Oxygen Reactions in *p*-Hydroxybenzoate Hydroxylase Utilize the H-Bond Network during Catalysis†

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ABSTRACT: *para*-Hydroxybenzoate hydroxylase is a flavoprotein monooxygenase that catalyses a reaction in two parts: reduction of the flavin adenine dinucleotide (FAD) in the enzyme by reduced nicotinamide adenine dinucleotide phosphate (NADPH) in response to binding *p*-hydroxybenzoate to the enzyme and oxidation of reduced FAD with oxygen to form a hydroperoxide, which then oxygenates *p*-hydroxybenzoate. These different reactions are coordinated through conformational rearrangements of the protein and isoalloxazine ring during catalysis. Earlier research showed that reduction of FAD occurs when the isoalloxazine of the FAD moves to the surface of the protein to allow hydride transfer from NADPH. This move is coordinated with protein rearrangements that are triggered by deprotonation of buried *p*-hydroxybenzoate through a H-bond network that leads to the surface of the protein. In this paper, we examine the involvement of this same H-bond network in the oxygen reactions—the initial formation of a flavin-C4a-hydroperoxide from the reaction between oxygen and reduced flavin, the electrophilic attack of the hydroperoxide upon the substrate to form product, and the elimination of water from the flavin-C4a-hydroxide to form oxidized enzyme in association with product release. These reactions were measured through absorbance and fluorescence changes in the FAD during the reactions. Results were collected over a range of pH for the reactions of wild-type enzyme and a series of mutant enzymes with the natural substrate and substrate analogues. We discovered that the rate of formation of the flavin hydroperoxide is not influenced by pH change, which indicates that the proton required for this reaction does not come from the H-bond network. The rate of the hydroxylation reaction increases with pH in a manner consistent with a pK_a of 7.1. We conclude that the H-bond network abstracts the phenolic proton from *p*-hydroxybenzoate in the transition state of oxygen transfer. The rate of formation of oxidized enzyme increases with pH in a manner consistent with a pK_a of 7.1, indicating the involvement of the H-bond network. We conclude that product deprotonation enhances the rate of a specific conformational change required for both product release and the elimination of water from C4a-OH-FAD.

para-Hydroxybenzoate hydroxylase (PHBH)[1] from *Pseudomonas aeruginosa* and *P. fluorescens* (EC 1.14.13.2) has been used extensively as a model for the reactions catalyzed by flavoprotein monooxygenases, particularly for enzymes that insert an atom of oxygen into the aromatic ring of a substrate (*1*, *2*). The catalytic scheme for the reaction catalyzed by PHBH shown in Figure 1 is typical of this group of enzymes. The reaction is an important transformation in some of the major metabolic pathways for the degradation of aromatic compounds by microorganisms. PHBH and probably other enzymes of this type utilize a novel mechanism to link two rather different catalytic processes, the reduction of the flavin by reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H), followed by its reaction with oxygen to bring about hydroxylation of the substrate. The isoalloxazine of flavin adenine dinucleotide (FAD) is mobile in PHBH and can move some 7−8 Å from an "in" position to an "out" position (ref *3* and references therein). Before NADPH can reduce the flavin, the isoalloxazine of the FAD must move to the out position, where the N5 of the ring is exposed to solvent on the *re* side, and this movement requires *p*-hydroxybenzoate (pOHB) to be bound to the enzyme. The *pro*-R hydride from NADPH is then transferred to the exposed N-5 of the isoalloxazine. Without pOHB bound, reduction of the FAD in the enzyme by NADPH is 10^5-fold slower because the protein does not adopt the conformation necessary for hydride transfer. This conformational change is stimulated by formation of the phenolate of the buried pOHB by removal of a proton through a H-bond network (Figure 2) connecting the phenolic proton to His72 at the surface of the enzyme (*3*, *4*). After it is reduced, the isoalloxazine moves back to the in conformation, where the reduced flavin reacts with oxygen in an environment almost solvent-free to initiate the second half of the catalysis, oxygenation of the substrate. This effectively gives PHBH two active sites to carry out two diverse reactions—one with flavin exposed to solvent and the other with flavin sequestered from solvent. This conformational flexibility of PHBH enables the enzyme to catalyze two

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[1] Abbreviations: PHBH, *p*-hydroxybenzoate hydroxylase; WT-PHBH, *p*-hydroxybenzoate hydroxylase derived from *Pseudomonas aeruginosa*; Arg220Gln-PHBH (and similar abbreviations), mutant forms of *p*-hydroxybenzoate hydroxylase; pOHB, 4-hydroxybenzoate; 2,4-DOHB, 2,4-dihydroxybenzoate; pAB, 4-aminobenzoate.

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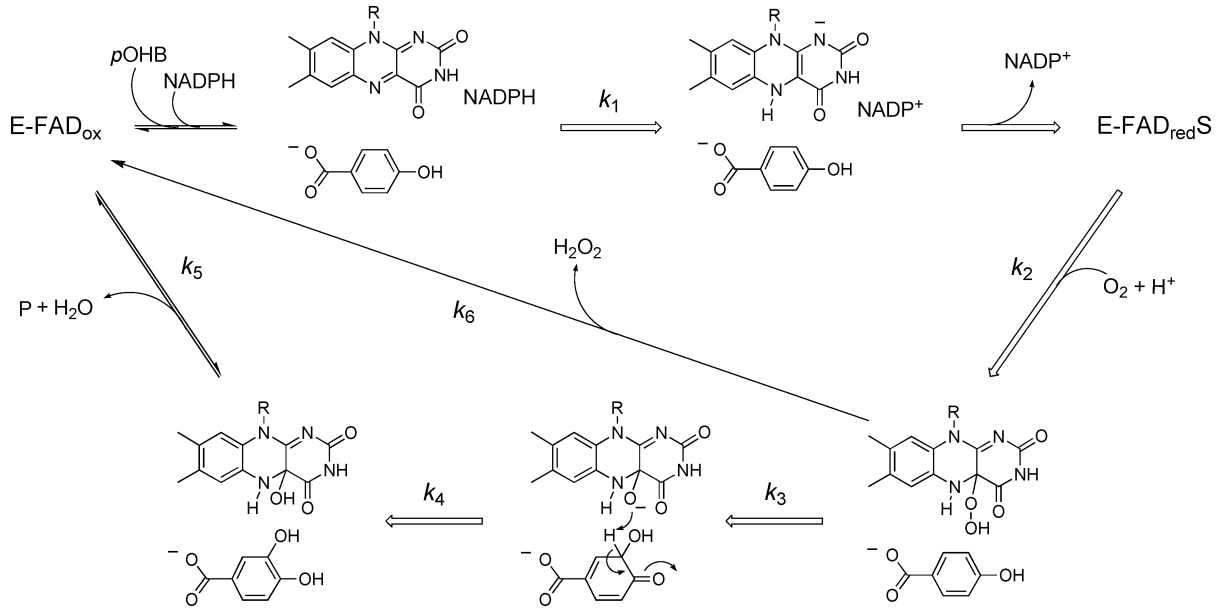

