Studies of the Mechanism of Phenol Hydroxylase: Effect of Mutation of Proline 364 to Serine†

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Received July 1, 2002; Revised Manuscript Received September 11, 2002

ABSTRACT: An active site residue in phenol hydroxylase (PHHY), Pro364, was mutated to serine to investigate its role in enzymatic catalysis. In the presence of phenol, the reaction between the reduced flavin of P364S and oxygen is very fast, but only 13% of the flavin is utilized to hydroxylate the substrate, compared to nearly 100% for the wild-type enzyme. The oxidative half-reaction of PHHY using *m*-cresol as a substrate is similarly affected by the mutation. Pro364 was suggested to be important in stabilizing the transition state of the oxygen transfer step by forming a hydrogen bond between its carbonyl oxygen and the C4a-hydroperoxyflavin [Ridder, L., Mullholland, A. J., Rietjens, I. M. C. M., and Vervoort, J. (2000) *J. Am. Chem. Soc. 122*, 8728−8738]. The P364S mutation may weaken this interaction by increasing the flexibility of the peptide chain; hence, the transition state would be destabilized to result in a decreased level of hydroxylation of phenol. However, when the oxidative half-reaction was studied using resorcinol as a substrate, the P364S mutant form was not significantly different from the wild-type enzyme. The rate constants for all the reaction steps as well as the hydroxylation efficiency (coupling between NADPH oxidation and resorcinol consumption) are comparable to those of the wild-type enzyme. It is suggested that the function of Pro364 in catalysis, stabilization of the transition state, is not as important in the reaction with resorcinol, possibly because the position of hydroxylation is different with resorcinol than with phenol and *m*-cresol.

Phenol hydroxylase (PHHY,[1] EC 1.14.13.7) is a flavoprotein monooxygenase from the aerobic topsoil yeast *Trichosporon cutaneum* (*1, 2*). It catalyzes a reaction that incorporates an atom from molecular oxygen into the ortho position of phenol or its simple derivatives (*3*−*6*). In the proposed mechanism of PHHY (Scheme 1), oxygen reacts with enzyme-bound FAD, which has been reduced by NADPH, to form C4a-hydroperoxyflavin (intermediate I). The flavin intermediate then undergoes nucleophilic attack by the phenolic substrate. As a result, the distal hydroxyl group of the hydroperoxyflavin is transferred to the substrate to form intermediate II, a complex of C4a-hydroxyflavin and a cyclohexadienone, the nonaromatic oxygenated product. The reaction cycle is completed by rearomatization of the product and dehydration of intermediate III (C4a-hydroxyflavin).

The structure of PHHY with phenol bound (*7*) reveals that Pro364 could be important in enzymatic catalysis (Figure 1A). According to quantum and molecular mechanical computations (*8*), the carbonyl oxygen of Pro364 forms a hydrogen bond with the hydroperoxide of intermediate I (Scheme 2, top). The partial negative charge on the oxygen atom of the residue is suggested to stabilize the hydroxyl group of intermediate I that is attacked by substrate in the transition state. This interaction is suggested to lower the energy barrier for the oxygen transfer (phenol hydroxylation) step by 3 kcal/mol (*8*).

To test the hypothetical function of Pro364 in the catalysis of PHHY, the residue was changed by mutation to serine to allow more flexibility of the carbonyl oxygen. Kinetic and other studies of the P364S mutant enzyme show how the added flexibility affects catalysis.

MATERIALS AND METHODS

Mutagenesis. The plasmid (pRJ6C) that encodes the gene of phenol hydroxylase (*phyA*) (*9*) was provided by J. Reiser (National Institutes of Health, Bethesda, MD). In the plasmid, *phyA* is ligated to the *Escherichia coli* expression vector pKK223-3 pelB. The plasmid includes the *tac* promoter for

† Financial support was received from the U.S. Public Health Service (Grants GM 20877 and GM 64711 to D.P.B. and Grant GM 11106 to V.M.).

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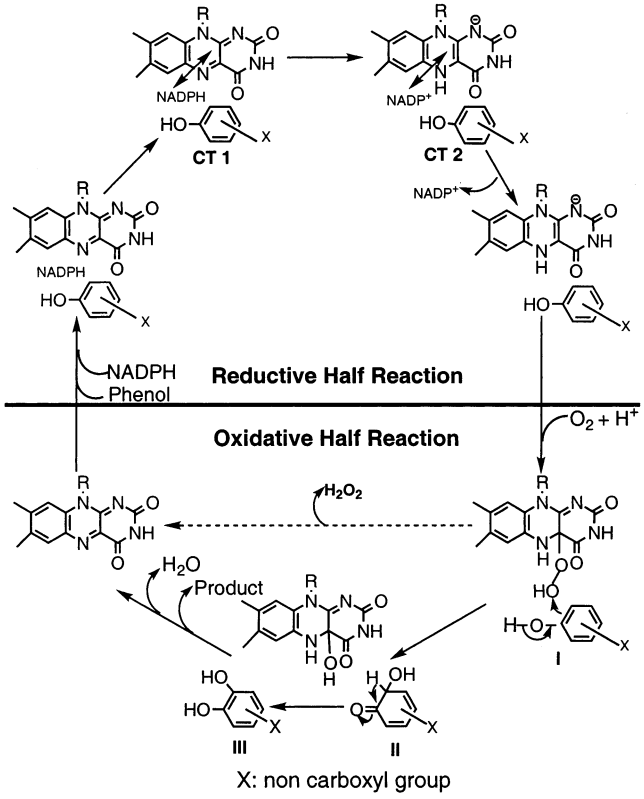
[1] Abbreviations: PHHY, phenol hydroxylase from *T. cutaneum*; PHBH, *p*-hydroxybenzoate hydroxylase from *Pseudomonas aeruginosa*; CDO, catechol 2,3-dioxygenase; SOD, superoxide dismutase; FAD, flavin adenine dinucleotide; IPTG, isopropyl 1-thio-β-D-galactopyranoside; P_i, phosphate; FlHOOH, enzyme-bound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

10.1021/bi020446n CCC: $22.00
Published on Web 10/26/2002

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



controlling the expression of phenol hydroxylase, as well as the sequence encoding β-lactamase, which enables selection of transformants by their resistance to ampicillin-related antibiotics. The QuickChange site-directed mutagenesis kit from Stratagene was used to construct the mutant. A pair of primers containing the desired mutation was used in the PCR procedure. The sequence of the sense strand is GCTTGCCACACCCATTCG*TCC*AAGGCCGGCCAGGGCATG (the underlined codon with the italicized substitution encodes the mutation from proline to serine). After the PCR was finished, *Dpn*I, a restriction enzyme that is specific for methylated and hemimethylated DNA, was introduced to specifically degrade the original template plasmid for the wild-type phenol hydroxylase. Then the synthesized mutant plasmid was transformed into *E. coli* XL-1 Blue supercompetent cells so that the staggered nicks could be ligated.

Enzyme Purification. Both the wild-type and mutant phenol hydroxylases were expressed and purified as recombinant enzymes (*10*). 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 carbenicillin (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 ultrasonication. The enzyme was purified by the two-step chromatography procedure described previously (*10*), 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 the 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 (*11*). Excitation light was selected with a monochromator; fluorescent emission of light with wavelengths longer than 500 or 530 nm was selected with yellow filters. The Hi-Tech instrument is also equipped with a diode array detector for recording absorption spectra of the reaction mixture. 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 (*12*). All buffer and substrate solutions were made anaerobic by bubbling for 15 min with purified argon before being used. For the oxidative half-reaction, solutions with various oxygen concentrations were obtained by bubbling with a standardized O_2/N_2 gas mixture 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 from a sidearm, after the tonometer had been made anaerobic, an NADPH regenerating system containing glucose 6-phosphate, $NADP^+$, and glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Sigma). The amounts of $NADP^+$ and glucose-6-phosphate dehydrogenase were adjusted so that the complete reduction takes 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 (*13*) incorporated into Program A (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 (13), also coded in Program A.

