Studies of the Mechanism of Phenol Hydroxylase: Mutants Tyr289Phe, Asp54Asn, and Arg281Met†

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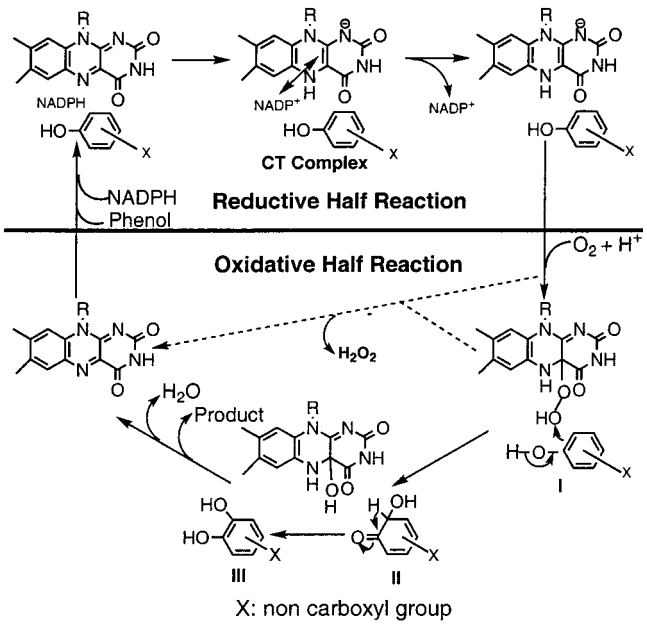
Received May 10, 2001; Revised Manuscript Received August 22, 2001

ABSTRACT: Three residues in the active site of the flavoprotein phenol hydroxylase (PHHY) were independently changed by site-directed mutagenesis. One of the mutant forms of PHHY, Tyr289Phe, is reduced by NADPH much slower than is the wild-type enzyme, although it has a slightly higher redox potential than the wild-type enzyme. In the structure of the wild-type enzyme, residue Tyr289 is hydrogen-bonded with the FAD when the latter is at the "out" position but has no direct contact with the flavin when it is "in". The oxidative half-reaction of PHHY is not significantly affected by this mutation, contrary to the concept that Tyr289 is a critical residue in the hydroxylation reaction [Enroth, C., Neujahr, H., Schneider, G., and Lindqvist, Y. (1998) *Structure 6*, 605−617; Ridder, L., Mullholland, A. J., Rietjens, I. M. C. M., and Vervoort, J. (2000) *J. Am. Chem. Soc. 122*, 8728−8738]. Tyr289 may help stabilize the FAD in the out conformation where it can be reduced by NADPH. For the Asp54Asn mutant form of PHHY, the initial step of the oxidative half-reaction is significantly slower than for the wild-type enzyme. Asp54Asn utilizes less than 20% of the reduced flavin for hydroxylating the substrate with the remainder forming H_2O_2. Similar changes are observed when Arg281, a residue between Asp54 and the solvent, is mutated to Met. These two residues are suggested to be part of the active site environment the enzyme provides for the flavin cofactor to function optimally in the oxidative half-reaction. In the construction of the mutant forms of PHHY, it was determined that 11 of the previously reported amino acid residues in the sequence of PHHY were incorrect.

Aromatic compounds are the second largest organic group existing in nature, next only to the carbohydrates. They are mainly found in the plant kingdom, and derive in large measure from lignin of plants (*1*). With the development of modern industry, phenolic compounds are also produced as plastics, resins, dyes, etc., many of which become toxic waste after their usage. Hence, the degradation of aromatic materials is very important for the carbon cycle and for detoxification of waste products produced by humans. Phenol hydroxylase (PHHY,[1] EC 1.14.13.7) is a flavin-containing monooxygenase from the aerobic topsoil yeast *Trichosporon cutaneum* (*2*). It is the first enzyme of a pathway this species uses for the degradation of phenolic compounds (*3*). Because of this pathway, *T. cutaneum* is able to utilize phenol as its sole carbon and energy source.

PHHY is one of the more fully characterized flavoprotein aromatic hydroxylases (*4*). In the reaction it catalyzes (*5*, *6*) (Scheme 1), the flavin of phenol hydroxylase is first reduced by NADPH. After releasing $NADP^+$, the reduced flavin reacts with oxygen to form intermediate I (C4a-hydroperoxyflavin) that is used to hydroxylate the substrate, phenol, or its derivatives, at the ortho position. The immediate product of the hydroxylation is termed intermediate II, which undergoes further change to give intermediate III (C4a-

Scheme 1: Reaction Cycle and Intermediates of Phenol Hydroxylase (*5*, *6*)



† Financial support was received from U.S. Public Health Service (Grant GM 20877 to D.P.B. and Grant GM 11106 to V.M.).

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[1] Abbreviations: PHHY, phenol hydroxylase from *T. cutaneum*; PHBH, *p*-hydroxybenzoate hydroxylase from *Pseudomonas aeruginosa*; CDO, catechol 2,3-dioxygenase; FAD, flavin adenine dinucleotide; IPTG, isopropyl 1-thio-β-D-galactopyranoside; P_i, phosphate; FlHOOH, enzyme-bound flavin hydroperoxide; FlHOH, enzyme-bound flavin hydroxide; WT, wild-type; TCA, trichloroacetic acid; CT, charge transfer; 2,4-D, 2,4-dihydroxybenzoate.

10.1021/bi010962y CCC: $20.00
Published on Web 09/22/2001

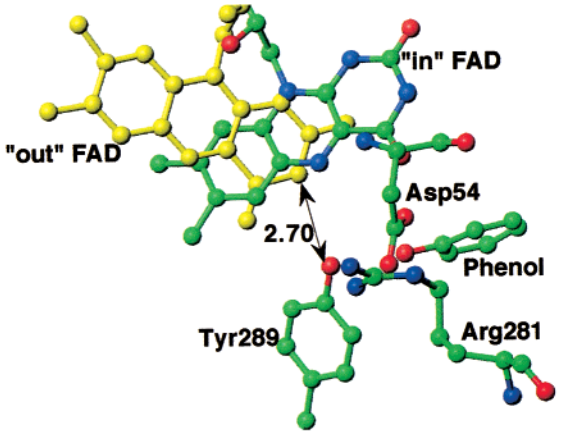


FIGURE 1: Structure of the active site of PHHY. The figure shows the two conformations (in and out) of FAD. The two flavins are aligned using their adenine parts as references. The out FAD is yellow. Also shown are the substrate (phenol) and three amino acid residues mutated in this paper. The hydrogen bond formed by Tyr289 and the N(3) atom of the out FAD is represented as a double-headed arrow. The distance (in angstroms) is calculated on the basis of the coordinates of Enroth et al. (*8*).

hydroxyflavin). The spectrum of intermediate II has been shown to be the sum of the spectra of the C4a-hydroxyflavin and the presumed dienone form of the product (*6*). The enzyme finishes the reaction cycle by releasing a molecule of H_2O to return the flavin to the oxidized state. Phenol hydroxylase has relatively broad substrate tolerance, permitting it to accept simple derivatives of phenol with amino, hydroxyl, halogen, or methyl substitutions (*7*). For all substrates, a fraction of the reduced flavin in PHHY is not utilized to hydroxylate the substrate, but instead returns to the oxidized state by releasing H_2O_2 (dashed lines in Scheme 1).