FIGURE 1: Catalytic cycle of PHBH. In the figure, E represents the enzyme, S represents pOHB, and P represents 3,4-dihydroxybenzoate, the oxygenated product. Note that k_1-k_6 represent the rate constants for the chemical reactions in the catalytic cycle.

different reactions while exhibiting a ping-pong kinetic mechanism.

Our understanding of the complexity of the function of this protein has been enhanced by a structure of the mutant enzyme Arg220Gln-PHBH with NADPH bound (*5*). PHBH exhibits a motif for binding NADPH that has not been previously recognized in other flavoproteins. The structure shows that the adenine of NADPH binds over the *si* side of the isoalloxazine ring with the nicotinamide ring extended many angstroms away from the flavin. For reduction to occur in the enzyme, the isoalloxazine has to move out and the nicotinamide ring of bound NADPH must rotate to form a hooked conformation with the nicotinamide ring within 4 Å of the *re* side of the isoalloxazine (*5*). Arg220Gln-PHBH formed high-quality crystals without ligands. As a result, the first high-resolution structure of PHBH without a bound ligand (*5*) was solved. This structure reveals an open conformation of the enzyme that has not previously been observed. This open conformation has a clear solvent path into the interior of the protein where the oxygen reactions occur and thereby provides a structural rationale for a pathway of binding and release of substrate and product from the enclosed active site. Thus, there are two potentially important protein movements to consider in the catalytic function of PHBH. First, the isoalloxazine ring is mobile, and this mobility is known to be fundamental to the two halves of catalysis, reduction and oxygenation (ref *3* and references therein). Second, there is the movement between "open" and "closed" conformations that is important for substrate and product exchange and even for NADPH binding (*5*, *6*). PHBH thus provides an outstanding demonstration of the role of conformational states in protein function.

As can be seen in Figure 1, the oxygen reactions of PHBH (and other enzymes of this family) are complex. Under appropriate experimental conditions, three unique consecutive transient chemical species are observed upon reaction of PHBH with oxygen (*7*). The first reaction (k_2, Figure 1) is the second-order formation of a flavin-C4a-hydroperoxide

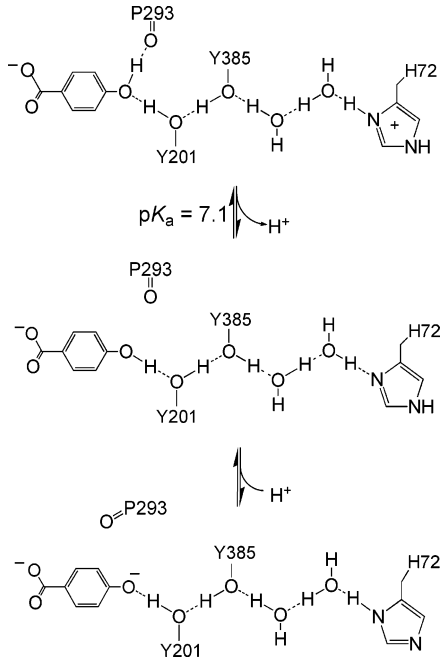

FIGURE 2: The H-bond network linking the substrate (pOHB) to the surface of PHBH. The arrangement of Tyr201, Tyr385, and His72 with structural waters to form a connection of H-bonds that can exchange protons with solvent was described in detail in ref *4*. The figure illustrates a modification of the scheme presented in ref *4* and shows that the pK_a of His72 can control the ability of the network to protonate or deprotonate pOHB in the active site. The model presented is consistent with the data for pH dependence in this paper. It has been shown that this pK_a is also essential for control of high rates of reduction [k_1 in Figure 1 and ref *3*].

from reduced FAD and oxygen. The rate of this reaction in PHBH has been changed by replacement of FAD with modified forms of FAD that have varying reactivity with oxygen (*8*). The second reaction is a first-order process (k_3, Figure 1) where the terminal oxygen of the hydroperoxide is transferred to the substrate in an electophilic substitution reaction that forms the flavin-C4a-oxide anion and the nonaromatic product (*8*). The third reaction (k_4 in Figure 1)

is a first-order reaction where the product rearranges to an aromatic structure, probably by transfer of the proton from the tetrahedral carbon in the product to the flavin to form the flavin-C4a-hydroxide. The last chemical reaction (k_5 in Figure 1) is a first-order reaction where water is lost from the flavin-C4a-hydroxide to reform the oxidized enzyme. This process is commonly indistinguishable kinetically from the dissociation of product and binding of substrate to the oxidized enzyme. However, this final combination of events can be observed to occur in more than one phase under certain conditions. The most spectacular example is the trapping of flavin-C4a-hydroxide in phenol hydroxylase by rebinding of substrate before loss of water from the flavin (*9*). In this case, the enzyme containing the flavin hydroxide can be isolated by column chromatography. By contrast, in the cases of the mutant enzyme Glu49Gln-PHBH (*6*) and WT-PHBH containing 8-SH-FAD (*10*), the flavin-C4a-hydroxide loses water faster than the enzyme releases product and rebinds substrate.