(4*R*)-[4-^{2}H]NADPH (NADPD) was prepared as previously described (*14*). 2,4,5,6-[^{2}H]$_4$Resorcinol was synthesized and characterized by methods previously reported (*15*, *16*).

Measurement of the Hydroxylation Efficiency. The catechol 2,3-dioxygenase (CDO) reaction was used to quantify the product formed by the P364S mutant enzyme using phenol as the substrate (*11*, *16*). In a stopped-flow apparatus, the reduced enzyme with phenol bound was mixed with aerobic buffer containing CDO, superoxide dismutase (SOD), and phenol. SOD is introduced into the system to eliminate superoxide ion formed in the reaction that might also oxidize catechol, the product of phenol hydroxylation. The product of the catechol 2,3-dioxygenase (2-hydroxymuconic semialdehyde) has an intense absorption peak at 375 nm (*17*, *18*). As a control experiment, the reaction was carried out in the absence of CDO. The additional absorbance increase at 400 nm caused by the reaction catalyzed by catechol 2,3-dioxygenase, where the product has an extinction coefficient of 21 200 M^{-1} cm^{-1}, was used to measure the amount of catechol formed in the system (*11*). The coupling between the hydroxylation of phenol and the reoxidation of enzyme was calculated from the concentration of catechol formed compared with that of the reduced enzyme initially in the reaction system. The hydroxylation efficiency of PHHY with *m*-cresol as the substrate was quantified by the

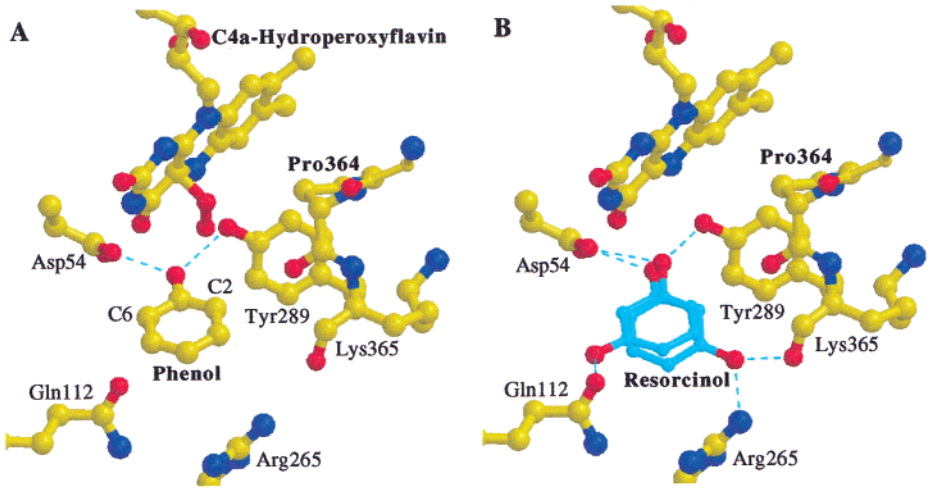
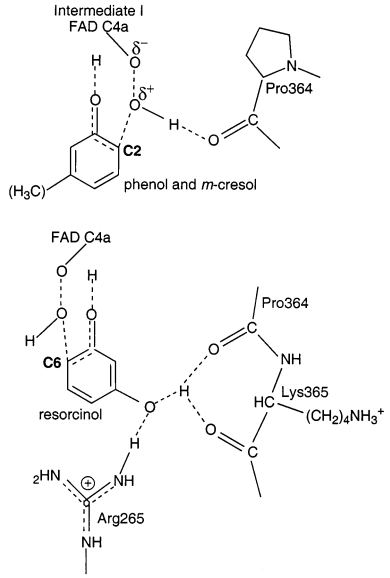


FIGURE 1: Active site of phenol hydroxylase. (A) Structure of PHHY with phenol bound (*7*). The modeled intermediate C4a-hydroperoxyflavin is shown in the figure as well as phenol and several residues, including Pro364 (mutated to serine in this work). Ortho carbon atoms of phenol are numbered C2 and C6 as explained in the text. (B) Simulated structure of enzyme with resorcinol bound. Resorcinol (light blue) is shown in two potential conformations with respect to the rest of the enzyme. Hydrogen bonds that are thought to be important for substrate binding are shown as dashed lines.

Scheme 2: Revised Hypothetical Function of Pro364 in the Hydroxylation Step of PHHY



same method. The extinction coefficient at 375 nm (42 800 M^{-1} cm^{-1}) of the final product of the CDO reaction on *m*-cresol was used.

The dioxygenase method is not applicable for the resorcinol reaction because of the poor stability of the product of the dioxygenase reaction. Therefore, the hydroxylation efficiency was measured as resorcinol consumption versus NADPH oxidation in the steady-state reaction. A limited amount of resorcinol was mixed to react (room temperature, air-saturated solution) with excess NADPH, catalytic amounts of the P364S mutant form or wild-type PHHY, CDO, and SOD. Catechol dioxygenase was included in the reaction system to consume the hydroxylated product [1,2,4-trihydroxybenzene (THB)] so that THB could not stimulate the oxidation of NADPH. [Over the time course of the assay (~15 min), the unstable product of the CDO reaction decomposes to compound(s) with negligible absorbance at 340 nm.] Thus, the oxidation of NADPH very nearly stops when resorcinol is fully hydroxylated. The amount of NADPH oxidized was calculated by the absorbance decrease at 340 nm ($\epsilon_{340} = 6220$ M^{-1} cm^{-1}) in the reaction. The ratio (total resorcinol/NADPH consumed) was taken to be the hydroxylation efficiency.

Table 1: Comparison of the Reductive Half-Reaction with NADPH between Wild-Type and P364S Mutant PHHY[a]

	phenol	resorcinol	*m*-cresol	no substrate
k_{red} (s^{-1})				
wild type	52	22	1.8	0.065
P364S mutant	164	25	5.2	2.4
$K_{d(NADPH)}$ (μM)				
wild type	65	140	60	570
P364S mutant	60	170	50	430

[a] The k_{red} values that are shown were obtained in the presence of saturating concentrations of phenolic substrates and represent the limiting rate constants extrapolated to an infinite concentration of NADPH. The K_d values correspond to the concentrations of NADPH equivalent to that which would give half the maximal extrapolated rate constants.

RESULTS

Reductive Half-Reaction of the Pro364Ser Mutant. Like wild-type PHHY (*2*), the mutant form binds its substrate (phenol) tightly, with a dissociation constant (K_d) of ~1 μM. However, the binding of FAD to P364S is weaker ($K_d \sim 2$ μM) than to the wild-type enzyme ($K_d \sim 10$ nM).

