rate constant for hydroxylation of *p*OHB by WT PHBH reconstituted with 8-Cl-FAD is at least 4.8-fold faster than that of the WT enzyme with FAD (*11*) (Table 3). The rate of decay for 8-Cl-FADHOH (k_7) for this mutant form is ~2-fold faster than for the mutant enzyme with FAD (10 s^{-1} for FAD and 19.2 s^{-1} for 8-Cl-FAD).

Oxidative Half-Reaction of WT PHBH with Native FAD and Enzyme Reconstituted with 8-Cl-FAD, in the Presence of F4-pOHB. Hydroxylation of F_4-*p*OHB by the WT enzyme with FAD or with enzyme reconstituted with 8-Cl-FAD is fully coupled at limiting concentrations of the substrate, as determined by NADPH consumption in enzymatic assays [containing 2 mM ascorbate to prevent nonenzymatic reaction

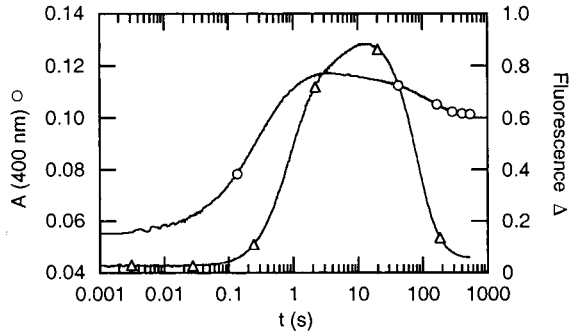


FIGURE 7: Kinetic traces from the oxidative half-reaction of 8-Cl-FAD PHBH in the presence of F_4-*p*OHB. The reduced enzyme with bound F_4-*p*OHB (40 mM) in 50 mM KP_i (pH 6.5) was reacted with oxygen at 4 °C in the same buffer solution using a stopped-flow spectrophotometer. Absorbance at 400 nm (○) ([enzyme] = 15 μM; [oxygen] = 0.98 mM) and fluorescence (△) with a λ_{ex} of 400 nm and a λ_{em} of >510 nm ([enzyme] = 11 μM; [oxygen] = 1.7 mM) are shown. The fluorescence experiment was carried out with asymmetrical mixing as described in Experimental Procedures.

of NADPH with the 3,5,6-trifluoro-4-carboxyorthobenzoquinone product (*22*) (Scheme 2)]. Previous work (*23*) demonstrated that the quinone is the primary observed product of F_4-*p*OHB hydroxylation, and it results from fluoride elimination from the nonaromatic precursor as shown in Scheme 2. A second equivalent of NADPH is consumed in a nonenzymatic reaction to reduce the quinonoid intermediate to the F_3-DOHB product (*23*). Quinonoid trapping agents such as DTT, cytochrome *c*, or ascorbate can be used to prevent the extra consumption of NADPH (*23*).

The reactions of oxygen with the WT enzyme containing reduced forms of FAD or 8-Cl-FAD were assessed in the presence of 20 mM F_4-*p*OHB, a less reactive substrate. Four reaction phases were detected at wavelengths between 330 and 490 nm. The first phase is complete at ~100 ms, the second phase at ~1.4 s, the third phase at ~20 s, and the last phase at ~500 s (Figure 7). The first two phases show first-order dependence on oxygen concentration (k_4 and k_{4a}) (Table 4) and generated the 8-Cl-FADHOOH intermediate. The larger second-order rate constant (5.8×10^3 M^{-1} s^{-1}) represents the formation of the 8-Cl-FADHOOH in enzyme with bound F_4-*p*OHB (k_4); this intermediate is capable of hydroxylating this substrate. The slower second-order rate constant (1.5×10^3 M^{-1} s^{-1}), due to the formation of the 8-Cl-FADHOOH intermediate (k_{4a}) detected during the oxidation reaction, presumably represents enzyme with no bound ligand. The concentration of F_4-*p*OHB used in these experiments was below saturation levels because of the high K_d for this substrate binding to the reduced enzyme [17 mM for WT PHBH (*23*)].[2] The rate of hydroxylation cannot be