Extensive experimental work has shown how the H-bond network of PHBH controls the reduction of the enzyme flavin by NADPH (*3*−*5*, *11*). Up to the present, there is no clear evidence for a similar role of a H-bond network in other flavoprotein hydroxylases. It has been suggested that this finely tuned mechanism in PHBH for control of reduction may have evolved to provide a mechanism of discrimination between pOHB (the natural substrate) and attack upon an essential cellular component, *p*-aminobenzoate [pAB] (*3*). It would be interesting to know whether the same H-bond network has evolved to play some function in the oxygen half of the catalytic cycle of PHBH. From the earliest days of research into PHBH, it was discovered that pH had a large effect upon the magnitude of some of the rate constants in the oxygen half-reaction (*7*). This observation was confirmed in more quantitative terms by a later study of PHBH in the hydroxylation of the substrate analogue 2,4-dihydroxybenzoate [2,4-DOHB] (*12*). That study showed pH dependence of the rates k_3, k_4, and k_5 (Figure 1) with the indication of ionization processes with pK_a values of approximately 7.4. From a combination of more recent work, knowledge of the H-bond network (*4*), and the availability of many structures of PHBH, it is clear that some of the postulates in ref *12* need to be revised. Only one other flavoprotein hydroxylase (phenol hydroxylase) has been studied to determine the effects of pH upon the oxygen half-reaction (*13*), and both similarities and differences between phenol hydroxylase and PHBH were found. However, in phenol hydroxylase, there is no indication of a H-bond network connecting the substrate phenolic group to the surface of the protein (*14*). Phenol hydroxylase is from a eukaryotic organism (a yeast), and PHBH is from a eubacterium. There is very little sequence similarity between these proteins, but there is a remarkably similar 3D folding pattern over much of the proteins. However, although the active sites are quite different, both enzymes achieve the same basic catalytic function.

In this paper, we report new results about the effect of pH upon the oxygen half-reaction of PHBH that clearly show that the H-bond network in the protein works to enhance both the hydroxylation reaction and the process that completes the catalytic cycle (k_5 in Figure 1). The results come particularly from a careful analysis of the oxygen reactions with the natural substrate bound in the active sites of WT enzyme and mutants that modify the hydroxylation process. A preliminary report on this research was presented at the Fourteenth International Symposium on Flavoproteins in Cambridge (*15*).

MATERIALS AND METHODS

All common reagents used in this work were analytical reagent grade. Substrates for PHBH were from commercial sources and were recrystallized before use.

The construction of plasmids and the methods for expression of PHBH in *Escherichia coli* have been described previously (*16*−*18*). The WT, Glu49Gln, Lys297Met, Tyr201Phe, Tyr385Phe, and His72Asn forms of PHBH were isolated and purified as described in earlier work (*16*, *19*).

All experimental measurements with PHBH were carried out at 3.5−4 °C to slow reactions to facilitate quantitative analysis. Methods used to carry out oxygen half-reactions were similar to those described in previous publications from this laboratory (*7*, *17*, *20*). Of particular importance were the buffers used for setting pH values with PHBH. For the pH range of 6.0−7.5, 50 mM potassium phosphate with 0.5 mM EDTA was used; for the range of 7.8−8.7, 50 mM Tris-sulfate was used; for the range 9.0−9.5, 50 mM glycine adjusted with NaOH was used. These buffer ions were found to have no significant differential effects upon the oxygen reactions studied. All kinetic data were collected with a Hi-Tech Scientific model SF-61 stopped-flow spectrophotometer (controlled by KISS software from Kinetic Instruments) in either absorbance or fluorescence modes. Rapid reaction kinetic traces were analyzed and simulated with the software Program A, an MS-DOS based series of programs developed in our laboratory by Rong Chang, Chung-Yen Chiu, Joel Dinverno, and David Ballou, University of Michigan. Analysis is based upon the Marquardt algorithm for fitting data to sums of exponentials (*21*).

RESULTS

Oxygen Half-Reaction of WT-PHBH. To study this process, WT enzyme was mixed with 0.5 mM pOHB [approximately 25K_d (*7*)] and then was reduced by dithionite under anaerobic conditions. The reduced enzyme must be in complex with pOHB before reaction with oxygen because the reduced enzyme does not exchange pOHB at a significant rate compared to the rate of catalysis (*7*). The reduced enzyme was then reacted with a range of oxygen concentrations in the stopped-flow spectrophotometer/fluorimeter. This process was repeated at several pH values over a range of 6−9. Previous research has shown that this reaction simplifies to three phases because $k_4 \gg k_3$ (Figure 1) when pOHB is the substrate bound (*7*). Thus, the nonaromatic product is not observed. The first reaction (k_2, formation of the hydroperoxide) was observed by the increase in absorbance at approximately 385 nm (an appropriate isosbestic point) and the last reaction (k_5) by absorbance changes at 470 nm (where absorbance increases are due to formation of oxidized FAD). To measure the hydroxylation reaction (k_3), fluorescence was used, because the flavin hydroxide in PHBH is considerably more fluorescent than other species in the reaction (*20*). The large fluorescence signal from the flavin hydroxide provided accurate and specific data that could not

