Role of Protein Flexibility in the Catalytic Cycle of *p*-Hydroxybenzoate Hydroxylase Elucidated by the Pro293Ser Mutant†

Bruce A. Palfey,‡ Rajit Basu,‡,|| Kendra King Frederick,‡,¶ Barrie Entsch,*,§ and David P. Ballou*,‡

Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109-0606 USA, and Molecular and Cellular Biology, School of Biological Sciences, University of New England, Armidale, NSW 2351, Australia

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ABSTRACT: Proline 293 of *p*-hydroxybenzoate hydroxylase from *Pseudomonas aeruginosa* is in a highly conserved region of the flavoprotein aromatic hydroxylases. It is thought to impart rigidity to the backbone, as it partially cradles the FAD in these hydroxylases. Thus, this residue has been substituted with serine by site-directed mutagenesis to investigate the importance of flexibility of the peptide segment in catalysis. Differential scanning calorimetry demonstrated that the mutation has decreased the stability of the folded mutant protein compared to the wild-type PHBH. The increased flexibility in the protein backbone enhanced the accessibility of the flavin hydroperoxide intermediate to the solvent, causing an increase in the elimination of H_2O_2 from this labile intermediate and, consequently, a decrease in the efficiency of substrate hydroxylation. Additionally, the increased accessibility of this mutant form of the enzyme makes it more susceptible than the wild-type enzyme to being trapped in the hydroxyflavin intermediate form in the presence of high levels of *p*-hydroxybenzoate. The mutation also lowers the pK_a of the phenolic oxygen of bound *p*-hydroxybenzoate, and eliminates the pH dependence of the rate constant for flavin reduction by NADPH. These experimental observations lead to a model that explains how the wild-type protein can sense the charge of the 4-substituent of the aromatic ligand and link this charge to a flavin conformational change that is required for reaction with NADPH: (i) The peptide oxygen of Pro 293 is repelled by the negative charge of the phenolic oxygen of *p*-hydroxybenzoate. (ii) This repulsion is transmitted through the peptide backbone, causing the movement of Asn 300. (iii) The change in the position of Asn 300 triggers the movement of the flavin from the largely buried "in" conformation to the exposed, reactive "out" conformation.

p-Hydroxybenzoate (pOHB)[1] is generated by the degradation of lignin and serves as a carbon source for a variety of bacteria and fungi. The first step in pOHB catabolism is the hydroxylation reaction catalyzed by *p*-hydroxybenzoate hydroxylase (PHBH; E.C. 1.14.13.2). The enzymes from *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, which differ by only two surface residues, have been studied most thoroughly (*1*). PHBH catalyzes the reaction of pOHB, O_2, and NADPH to give protocatechuic acid (PCA), water, and $NADP^+$ according to the mechanism shown in Scheme 1. The hydroxylating intermediate is an enzyme-bound flavin hydroperoxide, which is generated by the reduction of the oxidized flavin by NADPH (k_1) and the subsequent reaction with O_2 (k_2). The flavin hydroxide formed in the hydroxylation reaction dehydrates (k_4) to regenerate the oxidized flavoenzyme (Scheme 1). The steady-state kinetic mechanism is ping−pong (*2*), with a reductive half-reaction generating the reduced flavin and an oxidative half-reaction generating and utilizing the flavin hydroperoxide. Each of the half-reactions may be studied directly and independently of the other by stopped-flow methods, allowing the detailed dissection of catalytic events.

The reductive half-reaction serves as a key control point in the catalytic cycle. Although not involved chemically, when substrate is present the rate of flavin reduction by NADPH (k_1) is stimulated by a factor of $\sim 10^5$ over that of the free enzyme (*2*). This serves to prevent the wasteful oxidation of NADPH in the absence of pOHB. The discrimination between potential aromatic substrates occurs in the flavin reduction reaction (*3*). Good substrates such as pOHB are ionized by the enzyme to form a negative charge on the 4-substituent, and this leads to the rapid reduction of the flavin by NADPH. By contrast, aromatic molecules that cannot be ionized stimulate flavin reduction by a much smaller factor (∼100−1000-fold). The control of reduction by the ionization state of the aromatic ligand is linked to a conformational change in the flavin. Most crystal structures show the isoalloxazine of the flavin in a largely buried position near pOHB, termed the "in" conformation (Figure 1). However, in some structures the isoalloxazine is in a more

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* To whom correspondence should be addressed. Phone: (734) 764-9582; fax: (734) 763-4581; e-mail: dballou@umich.edu

‡ University of Michigan.

§ University of New England.

|| Present Address: Indiana University School of Medicine, Indiana University Purdue University Indianapolis, Indianapolis, IN 46202

¶ Present Address: Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104

[1] Abbreviations: PHBH, *p*-hydroxybenzoate hydroxylase; pOHB, *p*-hydroxybenzoate; PCA, protocatechuic acid; 2,4-diOHB, 2,4-dihydroxybenzoate; DSC, differential scanning calorimetry.

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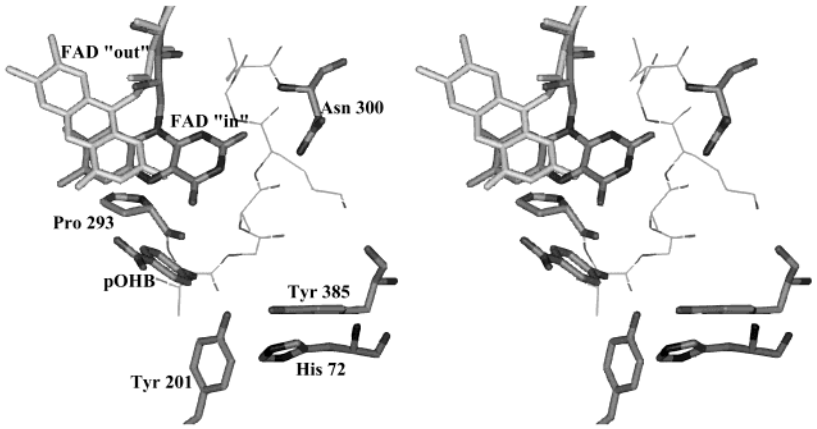
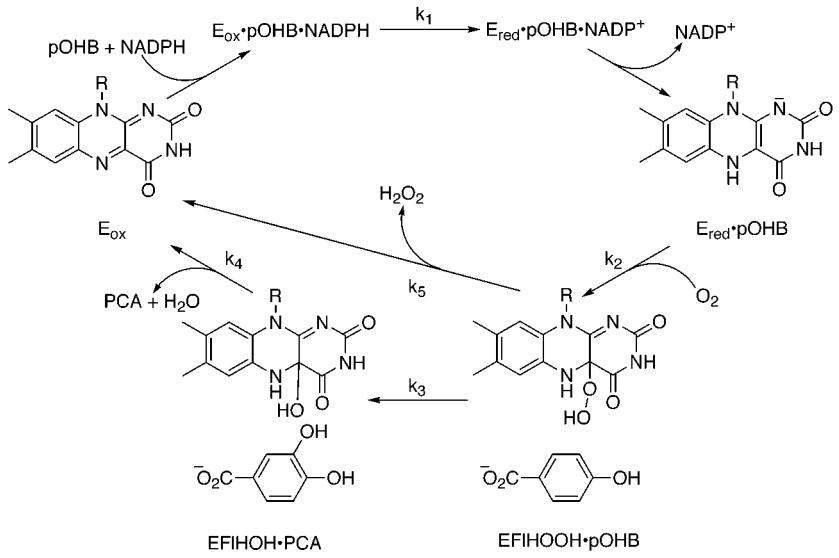