The crystal structure of PHHY with phenol bound has been determined at a resolution of 2.4 Å (*8*). The protein is a homodimer, and each monomer has 664 amino acid residues (molecular mass of 76 kDa) and a noncovalently bound FAD cofactor (Figure 1). In the structure of the dimer, the flavins are found to adopt two distinct conformations. In one monomer, the FAD is in a position termed "in", which is assumed to be the conformation necessary for hydroxylating the substrate. The FAD is buried inside the protein, close to the phenol bound at the active site, and is shielded from the solvent. In this conformation, the protein is poised to stabilize the reaction intermediates that otherwise would be short-lived in water solution. The flavin has a number of interactions with protein residues through hydrogen bonds, especially through its N(1) and O(2) atoms. Curiously, the FAD in the second monomer is shown to be in the "out" position. This is accompanied by a significantly different conformation of a peptide segment. In the out position, solvent is accessible to the *re* side of the FAD. Thus, NADPH could stack with the flavin to promote hydride transfer to the *re* side.

The active site geometry of PHHY is quite different from that of *p*-hydroxybenzoate hydroxylase (PHBH) (*9*), another FAD-containing enzyme that hydroxylates aromatic compounds (*4*), yet the reaction mechanisms of these two enzymes are very similar (*6*, *10*). The substrate of PHHY, phenol, is bound with its hydroxyl group pointing toward the dimethylbenzene part of the FAD (Figure 1), whereas with *p*-hydroxybenzoate in PHBH, the hydroxyl is oriented toward the flavin pyrimidine ring (*9*). In PHBH, there is a proton-transferring network that is composed of Tyr201, Tyr385, and His72. This network has been shown to be responsible for both the deprotonation of the substrate to enhance its nucleophilicity (*11*) and triggering the flavin movement (*12*, *13*) that is necessary for reduction of the flavin. Considerable evidence suggests that reduction of the flavin in PHBH occurs when the flavin is out. Because there is no direct counterpart of the proton-transferring network in PHHY, it is interesting to elucidate the mechanism of the flavin movement and what role such movement plays in the catalysis of phenol hydroxylase. It is equally important to find out what residues in this enzyme are involved in promoting catalysis of the chemical reaction. In these studies, we have constructed three mutant forms of PHHY that on the basis of the crystal structure (Figure 1) are likely to be involved in these catalytic processes. The residues that are changed are Tyr289, Asp54, and Arg281. The results of kinetics and other studies of these mutant forms are summarized in this paper.

Table 1: Mutagenic Primers

mutation	original codon	mutated codon
Asp54Asn	GAC	AAC
Arg281Met	CGC	ATG
Tyr289Phe	TAC	TTC

MATERIALS AND METHODS

Mutagenesis. The plasmid encoding the gene of phenol hydroxylase (*14*) (pRJ6C) was provided by J. Reiser (National Institutes of Health, Bethesda, MD). In the plasmid, *phyA*, the cDNA encoding phenol hydroxylase, is ligated to the *Escherichia coli* expression vector pKK223-3 pelB. The expression of phenol hydroxylase is controlled by the regulatable *tac* promoter. The plasmid also includes the sequence encoding β-lactamase to enable selection of transformants by their resistance to ampicillin. The QuickChange Site-Directed Mutagenesis Kit from Stratagene was used to construct the mutants. Pairs of primers containing the desired mutations were used in the PCR. The primers had the following sequences (the underlined codons include the italicized substitutions that encode the mutations): D54N (GTCTACAATGGCCAGGCA*AAC*GGTCTCCAGTGC), R281M (GGCTCGATCATGATCATCCCG*ATG*GAGAACAATCTCGTCCGC), and Y289F (CAATCTCGTCCGCTTCT*TC*GTTCAGCTCCAGGCCCG) (Table 1). After the chain reaction was finished, *Dpn*I, a restriction enzyme that is specific for methylated and hemimethylated DNA, was introduced to specifically degrade the original template plasmid encoding the gene for the wild-type phenol hydroxylase. Then the synthesized mutated plasmid was transformed into *E. coli* XL-1 Blue supercompetent cells to have the staggered nicks ligated.

Enzyme Purification. Both the wild-type and the mutated phenol hydroxylases were expressed and purified as recombinant enzymes (*15*). The plasmid was transformed into BL21 (DE3) *E. coli* cells from Novagen. The cells were allowed to grow in LB medium in the presence of ampicillin (50 μg/mL) at 25 °C. When the OD_{600} of the medium reached 0.6, the expression of the desired protein was induced by adding IPTG to a final concentration of 50 μM. The cells

were collected by centrifugation and broken by sonication. The enzyme was purified by the two-step chromatography procedure described in Waters and Neujahr (*15*), which uses phenyl-Sepharose (from Pharmacia Biotech), and DEAE-Sepharose ion exchange (from Sigma). The purified protein was manifest as a single band in SDS−PAGE with an apparent molecular mass of 76 kDa.

Stopped-Flow Studies of Half-Reactions. The UV−visible absorption changes in the reaction of phenol hydroxylase were monitored with a Kinetic Instruments stopped-flow spectrophotometer. The same reactions were monitored with a Hi-Tech SF-61 stopped-flow instrument to detect fluorescent intermediates (*6*). Fluorescent emitted light with wavelengths of >500 nm was detected and recorded. The Hi-Tech instrument is also equipped with a diode array detector to record absorption spectra in the reaction. Unless specifically mentioned, all stopped-flow experiments were conducted at 4 °C in 50 mM KP_i buffer (pH 7.6). The stopped-flow system was made anaerobic by incubating it overnight with an anaerobic solution of 3,4-dihydroxybenzoate (protocatechuate) and protocatechuate 3,4-dioxygenase (*16*). All buffer and substrate solutions were made anaerobic by bubbling for 15 min with purified argon before use. For the oxidative half-reaction, solutions of various oxygen concentrations were obtained by bubbling with standardized O_2/N_2 gas mixtures from Matheson. The enzyme solution was made anaerobic in a glass tonometer by alternate steps of evacuating and purging with argon. In the study of the oxidative half-reaction, the enzyme was reduced by adding an NADPH-generating system containing glucose 6-phosphate, $NADP^+$, and glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Sigma), from a sidearm after the tonometer had been made anaerobic. The amounts of $NADP^+$ and glucose-6-phosphate dehydrogenase were adjusted so that the complete reduction of the enzyme was slow, requiring more than 15 min, so as not to interfere with the oxidative reaction being studied. Analyses were conducted by fitting data to exponential equations using the Marquardt algorithm (*17*) incorporated into Program A and developed by C. J. Chiu, R. Chang, J. Diverno, and D. P. Ballou at the University of Michigan. Simulations were carried out by using numerical integrations of the differential equations for the reaction sequences using a fourth-order Runge−Kutta algorithm (*17*), also in Program A. The spectra of intermediates in the reactions are determined by deconvolution methods as described previously (*10*).