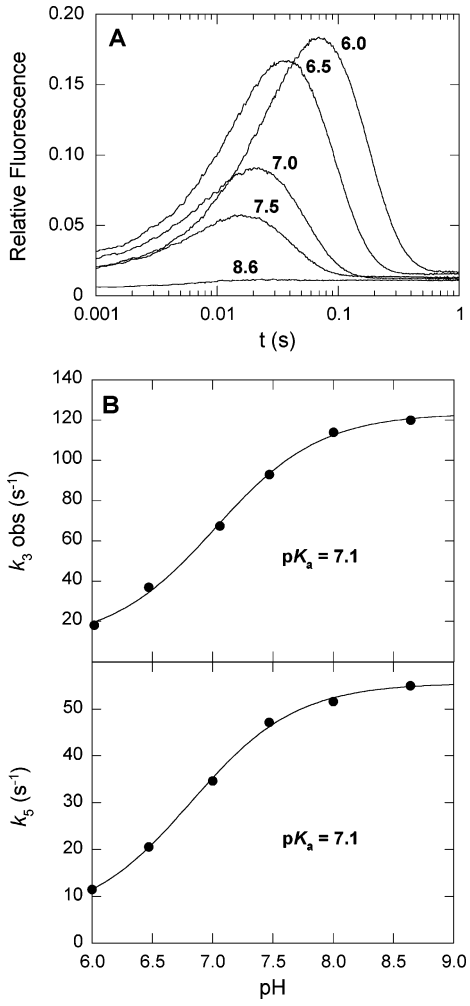

FIGURE 3: The effect of pH upon the values of k_3 and k_5 for the oxygen reactions of WT-PHBH in complex with pOHB. In panel A, reduced enzyme (10 μM) in complex with pOHB (0.5 mM) was reacted with oxygen (0.63 mM) at 4 °C in a variety of buffer solutions (see Materials and Methods), and the reaction was followed by fluorescence excitation at 390 and 480 nm with emission beyond 510 nm. The traces for selected reactions with excitation at 390 nm and at pH values on the figure are plotted. With 390 nm excitation, the reaction traces are almost exclusively due to flavin-C4a-hydroxide. The data from excitation at 480 nm and separate absorbance data were used to help obtain all the rate constants at all pH values. Panel B shows a plot of observed rate constants for k_3 and k_5 (see Figure 1) against pH. Solid dots are data points. The lines in the diagram represent the behavior of a single ionization process with a pK_a of 7.1.

be obtained with the same precision from absorbance changes.

The rate of formation of the flavin-C4a-hydroperoxide (k_2) was independent of pH and agreed with the published value of 3×10^5 M^{-1} s^{-1} at 4 °C and pH 6.5 (*7*). The effect of pH upon the other oxygen reactions is illustrated by the fluorescence traces in Figure 3A. Comparison of the traces at pH 6.0 and 6.5 shows that the hydroxylation process (fluorescence increase) and the decomposition of the transient species (fluorescence decrease) both increase in rate as pH rises. These changes continue up to pH 8.6 but are not seen immediately in the traces because the amplitude of fluorescence declines as pH rises (see below). The relationship between pH and the observed rate constants is plotted in Figure 3B. The data points (in both reactions) show an excellent correlation with a single pK_a of approximately 7.1. Each reaction increases in rate by roughly an order of magnitude in response to the change of an ionizable group. Although the fluorescence measured at pH 8.6 (Figure 3A) is very small, it does (when the signal is amplified) increase in a single phase with a rate equal to k_2, the first reaction with oxygen. At pH 8.6, the enzyme forms product normally, just as at lower pH. Based upon the measured rate constants at pH 8.6 (from absorbance traces), there should have been 40−45% of the fluorescent intermediate formed during the reaction, but no fluorescence is observed from this species. It can be concluded that the fluorescence of the flavin hydroxide is quenched coincident with the change in protonation state. Either the flavin or a chemical species near the flavin must change its protonation state to cause this quenching. The most likely candidate is the dianion of the aromatic product.

Without pOHB, the reaction of the enzyme with oxygen simplifies to a single phase—the initial second-order reaction between reduced flavin and oxygen [ref *7* and k_2 in Figure 1]. This phase is an order of magnitude slower than it is with pOHB but is also independent of pH. Because the rate constant for the release of H_2O_2 (k_6 in Figure 1) is much larger than that for formation of the flavin hydroperoxide under the conditions of study, no flavin hydroperoxide is observed.

Another series of oxygen reactions with WT enzyme was carried out with pAB as the substrate over a similar range of pH. This compound is a close analogue of pOHB. Even though pAB is a better nucleophile than protonated pOHB, the enzyme seems to have evolved to avoid hydroxylation of pAB (*3*). Discrimination occurs in the reductive half-reaction, where pAB does not promote reduction of FAD by NADPH. The hydrogens of the aromatic amino group of pAB cannot dissociate to form an anion like the hydrogen of the phenolic group of pOHB. Thus, pAB fails to stimulate the out conformation for reduction and it provides a contrasting substrate for the oxygen half-reaction, where oxygen reactions cannot be influenced by deprotonation of pAB. However, when the oxygen half-reaction is carried out with pAB bound to PHBH, it is hydroxylated by the enzyme (*7*). The fraction of pAB that is hydroxylated decreases with increasing pH (*22*). Because pAB cannot form a dianion, this pH effect is likely due to increased ionization of the N5 of the isoalloxazine causing loss of H_2O_2 as shown by k_6 in Figure 1. This "accidental" hydroxylation of pAB has been very helpful to our understanding of PHBH.

The initial reaction between the pAB complex with reduced PHBH and oxygen was, like that with pOHB, independent of pH, and the value of k_2 (Figure 1) was 3.3×10^5 M^{-1} s^{-1}—the same as the published value at pH 6.5 and almost the same as that with pOHB present (*7*). In Figure 4, the initial reaction can be seen in the fast increase of absorbance at 390 nm (between 1 and 15 ms) with no change in absorbance at 480 nm. The hydroxylation reaction rate (k_3 in Figure 1) was different from that with pOHB; it was independent of pH with a first-order rate constant of 5 s^{-1}. Note that this value is similar to the rate constant for hydroxylation of pOHB at pH 6.0 (where pOHB is protonated) but is 25-fold smaller than the rate constant with pOHB at pH 8.6 (Figure 3B). In contrast to the reaction with pOHB bound, the nonaromatic product of hydroxylation of