FIGURE 1: Flavin conformation in PHBH. The in and out conformations of the FAD of PHBH are compared using the structures of the wild-type−pOHB complex (1pbe) and the Tyr222Phe−pOHB complex (1dor). Additionally, the residues of the proton-transfer network (Tyr 201, Tyr 385, and His 72) are shown, as are two residues, Pro 293 and Asn 300, that appear to be important in controlling the interconversion of the two conformations.

Scheme 1: Catalytic Cycle of PHBH



solvent-exposed conformation, termed the "out" conformation (*4*, *5*). Evidence has accumulated that the reduction of the flavin occurs in the out conformation, and this conformation is not attained rapidly unless a negative charge is present on the 4-substituent (*3*, *6*−*8*). This negative charge on the phenolate of pOHB is generated because the enzyme lowers the pK_a of the group by ∼2 units (*9*). Rapid deprotonation of the substrate is ensured by an internal proton-transfer network, extending from pOHB to Tyr 201, Tyr 385, two internal water molecules, and His 72, a surface residue ∼12 Å from pOHB (*10*).

Besides regulating the reductive half-reaction, the phenolate of pOHB accelerates the catalytic cycle because of its higher reactivity with the flavin hydroperoxide (*9*). When the proton-transfer network is disrupted by mutagenesis, hydroxylation is significantly slower or abolished altogether. The importance of the proton transfer network in the hydroxylation reaction was demonstrated by the Tyr201Phe mutant, whose flavin hydroperoxide hydroxylated pOHB with only ∼5% success (*9*), the remainder decaying to form oxidized flavin and H_2O_2. The proton-transfer network allows the phenolate of pOHB to be generated even though the flavin hydroperoxide is sequestered from solvent; thus, solvent-catalyzed elimination of hydrogen peroxide is prevented. Although the elimination of hydrogen peroxide (k_5 in Scheme 1) is not significant when wild-type PHBH uses pOHB as substrate, this side-reaction can become significant when other substrates are used, or when the protein has been altered by mutagenesis.

The phenolic oxygen of pOHB is within hydrogen bonding distance to the peptide carbonyl oxygen of Pro 293 (*11*). This Pro is conserved among all known PHBHs, and throughout the family of flavin-utilizing aromatic hydroxylases (*12*, *13*). In PHBH, Pro 293 is the second proline in a pair (Pro 292−Pro 293) that is likely to confer a degree of local rigidity to the protein backbone in a region that neighbors not only the aromatic substrate, but also the isoalloxazine of FAD. Crystallographic studies have suggested that the protein backbone in this region can respond to the ionization state of pOHB, and molecular dynamics calculations indicate that the phenolic hydrogen of pOHB fluctuates between the carbonyl oxygen of Pro 293 and the phenolic oxygen of Tyr 201 as hydrogen bonding partners (*14*). The Pro293Ser mutant was constructed to explore the

importance of backbone rigidity at residue 293 and to probe its effects on the protonation state of pOHB. We find that increasing the protein flexibility alters properties of the enzyme in both the reductive and oxidative half-reactions. These results suggest that this residue plays an important role in controlling the conformation of the flavin that is central to coordinating the complexities of catalysis.

MATERIALS AND METHODS

The Pro293Ser mutant of *P. aeruginosa* PAO1 PHBH was constructed using the *poba* gene using published procedures (*15*). Enzymes were expressed in *Escherichia coli* (*9*) and purified as described (*10*). All reagents were obtained from commercial sources. Differential scanning calorimetry (DSC) was performed with a CSC nano-differential scanning calorimeter II instrument. Wild-type PHBH and the Pro293Ser mutant were dialyzed against 50 mM KPi, 0.5 mM EDTA, pH 6.5 buffer, and the dialysate was used as the reference solution. Spectrophotometric titrations and product stoichiometry measurements were performed using a temperature-controlled Shimdazu UV-2501PC instrument. Reduction potentials were obtained using the method of Massey (*16*). Enzyme solutions were made anaerobic by repeatedly evacuating the atmosphere over the solution, replacing it with oxygen-free argon, and equilibrating (*17*, *18*). Substrate solutions in 10-mL syringes for stopped-flow experiments were made anaerobic by bubbling for 10 min with water-saturated oxygen-free argon. Stopped-flow spectrophotometric studies were conducted on either an apparatus from Kinetic Instruments, or an SF-61 instrument from Hi-Tech Scientific, Ltd. Absorbance data were collected from both instruments using a tungsten−halogen lamp and a photomultiplier detector. Fluorescence stopped-flow data were collected on a Hi-Tech SF-61 instrument equipped with a xenon lamp and a 515 nm emission cutoff filter. Diode array data were collected on a Hi-Tech SF-61 DX-2 instrument using a 1.5 ms integration time. Stopped-flow instruments were made anaerobic using the protocatechuate/protocatechuate dioxygenase system (*19*).

RESULTS

Thermal Denaturation. The Pro to Ser substitution was expected to increase the flexibility of the protein backbone in a region of low flexibility. Evidence was sought that this was actually the effect. Replacing rigid proline residues by more flexible amino acids has been shown to increase the flexibility of the backbones of proteins, thereby preferentially stabilizing the unfolded state of the protein (*20*). We compared the stability of wild-type PHBH with that of the Pro293Ser enzyme by differential scanning calorimetry (DSC). Thermal denaturation was irreversible for both enzymes, preventing a quantitative thermodynamic analysis (Figure 2). However, scanning at the same rate (1 °C/min) allowed a qualitative comparison of stability. Denaturation was a two-step process. First, a transition with a peak in the heat capacity at 61.5 °C was present in both the wild-type and the mutant enzymes. A significantly larger transition occurred afterward at 71.5 °C in the wild-type enzyme and 69.7 °C in the Pro293Ser mutant. We tentatively attribute the first transition to the dissociation of the PHBH dimer, and the second transition to the unfolding of the tertiary structure of the enzyme.