Product Analysis. To quantify the products formed by phenol hydroxylase or its mutant forms, the enzyme with phenol bound was prereduced slowly in a tonometer by the glucose-6-phosphate dehydrogenase system. The reduced enzyme was mixed with oxygen-containing buffer in the stopped-flow instrument. After completion of the oxidation, the out-flow solution was collected with tubes containing 10% TCA to quickly quench the reaction and release the hydroxylated product and FAD cofactor. Only a single turnover of the enzyme reaction is possible by this method. The denatured protein was separated by centrifugation, and the supernatant solution was neutralized by adding solid sodium bicarbonate. The enzyme concentration was determined by recording the spectrum of the solution with a Hewlett-Packard 8452 diode array spectrophotometer. The extinction coefficient of free FAD at 450 nm (11 300 M^{-1} cm^{-1}) is used in the calculation. Then catechol 2,3-dioxygenase (*18*) was added to the solution. The product of the dioxygenase reaction (2-hydroxymuconic semialdehyde) has significant absorption with a peak at 375 nm (*19*). The absorbance change at 400 nm, where the product has an extinction coefficient of 21 190 M^{-1} cm^{-1}, was measured after adding the dioxygenase. This allowed calculation of the amount of 2-hydroxymuconic semialdehyde, which was assumed to be equivalent to the amount of catechol formed (Scheme 2). The coupling percentage between the hydroxylation of phenol and the enzyme reoxidation was obtained by comparing the quantity of the catechol formed and the quantity of FAD in the enzyme. The coupling efficiency was also determined by analysis of stopped-flow traces at 440 nm where only the oxidized enzyme has significant absorbance. Only the absorbance increase after the formation of intermediate II (the transfer of oxygen to phenol/resorcinol) represents the fraction of the enzyme that hydroxylates the substrate. The percentage of coupling was calculated by dividing this absorbance by the total absorbance change observed at 440 nm in the oxidative half-reaction.

Scheme 2: Reaction of Catechol 2,3-Dioxygenase (CDO) (*18, 19*)

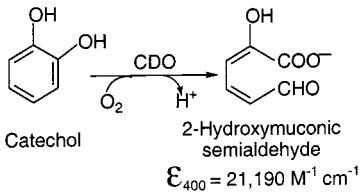


Table 2: Discrepancies in the Identities of Specific Nucleotides and Derived Amino Acids between That Found in This Work and That Reported Previously (*14*)

amino acid	in publications	in plasmid
19	Gly [GGT]	Gly [GGG]
123	His [CAC]	Arg [CGC]
171	Asp [GAC]	Glu [GAG]
172	His [CAC]	Asp [GAC]
177	Leu [CTA]	Leu [CTG]
186	Ser [AGC]	Gly [GGC]
189	His [CAC]	Arg [CGC]
405	His [CAC]	Gln [CAG]
406	Ala [GCA]	Pro [CCA]
532	Ser [TCC]	Ala [GCC]
544	Leu [CTC]	Arg [CGC]
549	Val [GTC]	Gly [GGC]
550	Ser [TCT]	Ala [GCT]
265[a]	Pro [CCG]	Arg [CGC]

[a] Position 265 is reported as Pro in the cloning paper (*14*), but was recognized as Arg in the structure paper (*8*).

RESULTS

Amino Acid Sequence of Phenol Hydroxylase. The gene encoding phenol hydroxylase was sequenced. In three separate sequencing results (for the WT as well as D54N and Y289F mutant forms), it was found that 13 nucleotides were different from those previously reported (*8, 14*). The single base discrepancies correspond to 11 amino acids, and the changes are listed in Table 2. Fortunately, none of these residues is close to the active site so that the original interpretation of the X-ray electron density map is probably mostly correct (*8*). C. Enroth and Y. Lindqvist are refining

Table 3: Oxidative Half-Reaction of the Wild-Type PHHY and Three Mutant Forms Using Phenol as a Substrate

	WT	Y289F	D54N	R281M
k_{obs1} (M^{-1} s^{-1})	1.6×10^5	2.0×10^5	2.1×10^3	2.9×10^4
k_{obs2} (s^{-1})	5.3	32	—[c]	10
coupling of hydroxylation and NADPH consumption				
1[a]	88%	65%	low[c]	14%
2[b]	91%	62%, 67%	19%	17%

[a] Estimation from the reaction trace. [b] Catechol 2,3-dioxygenase reaction (*18*, *19*). [c] Single-phase reaction.

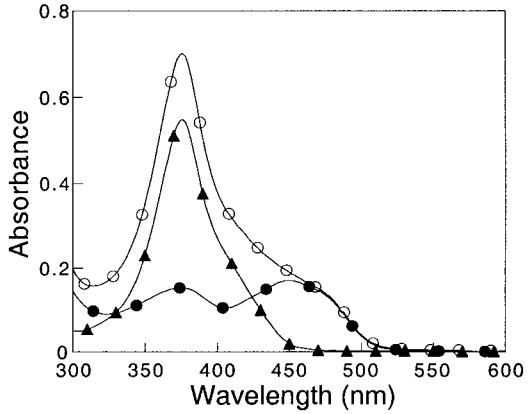


FIGURE 2: Coupling between the hydroxylation of phenol and FAD reoxidation in a single-turnover experiment for Y289F. Experimental details and conditions are described in Materials and Methods. The spectrum of FAD released from the enzyme is shown (●). The spectrum of the solution after the addition of catechol 2,3-dioxygenase is shown (○). The difference spectrum (▲) was used to quantify the amount of catechol formed.

the structure on the basis of the corrected sequence (personal communication).

Transient Kinetic Studies on the Tyr289Phe Mutant. Tyrosine 289 was postulated to be a critical residue for the catalytic function of PHHY (*8*). Tyr289 is hydrogen bonded with the hydroxyl group of the substrate (phenol), similar to how Tyr201 hydrogen bonds to *p*-hydroxybenzoate in PHBH. Tyr201 has been shown to be a general base in the oxidative half-reaction of PHBH (*11*). It deprotonates the substrate, *p*-hydroxybenzoate, to facilitate nucleophilic attack on the flavin C4a-hydroperoxide intermediate. To test the possibility that tyrosine 289 of PHHY was similarly responsible for deprotonation of the substrate, it was changed to phenylalanine by site-directed mutagenesis to remove the hydrogen bond interaction with the substrate. The mutant enzyme, Y289F, binds phenol more weakly than does the wild-type enzyme, with the dissociation constant increased from 1.8 to 27 μM; this is consistent with the loss of a hydrogen bond between the protein and the substrate. The dissociation constants were determined from equilibrium titrations by monitoring the changes to the spectrum of the enzyme-bound flavin caused by binding the substrate.

The oxidative half-reaction was first carried out using phenol as the substrate. The reaction of reduced Tyr289Phe with O_2 to form the hydroperoxyflavin is slightly faster than that with the wild-type enzyme (Table 3). The coupling between the hydroxylation of phenol and enzyme reoxidation was estimated to be ~66% toward the formation of the catechol product and ~34% toward the formation of H_2O_2. This was determined both on the basis of stopped-flow