Table 4: Oxidative Half-Reaction Rate Constants[a] of WT PHBH Forms in the Presence of F_4-*p*OHB[b]

EFlH$^-$·S + O_2	k_4 (M^{-1} s^{-1}) →	EFlHOOH·S	k_5 (s^{-1}) →	EFlHOH·P	k_7 (s^{-1}) →	E_{ox}·S
WT	1.1×10^5		0.52 ± 0.5		0.014	
8-Cl WT	5.8×10^3		1.17 ± 0.1		0.015	
EFlH$^-$ + O_2	k_{4a} (M^{-1} s^{-1}) →	EFlHOOH	→	E_{ox}·S		
WT	1.0×10^4		fast		25% of total enzyme	
8-Cl WT	1.5×10^3		fast		25% of total enzyme	

[a] The estimated uncertainty for the rate constants is ≤10%. [b] Concentration of F_4-*p*OHB = 20 mM.

measured accurately by following the reaction by UV−visible absorbance because the spectra of the intermediates, FADHOOH and FADHOH, are very similar to each other. However, the rate constant for hydroxylation (k_5) could be determined accurately by measuring the fluorescence emission traces (515 nm cutoff filter) resulting from excitation at 400 nm (Figure 7), as described in Experimental Procedures. The 8-Cl-FADHOOH intermediate hydroxylates F_4-*p*OHB 2.3-fold faster than does the native FADHOOH intermediate (Table 4). Finally, elimination of water from the FADHOH intermediate (k_7) was not significantly altered by replacing FAD with 8-Cl-FAD in WT PHBH (Table 4).

DISCUSSION

Model chemistry for the hydroxylation of aromatic compounds by flavins has not been successful. We propose that simple models are not likely to give the whole set of properties necessary for hydroxylating phenols. Many limitations have prevented models from mimicking the reactions of enzymes. Factors known to be important for the hydroxylation of phenolic compounds by flavins include (a) formation and stabilization of a flavin-C4a-hydroperoxide, (b) binding of substrate in proper orientation for transfer of oxygen from the hydroperoxide to the substrate, (c) activation of the phenol by deprotonation, (d) stabilization of the flavin alkoxide leaving group, and (e) promotion of the proton transfers required for these reactions without allowing direct interaction with solvent. Interactions with solvent would lead to the breakdown of the hydroperoxide. To satisfy these requirements, an exquisitely tailored active site is probably required. For example, PHBH has a proton network that assists in the ionization of substrate to increase its nucleophilic character, an important step for the hydroxylation reaction (*6*). The proton network allows transfer of protons without direct contact with solvent. The proton network also serves to control flavin dynamics that are important for the reduction reaction (*24*). Furthermore, the positive electrostatic potential of the active site is essential for stabilizing the leaving group during the monooxygen transfer step (*11*). PHBH is used in this study as a model to expand our knowledge about how this class of enzymes controls the individual steps of the hydroxylation reaction, especially the oxygen transfer step. The mechanism is complex, so single or multiple modifications in enzymatic structure must be evaluated by examining the individual steps in the reaction mechanism. Because of possible changes in the rate-limiting step of the overall reaction, steady-state kinetic methods are not likely to give us a complete picture of the effects of such modifications. Therefore, we have employed rapid reaction techniques to monitor individual steps in the catalysis.