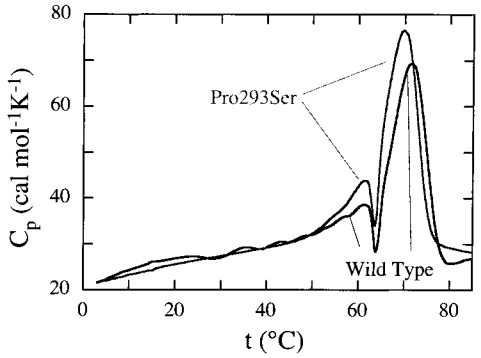


FIGURE 2: Thermal denaturation of PHBH. DSC traces of the irreversible melting of the wild type and Pro293Ser enzymes are shown. The enzyme concentration (900 μM) was the same in both experiments. DSC experiments were conducted in 50 mM KPi, 0.5 mM EDTA, pH 6.5, using a scan rate of 1 °C/min.

Table 1: Selected Properties of the Pro293Ser Mutant Form Compared to the Wild-Type Enzyme

	WT[a]	Pro293Ser
pK_a of bound pOHB[b]	7.4	6.7
K_d (pOHB) (μM)[c]	9.5	70
K_d (2,4-diOHB) (μM)[c]	22	160
K_d (PCA) (μM)[c]	230	110
E_{m7}, free enzyme (mV)[d]	−163	−181
E_{m7}, enzyme + pOHB (mV)[d]	−165	−183
ϵ_{450}, free E_{ox} (mM^{-1} cm^{-1})[e]	10.3	10.3

[a] Values for the wild-type enzyme were taken from ref *9*. [b] The values for the phenolic pK_a of pOHB were determined from the phenolate absorbance at 290 and 300 nm in spectral titrations across a range of pH values. [c] Dissociation constants were determined at 4 °C in 50 mM KPi, pH 6.5, from the changes in flavin absorbance in titrations of enzyme with concentrated ligand solutions. [d] Redox potentials were determined at 25 °C in 0.1 M KPi, pH 7.0, using 1-hydroxyphenazine as a reference dye. [e] The extinction coefficient of enzyme-bound FAD was determined by comparing the absorbance of enzyme that had been denatured with SDS with the absorbance of the sample prior to the addition of SDS. The measurements were conducted at 25 °C in 50 mM sodium phosphate, pH 7.0.

Table 2: A Comparison of Rate and Dissociation Constants Determined at pH 6.5, 4 °C[a]

	WT[b]	Pro293Ser
K_d (NADPH) (μM)	210	140
k_1 (s^{-1})	50	16
k_2 (M^{-1} s^{-1})	2.6×10^5	1.2×10^5
k_3 (s^{-1})	48	6.5
k_4 (s^{-1})	14.5	N.M.[c]
k_5 (s^{-1})	N.M.[c]	6.5

[a] The reactions in Scheme 1 define the rate constants. [d] Values for the wild-type enzyme are from refs *3* and *26*. [c] N. M., not measurable.

Ligand Binding and Spectral Properties. The Pro293Ser mutant was titrated at pH 6.55, 4 °C, with pOHB, 2,4-diOHB, or PCA. Ligand binding was monitored by changes in the absorbance spectrum of the enzyme-bound FAD, enabling dissociation constants to be determined (Table 1). Both pOHB and 2,4-diOHB are substrates of PHBH, and binding of each of these compounds was about 1 order of magnitude weaker (K_d = 70 and 160 μM, respectively) to the Pro293Ser mutant than it was to the wild-type enzyme. The weaker binding of pOHB and 2,4-diOHB can be rationalized by considering the changes to the free enzyme and its complexes caused by the mutation. In the absence of substrate, the active site is accessible to water, allowing Ser 293 (but not Pro

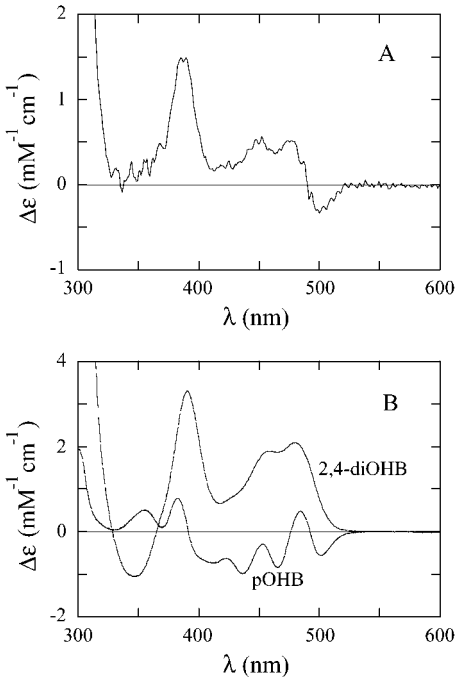


FIGURE 3: Difference spectra caused by the binding of benzoate ligands. Panel A shows the spectral perturbations caused by the binding of pOHB to the Pro293Ser mutant (9.1 μM) in 50 mM KPi, 0.5 mM EDTA, pH 6.5, at 4 °C. Panel B shows the difference spectra for the wild-type enzyme caused by the binding of pOHB and 2,4-diOHB.

293 in wild type) to form hydrogen bonds to the solvent. Ligand binding to PHBH drives solvent molecules from the active site, leaving the hydroxyl of Ser 293 in van der Waals contact with the hydrophobic edge of the aromatic ring of the ligand. Thus, compared with wild type, Pro293Ser, upon forming complexes with pOHB or 2,4-diOHB, has a greater net loss of favorable interactions for the side-chain of Ser 293 with solvent, resulting in a larger K_d for Pro293Ser than for wild type for these two ligands.

In contrast, the binding of PCA (the hydroxylation product of pOHB) was tighter to the mutant enzyme than to the wild-type enzyme by about a factor of 2. This change may be explained by considering the likely effects of the mutation on the structure of the PCA complex. The distance from the β-carbon of Pro 293 to the 3-OH of PCA in the wild-type-PCA complex (1phh; ref *21*) is 3.69 Å. The Pro293Ser mutation extends the protein side-chain at the β-carbon with a hydroxyl group, which can form a new hydrogen bond with the 3-hydroxyl of PCA. This new hydrogen bond very likely accounts for the slightly tighter binding of PCA to the mutant form of PHBH.