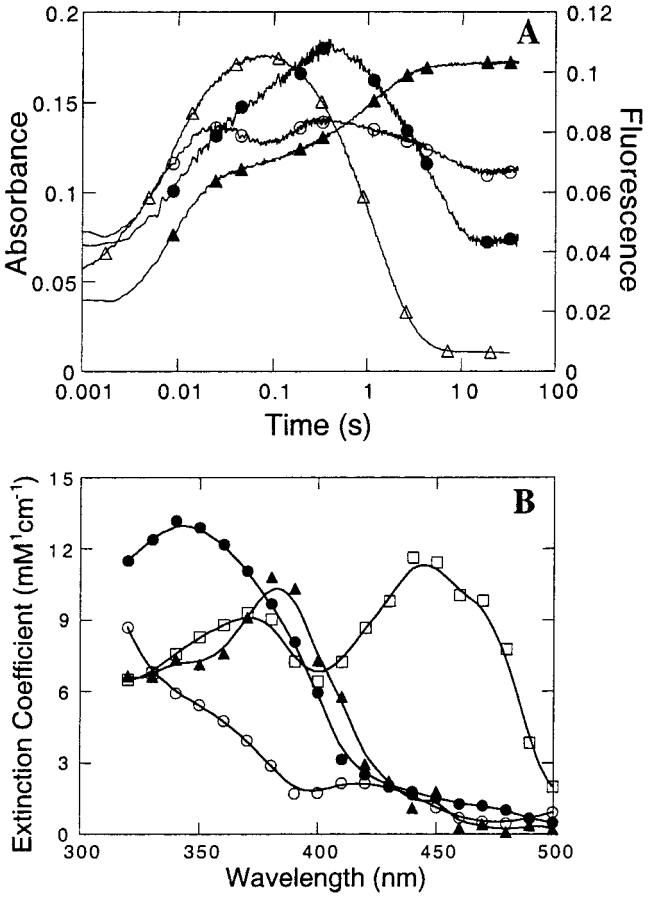
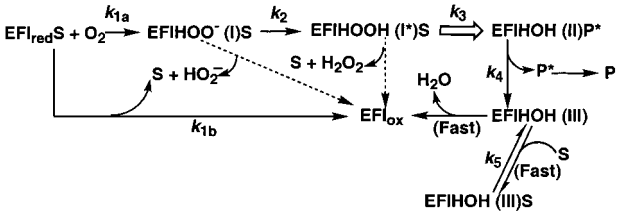


FIGURE 3: Oxidative half-reaction of Tyr289Phe with 2 mM resorcinol and 975 μM O_2 (conditions after mixing). The reduced enzyme (32 μM) with resorcinol bound (2 mM) was mixed with phosphate buffer equilibrated on ice with O_2 and 2 mM substrate. (A) Absorbance changes in the reaction at 340 (●), 380 (○), and 440 nm (▲) as well as fluorescence changes (△, excitation wavelength of 385 nm, emission wavelength of >500 nm) are shown. There are five discernable phases, the same as with the wild-type enzyme (*6*). See Scheme 3 for a detailed analysis of the reaction. (B) The spectra of intermediate I [C4a-peroxyflavin (▲)] and intermediate II [C4a-hydroxyflavin and dienone form of the product (●)]. Spectra of the fully reduced (○) and oxidized (□) Y289F mutant enzymes are also shown in the figure. All four spectra were acquired through deconvolution of the stopped-flow experimental results.

reaction traces (data not shown; see Materials and Methods) and by monitoring catechol formation with catechol 2,3-dioxygenase (Scheme 2 and Figure 2) (*18*, *19*). With the wild-type enzyme under the same conditions, the extent of coupling was ~90% (Table 3). These results show that the oxidative half-reaction of PHHY is not seriously affected by the mutation of Tyr289 to Phe when phenol is the substrate. By contrast, the apparently analogous mutation with PHBH, Tyr201Phe, causes complete uncoupling of the reaction so that no hydroxylated product is observed.

For kinetic reasons, when using phenol as the substrate, intermediate II cannot be observed in the oxidative half-reaction for either the wild type (*20*) or the Tyr289Phe mutant form of PHHY (this study). The oxidative reaction with resorcinol (1,3-dihydroxybenzene) as the substrate permitted detection of intermediate II, as was true with the WT enzyme (*6*) (Figure 3 and Scheme 3). As with the wild-type enzyme, five steps were also observed with Y289F in this reaction. The first step is a second-order reaction with a rate directly

Scheme 3: Oxidative Half-Reaction of the Wild-Type (*6*) and Tyr289Phe Mutant PHHY Using Resorcinol (S) as a Substrate



	k_1 (M^{-1} s^{-1})	k_2 (s^{-1})	k_3 (s^{-1})	k_4 (s^{-1})	k_5 (s^{-1})
PHHY-WT	1.1×10^5	44.2	6.9	1.85	0.164
PHHY-Y289F	1.2×10^5	24.5	10.6	4.29	0.17
PHHY-WT (with NaN_3)	8.8×10^4	45.6	1.16	0.43	0.001
PHHY-Y289F (with NaN_3)	8.7×10^4	34	6.7	0.65	0.03

k_1 is the sum of k_{1a} and k_{1b}

dependent on the oxygen concentration, and it is assumed to be the formation of intermediate I (flavin C4a-peroxide) whose spectrum was determined by deconvolution methods developed previously (*10*) and shown in Figure 3B. This step is characterized by increases in absorbance around 380−400 nm and by an increased fluorescence. At 975 μM O_2, the rate is 117 s^{-1}; therefore, this step is nearly complete by 25 ms. The absorbance at 440 nm also increases in the first 25 ms, consistent with the formation of oxidized FAD. This corresponds to a fraction of the reduced flavin being uncoupled to form H_2O_2 rather than hydroxylated product. The second step has been suggested to be the protonation of the C4a-peroxide (*6*). It is shown in Figure 3A as a further increase in fluorescence and a slight decrease in absorbance at 380 nm (between 20 and 100 ms) with a rate constant of 24.5 s^{-1}. The fluorescent properties of the mutant are different from those of the wild-type enzyme where the C4a-hydroperoxide (I*) is less fluorescent than the nonprotonated C4a-peroxide (I) (*6*). The third step (Scheme 3, $k_3 = 10.6 \pm 0.1$ s^{-1}) is the transfer of oxygen from intermediate I* to the substrate to form intermediate II (C4a-hydroxyflavin and the nonaromatic hydroxylated product) with an absorption peak at 340 nm (spectrum is shown in Figure 3B). This is characterized by an increase in absorbance at 340 nm, as well as a decrease in fluorescence (Figure 3A). In the fourth step, part of the hydroxyflavin returns to the oxidized state by releasing H_2O (manifested as an increase in absorbance at 440 nm). At the same time, the immediate nonaromatic product of oxygen transfer is rearranged to give 1,2,4-trihydroxybenzene. This is shown as decreases in absorbance at 340 nm from 0.5 to 10 s.

When the resorcinol concentration is high, as soon as the product is released, resorcinol can bind to the enzyme and stabilize intermediate III (C4a-hydroxyflavin) before H_2O is released. The slow dissociation of this substrate−intermediate III complex is suggested to be the cause of the fifth observed step (*6*), which occurs at a rate of 0.17 s^{-1}. As mentioned above, part of the reduced enzyme does not go through the hydroxylation steps, but releases H_2O_2 to return to the oxidized form. This is seen for both the Y289F mutant and the wild-type enzyme (with ~50 and 40% uncoupling, respectively). It is represented in Figure 3A as the increase in absorbance at 440 nm that occurs before the substrate is hydroxylated and coinciding with formation of intermediate I (in the first 25 ms of the reaction). This is a good diagnostic of the uncoupling reaction because oxidized flavin is the only species that has significant absorption at this wavelength. The spectra of intermediate I (C4a-peroxyflavin) and of intermediate II (C4a-hydroxyflavin and the dienone form of product) obtained through deconvolution (*10*) as well as the reduced and oxidized Y289F are presented in Figure 3B. They closely resemble those found in the reaction of the wild-type enzyme (*6*, *20*).