The P364S mutant form of phenol hydroxylase with phenol bound at the active site is reduced by NADPH faster than is the wild-type enzyme, with a maximum reduction rate of 164 s^{-1} compared to a rate of 52 s^{-1} for the wild-type phenol hydroxylase (*11*) (Table 1). The higher reactivity cannot be explained by differences in redox potential, as $E°$ of this mutant form in the presence of phenol (−220 mV, results not shown) is almost identical to that of the wild-type enzyme (−222 mV) in the presence of phenol (*19*). Likewise, the apparent dissociation constant for NADPH is not significantly affected by the mutation (60 μM for the mutant form compared to 65 μM for the wild-type phenol hydroxylase). The rapid hydride transfer with the mutant form depletes the first charge-transfer complex, CT 1

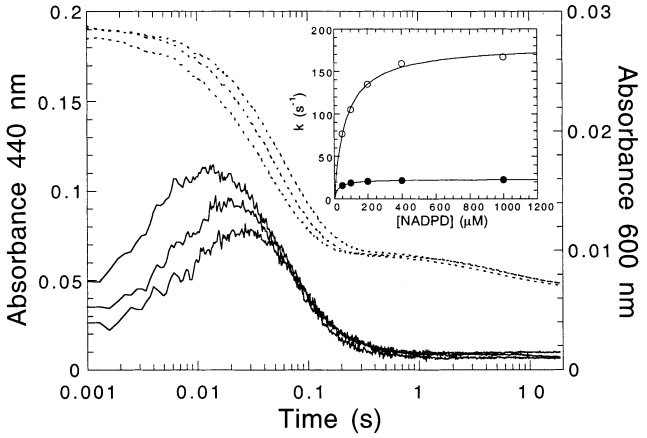


FIGURE 2: Reductive half-reaction of P364S PHHY by NADPD in the presence of phenol. The enzyme (35 μM) solution was mixed with NADPD (both contained 1 mM phenol) in a stopped-flow spectrophotometer under anaerobic conditions. The absorbance changes in the reaction at 440 nm (- - -, left axis) and 600 nm (—, right axis) were followed. The reaction traces at three final concentrations of NADPD (50 μM, 100 μM, and 1 mM) are shown for each wavelength. In the inset, the rate constants for reduction of FAD (●) and those for the formation of CT 1 (○) are plotted vs the concentration of NADPD.

Table 2: Kinetic Isotope Effect (KIE) (NADPH vs NADPD) in the Reductive Half-Reaction of Wild-Type and P364S Mutant PHHY[a]

	wild type		P364S mutant	
	phenol	resorcinol	phenol	resorcinol
KIE	9.6	8.3	7.1	2.2

[a] KIE values are calculated as the ratio of the limiting rate constant of reduction by NADPH to that of reduction by NADPD.

(NADPH-oxidized enzyme, Scheme 1), nearly as quickly as it is formed so that only the second charge-transfer complex, CT 2, that between the reduced enzyme and $NADP^+$, is observed. This charge-transfer complex, which is characterized by a low absorbance in the 400−500 nm region, and a broad band between 700 and 800 nm, is similar to that observed with PHBH (*20*) and decomposes at 63 s^{-1} as $NADP^+$ dissociates from the reduced enzyme (the analogous complex for the wild-type enzyme decays at ∼230 s^{-1}). Consistent with this faster reductive reaction and slower dissociation of $NADP^+$ from the mutant enzyme, the charge-transfer complex of the reduced enzyme and $NADP^+$ (CT 2 in Scheme 1) is more intense with the mutant form than that with wild-type PHHY.

The P364S mutant enzyme is reduced by saturating concentrations of NADPD at a rate of 23 s^{-1}, and a kinetic isotope effect of 7.1 was calculated (Figure 2 and Table 2). In the reaction, a charge-transfer complex characterized by a relatively small absorbance decrease in the 400−500 nm range, and an absorption maximum at 600 nm, consistent with CT 1 in Scheme 1, was observed. The rate of formation of this complex is dependent on the concentration of NADPD (apparent dissociation constant of 70 μM) with a limiting value of 180 s^{-1}. This complex (CT 1) of the oxidized flavin and reduced pyridine nucleotide accumulates during the reaction because of the slower reduction of enzyme-bound FAD by NADPD than by NADPH. This charge-transfer complex is also formed in the wild-type enzyme with a similar rate constant (140 s^{-1}) upon reaction with NADPD (data not shown). However, because of the slower reduction

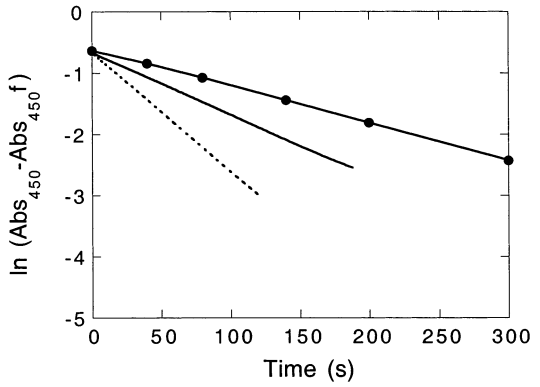


FIGURE 3: Flavin reductase activity of the P364S mutant form of PHHY. The mutant enzyme in the presence of 1 mM phenol was mixed with buffer containing the same concentration of phenol as well as 0.1 mM FAD and 1 mM NADPH in a stopped-flow spectrophotometer under anaerobic conditions. The reaction was followed by the absorbance change at 450 nm as FAD is reduced. The final enzyme concentrations were 2 (—) and 4 μM (- - -). The FAD reductase activity was also measured in an anaerobic cuvette experiment (●). The enzyme (final concentration of 1.3 μM) solution contained 50 μM FAD and 1 mM phenol. The reaction was started by adding a small volume of NADPH (final concentration of 0.2 mM) from the sidearm of the cuvette and followed by the absorbance change at 450 nm. For each trace, the logarithm of flavin absorbance minus that of the final reduced flavin (both at 450 nm) is plotted vs the reaction time. All reactions were carried out at 25 °C.

of the FAD in WT (KIE of 9.6, Table 2), coupled with the presumably unchanged dissociation rate constant of ∼230 s^{-1} from $NADP^+$, CT 2 cannot be observed with NADPD as the reductant.

Either with *m*-cresol bound or in the absence of any phenolic substrate, the P364S mutant form is also reduced faster than the wild-type enzyme (Table 1). In contrast, the reduction of P364S with resorcinol bound (25 s^{-1}) is very similar to that for the wild-type enzyme (22 s^{-1}). For all the reactions with NADPH, the apparent K_d values for NADPH are similar to those of the wild-type enzyme.

To test whether the similarity of the mutant and WT rates with resorcinol bound was due to some step occurring before hydride transfer, we determined the isotope effect with the P364S mutant enzyme. A saturating rate constant for reduction with NADPD of 11.6 s^{-1} was found, yielding a kinetic isotope effect of only 2.2 (Table 2). This KIE, which is much smaller than that for the reductive reaction in the presence of phenol (7.1), suggests that another step masks the true KIE. KIE values for flavoprotein hydroxylases usually equal 8−10 (*14*). In the presence of resorcinol, the wild-type enzyme is reduced by NADPD with maximum rate of 2.6 s^{-1}, which gives a KIE of 8.3 (Table 2), suggesting that in the wild-type enzyme no other step contributes significantly to the overall rate of reduction.