Substitution of FAD with 8-Cl-FAD in the PHBH forms that were studied does not cause any large perturbations to the enzyme structure or to its activities. For example, the binding of substrate to the enzyme forms is similar to that for WT, and as expected, the redox potential of the flavin analogue is increased when it is bound to the enzyme in accordance with the previously observed correlation with its potential *free* in solution (*11*). The rate of reduction of the altered enzyme forms containing 8-Cl-FAD with NADPH (k_2, Table 2) increased ∼1.7-fold compared to those for enzyme forms with native FAD. This increase in the reduction rate caused by substituting 8-Cl-FAD in the altered enzyme forms is similar to the 1.6-fold increase observed for WT PHBH reconstituted with the flavin analogue. Largely because of the higher redox potential of 8-Cl-FAD relative to that of FAD, enzyme forms reconstituted with 8-Cl-FAD react with oxygen ∼6.5-fold slower (k_4, Table 3) to form the flavin-C4a-hydroperoxide. The electron-withdrawing effect of the chloro group also results in a ∼2.5-fold faster dehydration of the flavin-C4a-hydroxide as compared to enzyme forms with FAD (k_7, Table 3), probably due to the higher acidity of the N(5)-H group of the flavin. All of the intermediates observed with FAD in these various mutated forms of PHBH are also observed with 8-Cl-FAD, evidence that the reaction mechanism for the 8-Cl-FAD-reconstituted mutant enzymes is substantially the same as that with enzyme forms with FAD. However, on substitution of 8-Cl-FAD for FAD, the rate of hydroxylation (k_5, Table 3) increases ≥4.8-fold for the WT enzyme, while it increases only ∼1.7-fold for the mutationally modified enzyme forms. The following discussion attempts to rationalize this unexpected difference in activation by 8-Cl-FAD for the various enzyme forms.

The results presented in this study show that substrate activation by PHBH and stabilization of the flavin-C4a-alkoxide leaving group are both important processes that are required for efficient and competent hydroxylation. The transition state of the hydroxylation reaction (k_5) in the WT enzyme is partly stabilized by the hydrogen-bonding network. The binding of substrate to oxidized enzyme results in a decrease in the pK_a of the phenolic substrate from 9.3 to 7.4, which is equivalent to ∼2.6 kcal/mol of stabilization of the phenolate. It should be noted, however, that the pK_a of *p*OHB was measured for oxidized PHBH, whereas the

[2] Probably because of the low pK_a of F_4-*p*OHB, the phenolate binds poorly to the reduced anionic state of the enzyme. It is known that the substrate must be protonated to bind to the reduced state of the enzyme (*24*).

Scheme 3

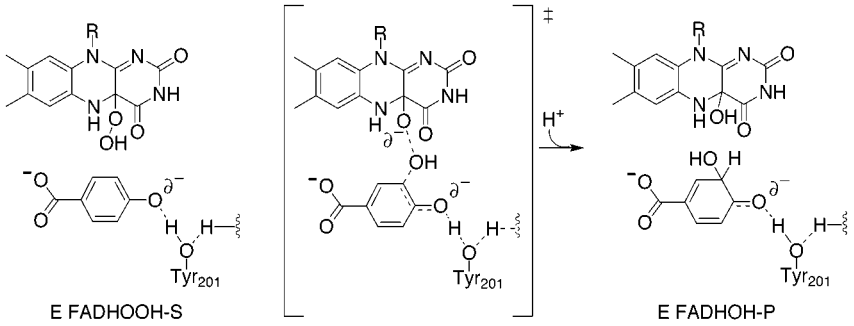


Table 5: Transition State Free Energy Changes of the Monooxygen Transfer Step Caused by Single and Double Modifications to PHBH

	$\Delta\Delta G^{\ddagger}$ (kcal/mol)[a]			
	measured		predicted	
PHBH mutant	FAD-mutant/FAD-WT	8-Cl-FAD-mutant/FAD-WT	8-Cl-FAD-WT/FAD-WT + FAD-mutant/FAD-WT[b]	$\Delta\Delta G^{\ddagger}_{I}$ (kcal/mol)[c]
Lys297Met	1.93 ± 0.07	1.69 ± 0.06	1.00 ± 0.09	0.69 ± 0.08
Asn300Asp	2.22 ± 0.07	1.79 ± 0.06	1.30 ± 0.09	0.49 ± 0.09
Tyr385Phe	1.87 ± 0.06	1.63 ± 0.08	0.94 ± 0.09	0.7 ± 0.1
WT·F_4-*p*OHB[d]	2.7 ± 0.1	2.2 ± 0.1	1.7 ± 0.1	0.4 ± 0.1