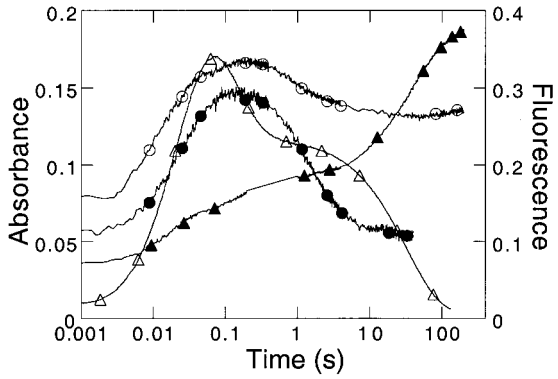


FIGURE 4: Oxidative half-reaction of the Tyr289Phe mutant with resorcinol and O_2 in the presence of azide. The reduced enzyme (32 μM) in the presence of resorcinol (2 mM) was mixed with equal volumes of KP_i buffer containing 1.95 mM O_2, resorcinol (2 mM), and 0.4 M NaN_3. The reaction is followed by the absorbance changes (left axis) at 340 (●), 380 (○), and 440 nm (▲) as well as fluorescence change (△, excitation wavelength of 385 nm, emission wavelength of >500 nm, right axis). The kinetic parameters are listed in Scheme 3. The absorbance and fluorescence changes are directly comparable with those of Figure 3.

The presence of azide in the oxidative reaction of several flavoprotein hydroxylases, including PHHY and PHBH, helps to resolve the kinetics of the intermediates (*5*, *6*, *10*). The influence of monovalent ions, such as azide, cannot be attributed simply to the increase in ionic strength in the reaction system because the oxidative half-reaction of the wild-type phenol hydroxylase is not affected by the addition of sodium sulfate at the same ionic strength. Azide has very little effect on the reaction with oxygen to form the C4a adduct, but it slows the ensuing steps significantly and thereby helps in characterizing intermediates of the oxidative reactions. Therefore, the oxidative half-reaction of the Tyr289Phe form was also studied in the presence of 0.2 M sodium azide (Figure 4 and Scheme 3). It is clear that azide does not significantly alter the rates for the initial reaction phases. However, the later steps are considerably slower in the presence of sodium azide, as shown by the fluorescence changes in the oxidative half-reaction. As with the wild-type enzyme, it appears that monovalent anions, such as azide, can stabilize the abortive complex of the substrate and intermediate III (flavin hydroxide) that is otherwise converted to the oxidized enzyme quickly by dehydration (*5*, *6*). The second phase of fluorescence decay probably represents the fraction of enzyme without substrate bound, and is characterized by an apparent rate constant of 0.65 s^{-1}. The last phase of fluorescence decay is due to the fraction of enzyme that has both resorcinol and azide bound at the active site. This state is termed the abortive substrate complex. The rate constant of 0.03 s^{-1} is explained as the slow release of the substrate from intermediate III (Scheme 3, k_5). As is the case for the wild-type phenol hydroxylase, the presence of azide ion increases the fluorescent quantum yields for the C4a-

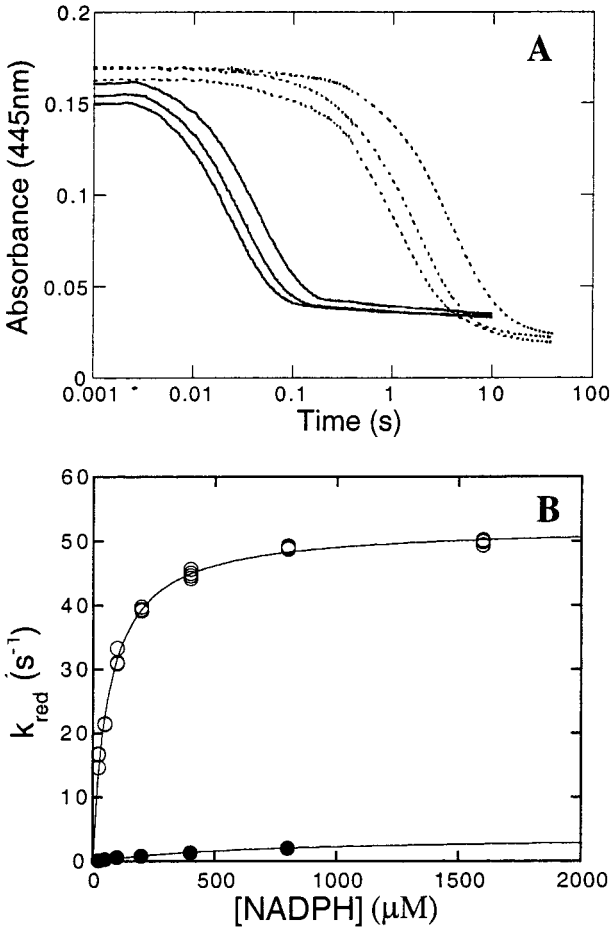


FIGURE 5: Comparison of the reductive half-reaction of wild-type PHHY and the Tyr289Phe mutant (both in the presence of 1 mM phenol). A solution containing the oxidized enzyme was mixed with NADPH anaerobically using the stopped-flow spectrophotometer. (A) The reactions for WT (—) and the Y289F mutant form (- - -) followed by the absorbance change at 445 nm. The reaction traces at three final concentrations of NADPH (50, 100, and 200 μM) are shown for each form of the enzyme. (B) Dependence of the reductive reaction on the NADPH concentration for wild-type (○) and Y289F PHHY (●).

flavin intermediates and enhances inhibition by the substrate of the Tyr289Phe mutant form. The results described above show that the steps in the reaction of oxygen with the reduced form of Tyr289Phe PHHY are very similar to those with WT (*5*, *6*) (Scheme 3).

The reductive half-reaction with NADPH, however, is significantly affected by the mutation. The rate constant is 52 s^{-1} for the wild-type enzyme, but only 3.9 s^{-1} with Tyr289Phe. In addition, the apparent dissociation constant for NADPH is more than 10-fold higher for the mutant form (65 μM for WT and 740 μM for Tyr289Phe). Thus, the efficiency of the reduction for the Y289F mutant, measured by k_{red}/K_d, is only 1/150 of that of the wild-type PHHY (Figure 5 and Table 4). The redox potential of this mutant enzyme was measured by using xanthine and xanthine oxidase to mediate the reduction of the enzyme in the presence of the redox dye, phenosafranin (*21*). The potential of Tyr289Phe was determined to be slightly higher (−192 mV) than that of the wild-type enzyme (−222 mV) (both determined with phenol bound). A higher redox potential would suggest that the FAD might be reduced more quickly, rather than more slowly, as observed. Therefore, a reason

Table 4: Reductive Half-Reaction of the Wild-Type PHHY and Mutants (with Phenol Bound)

	WT	Y289F	D54N	R281M
k_{red} (s^{-1})	52	3.9	9.4	12
K_d (μM) (NADPH)	65	740	79	40
k_{red}/K_d (s^{-1} M^{-1})	8.0×10^5	5.3×10^3	1.2×10^5	3.0×10^5
K_d (μM) (phenol)	1.8	27	124	~1.0

or reasons other than thermodynamic drive must be the cause of the FAD being reduced more slowly in Y289F. A comparison between the two conformations of the enzyme-bound FAD (*8*) suggests a possible explanation. The Tyr289 residue is hydrogen bonded through its side chain oxygen atom to the N (*3*) of the FAD when the latter adopts the out conformation. However, it does not have this interaction when the flavin moves to the in position (*8*) (Figure 1). Judging from the crystal structure, we conclude that NADPH could be easily accessible to the isoalloxazine ring of the out FAD to permit hydride transfer to the enzyme flavin. It is possible that the mutation from tyrosine to phenylalanine favors the "flavin in" conformation by annulling the hydrogen bond with the "flavin out" conformation. In other words, Tyr289 might affect the equilibration between the two conformations and help the flavin to move out to be reduced by NADPH.