Flavin Reductase Activity of P364S Mutant PHHY. The mutant enzyme has considerable flavin reductase activity. At 25 °C and pH 7.6, free FAD is reduced by NADPH in a second-order reaction (i.e., it is directly dependent on the FAD concentration). The P364S mutant enzyme (in the presence of saturating concentrations of NADPH and phenol) has an NADPH−FAD reductase activity of 5.0×10^3 M^{-1} s^{-1} (Figure 3). By comparison, the values for wild-type PHHY (350 M^{-1} s^{-1}) and the Arg281Met mutant enzyme (380 M^{-1} s^{-1}) are both more than 1 order of magnitude

Table 3: Oxidative Half-Reaction of Wild-Type PHHY and the P364S Mutant Form

		phenol	resorcinol	*m*-cresol
				
k_{obs1} (M^{-1} s^{-1})	WT	1.6×10^5	1.2×10^5	9.7×10^4
	P364S	9.4×10^4	7.5×10^4	6.6×10^4
coupling of hydroxylation and NADPH oxidation (%)	WT	100	58	85
	P364S	13	55	12

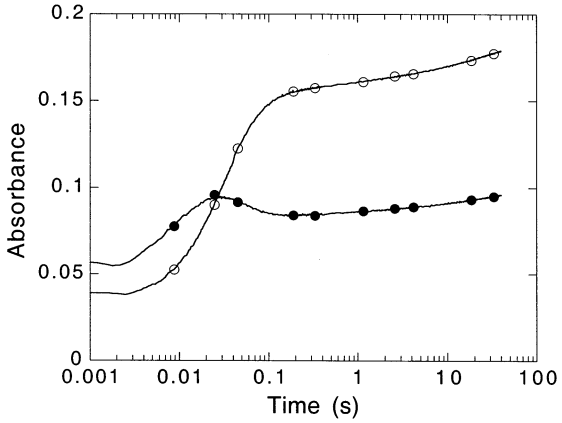


FIGURE 4: Oxidative half-reaction of P364S PHHY with phenol as the substrate. The reduced mutant enzyme (32 μM) in the presence of 1 mM phenol was mixed in a stopped-flow spectrophotometer with buffer solution containing the same concentration of phenol and 1.95 mM oxygen. The reaction was followed by absorption changes at 400 (●) and 440 nm (○).

smaller. Under the same conditions, P364S also mediates efficiently the reduction of either riboflavin or FMN by NADPH, with rate constants of 2.3×10^4 and 1.1×10^4 M^{-1} s^{-1}, respectively.

Oxidative Half-Reaction of P364S Mutant PHHY Using Phenol as a Substrate. The initial reaction between enzyme-bound reduced FAD and oxygen (Figure 4) is fast (9.4×10^4 M^{-1} s^{-1}), only marginally slower than that with the wild-type enzyme (1.6×10^5 M^{-1} s^{-1}) (Table 3) (*11*). The reaction is similar to that of the wild-type enzyme with the appearance of a transient intermediate absorbing at 400 nm due to the formation of intermediate I (C4a-hydroperoxyflavin) (*5*, *6*). When the maximal amount of intermediate I is formed at 25 ms, the absorbance increase at 440 nm is ~40% of the total increase in the oxidative half-reaction. Since the oxidized enzyme is the only species that has significant absorbance at 440 nm, it is estimated that approximately 60% of the reduced P364S mutant form of the enzyme reacts with oxygen to form intermediate I. This value, therefore, is the maximal fraction of enzyme capable of hydroxylating phenol bound at the active site. However, the coupling between product formation (measured by the catechol 2,3-dioxygenase reaction) and reoxidation of the enzyme-bound flavin is only 13% at 4 °C and 8% at 25 °C (the numbers for wild-type enzyme are 100 and 91%, respectively). Therefore, most of the intermediate I formed in the reaction is not utilized by the mutant enzyme to hydroxylate phenol, but probably decomposes nonproductively to hydrogen peroxide and the oxidized enzyme with a rate constant of 35 s^{-1}.

To better enable detection of intermediate III (C4a-hydroxyflavin), the oxidative half-reaction was also studied

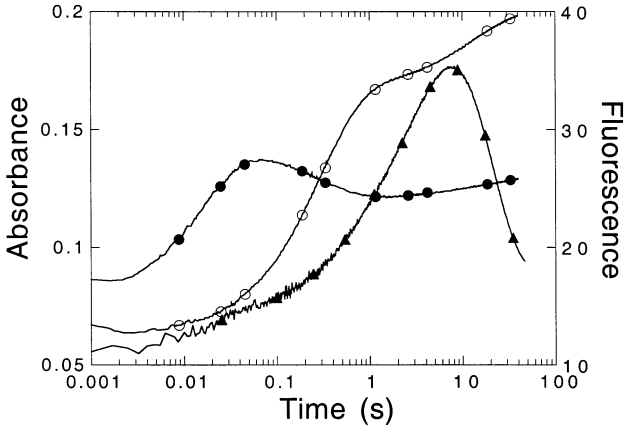


FIGURE 5: Oxidative half-reaction of P364S PHHY using phenol as the substrate and in the presence of sodium azide. The same reduced enzyme solution as in Figure 4 was mixed with buffer that included 1 mM phenol, 1.95 mM O_2, and 0.4 M NaN_3. The reaction was followed by absorption changes (left axis) at 400 (●) and 440 nm (○). The reaction was also studied by following the fluorescence change (right axis). In this case, the final enzyme concentration was 9 μM, the excitation wavelength was 380 nm, and emission was detected at wavelengths of >500 nm (▲).

in the presence of sodium azide, which is known to slow steps in the hydroxylation reaction (*5*, *6*). On the basis of absorbance and fluorescence, four steps could be distinguished (Figure 5). The first is dependent on the concentration of O_2 and is characterized by an absorbance increase around 400 nm with a rate constant of 6.2×10^4 M^{-1} s^{-1}. Very little absorbance change occurs at 440 nm in this phase, indicating that less direct formation of oxidized flavin occurs than in the absence of azide. This phase can be attributed to the formation of intermediate I (C4a-hydroperoxyflavin), because the observed rate constant is directly proportional to oxygen concentration. The second phase, which occurs at 3.6 s^{-1}, is associated with a significant absorbance increase at 440 nm (80−85% of the total change in the reaction). This increase is due to the formation of oxidized flavin, because it is the only species that has significant absorbance at this wavelength. In the same step, the absorbance at 400 nm decreases as intermediate I is converted to oxidized enzyme without hydroxylation of the phenolic substrate (dashed arrow in Scheme 1). The third phase of the reaction ($k_3 = 0.24$ s^{-1}) is detected as an increase in fluorescence. The strongest fluorescence signal is acquired when the excitation beam has a wavelength of 380 nm, the wavelength for the absorption peak of intermediate III (C4a-hydroxyflavin). The final step is characterized by a decrease in fluorescence at 0.06 s^{-1} and a small increase in absorbance at 440 nm (10−15% of the total change in the reaction). Thus, only a small amount (10−15%) of intermediate III accumulates at any time in the reaction (Scheme 1). It is clear from these results that intermediate III is highly fluorescent. Because intermediate III is a direct product of the hydroxylation reaction, these results are consistent with independent product measurements using the catechol 2,3-dioxygenase reaction that show only ~11% of the enzyme-bound phenol is hydroxylated in this reaction. Thus, most of the reduced P364S mutant enzyme forms intermediate I in the oxidative half-reaction with phenol bound at the active site, but only a small fraction of the intermediate is utilized to hydroxylate the substrate.