The difference spectra produced by the binding of aromatic ligands to PHBH have been correlated with the conformation of FAD observed in crystal structures (*4*, *5*, *22*). The binding of pOHB to the Pro293Ser mutant produced a difference spectrum with characteristics of both the in and out conformations (Figure 3). Thus, the Pro293Ser mutation appears to alter the equilibrium position of the isoalloxazine in favor of the out conformation, suggesting that although residue 293 does not interact directly with the flavin, it influences the flavin conformation.

It was anticipated that the increased flexibility of PHBH caused by changing Pro293 to Ser would result in an increase

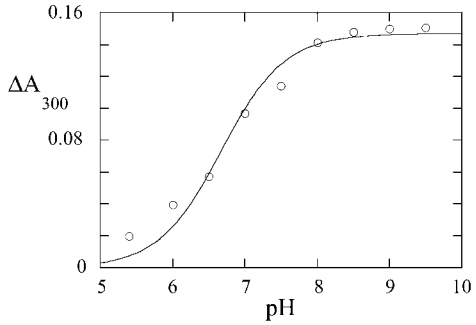


FIGURE 4: Ionization of pOHB bound to Pro293Ser. The maximum absorbance changes due to the formation of the phenolate upon binding to the enzyme were obtained from spectral titrations at different pH values, enabling a pK_a value of 6.7 to be determined as described in Results.

in the rate of substrate binding. An attempt was made to investigate the kinetics of pOHB binding to the Pro293Ser enzyme. Various concentrations of pOHB from 50 μM to 50 mM were mixed with ligand-free enzyme at pH 6.5, 4 °C, in a stopped-flow spectrophotometer. The absorbance changes caused by ligand binding were complete in the dead-time of the instrument (~2 ms) and were therefore too rapid for kinetic analysis. This is also true of the wild-type enzyme (*23*), so we cannot distinguish whether binding is faster or slower than for wild type; it is clear that it is fast. Thus, the rate of pOHB binding might be unaltered or slower than wild type, yet still faster than the limit of detection, or it is possible that the increased protein flexibility has increased the rate of pOHB binding.

pOHB Activation. Wild-type PHBH activates pOHB for reaction with the electrophilic flavin hydroperoxide by deprotonating the phenolic oxygen of the substrate. The enzyme facilitates this by lowering the pK_a of the phenolic oxygen of bound pOHB to 7.4 (when bound to the oxidized enzyme), almost two units lower than its value in solution (*9*). The pK_a of pOHB in the Pro293Ser−pOHB complex was measured by titrating ~10 μM samples of the enzyme with pOHB at pH values from 5.4 to 9.5 at 25 °C. The ionization of the phenolic oxygen of pOHB causes a significant increase in ultraviolet absorbance in the 290−300 nm range ($\Delta\epsilon_{300} \sim 15$ mM^{-1} cm^{-1}), allowing the amount of phenolate present on the saturated complex to be measured at each pH (*9*). A pK_a value of 6.7 for pOHB bound to Pro293Ser was obtained at 300 nm (Figure 4). Thus, the mutation of Pro to Ser enhances the stability of the phenolate relative to the phenol by 0.7 units compared to the wild-type enzyme, equivalent to 1 kcal mol^{-1}.

Catalytic Properties. The behavior of the mutant enzyme in steady-state turnover was investigated. As with other mutant forms, the flavin hydroperoxide intermediate partitions between a productive hydroxylation pathway and the elimination of H_2O_2 (Scheme 1). The extent of hydroxylation, measured as the ratio of PCA produced to NADPH consumed, provides the value of the ratio of the rate constants for these two competing reactions. Hydroxylation was measured by monitoring the change in A_{340} in assays of the enzyme using an excess of NADPH and O_2 over pOHB. Protocatechuate dioxygenase was included to consume the PCA product and prevent PCA-instigated NADPH consumption. Thus, the only aromatic compound capable of stimulating NADPH oxidation over the course of an assay was

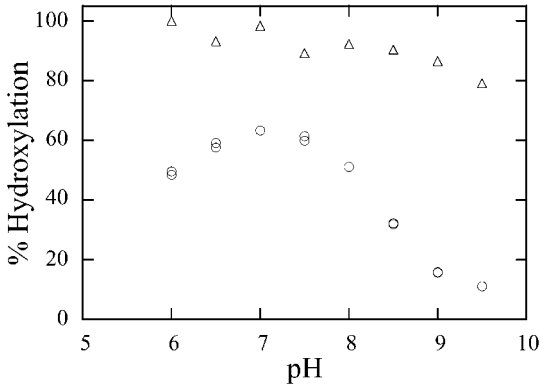


FIGURE 5: Variation of hydroxylation efficiency with pH. Hydroxylation efficiency, defined as 100 × (pOHB hydroxylated)/(NADPH consumed), was determined by measuring the amount of NADPH consumed in steady-state assays at 25 °C in the presence of limiting amounts of pOHB. Protocatechuate dioxygenase was included to prevent PCA-stimulated NADPH consumption by PHBH. Values obtained for the wild-type enzyme are shown by triangles, while values obtained for the Pro293Ser mutant are shown by circles.

pOHB, and when that was completely consumed, NADPH consumption slowed dramatically, allowing the concentration of NADPH required to consume the limiting concentration of pOHB to be measured. At pH 6.5, 4 °C, a value of 50% coupling was obtained. At 25 °C, this value increased somewhat to 60%. The dependence on pH of the coupling of reduction to hydroxylation was determined from pH 6.0 to pH 9.5 for both the wild type and Pro293Ser mutant. The wild type enzyme hydroxylates 100% effectively at pH 6.0 and about 95% effectively from pH 6.5 to 8.0. Above pH 8.0, the coupling decreases slightly, approaching 80% at pH 9.5. For the Pro293Ser mutant, a bell-shaped dependence was obtained (Figure 5), with a maximum value of 65% coupling being obtained at pH 7.0. These data imply that there is one group on the enzyme that must be deprotonated for optimal hydroxylation and another that must be protonated. The most likely candidate for the group that must be deprotonated for optimal hydroxylation is the phenolic oxygen of pOHB. When protonated, pOHB should be relatively unreactive toward the flavin hydroperoxide. However, because the pK_a of this group is 6.7 (see above), the reactive phenolate state is accessible near neutral pH. The group that becomes deprotonated at high pH and causes elimination of H_2O_2 to compete with hydroxylation is not as readily assigned. N5 of the flavin hydroperoxide must be deprotonated before or during the elimination of H_2O_2. Therefore, either the direct deprotonation of N5 or of a basic group that is necessary for preventing the elimination of H_2O_2 may be involved in the decreased coupling. Increased protein flexibility could facilitate access to solvent. It is worth noting that qualitatively similar pH dependencies of hydroxylation stoichiometry have been observed with other PHBH mutants, including Tyr222Phe, Tyr222Ala, and Arg220Lys (*24*, *25*).