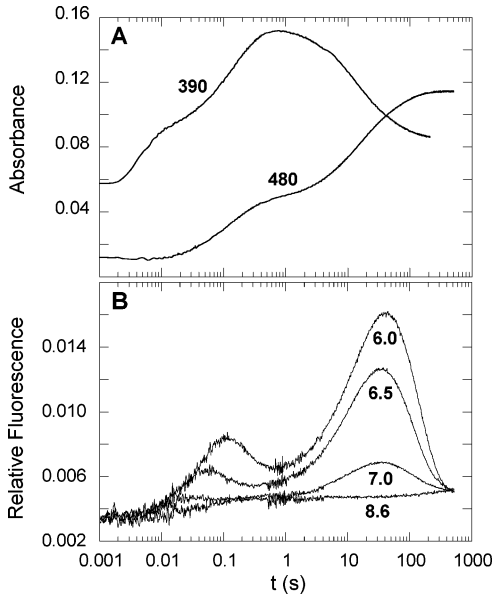

FIGURE 4: The effect of pH upon the kinetics of the oxygen reactions of WT-PHBH with the "abnormal" substrate pAB. Panel A shows reaction of reduced PHBH (15 μM) in complex with pAB (0.5 mM) and oxygen (0.63 mM) at 4 °C in potassium phosphate buffer at pH 7.5. Wavelengths of absorbance traces are shown on the figure. At 390 nm, note the fast increase in absorbance in the first 10 ms (k_2 in Figure 1), followed by a larger increase in absorbance up to 1 s (k_3 in Figure 1), which illustrates the large absorbance contribution from the primary nonaromatic product. At 480 nm, note that the absorbance increase from 1 to 100 s (formation of oxidized FAD, k_5) corresponds with the decrease in absorbance at 390 nm (rearrangement of enamine product to the aromatic structure, k_4). The increase in absorbance at 480 nm between 10 ms and 1s corresponds to enzyme that fails to hydroxylate at this pH (k_6 in Figure 1). Panel B shows reactions such as those in panel A but with 10 μM enzyme and over a range of pH (shown on the figure) using fluorescence detection with excitation at 390 nm, which measures almost exclusively flavin-C4a-hydroxide. Note that the hydroxylation reaction (k_3, between 10 ms and 1 s) forming flavin-C4a-hydroxide occurs in two phases by fluorescence. Fluorescence increases at the rate of hydroxylation as determined by absorbance but is quenched in the following process, which has a larger rate constant than hydroxylation and is pH dependent; therefore, fluorescence is completely quenched as pH rises. Then, the fluorescence reappears (at lower pH values) at the same rate that oxidized enzyme is formed and finally decreases after most of the enzyme is oxidized. This high fluorescence when the enzyme is largely oxidized is typical of a small portion of the enzyme trapped in a highly fluorescent form as the flavin-C4a-hydroxide with substrate bound (*9*).

pAB does not rearrange fast to the aromatic product, 3-hydroxy-4-aminobenzoate. This can be seen by the high absorbance at 390 nm after 1 s of reaction (Figure 4) — considerably greater than the absorbance due to the flavin hydroxide or hydroperoxide alone. Hydroxylation occurs with the absorbance increase from 10 ms to 1 s that is partly due to the nonaromatic intermediate. The subsequent rearomatization to form the product is very slow and independent of pH; the observed value of k_4 was 0.06 s^{-1}. With pAB, the k_4 reaction forms mostly oxidized FAD and a small amount of flavin hydroxide (absorbance changes between 1 and 100 s in Figure 4A). The most likely explanation for this observation is that the nonaromatic product dissociates from the enzyme, followed by rapid rearomatization in free solution. This dissociation of product enables a major fraction of the flavin to eliminate water by exposure to solvent, but some is trapped as the flavin hydroxide in complex with pAB (as observed in the reaction of the pOHB complex at higher pOHB concentrations). When the oxygen reaction with pAB is followed by fluorescence (Figure 4B), a small transient peak in fluorescence (at 0.1 s) due to the flavin hydroxide (formed upon hydroxylation) is observed at pH 6.0. However, this fluorescence is quenched at higher pH as can be seen in the traces in Figure 4 (see 0.01 to 1 s). Analysis of the traces in Figure 4B up to 1 s showed that the fluorescence increase occurred with a fixed rate constant equal to the rate of hydroxylation, while the loss of fluorescence occurred with a larger rate constant and did not correspond to any observable absorbance change in the reaction. Thus, the amount of the fluorescent species transiently formed was always small, and by pH 7.5, no fluorescence due to the flavin hydroxide can be detected, even though approximately 70% of the enzyme hydroxylates the substrate (based upon the observation that about 30% of oxidized enzyme formed in the hydroxylation step at 480 nm, followed by about 70% of oxidized formed after hydroxylation was complete [Figure 4A]). A similar pH-dependent quenching of fluorescence occurs in the reactions with pOHB as the substrate (Figure 3A) but is shifted to higher pH. With pOHB as substrate, fluorescence quenching with increasing pH is coincident with hydroxylation (described above), but with pAB, the quenching is observed as a separate process following hydroxylation.

Oxygen Half-Reaction of Tyr201Phe-PHBH. Removal of the phenolic group of Tyr201 in PHBH removes the primary H-bond in the network from pOHB to the surface of the enzyme (Figure 2). This single change prevents hydroxylation of pOHB. Instead, H_2O_2 is formed from decomposition of the flavin hydroperoxide [ref *17* and k_6 in Figure 1]. We predicted that this mutant enzyme should be capable of hydroxylation of pAB, since this compound works with WT enzyme (above) and cannot respond to the H-bond network. It was found that pAB was hydroxylated by Tyr201Phe-PHBH with a rate constant of 2.6 s^{-1} at both pH 6.5 and 8.0—only a factor of 2 different from WT enzyme. This result is very different from the reaction of pOHB bound to the Tyr201Phe mutant enzyme (*17*), where the rate of hydroxylation is 10^3-fold smaller than that for WT enzyme under the same conditions. After hydroxylation of pAB by the mutant enzyme, the reaction diverged significantly from WT enzyme. Rearomatization of the product (k_4) was at least an order of magnitude faster than that in WT enzyme. Associated with hydroxylation and rearomatization of pAB in the mutant enzyme, there was a large fluorescence increase due to the formation of the flavin hydroxide, in contrast to the result with WT enzyme (Figure 4B). This fluorescence formed and then decayed in a manner very similar to WT enzyme in reactions with pOHB but with very different rate constants.

Oxygen Half-Reaction of Lys297Met-PHBH. This mutation decreases the positive charge in the active site of PHBH but does not interfere with the H-bond network (*20*). The less positive electrostatic field at the flavin causes a 25-fold decrease in the rate of hydroxylation compared to WT enzyme, but the same mechanism of hydroxylation is retained. The effect of pH on this mutant enzyme was studied (in a manner similar to WT enzyme above) to test the potential interaction between the H-bond network and the electrostatic field in the mechanism of hydroxylation.