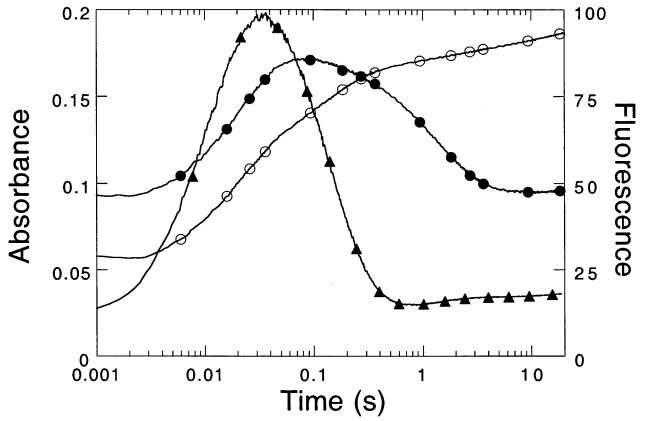
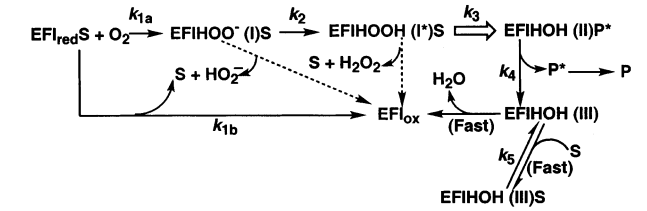


FIGURE 6: Oxidative half-reaction of P364S PHHY with resorcinol as the substrate. In a stopped-flow apparatus, the reduced mutant enzyme (32 μM) in the presence of 2 mM resorcinol was mixed with a buffer solution containing 2 mM resorcinol and 1.95 mM O_2. The reaction was followed by absorbance changes (left axis) at 340 (●) and 440 nm (○). The reaction was also studied by following the fluorescence change (right axis) (final enzyme concentration of 9 μM, excitation wavelength of 380 nm, and emission wavelength of >530 nm) (▲).

Scheme 3: Oxidative Half-Reaction of the Wild-Type and Pro364Ser Mutant PHHY Using Resorcinol (S) as the 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	1.2×10^5	46	5	0.54	NM
PHHY-P364S	7.5×10^4	60	7.4	0.76	NM
PHHY-WT (with NaN_3)	8.6×10^4	46.5	4.1	1.6	0.001
PHHY-P364S (with NaN_3)	4×10^4	47	4.2	0.7	0.16

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

Oxidative Half-Reaction with Resorcinol as a Substrate. The oxidative half-reaction of the wild-type enzyme with resorcinol (1,3-dihydroxybenzene) permits clear observation of intermediate II (complex of C4a-hydroxyflavin and the immediate nonaromatic product of hydroxylation) (Scheme 1) (*5*, *6*, *15*). Therefore, to test whether intermediate II can be resolved with P364S, resorcinol was used in the study of the oxidative half-reaction. Indeed, the reaction proceeds in a manner very similar to that of the wild-type enzyme, with four apparent steps (Figure 6 and Scheme 3). The first and second phases (the first 40 ms) are evidenced by an absorbance increase at short wavelengths (340−380 nm) and an intense fluorescence increase (excitation at 380 nm) with rate constants of 7.5×10^4 M^{-1} s^{-1} and 60 s^{-1}. On the basis of the results with the wild-type enzyme (*6*), the initial phases are suggested to be the formation of intermediate I (C4a-peroxide anion) and its protonation to C4a-hydroperoxide (Scheme 3). The third step (40 ms to 0.2 s) ($k_3 = 7.4$ s^{-1}) is distinguished by a decrease in fluorescence while the absorbance remains high in the short wavelength range. Intermediate I with resorcinol present is known to have intense fluorescence (*6*, *11*). Thus, the results are consistent with the third step being oxygen transfer from the C4a-hydroperoxyflavin to resorcinol to form intermediate II (C4a-hydroxyflavin and the dienone form of the product). The last step with a rate constant of 0.76 s^{-1} is characterized by large absorbance decreases at short wavelengths and an increase at 440 nm between 0.4 and 3 s. In this step, the product dissociates from the enzyme and rearomatizes (*6*) (Scheme 3). Very little intermediate III (C4a-hydroxyflavin) is observed because it quickly dehydrates to form the oxidized enzyme. Stopped-flow experiments using a diode array detector show that in this final step the absorption peak shifts from 340 nm, which is typical for intermediate II, to 375 nm, that of the oxidized enzyme (results not shown).

Because the absorbance at 440 nm increased in all four steps of the reaction, it was difficult to judge simply from the reaction traces how much resorcinol is hydroxylated by the P364S mutant enzyme. Therefore, the coupling between substrate hydroxylation and enzyme oxidation was measured by a different method (see Materials and Methods). A catalytic amount of the mutant enzyme was included in an aerobic reaction mixture containing excess NADPH and a limited amount of resorcinol. The oxidation of NADPH was monitored directly by the absorbance decrease at 340 nm. Under these experimental conditions, the oxidative consumption of NADPH in the absence of the phenolic substrate is much slower than in the presence of resorcinol because reduction of the flavin is at least 10-fold slower (Table 1). Therefore, the reaction rate is dramatically slower at the point when resorcinol is depleted. The coupling between substrate (resorcinol) hydroxylation and NADPH oxidation (enzyme reduction) could be calculated from the amount of resorcinol initially present to that of NADPH consumed. The value is 55% for the P364S mutant form and 58% for wild-type PHHY (Table 3), and the latter compares closely to the previously documented value (62%) found by quantification of the product through HPLC (*5*). The experiments show that the oxidative half-reaction of PHHY using resorcinol as a substrate is essentially unaffected by the mutation, which is remarkably different from the reaction with phenol as the substrate.

The reaction was also studied in the presence of 0.2 M NaN_3. As with the wild-type enzyme, the reaction with P364S has five steps (Figure 7A and Scheme 3) (*6*). The first two steps (the initial 70 ms) are similar to the reaction in the absence of azide, and are considered to be the formation and protonation of the flavin C4a-peroxide anion, with rate constants of 4×10^4 M^{-1} s^{-1} and 47 s^{-1}. Similar to the reaction without azide, there are increases of both the absorbance in the short wavelength range and the fluorescence in these steps. The third step (between 70 ms and 0.2 s) is the oxygen transfer reaction (the substrate hydroxylation), which is discerned as a small fluorescence decrease at 4.2 s^{-1}. The fourth observed step is the decrease in absorbance at 340 nm between 0.2 and 2 s, suggested to be due to the dissociation and conversion of the product from the dienone form to the aromatic form. Its rate constant of 0.7 s^{-1} is very close to that for the reaction without sodium azide (Scheme 3). In this step, the fluorescence does not decrease significantly in contrast to the reaction in the absence of azide. The difference appears to be due to the C4a-hydroxyflavin (a major fluorescent species and the result of oxygen transfer) being stabilized by the binding of azide ion and the substrate, resorcinol (*5*, *6*, *21*). Previously, it was