The saturating turnover number of the mutant enzyme could not be determined because of inhibition by pOHB. Steady-state velocities were measured in a stopped-flow instrument by monitoring the disappearance of the absorbance of NADPH using 1 μM Pro293Ser at 4 °C, pH 6.5. When 500 μM NADPH and either 256 μM or 740 μM O_2 were used, the initial velocity reached a maximum at 25 μM pOHB and declined at higher pOHB concentrations (Figure

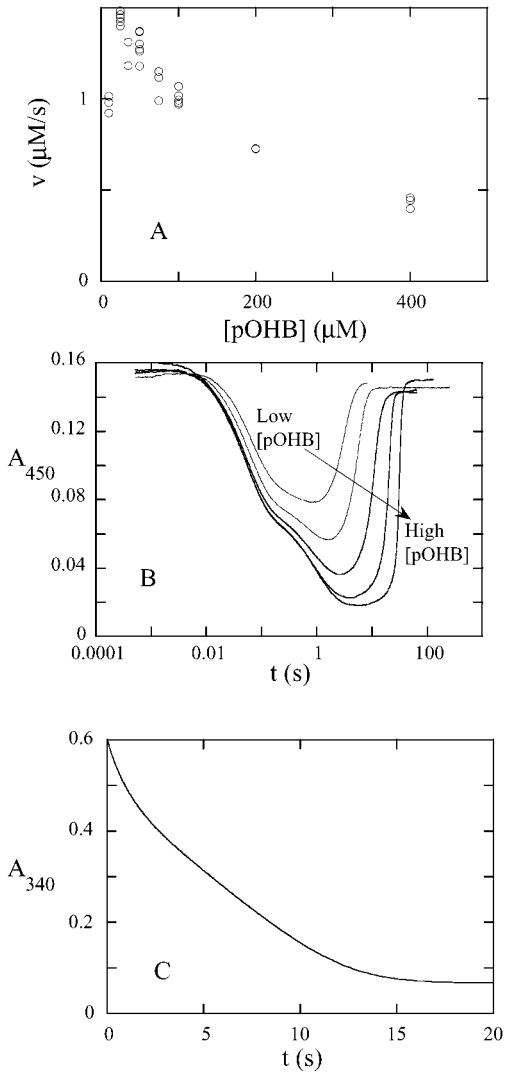


FIGURE 6: Substrate inhibition in Pro293Ser. Steady-state turnover reactions were monitored in a stopped-flow spectrophotometer at pH 6.5, 4 °C. (A) Initial velocities were obtained as a function of pOHB concentration from the second linear region of curves in experiments such as that in panel C. Marked substrate inhibition is evident. (B) The state of the enzyme-bound FAD (15 μM) was monitored by its absorbance at 450 nm. As the pOHB concentration was increased, a larger proportion of the enzyme was trapped in the hydroxyflavin form and turnover was inhibited. Note the logarithmic time scale. Concentrations of pOHB were 31, 62, 125, 250, and 500 μM. (C) When NADPH consumption was monitored by the absorbance at 340 nm, an initial approach to steady state was superseded by a new, slower steady state. Concentrations were 1 μM Pro293Ser, 100 μM NADPH, and 500 μM pOHB.

6A). The maximum turnover number measured was 1.4 s^{-1}, but this clearly does not represent k_{cat}. Substrate inhibition is a frequent phenomenon among the flavin aromatic hydroxylases (*26*, *27*) and is attributable to the binding of the aromatic substrate to a fraction of the hydroxyflavin form of the enzyme, stabilizing the C4a-intermediate. Thus, in each catalytic cycle a portion of active enzyme is siphoned off to the inhibited form. The inhibitory concentration of pOHB is much lower for the Pro293Ser mutant than for the wild-type enzyme (*28*), indicating that the mutation has somehow either enhanced the rate of exchange of pOHB onto the hydroxyflavin intermediate form of the enzyme or that, once bound, the dead-end complex is much more stable than in the case of the wild type enzyme. However, it should be

noted that the wild type enzyme can be extremely sensitive to substrate inhibition, as with 2-fluoro-*p*-hydroxybenzoate (*29*). Therefore, not only the properties of enzyme, but also those of the substrate or product can facilitate substrate inhibition.

The state of the enzyme during turnover was monitored by recording the absorbance of the flavin when 13 μM enzyme was mixed with various amounts of substrates in a stopped-flow spectrophotometer (Figure 6B). The initial turnover of the enzyme led to a steady-state distribution of enzyme species that had an absorbance at 450 nm of about half that of fully oxidized enzyme. However, as the hydroxyflavin form of the enzyme (which does not absorb at 450 nm) was trapped by pOHB, a new steady-state distribution of enzyme was established, characterized by a further decrease in absorbance at 450 nm, indicating that more of the hydroxyflavin intermediate was trapped. When NADPH consumption was monitored at 340 nm, an initial steady-state evolved into a slower steady state, as predicted by this model (Figure 6C).

Redox Potentials. The redox potential of the enzyme-bound flavin was determined by slowly reducing the enzyme with xanthine and xanthine oxidase in the presence of the redox indicator dye 1-hydroxyphenazine ($E_{m7} = -172$ mV) (*15*). Values were determined in 0.1 M KPi, pH 7.0, at 25 °C in the absence of ligands and in the presence of 5 mM pOHB. Linear Nernst plots with unit slopes were obtained, giving redox potentials of −181 mV for the free mutant and −183 mV for the pOHB complex. These values are about 20 mV more negative than the corresponding values for the wild-type enzyme. The potential of free FAD is significantly lower, at −207 mV. Therefore, although both mutant and wild type forms of apo-PHBH bind reduced FAD more tightly than oxidized FAD, the substitution of Pro293 by Ser decreases the preference for reduced flavin. The structure of the reduced wild-type enzyme−pOHB complex shows that the isoalloxazine is in the in conformation and makes the same interactions with the protein observed in the oxidized enzyme−substrate complex (*30*). Electrostatic calculations have demonstrated that the pyrimidine-edge of the isoalloxazine is in a region of positive electrostatic potential (*7*); this positive potential is likely to be a factor in stabilizing the anionic reduced flavin. The difference spectra obtained for ligand binding to the Pro293Ser enzyme indicate that a substantial fraction of the oxidized flavin is in the out conformation; however, the rapid rate of reaction of the reduced enzyme with O_2 (see below) suggests that the flavin is in the in conformation after it is reduced. By contrast, in the wild-type enzyme the flavin is in both before and after reduction. If the reduced flavin of the Pro293Ser enzyme occupies the in position, as is the case with wild type, then reduction of Pro293Ser will be less favorable than wild type by the amount of energy required to move the oxidized flavin from out to in. This is reflected in the 20 mV decrease in the redox potential, which is equivalent to 0.9 kcal mol^{-1}.