[a] Values were derived from the rate constants in Table 3 using eq 1. [b] Calculated by adding $\Delta\Delta G^{\ddagger}_{(8-Cl-FAD-WT/FAD-WT)}$ (= −0.93 ± 0.06 kcal/mol) to values given under the heading FAD-mutant/FAD-WT. [c] Values were calculated using eq 1. [d] WT·F_4-*p*OHB is generalized as a PHBH mutant for simplicity.

relevant pK_a value is that for the substrate bound to the transient FADHOOH form of the enzyme; this pK_a cannot be determined experimentally. The positive electrostatic potential of the active site in the WT enzyme may facilitate ionization of the substrate phenol as well as stabilize the developing negative charge on the flavin alkoxide leaving group during the formation of the transition state (Scheme 3; dashed lines in the reactant and product states represent the hydrogen-bonding network between *p*OHB and residues Tyr201, Tyr385, and His72). Thus, residues at the active site that decrease the positive electrostatic potential may have multiple synergistic negative effects on the stabilization of the transition state. The decreased electrostatic potential of the active site in the Lys297Met and Asn300Asp variants resulted in a ∼32-fold decrease in the observed rate of hydroxylation (*7*, *8*), which corresponds to an increase of 2.1 kcal/mol in transition state energy (Table 5). This higher-energy transition state is partly due to the decreased level of ionization of the phenolic substrate in these mutant enzymes (leading to an earlier or reactant-like transition state). The Tyr385Phe modification, which also impedes ionization of the phenol, leads to decreased level of activation of the substrate; however, the positive character of the active site is maintained. Nevertheless, there was a ∼28-fold decrease in the rate of hydroxylation (2.0 kcal/mol) (Table 5). Thus, the mutant forms, Lys297Met, Asn300Asp, and Tyr385Phe, all demonstrate the importance of substrate activation during the formation of the transition state in the hydroxylation reaction.The flavin-C4a-hydroperoxide intermediate formed with 8-Cl-FAD is more electrophilic than that with FAD (see the Supporting Information). The hydroxylation step in the WT enzyme thereby proceeds ≥4.8-fold faster than with FAD, corresponding to a decrease in the transition state energy of at least −0.93 kcal/mol. This can be attributed to the leaving flavin C4a-alkoxide being stabilized by the electron-withdrawing effect of the 8-Cl substituent (*11*, *25*−*28*).

When active site amino acid residues are changed by site-directed mutagenesis, as in this study, replacement of the flavin in PHBH with 8-Cl-FAD only increases the rate of hydroxylation by ∼1.7-fold, contrasting with the ≥4.8-fold increase observed when the WT enzyme is substituted with the flavin analogue. One way of thinking about this discrepancy is in energetic terms describing the transition state. Table 5 lists in the first column the values for destabilization of the transition state (k_5) relative to the WT enzyme caused by various amino acid substitutions in PHBH. Summing these with the value for $\Delta\Delta G^{\ddagger}_{(8-Cl-FAD-WT/FAD-WT)}$ of −0.93 kcal/mol for simple substitution of the flavin in the WT enzyme might be expected to give the net stabilization/destabilization effect caused by the two perturbations (column 3, predicted). However, as shown in column 2, which gives the measured overall effect, the predicted values are poor. The differences in $\Delta\Delta G^{\ddagger}$ values between the WT and mutant enzymes are small, but they indicate significant changes in the structure of the transition state. For example, for the Lys297Met mutant form with 8-Cl-FAD, we would predict a net destabilization of 1.0 kcal/mol, but we observed 1.7 kcal/mol of destabilization. One way of accounting for the difference (0.7 kcal/mol) between these two numbers would be to consider a coupling or interaction free energy ($\Delta G^{\ddagger}_{I}$) (eq 1) between the two perturbations (*29*, *30*). This interaction free energy could account for possible structural differences in the active site caused by the mutation, lack of conformational flexibility required for efficient catalysis, and/or change in the electrostatic environment of the active site involved in stabilization of the transition state.