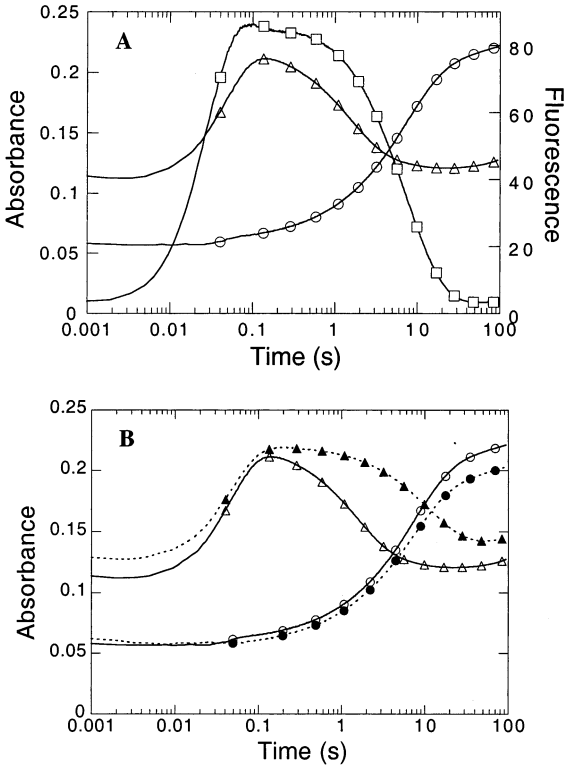


FIGURE 7: Oxidative half-reaction of P364S PHHY with resorcinol as the substrate and in the presence of sodium azide. (A) The reduced P364S mutant enzyme (40 μM) in the presence of 2 mM resorcinol was mixed with buffer containing 1.23 mM oxygen, 0.4 M NaN_3, and 2 mM resorcinol in a stopped-flow spectrophotometer. The reaction was followed by absorption changes (left axis) at 340 (△) and 440 nm (○). The reaction was also monitored by recording the fluorescence change (□) (right axis, final enzyme concentration of 9 μM) with excitation at 380 nm and emission at >530 nm detected. (B) The reaction conditions were the same as for panel A, except that both fully deuterated and protonated resorcinol were used as substrates. The absorbance changes at 440 nm (○ for [^{1}H]resorcinol and ● for the ^{2}H substrate) and 340 nm (△ and ▲ for [^{1}H]- and [^{2}H]resorcinol, respectively) are shown.

shown that after the product dissociates, intermediate III can rebind substrate (resorcinol) to form a quite stable abortive complex (*6*) (Scheme 3). In the last phase, resorcinol dissociates from its abortive complex with intermediate III with a rate constant of 0.16 s^{-1}, and the uncomplexed intermediate III (C4a-hydroxyflavin) dehydrates quickly to form the oxidized flavin, as shown by the absorbance increase at 440 nm and the decrease in fluorescence. It is clear that rate constants for four of the five steps are comparable to their counterparts for the reaction of the wild-type enzyme (*6*, *11*) (Scheme 3). The exception is the last step, the dissociation of resorcinol from its abortive complex with intermediate III, which is much slower in the presence of azide with the wild-type enzyme than with the mutant enzyme.

To probe further the step(s) involved in product formation, 2,4,5,6-[^{2}H]$_4$resorcinol was used in the study of the oxidative half-reaction with azide, as had been done with the wild-type enzyme (*6*, *15*, *16*) (Figure 7B). Only the fourth step in the reaction, the rearomatization of the product (seen as the decrease in absorbance at 340 nm), was affected. The phase of decreasing absorbance at 340 nm was measured with a rate constant of 0.1 s^{-1} (compared with a value of 0.7 s^{-1} for normal resorcinol). The kinetic deuterium isotope effect

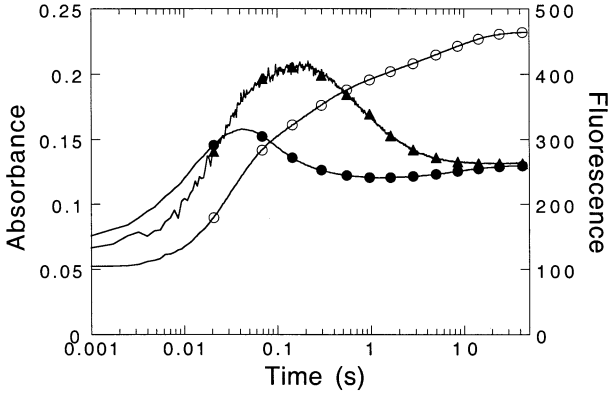


FIGURE 8: Oxidative half-reaction of wild-type PHHY with *m*-cresol as the substrate. The prereduced wild-type enzyme (40 μM) in the presence of 2 mM *m*-cresol was mixed with a buffer solution containing the same concentration of *m*-cresol and 1.23 mM oxygen in a stopped-flow apparatus. The absorbance changes (left axis) at 390 (●) and 440 nm (○) in the reaction are shown. The reaction was also studied by following the fluorescence change (▲) in the reaction (final enzyme concentration of 10 μM). The excitation wavelength was 370 nm, and emission at wavelengths of >500 nm was recorded.

([^{1}H]- vs [^{2}H]resorcinol) is thus calculated to be 7, similar to that for the wild-type enzyme (6.6) (*6*). The fluorescence traces and the absorbance changes at 440 nm, which both monitor the chemical status of the enzyme-bound flavin in the reaction using deuterated substrate, are the same as those using [^{1}H]resorcinol, like the results previously reported for wild-type PHHY (*6*). This KIE is expected for the tautomerization of the dienone (Scheme 1).

In summary, the oxidative half-reaction of the P364S mutant with resorcinol as the substrate is basically the same as that of the wild-type enzyme, even though the reaction using phenol as the substrate is dramatically influenced by the mutation.

Oxidative Half-Reaction of Wild-Type and P364S Mutant PHHY Using m-Cresol (3-Methylphenol) as a Substrate. In the oxidative half-reaction of wild-type PHHY with *m*-cresol, four phases of the reaction were observed (Figure 8). The first phase is characterized by absorbance increases at 380−400 nm with observed rate constants directly dependent on oxygen concentration. These data yield a rate constant of 9.7×10^4 M^{-1} s^{-1} for the formation of the flavin C4a-peroxide. With an oxygen concentration of 0.615 mM, the first phase is completed at 40 ms. The newly formed species is protonated to form the flavin hydroperoxide in the next 60 ms with a rate constant of 15 s^{-1}, evidenced by an absorbance decrease at the same wavelengths. Both of the phases are also manifested by increases in fluorescence, similar to the reaction with resorcinol as the substrate. The third step is characterized by an absorbance increase around 440 nm at 5.1 s^{-1} and finishes at ∼1 s. The rate constant for this phase is suggested to be associated with *m*-cresol hydroxylation and is very close to that with phenol as the substrate (5.3 s^{-1}) (*11*). For kinetic reasons, the formation of intermediates II and III could not be clearly discerned by absorbance changes. Instead, mostly oxidized enzyme is formed in this step, evidenced by the absorbance increase at 440 nm. The last step of the reaction (1.0 s^{-1}), associated with a fluorescence decrease and a further small increase in absorbance at 440 nm, is proposed to be due to the

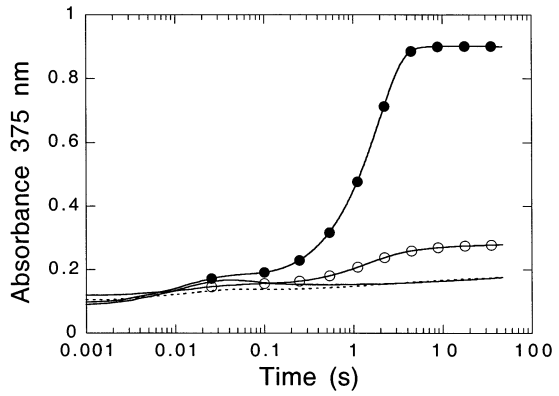


FIGURE 9: Measurement of the hydroxylation efficiencies for P364S mutant and wild-type PHHY using *m*-cresol as the substrate. The reaction conditions were the same as in Figure 8. The absorbance changes at 375 nm in the oxidative half-reaction with *m*-cresol as the substrate were followed for the wild-type enzyme (lower solid line without symbols) and for the P364S mutant form (dashed line). To calibrate the product formed in the reaction, the reduced enzyme was also mixed with the oxygen-containing buffer that included catalytic amounts of catechol 2,3-dioxygenase (Materials and Methods). The absorbance changes at 375 nm are shown in the figure for the wild-type (●) and mutant forms of the enzyme (○).

dissociation of the substrate from a small amount of intermediate III (C4a-hydroxyflavin), and is qualitatively similar to the reactions using phenol and resorcinol as substrates (*6*).