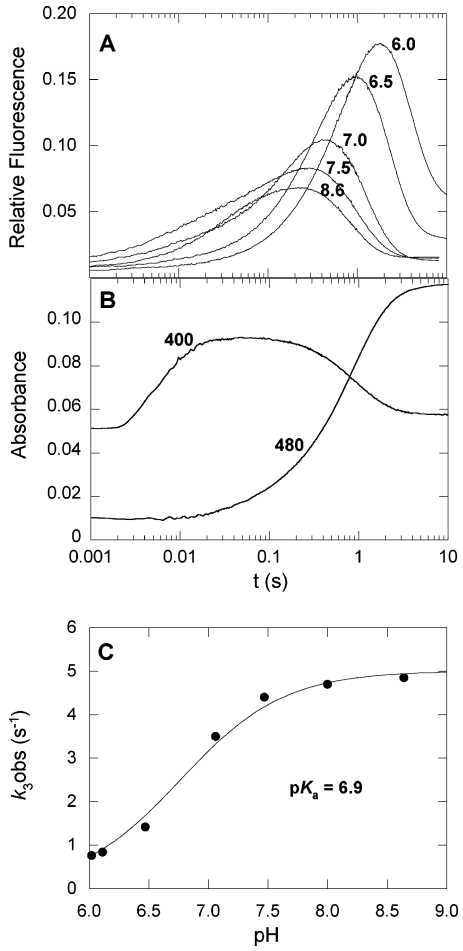

FIGURE 5: The effect of pH upon the rate constants for the reactions of oxygen with Lys297Met-PHBH in complex with pOHB. In panel A, reactions were carried out under the same conditions as those described in the legend for Figure 3A; pH values are shown on the figure. Panel B shows sample reactions carried out at pH 8.0 and 0.63 mM oxygen and monitored by absorbance at wavelengths marked upon the figure. Note the fast increase in absorbance at 400 nm up to about 20 ms (k_2 in Figure 1) followed by a lag and subsequent decrease in absorbance coincident with the increase in absorbance at 480 nm (k_5). In panel C, the dependence upon pH of the rate constant k_3 that was obtained from exponential fits to fluorescence traces such as those in panel A. Solid dots are data points. A theoretical curve for a single ionization process with a pK_a of 6.9 is fit to the data.

The first reaction with oxygen (k_2 in Figure 1) occurred at the same rate as for WT enzyme (based upon absorbance changes in the formation of flavin-C4a-peroxide, see Figure 5B) and was also independent of pH. The values for k_3 and k_5 (Figure 1) were obtained from fluorescence data that are shown in Figure 5A. The absorption spectra of the two transient oxygenated flavin species in the mutant enzyme (unlike WT enzyme) are almost identical, and thus the value of k_3 (conversion of the hydroperoxide to the hydroxide upon hydroxylation) could not be measured by absorbance. However, as with WT enzyme, the C-4a-hydroxide has substantially more fluorescence than other species in the reaction (*20*). The fluorescence traces are qualitatively similar to those for WT enzyme below pH 7, but k_3 and k_5 are at least 10-fold smaller. However, unlike WT enzyme above pH 7, the fluorescence traces (like those shown in Figure 5A) exhibit three phases. The extra phase is an increase in fluorescence in the first 100 ms that shows some oxygen dependence. This process is faster than hydroxylation, but slower than the initial reaction with oxygen (the increase in absorbance up to 20 ms at 400 nm in Figure 5B) and has no equivalent in absorbance. This extra phase between k_2 and k_3 for Lys297Met-PHBH decreases in rate as pH rises (compare fluorescence traces at pH 7.5 and 8.6 in Figure 5A) and can only be due to the flavin-C4a-peroxide/hydroperoxide. A fluorescence signal from this species is usually extremely small in PHBH compared to the signal from flavin-C4a-hydroxide. Above pH 7, hydroxylation by Lys297Met enzyme (k_3) occurs in the range from 10 to 300 ms as shown by some fluorescence increase in Figure 5A and by the lags in absorbance at 400 and 480 nm in Figure 5B. Observed rate constants for hydroxylation (obtained from the fluorescence traces) are plotted against pH in Figure 5C. As for WT enzyme, the data fit a single ionization event but with a pK_a of approximately 6.9 and with a similar factor in the change in rates with this pK_a. It should also be noted that the fluorescence of the flavin-C4a-hydroxide transient intermediate species decreased with increasing pH similarly to that with WT enzyme. Over the entire range of pH studied, Lys297Met-PHBH hydroxylated almost as effectively as WT, so the weaker fluorescence was not due to formation of less transient species in the reaction at higher pH.

The last reaction (k_5) in the oxygen half-reaction by Lys297Met enzyme is the increase in absorbance at 480 nm (characteristic of oxidized flavin), the decrease in absorbance at 400 nm (Figure 5B), and the decrease in fluorescence signal seen in Figure 5A, all at the same rate at one pH. As can be seen from the fluorescence data, this process is also pH-dependent with a pK_a of about 6.9. The magnitude of this rate change with pH is smaller than other examples in the results presented here. At pH 6.0, k_3 is only $1.5k_5$; this predicts the maximum fluorescence observed due to the C4a-hydroxyflavin (Figure 5A) is about 40−45% of the fluorescence from formation of 100% of the intermediate species. At pH 7.5, k_3 is $3k_5$ but the fluorescence signal at the maximum is about a third that at pH 6.0 (Figure 5A), when the ratio of rates predicts about 56% of the intermediate at the maximum. These observations illustrate clearly the quenching of the fluorescence of flavin-C4a-hydroxide with increasing pH.

Oxygen Half-Reaction of Glu49Gln-PHBH. In PHBH, Glu49 and Lys297 form a buried charge pair. In contrast to Lys297Met-PHBH, the Glu49Gln mutation enhances the positive charge in the active site of the enzyme by removing a negative charge near the flavin (*6*) without disturbing the H-bond network. Studies with Glu49Gln-PHBH strengthen the case for the function of positive electrostatic potential about the flavin by showing an increase in the rate of hydroxylation over WT enzyme (*6*). The effect of pH upon this reaction was studied as discussed above for WT enzyme.