$$\Delta\Delta G^{\ddagger}_{(\text{8-Cl-FAD-mutant/FAD-WT})} = \Delta\Delta G^{\ddagger}_{(\text{8-Cl-FAD-WT/FAD-WT})} + \Delta\Delta G^{\ddagger}_{(\text{FAD-mutant/FAD-WT})} + \Delta G^{\ddagger}_{\text{I}} \quad (1)$$

where

$$\Delta\Delta G^{\ddagger}_{(\text{8-Cl-FAD-mutant/FAD-WT})} = -RT \ln \frac{k_{(\text{8-Cl-FAD-mutant})}}{k_{(\text{FAD-WT})}}$$

$$\Delta\Delta G^{\ddagger}_{(\text{8-Cl-FAD-WT/FAD-WT})} = -RT \ln \frac{k_{(\text{8-Cl-FAD-WT})}}{k_{(\text{FAD-WT})}}$$

$$\Delta\Delta G^{\ddagger}_{(\text{FAD-mutant/FAD-WT})} = -RT \ln \frac{k_{(\text{FAD-mutant})}}{k_{(\text{FAD-WT})}}$$

and $\Delta G^{\ddagger}_{\text{I}}$ is the interaction free energy.

In electrophilic substitution reactions, the characteristics of the aromatic substrate [charge density and the energy coefficients of the highest occupied molecular orbital (HOMO)] strongly influence the position of the electrophilic attack (*9*). The fluorine substituents in F_4-*p*OHB decrease the electron density of the C(3) position, making this substrate a less reactive nucleophile than *p*OHB, even though the phenol is fully ionized [pK_a = 5.3 (*23*)]. Because the phenol is ionized under all the conditions that were employed, the hydrogen-bond network of the enzyme cannot significantly enhance its reactivity. As anticipated, F_4-*p*OHB with the WT enzyme behaved like enzymes with mutations that diminish the level of substrate activation. The rate of hydroxylation of F_4-*p*OHB by the WT enzyme substituted with 8-Cl-FAD was only slightly greater than that of the WT enzyme (2.3-fold). Thus, although 8-Cl-FADHOOH is a better electrophile than FADHOOH, this experiment also demonstrates the importance of the nucleophilicity of the substrate in the overall reaction. This is also consistent with the fact that no flavoprotein aromatic hydroxylases are known that can hydroxylate aromatic rings that have no electron-donating groups.

A possible representation of the transition state for the WT enzyme is shown in Scheme 3. The oxygen on the $FADHO^-$ leaving group and the 4-OH on the nucleophilic substrate carry partial negative charge (δ^-) in the transition state. From QSAR studies, which give a β_{lg} of −0.42, the transition state is likely to be nearly midway between the reactant and product states (*11*). The transition states for the altered enzyme forms that were studied differ from that of the WT enzyme because in the altered enzymes the 4-hydroxyl proton of *p*OHB cannot be easily ionized due to either electrostatic effects or impairment of the proton network facilitating substrate ionization. Thus, the altered enzyme forms require an early transition state (reactant-like transition state) that is less favorable for rapid hydroxylation than that for the WT enzyme. This is analogous to previous observations in which the oxidative half-reactions of the WT enzyme that had been reconstituted with nine derivatives of FAD were analyzed in the presence of 0.1 M sodium azide (*11*). Binding of azide near the flavin (*20*) decreases the effective positive electrostatic potential of the active site so that it is similar to that in the Lys297Met and Asn300Asp enzyme forms; this increases the pK_a of the 4-hydroxyl proton on bound *p*OHB.