The coupling between *m*-cresol hydroxylation and FAD reoxidation was measured by the catechol 2,3-dioxygenase reaction (Materials and Methods) to be 85% for the wild-type enzyme, but only 12% for the P364S mutant enzyme (Table 3 and Figure 9). The oxidative half-reaction for the mutant form also had four phases (results not shown). The first two steps resembled their counterparts in the wild-type reaction, with rate constants of 6.6×10^4 M^{-1} s^{-1} and 20 s^{-1}, respectively. However, the third step (2.3 s^{-1}) appears to be the sum of two reactions. One of them is due to the oxygen transfer from the flavin hydroperoxide (intermediate I) to *m*-cresol, while the other is due to the intermediate directly returning to the oxidized state by releasing hydrogen peroxide. Judging by the substrate hydroxylation efficiency, most of the enzyme passes through the latter branch of the reaction. The last phase is probably the "trapped" intermediate III−substrate complex decomposing at 0.4 s^{-1}. As in the case of phenol, intermediate I (C4a-hydroperoxyflavin) is formed in the oxidative half-reaction of the P364S mutant enzyme with *m*-cresol bound at rates comparable to those of the wild-type PHHY reaction, but only a small fraction of it is utilized for hydroxylation.

DISCUSSION

The P364S mutant form is reduced faster by NADPH than the wild-type phenol hydroxylase, with either phenol or *m*-cresol bound, as well as in the absence of substrate (Table 1). It has been shown crystallographically that in the presence of phenol wild-type PHHY forms an asymmetrical dimer with one FAD "out" near the surface and one FAD buried in the "in" position near phenol, where the hydroxylation occurs (*7*). Extensive studies of PHBH, which shares many similarities with PHHY, have shown that flavin movement is critical to catalysis (*22*−*24*). The isoalloxazine moiety of FAD swings to the out position to become reduced and then swings back in to react with oxygen and oxygenate the substrate (*14*, *24*, *25*). It is likely that the in and out conformations found with PHHY have functions similar to those with PHBH. According to the crystal structure of wild-type PHHY with phenol bound (*7*), several residues adjacent to Pro364 (Gly369, Met370, and Asn371) form multiple hydrogen bonds with the N(1)−O(2) atoms of the in flavin through their peptide oxygen and nitrogen atoms. The mutation from proline to serine would reduce the rigidity of the neighboring peptide segment to allow the isoalloxazine ring to move more freely between the in and out positions. Moreover, the hydrogen bonds to the flavin may be weaker in P364S due to the less rigid peptide being less constrained. Thus, the flavin in conformation may be less stable, resulting in a larger population of out FAD. Also, due to the greater flexibility of the peptide, the FAD in P364S might assume an out conformation that is more favorable for hydride transfer from NADPH. All of these effects could lead to an increased rate of reduction of enzyme-bound FAD by NADPH.

The suggestion that the conformation of the enzyme-bound flavin is affected by the mutation is also supported by other experimental results. The higher flexibility of the neighboring peptide segment is also a likely cause of the mutant PHHY having such low affinity for FAD ($K_d \sim 2$ μM). Thus, at the concentrations of enzyme used in our stopped-flow experiments, the enzyme solution always contains a small but significant amount of free FAD in equilibrium with the bound FAD. The free FAD is reduced by NADPH as manifested by the slow decrease in absorbance at 440 nm in Figure 2, due to the flavin reductase activity of the P364S mutant enzyme. The high flavin reductase activity of P364S PHHY (Figure 3) also is consistent with the notion that compared with the wild-type enzyme the mutant form has better accessibility to flavin free in the solution as well as to NADPH because it has a larger population of the flavin out conformation. Because FMN and riboflavin as substrates are at least as good as FAD, it seems clear that the flavin reductase activity is due to a viable FAD-containing PHHY reducing free FAD.

The mutation causing a change of Pro364 to Ser dramatically influences the oxidative half-reaction using phenol or *m*-cresol as the substrate. The hydroxylation of phenol by P364S is only 13% coupled, compared to 100% for the wild-type enzyme (Table 3). Interestingly, with this mutant form of the enzyme, at least 60% of the reduced enzyme forms intermediate I (C4a-hydroperoxyflavin), but only $\sim 1/5$ of the intermediate form leads to hydroxylation of phenol. In the presence of NaN_3, an even larger percentage of C4a-hydroperoxyflavin is formed, but still, very little product results. It can be seen in Figure 5 that much of the flavin is oxidized before the fluorescence due to intermediate III is observed. Thus, very little intermediate III (C4a-hydroxyflavin, the product of the oxygen transfer reaction) accumulates. With the wild-type enzyme, intermediate III is fully formed in the presence of phenol and azide, and is stable for hours (*5*, *21*). On the basis of the crystal structure and computational studies of the wild-type enzyme (*7*, *8*), the carbonyl oxygen of Pro364 may form a hydrogen bond to the distal oxygen of the flavin hydroperoxide (Scheme 2, top). This interaction could stabilize the partial positive

charge developed on the hydroxyl group in the transition state of the oxygen transfer step. Because of the increased mobility of the carbonyl group of Ser364 compared to that of Pro364, the hydrogen bond to the hydroperoxide might be weaker and hence provide less stabilization of the transition state in the mutant form of the enzyme. This could account for the poorer coupling of hydroxylation of phenol and *m*-cresol in Pro364Ser. The residue homologous to Pro364 in *p*-hydroxybenzoate hydroxylase (PHBH) is Pro293 (*7*). The P293S mutant form of PHBH also has poorer coupling to hydroxylation than wild-type PHBH (50−60% vs 100% for the wild type) (*26*). Thus, these proline residues may play similar roles in the two aromatic hydroxylases (*8*, *27*, *28*).

A surprising result was that in contrast to the case with phenol, when resorcinol is bound, the Pro364Ser enzyme behaves in a manner very similar to that of the wild-type enzyme (Figures 6−8). On the basis of the transient spectra obtained during the oxidative half-reaction when catechol 2,3-dioxygenase was included (data not shown), the hydroxylated product is 1,2,4-trihydroxybenzene, the same product as in the reaction catalyzed by the wild-type enzyme (*1*). Kinetic parameters for the oxidative half-reactions of Pro364Ser with resorcinol are also comparable to those of the wild-type enzyme, with or without sodium azide present (Scheme 3 and Table 3) (*6*). In addition, the coupling with resorcinol as the substrate (55%) is very similar to that with the wild-type enzyme (58%). In contrast, when phenol is used as the substrate, a coupling of only 13% is obtained, compared with 100% for the wild-type enzyme. During the reactions with either phenol or resorcinol bound to the enzyme, approximately 60% of the reduced Pro364Ser forms the C4a-hydroperoxyflavin intermediate; hence, it can be concluded that oxygen is more effectively transferred to resorcinol than to phenol with this mutant form.