The rate of the reaction between reduced Glu49Gln enzyme and oxygen was very similar to WT enzyme (2×10^5 compared to 3×10^5 M^{-1} s^{-1}) and was independent of pH. Samples of the fluorescence changes with pH were published previously (*6*). With this mutant, the rate of hydroxylation (k_3 in Figure 1) cannot be separated from the initial reaction with oxygen (k_2), except to a small degree at pH 6.0. No pH dependence could be measured for k_3, as explained previously (*6*). The enhanced positive electrostatic field boosts the rate of hydroxylation above that of WT

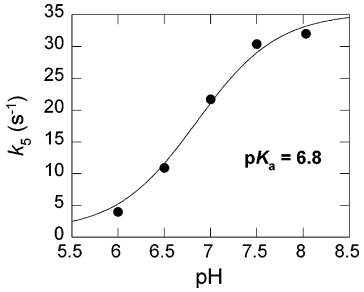

FIGURE 6: The effect of pH upon the rate constants for the oxygen reactions of Glu49Gln-PHBH in complex with pOHB. Reactions were carried out under the same conditions as those described in the legend for Figure 3A. Examples of absorbance and fluorescence traces for this mutant enzyme have been published (*6*) where it was shown that because k_3 (hydroxylation) is greater than k_2 in experiments, k_3 could not be determined accurately. The measured values for k_5 from the data are plotted against pH (●). A theoretical curve (solid line) for a single ionization process with a pK_a of 6.8 shows good agreement with the data.

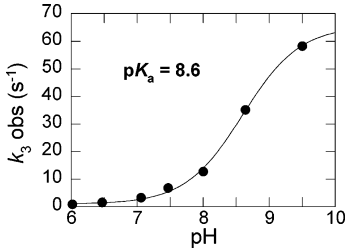

FIGURE 7: The effect of pH upon the rate constants for the oxygen reactions of Tyr385Phe-PHBH in complex with pOHB. A series of reactions were carried out as described in the legend to Figure 3 over a range of pH values using absorbance and fluorescence detection. With this mutant enzyme, only two phases are observed—the fast, oxygen-dependent formation of the flavin hydroperoxide and the oxygen-independent return to oxidized enzyme. The latter process was measured by absorbance (actually a measure of k_3 in Figure 1 with k_4 and k_5 being much faster than k_3) and was dependent upon pH as shown in the figure. The filled dots are experimental values. A theoretical curve for a single ionization event with a pK_a of 8.6 is plotted on the figure.

enzyme. The rate of decomposition of the flavin hydroxide intermediate (k_5 in Figure 1) changed with pH as shown in Figure 6 and could be described by a single ionization event with a pK_a of approximately 6.8. This result was similar to that obtained with WT enzyme (see Figure 3B). The oxygen reactions of Glu49Gln-PHBH are complicated by the unusual observation with this enzyme that the rate of release of product is slower than the rate of return of the flavin to the oxidized state (*6*). The release of product was directly measured as a small absorbance change in the oxidized enzyme spectrum, and the interpretation was confirmed by measuring directly the dissociation of 3,4-dihydroxybenzoate from the oxidized enzyme.

Oxygen Half-Reaction of Tyr385Phe-PHBH. The residue Tyr385 is in the middle of the H-bond network connecting the substrate phenolic group to His72 at the surface of the enzyme [ref *4* and Figure 2], and its substitution by phenylalanine disrupts the function of the H-bond network in the control of the reductive half-reaction [ref *3* and k_1 in Figure 1]. In the oxygen half-reaction, the rate of hydroxylation is 25-fold slower than that for the WT enzyme at pH 6.5 (*17*), and in contrast to WT enzyme, there was some formation of H_2O_2 in the reaction (approximately 20% of the amount of reduced enzyme). To test further the effects of this mutation, the oxygen reactions of the mutant enzyme, Tyr385Phe-PHBH, have been studied over a range of pH. Formation of the flavin hydroperoxide (k_2 in Figure 1) occurred at the same rate as that for WT enzyme, and the rate constant for this process was independent of pH. However, the behavior of this mutant enzyme diverged from that of WT in the hydroxylation reaction. Although not as effective as WT enzyme, the mutant enzyme does form a substantial fraction of product from reduced enzyme, from 0.8 to 0.65 as pH goes from 6.5 to 9.5. The relationship between pH and rate of hydroxylation is shown in Figure 7. Note that the rate constant approaches values similar to those for WT enzyme at high pH but only starts to increase significantly above pH 7.5. At low pH, the rate of hydroxylation is only about 1.5 s^{-1}—between 1% and 2% of WT rate. The fit of a single ionization event to the data shows a process with a pK_a of about 8.6 (Figure 7), unlike WT enzyme, where the pK_a is 7.1 (Figure 3B). Unlike WT enzyme, there is no detectable fluorescence signal at any pH from flavin-C4a-hydroxide in the reaction by Tyr385Phe enzyme. Hydroxylation occurs coincidently with the formation of oxidized enzyme. Thus, the rate of decomposition of the flavin-C4a-hydroxide must be much faster than the rate of its formation (both k_4 and $k_5 \gg k_3$). Disruption of the H-bond network in the enzyme has changed the pK_a of the hydroxylation reaction and its rate at lower pH but has not inhibited steps k_4 and k_5 in Figure 1.

Above pH 7.5 in the oxygen half-reaction of Tyr385Phe-PHBH, a process slower than the rate of hydroxylation was detected, particularly by fluorescence. At pH 8.5 and above, this slow reaction clearly occurred after formation of oxidized flavin. Separate experiments were carried out to examine this phenomenon. It was found that the slow reaction (at a rate of 1.8 s^{-1}) was not related to product release but was due to slow formation of a portion of the out conformation of the oxidized enzyme in complex with pOHB after pOHB had replaced the product in the active site at the end of the oxygen half-reaction. Thus, the slow reaction is not really part of the oxygen half-reaction but was the response of the enzyme with a damaged H-bond network after substrate binding, a part of the reductive half-reaction (*3*).

Oxygen Half-Reaction of His72Asn-PHBH. The residue His72 is the solvent interface for the H-bond network to the substrate phenol (Figure 2). Mutation of this residue to asparagine results in a stable enzyme that has provided many valuable insights into the role of the H-bond network in the reduction half-reaction of this enzyme (*23*). Because His72 is part of the H-bond network, the mutant enzyme might be expected to have a similar behavior to Tyr385Phe-PHBH in the oxygen reactions. This is partly true; the difference between the two mutant enzymes resides in the hydroxylation reaction (k_3, Figure 1).