A second hypothesis for the observed rate discrepancies considers an alternate kinetic scheme. This treats the oxygen transfer reaction as a two-step process, a deprotonation and/or conformational step (k_{5a}) and an oxygen transfer step (k_{5b}), as shown in the scheme below. The individual rate constants contribute to k_{obs} (for k_5) as shown in eq 2.

$$\text{A} \xrightarrow{k_{5a}} \text{B} \xrightarrow{k_{5b}} \text{C}$$

$$k_{obs} = \frac{k_{5a}k_{5b}}{k_{5a} + k_{5b}} \quad (2)$$

We assume that only k_{5b} is affected by the substitution on the flavin, whereas only k_{5a} is affected by changes in the structure of the enzyme (mutations). We can assume that proton transfer and conformational changes in the wild-type are rapid (*24*); i.e., $k_{5a} \gg k_{5b}$, and the chemical step is rate-determining. For example, if we assume $k_{5a} = 2000\ s^{-1}$, when $k_{obs} = 47\ s^{-1}$, we can calculate a k_{5b} of $48\ s^{-1}$. In this model, when the WT enzyme is substituted with 8-Cl-FAD, only the rate of oxygen transfer (k_{5b}) will be increased. Using a k_{obs} of $225\ s^{-1}$, which is the lowest possible value that from simulations[3] is consistent with the observed data (Table 3 and ref *11*) and a k_{5a} of $2000\ s^{-1}$, the calculated value for k_{5b} is $254\ s^{-1}$ with 8-Cl-FAD. Thus, the rate of hydroxylation with 8-Cl-FAD is calculated to be 5.3-fold greater than with FAD. These are not the only values that satisfy the data for the WT enzyme. Choice of a k_{5a} of $\gg 2000\ s^{-1}$ will give the value for the ratio of the rate constant for 8-Cl-FAD to that for FAD as 4.7. Values of less than $2000\ s^{-1}$ will give a ratio larger than 5.3 for these rate constants. However, with an observed value of $225\ s^{-1}$, the lower limit for k_{5a} must be at least $225\ s^{-1}$ because lower values would lead to negative rate constants for k_{5b} for 8-Cl-FAD in this model. On the basis of the above analysis for the WT enzyme, we assume that 8-Cl-FAD increases k_{5b} by at least 5.3-fold for each of the mutant enzymes. Mutated forms of the enzyme that have diminished levels of activation of substrate (e.g., Lys297Met) are assumed to have lower rates for the first step (k_{5a}), whether it be a deprotonation or a conformational change. For the Lys297Met variant, $k_{obs} = 1.8\ s^{-1}$ with FAD and $2.7\ s^{-1}$ with 8-Cl-FAD. These k_{obs} values can be fit with a k_{5a} of $3.1\ s^{-1}$, a k_{5b} of $4.4\ s^{-1}$ with FAD, and a k_{5b} of $23\ s^{-1}$ with 8-Cl-FAD. Calculated values for the other mutant enzymes are given in Table 6. Thus, the full effect of the increased reactivity of the 8-Cl-FAD can be masked when the modified residue makes the first step partially rate-determining in the overall oxygen transfer step. This model, if correct, permits us to separate the substrate activation or conformational effects and the flavin activation steps for the oxygenation reaction of *p*OHB instead of treating the phenomenon as an interaction term. However, experiments with the WT enzyme with two alternative substrates suggest that this situation may be oversimplified. For example, with F_4-*p*OHB as the substrate, the rate of hydroxylation by the WT enzyme with 8-Cl-FAD was only 2.3-fold greater than

[3] The reaction of 8-Cl-FAD PHBH with oxygen was simulated at 390, 440, and 470 nm. The rate constants used in the simulations (constants are numbered to be consistent with Scheme 1) for the reaction of the 8-Cl-FAD-labeled enzyme were as follows: $k_4 = 4.1 \times 10^4\ M^{-1}\ s^{-1}$ (the reaction with oxygen) and $k_7 = 40\ s^{-1}$ (dehydration of the pseudobase). Values for k_5 were varied between 50 and $3000\ s^{-1}$. Simulations were performed as previously described in ref *11*, and they are shown in the Supporting Information.