Reductive Half-Reaction. The reduction of the enzyme-bound FAD was studied at 4 °C by mixing anaerobic solutions of the Pro293Ser−pOHB complex with anaerobic solutions of NADPH in a stopped-flow spectrophotometer and observing the absorbance decrease at 450 nm. The observed rate constants, obtained from fits to single exponential functions, varied hyperbolically with NADPH concentration, allowing the rate constant for hydride transfer and the apparent K_d for NADPH to be determined (Table 2) (*31*). Experiments were performed at several pH values ranging from 6.0 to 9.0. The rate constant for hydride transfer had no systematic variation with pH, but was in the range of 16 ± 4 s^{-1} (mean ± standard deviation) (Figure 7). This is in contrast to the wild-type enzyme, whose reduction rate constant increases from 35 s^{-1} at pH 6.0 to 77 s^{-1} at pH 9, with a pK_a of about 6.2 (*3*). Thus, the Pro293Ser mutation has affected the rate of reduction by less than a factor of 6, but has largely removed the pH dependence. The K_d of NADPH for the mutant enzyme varied slightly from pH 6.5 to 9.0, with values ranging from 140 μM to 10 μM, respectively. At pH 6.0, a significant increase in the K_d value to 440 μM was observed. This could be due to the protonation of the 2′-phosphate of NADPH, which has a pK_a of 6.5 (*32*).

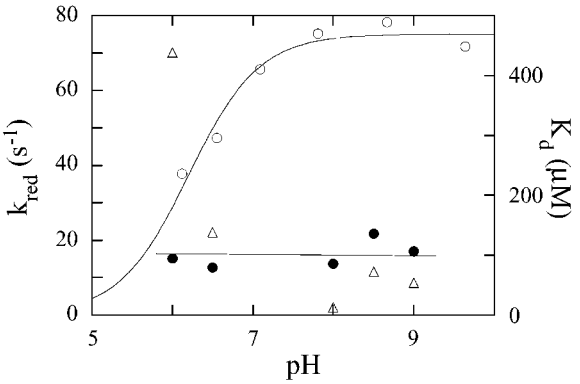


FIGURE 7: Kinetic parameters for flavin reduction as a function of pH. The rate constant for hydride transfer from NADPH to FAD bound to the Pro293Ser mutant was determined at 4 °C in anaerobic stopped-flow experiments from the limiting value of the observed rate constant for flavin reduction as the NADPH concentration was increased to high values. No systematic variation with pH of the rate constant for reduction of the mutant was observed (filled circles). For comparison, the rate constants for the reduction of the wild-type enzyme are also shown (open circles; data from ref *3*). The apparent dissociation constants for NADPH, obtained from the half-saturating value of the NADPH concentration, are shown by triangles.

Oxidative Half-Reaction. The oxidative half-reaction of Pro293Ser was studied in stopped-flow experiments at pH 6.5, 4 °C, by mixing the dithionite-reduced enzyme (10−20 μM) in complex with pOHB with buffer to give final concentrations of O_2 to be 0.061−0.61 mM (after mixing). Single wavelength absorbance traces were collected from 300 to 500 nm, while a diode-array spectrometer was used to record spectra from 325 to 650 nm within 1.5 ms. Fluorescence data were collected by exciting at either 400 nm, where both hydroxyflavin and oxidized flavin can be detected, or at 480 nm, where only oxidized flavin is excited.

Two intermediates are observed in the oxidative half-reaction of the wild-type enzyme when pOHB is the substrate (*23*). The first of these is the flavin C4a-hydroperoxide, formed in the reaction of the reduced enzyme with O_2. The second intermediate is the flavin C4a-hydroxide, which is formed upon substrate hydroxylation. With the Pro293Ser mutant, three reaction phases were observed by absorbance (Figure 8). The observed rate constant of the first phase was linearly dependent on oxygen concentration, giving a second-order rate constant for the reaction of the reduced enzyme−pOHB complex with O_2 of 1.2×10^5 M^{-1} s^{-1} (Table 2), a

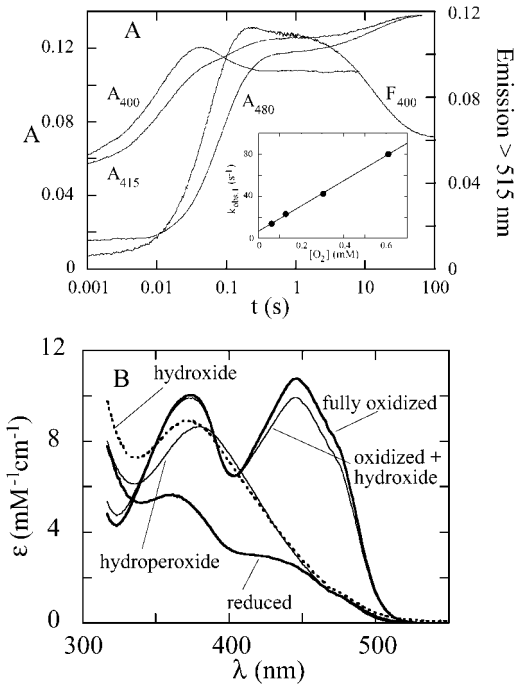


FIGURE 8: Oxidative half-reaction of Pro293Ser. The oxidative half-reaction was studied at 4 °C in 50 mM KPi, 1 mM EDTA, pH 6.5, in a stopped-flow spectrophotometer/fluorimeter by mixing the reduced enzyme−pOHB complex (19 μM) and 2 mM pOHB with buffer solutions containing various concentrations of oxygen and 2 mM pOHB. (A) Representative reaction traces are shown, illustrating the occurrence of intermediates. The trace obtained by fluorescence, marked F_{400}, was obtained by exciting at 400 nm and using a 500 nm emission cutoff filter. The inset shows the variation of the first observed rate constant with oxygen concentration. (B) Spectra of intermediates were obtained in a stopped-flow instrument equipped with a diode-array detector. The spectra shown are those calculated (see text) for the starting reduced enzyme, the flavin hydroperoxide (thin line), the mixture after hydroxylation (consisting of ~90% oxidized enzyme and ~10% enzyme in the flavin hydroxide form), and the final oxidized enzyme. The C4a-hydroxyflavin (dotted line) was calculated by assuming that the mixture was 90% oxidized and 10% flavin hydroxide.

value that is typical for most PHBH forms that have been studied. [In the absence of pOHB, a value similar to that of wild type (9.9×10^3 M^{-1} s^{-1}) was obtained for the mutant.] This was followed by a reaction with an apparent rate constant of 13.1 s^{-1} (occurring from ~0.02−0.1 s) to form a mixture of about 90% oxidized enzyme and about 10% enzyme with bound C4a-hydroxyflavin. Finally, a slow reaction at 0.07 s^{-1} (occurring from ~10−80 s) led to fully oxidized flavin.