Studies on the Asp54Asn Mutant Form of PHHY. The active site Asp54 was also regarded as a candidate for the catalytic base in the structure paper (*8*). The Asp54Asn mutant form of the enzyme exhibits remarkable differences from the wild-type PHHY. The dissociation constant for binding of phenol is 1.8 μM for WT, while it is 124 μM for Asp54Asn (Table 4). This is consistent with the crystal structure of WT, which shows that the carboxylic oxygen of Asp54 is hydrogen bonded with the substrate (Figure 1). No intermediates could be discerned in stopped-flow experiments when the reduced mutant enzyme in the presence of saturating concentrations of phenol was mixed with oxygen (Figure 6A). As shown in Figure 6, this is fundamentally different from the analogous reaction of the wild-type PHHY in which the formation of intermediate I could be observed by following the absorbance change at 400 nm. For Asp54Asn, the oxidized enzyme is formed in a second-order reaction with a rate constant of 2.1×10^3 M^{-1} s^{-1} (Figure 6B and Table 3). This is only about 1/80 of the value for the formation of the flavin hydroperoxide in the wild-type enzyme under the same conditions. It appears that the pseudo-first-order rate constant for the initial step is smaller than those for later steps so that the intermediates do not accumulate. The product, catechol, was quantified by the catechol 2,3-dioxygenase reaction (*18*, *19*) (Table 3). Only 19% of the reduced FAD is utilized by the mutant enzyme to hydroxylate phenol, while the rest enters the futile route to give hydrogen peroxide.

By contrast to the reaction with phenol as the substrate, the oxidative half-reaction of the D54N mutant enzyme using resorcinol as a substrate has four distinguishable steps (Figure 7). The kinetics are complicated by the fact that the rate constant for the first chemical step (Scheme 3, k_1) is smaller than those for the subsequent steps, k_2 and k_3. The first observed phase was a slight increase in fluorescence and in absorbance at 445 nm. This occurred at a rate of 9 s^{-1} and was independent of oxygen concentration. The next step gave

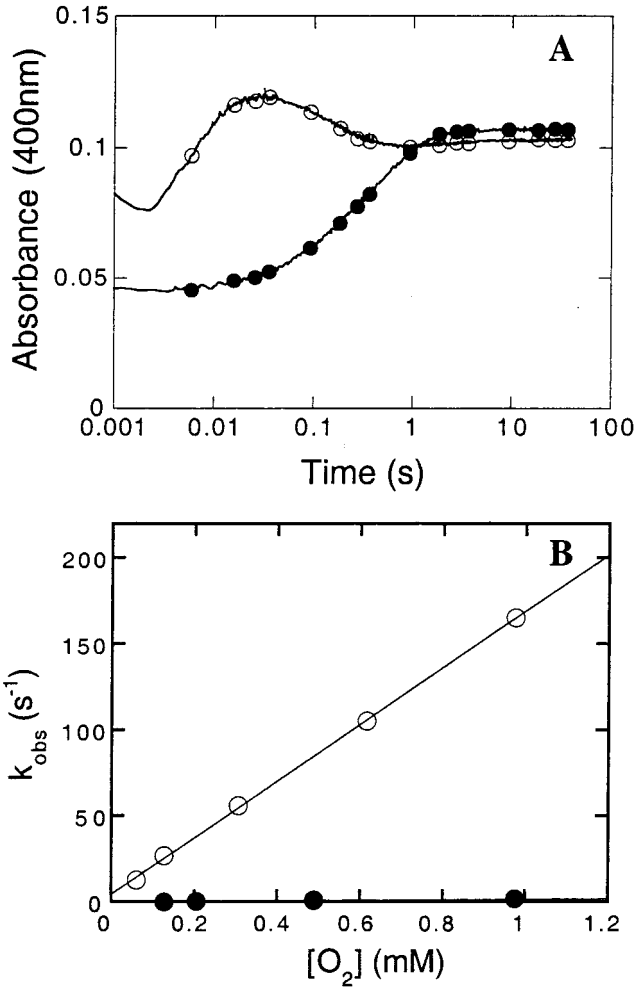


FIGURE 6: Oxidative half-reaction of the wild-type and Asp54Asn mutant forms of PHHY studied by stopped-flow experiments in the presence of 0.2 and 2 mM phenol, respectively. (A) Absorbance change at 400 nm (final oxygen concentration of 975 μM). For the wild-type PHHY (○), the reaction has two phases, with intermediate I formed in the first one. For the D54N mutant (●), the reaction is monophasic. (B) The observed rate constants of the first step in the oxidation of the wild-type (○) and the D54N mutant (●) PHHY plotted vs the concentration of oxygen. For both studies, the initial concentration of the reduced enzyme was 20 μM.

rise to a large increase in fluorescence at a rate of 4 s^{-1} and had a maximum quantum yield when the excitation wavelength was 385 nm. On the basis of studies of the oxidative half-reaction of the wild-type form of PHHY (*6*, *20*), such highly fluorescent intermediates are likely to be due to the peroxyflavin species, I and I*. Curiously, only the third observed step exhibits a dependence on oxygen concentration. This step is characterized by an increase in absorbance at 345 nm and a large decrease in fluorescence. This process can be characterized with a rate constant of 2×10^3 M^{-1} s^{-1} (1.9 s^{-1} at 0.975 mM oxygen used in the experiment of Figure 7), and is most likely the actual rate of formation of intermediate I. The rate constant is very close to that for the reaction using phenol as a substrate (Table 3). Thus, because the steps that follow have rate constants larger than that for the formation of intermediate I, only small amounts of the intermediates accumulate, and the first two observed steps are due to events that actually occur after formation of intermediate I. This type of phenomenon is described by Fersht (*22*). In the last step (0.3 s^{-1}), the fluorescence as

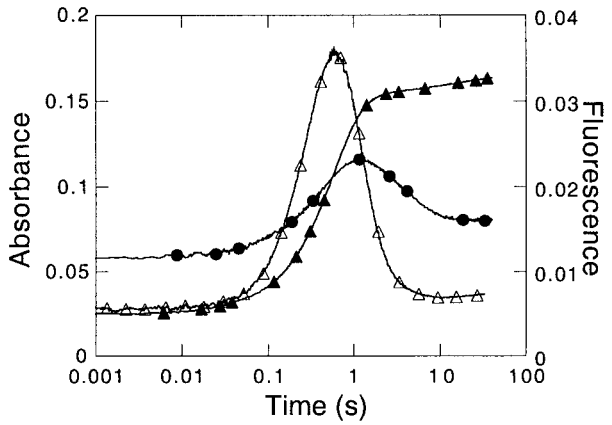


FIGURE 7: Oxidative half-reaction of 18 μM Asp54Asn with 975 μM O_2 in the presence of 5 mM resorcinol. Changes in absorbance at 345 (●) and 445 nm (▲) as well as fluorescence intensity (△, excitation wavelength of 385 nm, emission wavelength of >500 nm) are plotted vs time.