The distances between the C4a atom of FAD and the two ortho carbon atoms of phenol are nearly the same (5.4 and 5.5 Å) (*7*). However, in Figure 1A, C2 is 3.6 Å and C6 5.8 Å from the carbonyl oxygen atom of Pro364. Thus, Pro364 could direct the hydroperoxide to attack C2 (Scheme 2, top). In principle, resorcinol could bind in the active site in either of two orientations, one similar to that shown with *m*-cresol in Scheme 2 (top) and the other with its *m*-hydroxyl hydrogen bonded to Pro364 as shown in Scheme 2 (bottom). Although in the top conformation a hydrogen bond could form with Gln112, the other orientation is more likely because it can form multiple hydrogen bonds with Arg265, Pro364, and Lys365 (Figure 1B and Scheme 2). The structure of the enzyme with resorcinol bound in the latter conformation was simulated using the structure of the wild-type enzyme with phenol bound as a starting point. In the simulated structure, the distance between the C3 oxygen of resorcinol and the carbonyl oxygen of Lys365 is only 2.3 Å, suggesting that a very strong hydrogen bond could be formed between these two groups. In this conformation, it might be too crowded for the terminal oxygen of intermediate I to be transferred to C2, between the two hydroxyl groups of resorcinol. Therefore, there has been speculation that resorcinol may be hydroxylated at the C6 position with little or no participation of the distant Pro364, to yield the observed product, 1,2,4-trihydroxybenzene (*1*). With the additional electron-donating hydroxyl group, resorcinol is inherently more activated than phenol. It may not need as much enzymatic assistance as phenol in the nucleophilic attack on intermediate I to acquire the hydroxyl group.

We hypothesize that the oxidative half-reaction of PHHY with resorcinol is essentially the same in the mutant form as in wild-type enzyme because in contrast to the hydroxylation of phenol, Pro364 would not be involved in the transition state. Thus, the putative hydrogen bond(s) formed by the C3 hydroxyl group of resorcinol and the active site residues stiffens the structure of PHHY so that the neighboring peptide is less flexible than that of the enzyme with phenol bound. This causes the movement from the in position to the out position to be slower with resorcinol bound than with phenol bound (Table 1). For P364S, the movement of flavin could be partially rate-limiting in the reductive half-reaction. This could account for the very small kinetic isotope effect (2.2) measured for the reduction of the P364S enzyme with resorcinol bound (Table 2). For the wild-type enzyme, the hydride transfer reaction is suggested to be slower than that of P364S because the out FAD in wild-type PHHY is not as favorably positioned to interact with pyridine nucleotide as in the mutant enzyme. Hence, the hydride transfer is the rate-limiting step for the reductive half-reaction of the wild-type enzyme with resorcinol bound, and a larger KIE (8.3, NADPH vs NADPD) is observed (Table 2).

The third substrate, *m*-cresol, is suggested to be hydroxylated at the C2 position because hydrogen bonds similar to those with resorcinol are not possible with the nonpolar methyl substituent. *m*-Cresol may therefore bind to PHHY with its methyl group pointing away from Pro364. The C2 atom could acquire the hydroxyl group from the flavin intermediate through nucleophilic attack on the flavin hydroperoxide, with assistance from Pro364, similar to the hydroxylation of phenol. The product of the hydroxylation of *m*-cresol, 4-methylcatechol (*1*), is consistent with this mechanism. The hypothesis could also help to explain why the hydroxylation efficiency of resorcinol by wild-type PHHY (58%) is lower than that of phenol (100%) or *m*-cresol (85%) (Table 3), even though resorcinol is a more nucleophilic substrate due to the electron-donating hydroxyl group at the meta position. The lower hydroxylation efficiency of P364S may simply be because it is more difficult to transfer the hydroxyl group to the C6 atom without the stabilization effect on the transition state brought about by Pro364, which exists in the wild-type enzyme.

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. Bette Jo Brown on mutagenesis and protein purification. We give thanks to Dr. Bruce A. Palfey for his help in preparing the structure figure of PHHY and Dr. Kazuko Yorita for valuable discussions on the reaction mechanism of PHHY.

REFERENCES

1. Neujahr, H. Y., and Gaal, A. (1973) *Eur. J. Biol. 35*, 386−400.
2. Neujahr, H. Y. (1990) in *Chemistry and Biochemistry of Flavoenzymes* (Muller, F., Ed.) Vol. 2, pp 65−85, CRC Press, Boca Raton, FL.

3. Entsch, B., and van Berkel, W. J. H. (1995) *FASEB J. 9*, 476−483.
4. Neujahr, H. Y., and Kjellen, K. G. (1978) *J. Biol. Chem. 253*, 8835−8841.
5. Detmer, K., and Massey, V. (1984) *J. Biol. Chem. 259*, 11265−11272.
6. Maeda-Yorita, K., and Massey, V. (1993*) J. Biol. Chem. 268*, 4134−4144.
7. Enroth, C., Neujahr, H., Schneider, G., and Lindqvist, Y. (1998) *Structure 6*, 605−617.
8. Ridder, L., Mulholland, A. J., Rietjens, I. M. C. M., and Vervoort, J. (2000) *J. Am. Chem. Soc. 122*, 8728−8738.
9. Kalin, M., Neujahr, H. Y., Weissmahr, R. N., Sejlitz, T., Johl, R., Fiechter, A., and Reiser, J. (1992) *J. Bacteriol. 174*, 7112−7120.
10. Waters, S., and Neujahr, H. Y. (1994) *Protein Expression Purif. 5*, 534−540.
11. Xu, D., Ballou, D. P., and Massey, V. (2001) *Biochemistry 40*, 12369−12378.
12. Patil, P. V., and Ballou, D. P. (2000) *Anal. Biochem. 286*, 187−192.
13. Press, W. H., Teukolsky, S. A., Vetterling, W. T., and Flannery, B. P. (1992) *Numerical Recipes in C, the Art of Scientific Computing*, 2nd ed., Cambridge University Press, Cambridge, U.K.
14. Palfey, B. A., Moran, G. R., Entsch, B., Ballou, D. P., and Massey, V. (1999) *Biochemistry 38*, 1153−1158.
15. Detmer, K., and Massey, V. (1985) *J. Biol. Chem. 260*, 5998−6005.
16. Taylor, M. G., and Massey, V. (1991) *J. Biol. Chem. 266*, 8291−8301.
17. Lipscomb, J. D., and Orville, A. M. (1992) *Met. Ions Biol. Syst. 28*, 243−298.
18. Kojima, Y., Itada, N., and Hayaishi, O. (1961) *J. Biol. Chem. 263*, 2223−2228.
19. Massey, V. (1990) in *Flavins and Flavoproteins* (Curti, B., Ronchi, S., and Zanetti, G., Eds.) pp 59−66, Walter de Gruyter & Co., Berlin.
20. Howell, L. G., Spector, T., and Massey, V. (1972) *J. Biol. Chem. 247*, 4340−4350.
21. Taylor, M. G., and Massey, V. (1990) *J. Biol. Chem. 265*, 13687−13694.
22. Gatti, D. L., Palfey, B. A., Lah, M.-S., Entsch, B., Massey, V., Ballou, D. P., and Ludwig, M. L. (1994) *Science 266*, 110−114.
23. Schreuder, H. A., Mattevi, A., Obmolova, G., Kalk, K. H., Hol, W. G. J., van der Bolt, F. J. T., and van Berkel, W. J. H. (1994) *Biochemistry 33*, 10161−10170.
24. Palfey, B. A., Ballou, D. P., and Massey, V. (1997) *Biochemistry 36*, 15713−15723.
25. Frederick, K. K., Ballou, D. P., and Palfey, B. A. (2001) *Biochemistry 40*, 3891−3899.
26. Palfey, B. A., Basu, R., Frederick, K. K., Entsch, B., and Ballou, D. P. (2002) *Biochemistry 41*, 8438−8446.
27. Ridder, L., Mulholland, A. J., Rietjens, I. M. C. M., and Vervoort, J. (1999) *J. Mol. Graphics Modell. 17*, 163−175.
28. Ridder, L., Mulholland, A. J., Vervoort, J., and Rietjens, I. M. C. M. (1998) *J. Am. Chem. Soc. 120*, 7641−7642.

BI020446N