The consumption of the flavin hydroperoxide occurs through two competing reactions, yielding 50% hydrogen peroxide and oxidized flavin in the nonproductive pathway, and 50% PCA and C4a-hydroxyflavin in the hydroxylation pathway (see *Catalytic Properties* above). Thus, the rate constant for pOHB hydroxylation is 6.5 s^{-1}, half the value of the observed rate constant of the second phase. Substrate hydroxylation in the wild-type enzyme has a rate constant of 50 s^{-1} under these conditions (Table 2), so the Pro293Ser mutation has caused an 8-fold decrease in the rate constant of this reaction, equivalent to an increase of 1.1 kcal mol^{-1} in the activation energy of the reaction.

Fluorescence traces obtained by exciting at 480 nm (data not shown), where only oxidized flavin fluoresces, closely mimicked absorbance traces obtained at 480 nm, with the major increase in fluorescence (at 13 s^{-1}) coming after a lag, followed by a smaller increase with a rate constant of 0.07 s^{-1}. Traces obtained by exciting at 400 nm, where both the hydroxyflavin and oxidized flavin fluoresce, also showed a lag followed by a substantial increase at 13 s^{-1}. This fluorescence is due to the formation of the C4a flavin hydroxide. The fluorescence then decreased slightly with an apparent rate constant of 15 s^{-1} (occurring from ~0.1 to ~0.4 s), though this value must be considered approximate owing to the small amplitude of the phase and its poor resolution from the previous phase. This phase is attributable to dehydration of some of the C4a flavin hydroxide to form oxidized flavin. A substantial fluorescence decrease followed with a rate constant of 0.07 s^{-1}, corresponding to the slowest phase observed when exciting at 480 nm. This can be attributed to the remainder of the C4a-flavin hydroxide converting to oxidized flavin as discussed below.

The absorbance spectra of the species involved were obtained by analyzing diode-array data with a three-step irreversible model in Specfit (Figure 8). The calculated spectrum of the starting species matches the spectrum of the reduced enzyme, while the final calculated spectrum is that of the oxidized enzyme. The calculated spectrum of the first intermediate has an absorbance maximum near 380 nm, characteristic of a flavin C4a-oxygen adduct, with no contribution from oxidized enzyme, which would have been evident in the 450−480 nm range. Our data suggest that the C4a-adduct observed in the oxidative half-reaction is the flavin hydroperoxide rather than the flavin hydroxide. This conclusion follows from the finding that the pOHB hydroxylation is ~50% coupled. If the first observed intermediate were the flavin hydroxide (formed after a very rapid hydroxylation), then about 50% oxidized enzyme should also have been present, but none was observed. Therefore, we conclude that the first intermediate observed was the flavin hydroperoxide, that this species decomposes with an observed rate constant of 13 s^{-1}, and that significant quantities of the flavin hydroxide were not observed by absorbance because of its relatively rapid decomposition to the oxidized flavin.

The spectrum calculated for the second intermediate is only slightly different from that of fully oxidized enzyme and represents a mixture of species. The likely explanation is that while most of the flavin C4a-hydroxide has eliminated water to form oxidized flavin, some has been kinetically stabilized by exchanging pOHB for PCA at the active site, even at the low pOHB concentrations used in this experiment. The hydroxyflavin intermediate is generally a very fluorescent species (excitation at 400 nm), and the slow decomposition of the small amount of trapped hydroxyflavin accounts for the large final fluorescence decrease as it decomposes to oxidized enzyme. These observations are also consistent with the strong inhibition of the enzyme shown by the steady-state kinetics reported in Results. It can be noted that k_{cat} > 1.4 s^{-1} (Figure 6), whereas the decay of the pOHB-trapped C4a hydroxyflavin (0.07 s^{-1}) is much less than this. Therefore, this trapped species cannot be on the normal catalytic pathway.

DISCUSSION

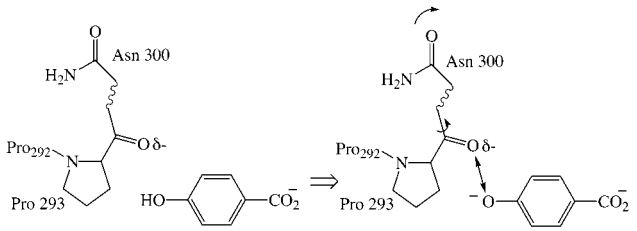
Our results demonstrate that proline 293 of PHBH has important functions in both the reductive and oxidative half-reactions, and that the mutation to serine increases the

flexibility of the active site causing effects throughout the catalytic cycle. The mutant enzyme allows greater access of solvent to the flavin hydroperoxide, causing loss of H_2O_2 and a decrease in the yield of the hydroxylation product. It more readily exchanges pOHB for PCA prior to eliminating H_2O from the hydroxyflavin than does wild type, causing the hydroxyflavin to be trapped in a dead-end complex manifested as severe substrate inhibition. In the wild type, ionization of pOHB is triggered by the binding of NADPH. The added flexibility of the protein backbone of the mutant enzyme uncouples the movement of the flavin from the ionization of pOHB (*8*), so that reduction is slightly slower.

Reductive Half-Reaction. Previously, it has been demonstrated that flavin reduction occurs when the flavin adopts the out conformation (*3*, *6*, *8*, *33*). The ability to rapidly adopt the out conformation requires the generation of a negative charge on the 4-substituent of the aromatic ligand. This linkage of aromatic ligand charge and flavin position constitute a novel mechanism for substrate recognition, enabling PHBH to prevent the wasteful consumption of NADPH in the presence of an inappropriate aromatic substrate such as *p*-aminobenzoate, a vital metabolite. Curiously, this discrimination occurs by inhibiting a step in which the aromatic ligand is not involved chemically (*3*). Central to this substrate recognition system is the internal hydrogen bond network, extending consecutively from the phenolic oxygen of pOHB to Tyr 201, Tyr 385, two internal water molecules, and terminating at His 72 some 12 Å away (Figure 1). It was shown that the binding of NADPH to the wild-type enzyme at low pH triggers the deprotonation of pOHB (*34*). Upon generating the phenolate of pOHB, the flavin adopts the out conformation, and a hydride is transferred from NADPH to FAD. Two critical events in this model have remained unexplained: the mechanism for NADPH-stimulated pOHB deprotonation, and the means by which the generation of the phenolate of pOHB causes flavin movement from the in to the out conformation. The data presented in this paper assist in resolving the latter issue.