well as the absorbance at shorter wavelengths decreased further as the enzyme returned to the oxidized state. It should be pointed out that the absorbance at 445 nm increased in all four steps of the reaction. Since no species other than the fully oxidized FAD has significant absorption at this wavelength (*6*), the increase in absorbance before the formation of intermediate II can be ascribed to the oxidation of flavin that has not transferred an oxygen atom to the phenolic substrate. In other words, only the absorbance increase in the fourth step (~17% of the total change) represents the fraction of flavin that is used to hydroxylate the substrate. This value is close to that for the coupling between the hydroxylation of phenol and the FAD reoxidation measured by the catechol 2,3-dioxygenase reaction for this mutant form (19%, Table 3). For both substrates, most of the reduced flavin with the D54N mutant form of enzyme enters the "futile" branches of the reaction cycle and produces H_2O_2 (Schemes 1 and 3). Because the formation of the peroxyflavin species is slower than the reaction of the hydroperoxide with substrate, and because of the large uncoupling to direct formation of oxidized enzyme and H_2O_2, only low concentrations of the intermediate species are formed with this mutant enzyme. The definite assignment of the first two observed rate constants to the corresponding chemical reaction steps, which is possible with the WT enzyme and the Y289F mutant form, is thus rendered impractical for the D54N mutant phenol hydroxylase. Simulations of the reaction sequence of Scheme 3 (*17*) using either set of rate constants ($k_2 = 9$ s^{-1} and $k_3 = 4$ s^{-1} or $k_2 = 4$ s^{-1} and $k_3 = 9$ s^{-1}) fit the experimental data equally well.

The presence of azide alters the oxidative half-reaction remarkably (Figure 8). The first phase becomes dependent on oxygen concentration. It is characterized by an increase in absorbance at 385 nm with a rate constant of 1.6×10^3 M^{-1} s^{-1}. Unlike that in the reaction without azide, the absorbance at 440 nm remained low in this step. It is reasoned that most of the reduced flavin reacts with oxygen to form C4a-peroxyflavin instead of being converted directly to the fully oxidized state. The second phase (rate constant of 0.65 s^{-1}) with a large increase in fluorescence is suggested to be the protonation of the flavin peroxide. The largest quantum yield was acquired at an excitation wavelength of

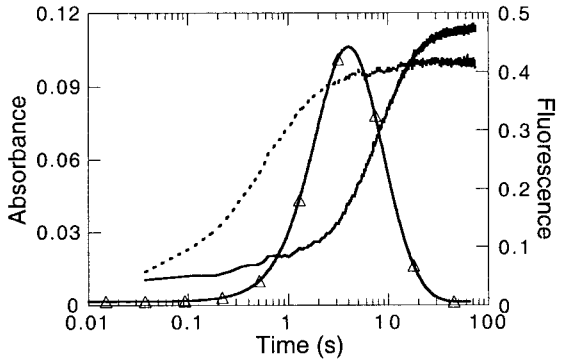


FIGURE 8: Oxidative half-reaction of the D54N PHHY mutant with 5 mM resorcinol, 975 μM O_2, and 0.2 M NaN_3 (all are final concentrations). The reaction was followed by the absorbance changes at 385 (- - -) and 440 nm (—) and the fluorescence change (△, excitation wavelength of 385 nm, emission wavelength of >500 nm).

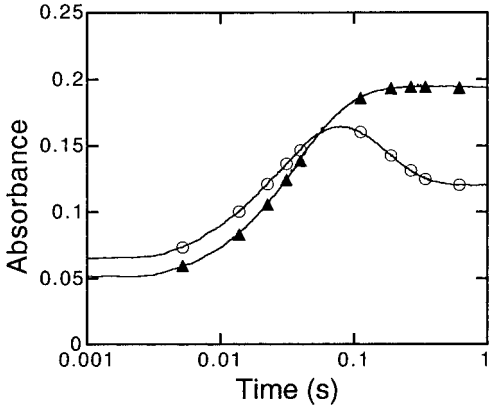


FIGURE 9: Oxidative half-reaction of Arg281Met. The reduced enzyme (17 μM) in the presence of 2 mM phenol was allowed to react with 975 μM O_2 (concentrations after mixing). Absorbance changes at 444 (▲) and 390 nm (○) are plotted vs time.

385 nm; this is also the absorbance peak of the C4a-hydroperoxyflavin. The fluorescence intensity was 12-fold greater than that for the reaction in the absence of azide (under the same conditions), possibly suggesting that considerably more flavin hydroperoxide was formed and/or the intermediate has a higher quantum yield. In the last observed step of the reaction, the enzyme returned to the oxidized form with a rate constant of 0.13 s^{-1}, as manifested by the increase in absorption at 440 nm, as well as by the decrease in fluorescence. These experiments show, as with the wild-type enzyme (*6*), that azide (final concentration of 0.2 M) increases the stability of the flavin hydroperoxide and that the fluorescence quantum yield of the hydroperoxide is significantly greater (Figure 8). It does not greatly change the rate for the initial reaction between oxygen and the reduced flavin (1.6×10^3 M^{-1} s^{-1} compared to 2×10^3 M^{-1} s^{-1} in the absence of azide) but retards subsequent reaction steps, as it does with the wild type and the Y289F mutant enzyme. The slowing of subsequent steps results in the first rate now being larger than those of the following steps.

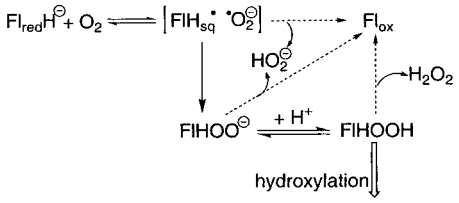
Studies on the Arg281Met Mutant of PHHY. Arg281, which lies between Asp54 and the solvent (Figure 1) (*8*), was changed to methionine to investigate the possible interactions that it has with Asp54. Consistent with the crystal structure, which shows that Arg281 has no direct contact with the substrate, the Arg281Met mutant form of the enzyme binds phenol with an avidity similar to that of WT, with a dissociation constant of ~1.0 μM. Like Asp54Asn, Arg281Met is reduced by NADPH reasonably fast with a limiting rate constant of 12 s^{-1} (Table 4). Neither of the mutations significantly affects the apparent dissociation constant for the binding of NADPH to the enzyme (79 and 40 μM, respectively, compared to 65 μM for the wild-type enzyme; see Table 4). As shown in the table, both mutant forms are reduced by NADPH with an efficiency (k_{red}/K_d) comparable to that for the wild-type enzyme.

The R281M mutant form of PHHY binds the FAD cofactor relatively weakly with a dissociation constant of ~1 μM. It is more difficult to distinguish and characterize intermediates in the oxidative half-reaction of this mutant than those of WT because of the free flavin in the enzyme solution. The R281M mutant form of the enzyme has lower reactivity toward molecular oxygen (Figure 9) than does WT. Judging by the absorbance change at 390 nm, a wavelength used to monitor the formation of intermediate I (*6*, *20*), we determined the rate constant for the initial step of the oxidative half-reaction using phenol as a substrate is 2.9×10^4 M^{-1} s^{-1}, which is $<^1/_5$ of that for wild-type PHHY (Table 3). By the time the absorbance at 390 nm reaches its maximum, most of the flavin is already oxidized, as shown by the increase in absorbance at 444 nm. This is a situation similar to the oxidative reaction of the Asp54Asn mutant form of the enzyme when resorcinol is the substrate. Comparison of these reaction traces shows that only ~14% of the reduced flavin is used to hydroxylate the bound phenol. In agreement, the coupling between substrate hydroxylation and FAD reoxidation measured by the catechol 2,3-dioxygenase reaction is only ~17% (Table 3), while that for the wild-type enzyme is ~90%. In general, this mutant form behaves in a manner similar to that of the Asp54Asn mutant, even though according to the structure Arg281 has no direct contact with the flavin or the substrate (*8*).