Table 6: Estimated Rate Constants for the Deprotonation or Conformational Change Step (k_{5a}) and the Oxygen Transfer Step (k_{5b}) of WT, Lys297Met, Asn300Asp, and Tyr385Phe Forms of PHBH in the Presence of *p*OHB (calculated from eq 2)

PHBH	flavin	k_{obs} (s^{-1})	k_{5a} (s^{-1})	k_{5b} (s^{-1})
WT	FAD	47	2000	48
	8-Cl-FAD	225	2000	254
Lys297Met	FAD	1.8	3.1	4.4
	8-Cl-FAD	2.7	3.1	23
Asn300Asp	FAD	1.1	3.1	1.7
	8-Cl-FAD	2.3	3.1	9.1
Tyr385Phe	FAD	2.0	3.4	4.9
	8-Cl-FAD	3.0	3.4	26

that of the WT enzyme with FAD. Because the phenol in F_4-*p*OHB is already fully ionized before being bound to PHBH, k_{5a} in the two-step mechanism cannot be simply due to deprotonation of the substrate. Moreover, the rate of hydroxylation of *p*-aminobenzoate (a more nucleophilic substrate than either F_4-*p*OHB or the protonated form of *p*OHB) by WT PHBH with 8-Cl-FAD was only slightly greater than that of the WT enzyme with FAD (1.4-fold; i.e., 7.1 vs 4.9 s^{-1}). It is clear that *p*-aminobenzoate (pK_a > 20) cannot ionize on the enzyme at pH 6.5 to increase its reactivity. This would suggest that the step characterized by k_{5a} is a conformational change that occurs prior to the monooxygen transfer step, and this might be limiting the rate of hydroxylation by the enzyme forms containing the reactive flavin-C4a-hydroperoxide.

The crystal structure of one of the altered enzyme forms that was studied, Asn300Asp PHBH, indicates that the atomic position of the side chain of Asp300 moves away from the flavin, compared to that of the original Asn residue (*31*). The substantial movement of the Asp300 side chain disrupts the interactions of the carboxamide group with the O(2) position of the flavin so that the α-helix H10 that begins at residue 297 is displaced by ~1.5 Å to the Cα of residue 300, altering its dipole interaction with the isoalloxazine ring of the flavin. Although the crystal structure for Lys297Met PHBH has not been successfully determined, similar atomic rearrangements might occur in the Lys-to-Met mutant. If movement of the altered residues in the enzyme forms reconstituted with 8-Cl-FAD differs from that of the enzyme forms with FAD, it could contribute to the observed interaction free energy proposed when the hydroxylation reaction is considered as a single step. Such minor structural changes might perturb the communication between the substrate and the proton network. A lack of communication between the substrate and the proton network (that permits substrate ionization) may prevent a necessary conformational change that allows the proper orientation between reactants or permits the essential protonation of the initially formed nascent 8-Cl-FADHOO$^-$. The interdependence between the conformational changes required during PHBH catalysis and proton movement may thus be essential for preparing the enzyme for proper hydroxylation. This interdependence could thereby account for not seeing the full effect of the flavin substitution. This is analogous to the idea of substrate recognition by password proposed in the reduction of the FAD in PHBH (*24*). In this case, deprotonation of the substrate via the proton network facilitates the movement of the flavin to the out position, where it becomes reduced by NADPH.

Overall, these studies show that in complex reactions such as these, simple summation of changes in activation energies caused by mutations or other factors may not be sufficient to fully describe the processes. Each of the perceived steps may actually consist of more than one event, so multiple activation energies may be involved. These detailed studies may caution us against making simple interpretations about site-directed mutations of complex systems.

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SUPPORTING INFORMATION AVAILABLE

Results of simulation experiments of the reaction of 8-Cl-FAD PHBH with oxygen. This material is available free of charge via the Internet at http://pubs.acs.org.

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