The following model can explain how PHBH can sense the ionization state of pOHB and control the flavin conformation. Both the phenolic oxygen of Tyr 201 (the first protein residue in the hydrogen bond network) and the peptide carbonyl oxygen of Pro 293 are within hydrogen bonding distance of the phenolic oxygen of pOHB. When pOHB is in the phenolic form, either protein residue may act as the acceptor in a hydrogen bond from pOHB (*14*). However, when in its phenolate form, pOHB will be the hydrogen bond acceptor from Tyr 201 and will repulse the negative dipole of the Pro 293 carbonyl oxygen to generate torque on the peptide backbone. The rigid platform of the diproline segment, Pro292−Pro293, would promote the movement of the residues further along the polypeptide, Thr 294, Gly 295, and Ala 296. This may trigger the flavin conformational change (Scheme 2). The only protein side-chain that interacts with the isoalloxazine moiety of FAD is that of Asn 300, which forms a hydrogen bond to the oxygen of the carbonyl at the 2-position. Therefore, the dipole-charge repulsion at residue 293 could be transmitted by the protein backbone to residue 300, whose movement would trigger the flavin conformational change. Consistent with this role for Asn300, the crystal structure of the Asn300Asp enzyme shows that the mutation causes small but widespread changes in the protein structure (*35*), and altering of the dynamic behavior of the enzyme, evidenced by markedly slower rates of both pOHB binding and flavin reduction (*10*).

Scheme 2: Linkage of pOHB Ionization to Flavin Movement



Two results support the model described above for translating the negative charge of the phenolate into flavin movement from in to out. First, the phenolic pK_a of pOHB bound to the Pro293Ser mutant is 0.7 units (∼1 kcal mol^{-1}) lower than in the wild-type enzyme. This stabilization of the phenolate by the mutant enzyme over the wild-type enzyme could result from increased flexibility of the peptide backbone, which allows the charge-dipole stress to be more easily relieved. Second, unlike the wild-type enzyme, the rate constant for the reduction of Pro293Ser was independent of pH. Thus, the increased flexibility in the peptide backbone uncouples flavin movement from the ionization state of the phenolic oxygen of pOHB.

Oxidative Half-Reaction. The reduced flavin of PHBH is converted to oxidized flavin in a series of steps initiated by the reaction of the enzyme with molecular oxygen (Scheme 1). The Pro293Ser mutation did not significantly alter the initial reaction of O_2 with the reduced enzyme, though the behavior of the mutant enzyme in subsequent steps is significantly different from the behavior of the wild-type enzyme. The mutation decreased the rate of oxygen transfer from the flavin hydroperoxide to pOHB and increased the rate of hydrogen peroxide elimination. Although the Pro293Ser enzyme lowered the pK_a of pOHB bound to the oxidized enzyme more than did wild type, its hydroxylation reaction was not as fast. This suggests that the mutation has decreased the reactivity of the hydroperoxide. QM/MM calculations suggest that in the hydroxylation transition state a hydrogen bond forms between the distal OH of the flavin hydroperoxide and the carbonyl oxygen of Pro 293 (*36*) to provide 1.7 kcal mol^{-1} of transition state stabilization. Although no mutation can remove this backbone carbonyl, replacing the rigid proline by the more flexible serine could alter the position of the peptide carbonyl to impose an energy cost for reorienting it to form the hydrogen bond in the transition state. Our data show that the mutation has destabilized the hydroxylation transition state by 1.1 kcal mol^{-1} relative to wild type, implying that the mutant enzyme has lost ∼60% of the theoretical stabilization that this interaction could provide.

The mutation has also increased the rate of hydrogen peroxide elimination from the flavin hydroperoxide compared to the wild-type enzyme. The wild-type enzyme prevents this wasteful side-reaction by restricting the access of solvent. Apparently, the flavin hydroperoxide is not as protected in the Pro293Ser mutant form. The DSC data indicates that Pro293Ser has increased the mobility of the peptide backbone, which would allow solvent to penetrate the active site.

Alternatively, if the mobility of the peptide backbone in the region of residues 293−300 is linked to flavin movement, as we hypothesized above, then the elimination of hydrogen peroxide from the flavin hydroperoxide may come from flavin that has adopted the out conformation. For either explanation, it appears that the rigidity conferred by a proline in position 293 is an important factor in preventing hydrogen peroxide elimination.

Function of Conserved Prolines in Hydroxylases. Proline 293 is conserved in PHBH from all species, and in all known flavin-utilizing aromatic hydroxylases (*12*, *13*), suggesting its role is important in all these enzymes. Phenol hydroxylase is the only other aromatic hydroxylase whose structure has been determined (*37*). The FAD in phenol hydroxylase can adopt in and out conformations, and Pro 364 in this enzyme corresponds structurally to Pro 293 in PHBH. Met 370 and Asn 371, which are a few residues further along in the sequence of phenol hydroxylase, form hydrogen bonds through the peptide nitrogens with the carbonyl oxygen of the 2-position of the isoalloxazine. In PHBH, similar interactions occur via the side-chain of Asn 300. Phenol hydroxylase may also couple movements of its active site proline to movements of its flavin via the intervening peptide backbone to control its catalytic cycle. While phenol stimulates the rate of flavin reduction by NADPH in phenol hydroxylase, there is no evidence that the ionization state of phenol bound to phenol hydroxylase has a large effect on the kinetics of reduction. Also, the crystal structure of phenol hydroxylase shows that the distance between the phenolic oxygen of bound substrate and the carbonyl oxygen of Pro 364 is 4.86 Å. Therefore, a sensing role for Pro 364 analogous to the role played by Pro 293 appears unlikely. Despite these important differences, Pro 364 does appear to play important roles in controlling both the reductive and oxidative half-reactions of phenol hydroxylase, as evidenced by effects on the kinetic behavior of the Pro364Ser mutant, and a large decrease in the efficiency of hydroxylation in this mutant enzyme (Dong Xu, personal communication). Therefore, we hypothesize that this conserved proline in aromatic hydroxylases performs roles common to this family of enzymes: the regulation of flavin movement, the stabilization of the flavin hydroperoxide through a hydrogen bond (*36*, *38*), and the prevention of solvent access to the flavin hydroperoxide. The recruitment of Pro 293 as a sensor of the ionization state of the aromatic ligand is likely to be a function that among the flavoprotein hydroxylases is unique to PHBH. However, it seems probable that as the chemistry and regulation of other flavoprotein hydroxylases are elucidated to a similar level of detail, new modes for coupling the conserved function of regulating active site dynamics to the particular chemical and physiological requirements of each hydroxylase will be discovered.

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