DISCUSSION

Phenol hydroxylase catalyzes an overall reaction involving three substrates (NADPH, phenol, and O_2); the FAD cofactor is involved in the reaction with each substrate. The requirements for it reacting with NADPH are different from those for reacting with O_2 and phenol. The crystal structure (*8*) shows that the FAD has two alternative conformations of its active site (Figure 1). By analogy to PHBH (*23*), it is proposed that the FAD can be reduced efficiently only when it occupies the out position where it is easily accessible to NADPH. For the oxidative half-reaction, however, the enzyme needs to accelerate the reaction between the reduced FAD and oxygen and to stabilize the flavin−oxygen intermediates that would be short-lived in free aqueous solution. This is only possible when the flavin is in the in position and isolated from the solvent. It is also the position where the flavin hydroperoxide could have direct contact with the bound substrate, phenol. Thus, there must be a mechanism to move the flavin from the out position to the in position. The N(1) and O(2) atoms of FAD can form several hydrogen bonds with amino acid residues Gly369, Met370, and Asn371 that are not possible when the flavin adopts the out conformation (*8*). What drives the oxidized FAD to move out to the surface to react with NADPH is

Scheme 4: Initial Reaction between Reduced Flavin and Oxygen (*25*−*30*)



less well understood. Binding of a substrate significantly accelerates the reductive half-reaction for all flavoprotein monooxygenases, including both phenol hydroxylase and *p*-hydroxybenzoate hydroxylase, that carry out hydroxylations of aromatic compounds. When PHBH binds one of its substrates, 2,4-dihydroxybenzoate (2,4-D), the flavin is mostly in the out position (*23*). From the crystal structure, it is clear that 2,4-D hydrogen bonds with the N(3) of FAD through its additional hydroxyl group, and hence, it favors the flavin out conformation (*24*). This hydrogen bond has a counterpart in the crystal structure of PHHY (*8*). The out but not the in flavin has its N(3) hydrogen bonded with the phenolic hydroxyl group of Tyr289 (Figure 1). As a result, by abolishing the hydrogen bond, the mutation of Tyr289 to phenylalanine very likely results in a less stable out conformation of FAD. This makes it more difficult for the flavin to move out to the surface of the protein, and the mutant is therefore reduced slowly by NADPH, even though it has a higher redox potential than the wild-type enzyme. In thermodynamic terms, the activation energy ($\Delta G^{\ddagger}$) for the reduction of the enzyme is proposed to be increased by the mutation, which may be expected to affect the equilibrium between the in and out conformations in favor of the flavin being in the in position.

The reaction between free reduced flavin and oxygen in aqueous solution is complex and autocatalytic (*25*−*30*). Because the reaction is spin-forbidden, the triplet oxygen has to accept one electron from the singlet reduced flavin in the first step to form a flavin semiquinone/superoxide anion radical pair that then can collapse to form peroxyflavin (Scheme 4). The formation of peroxyflavin is slow, with a rate constant of 250 M^{-1} s^{-1} at pH 6.5 and 20 °C (*26*) (when the oxygen concentration is 1 mM, the effective rate constant is 0.25 s^{-1}). The initial product (presumably the C4a-hydroperoxide) is unstable when the flavin is free in solution and cannot be directly observed because it decomposes rapidly to H_2O_2 and oxidized flavin, with a rate constant of 260 s^{-1} under the same conditions (*30*). The rate of formation of peroxyflavin in PHHY is 1.6×10^5 M^{-1} s^{-1} at pH 7.6 and 4 °C, nearly 10^3-fold faster than with free FAD at a temperature 16 °C higher. What the enzyme does to provide the active site for the oxidative half-reaction that can bring about such an increase in reactivity is an intriguing question that is not fully understood.

Studies on *p*-hydroxybenzoate hydroxylase (*11*, *12*, *24*) have identified three residues that are important in deprotonating the substrate and in forming a proton network from the active site to the solvent. They are Tyr201, Tyr385, and His72. By comparison, two residues at the active site of PHHY, Asp54 and Arg281, were originally considered to be the residues that facilitate the deprotonation of the substrate and pass the proton to the solvent, respectively (*8*). When these residues were changed in phenol hydroxylase, the rate of the initial step in the oxidative half-reaction was found to be significantly slower than that of the wild-type enzyme. If the roles of the two residues were as suggested, the conversion of the flavin hydroperoxide (intermediate I) to intermediate II (i.e., the oxygen transfer reaction), but not the formation of intermediate I, would be most severely affected by the mutations. However, a significant fraction (14−19% of the total, Table 3) of the flavin hydroperoxide of these mutant enzymes leads to hydroxylation of the substrate (phenol and resorcinol). Thus, the flavin hydroperoxide, although not efficiently formed, is utilized by the mutant enzyme to carry out its role in the catalysis. Previous ligand binding (*20*) and NMR (*31*) experiments suggest that phenolic substrates bound at the active site of PHHY are protonated, at least when bound to the oxidized enzyme. Recently, the catalytic reaction of phenol hydroxylase has been examined by computations that are based on quantum mechanics and molecular mechanics (*32*). These authors conclude that it is energetically unfavorable by 6 kcal/mol for the carboxylic group of Asp54 to accept the hydrogen from the hydroxyl group of the substrate. They suggest that even at the stage of intermediate I (flavin hydroperoxide), the majority of the substrate is still protonated. However, they reason that although the initial proton transfer step might be energetically unfavorable, the lower calculated energy barrier of the subsequent electrophilic attack of the flavin hydroperoxide on the phenolate would more than compensate for the energy barrier to deprotonation. Attractive though this argument might be, it is clear from the results presented here that if indeed the reactive species of the substrate is the phenolate, Asp54 is not responsible for its deprotonation. Instead, it would appear that Asp54 and Arg281 are more likely part of the environment that the enzyme provides at the active site for the oxidative half-reaction, especially the initial step between the reduced flavin and oxygen. However, they are not the counterparts of Tyr201, the catalytic base, or the residues involved in the proton transfer network of PHBH (*11*−*13*). When the residues are changed by mutation, the reaction with oxygen becomes closer to that of $FADH^-$ in free solution (slow and second-order) because it has lost the features that these residues must convey in the wild-type enzyme for efficient formation and stabilization of the peroxyflavin intermediates.

The results from computations (*32*) also suggest that Tyr289 of PHHY, through hydrogen bonding interactions, might stabilize the negative charge on the substrate (phenolate) that was deprotonated by Asp54 and hence would be important for the oxidative half-reaction (Scheme 1). This suggestion also is not supported by results of the studies on the Tyr289Phe mutant form of PHHY, which show that the mutation has little effect on the oxidative half-reaction of this enzyme. In summary, the residues involved in the catalytic functions of PHHY are very different from those of PHBH, even though the two enzymes have similar reaction intermediates (*6*, *10*).

ACKNOWLEDGMENT

We are grateful to Dr. Jakob Reiser from the National Institutes of Health who kindly provided the plasmid for PHHY and to Dr. John Lipscomb (University of Minnesota, Minneapolis, MN) for a gift of catechol 2,3-dioxygenase.

We appreciate the assistance from Dr. Betty Jo Brown on mutagenesis and protein purification. We are thankful to Drs. Dawei Sheng, Bruce A. Palfey, Kazuko Maeda-Yorita, and Mariliz Ortiz-Maldonado for helpful discussions on the reaction mechanism of PHHY and PHBH.

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BI010962Y