When reduced His72Asn-PHBH with pOHB bound is reacted with oxygen, the first step (k_2) occurs at a rate similar to WT and, as illustrated in Figure 8A, is independent of pH (like all of the examples above). As in the case of Tyr385Phe-PHBH, no fluorescence due to the flavin-C4a-hydroxide was observed, even though there was substantial product formation (90% at pH 6.5, declining to 68% at pH 9.5). This result implies that the magnitudes of k_4 and k_5

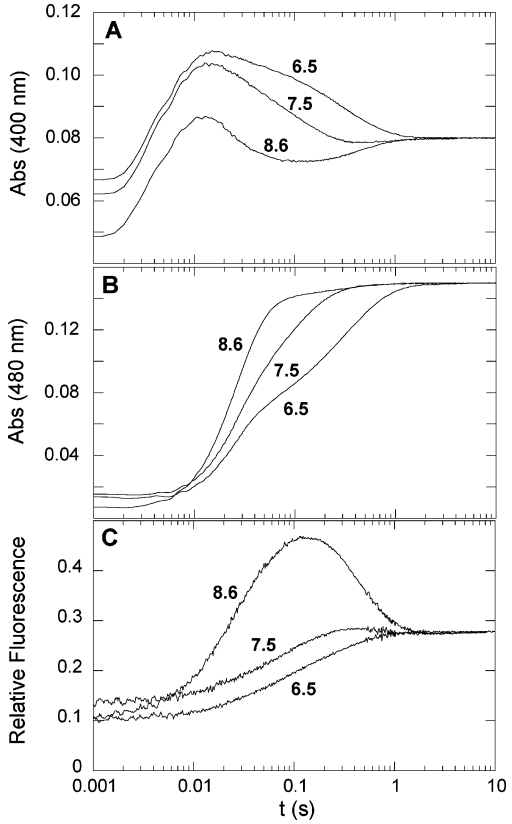

FIGURE 8: The effect of pH upon absorbance and fluorescence changes during the oxygen reactions of His72Asn-PHBH in complex with pOHB. Reactions were carried out as described in the legend to Figure 3. Panels A and B are absorbance traces at the pH values marked on the figure. Panel C is fluorescence traces (excitation at 450 nm) at the pH values on the figure. The raw data is shown to illustrate two remarkable features of this mutant enzyme. First, the hydroxylation reaction (the return to oxidized enzyme is the measure of k_3 in Figure 1) is biphasic, particularly at lower pH; this can be seen in the absorbance traces [400 and 480 nm in panels A and B] at pH 6.5 between 10 ms and 1 s. The faster phase has the same rate constant as WT enzyme at the same pH, and the slower phase is similar to the rate of hydroxylation by the Tyr385Phe mutant enzyme (Figure 7). A fluorescence signal (excitation at 390 nm) is not observed from the flavin-C4a-hydroxide (not shown) because k_4 and $k_5 \gg k_3$. The second feature of these reactions is the relatively slow reaction of the mutant enzyme after return to oxidized enzyme at higher pH values. This process is particularly noticeable in the fluorescence trace at pH 8.6 (panel C). Oxidized enzyme is formed by 0.15 s (see 480 nm absorbance change) and is followed by a fluorescence decrease between 0.15 and 3 s.

must be substantially greater than that of k_3 (as for the Tyr385Phe mutant above). Again, as seen with the Tyr385Phe mutant, the formation of oxidized enzyme at high pH is followed by a slow change in absorbance and fluorescence but with larger amplitude than seen with the Tyr385Phe mutant. This is clearly illustrated in Figure 8. At pH 8.6, the absorbance trace at 480 nm shows that the flavin is fully oxidized by 0.1 s, but at 400 nm, a small, slower absorbance change occurs between 0.1 and 1 s. This final phase (with a rate constant of 2.7 s^{-1}) is also observed in the fluorescence trace (Figure 8C) with excitation at 450 nm; the fluorescence of the oxidized enzyme decreases between 0.1 and 1 s. This phenomenon was studied in separate experiments. It was found to be due to the same process as that seen with the Tyr385Phe mutant (above): with the formation of oxidized enzyme, product is released and pOHB binds to the enzyme. After pOHB binds, the enzyme slowly forms the out conformation because of the disrupted H-bond network. However, with the His72Asn mutant enzyme, much more of the out conformation is formed at high pH than in the case of the Tyr385Phe mutant (*3*), leading to the larger observed absorbance and fluorescence changes.

It is in the hydroxylation reaction (k_3 of Figure 1) that the His72Asn enzyme differs from the Tyr385Phe enzyme and from WT enzyme. After formation of the flavin hydroperoxide in the first 10−15 ms (in Figure 8, see the rapid increase in absorbance at 400 nm compared to no change at 480 nm), there is a marked biphasic change in absorbance at both 400 and 480 nm (see particularly the pH 6.5 trace) coincident with the hydroxylation process. These biphasic changes are occurring with rate constants of 50 and 2.9 s^{-1} at pH 6.5. The faster process occurs at a rate similar to the rate of hydroxylation of WT enzyme at this pH, but the slower process is similar to the hydroxylation rate of Tyr385Phe enzyme at this pH. As pH increases, both phases increase in rate and merge at the highest pH values (see Figure 8B). At high pH, His72Asn and Tyr385Phe enzymes (with disrupted H-bond networks) hydroxylate as fast and as efficiently as WT enzyme in the oxygen half-reactions. At pH values below 7.5, in contrast to WT enzyme, the hydroxylation reaction for each mutant is potentially rate-determining in catalysis based upon the values of the rate constants for the steps in the oxygen half-reactions. In separate experiments, the absorption of the His72Asn mutant enzyme was measured at 450 nm during steady-state catalysis over a range of pH from 6.5 to 9.5. In steady state, the enzyme is mostly in the oxidized form. Thus, catalytic turnover was dominated by slow reduction (k_1 of Figure 1) with a smaller contribution from slow hydroxylation (k_3 of Figure 1). Thus, the overall effect of disruption of the H-bond network is to make the core reactions of catalysis (flavin reduction and pOHB hydroxylation) rate-determining, unlike WT enzyme. In the case of the His72Asn mutant enzyme, its rate of turnover is only about 8% of WT enzyme at all pH values.

DISCUSSION

The data presented in this paper, combined with some published work in past years, provide new insights into the role of proton exchanges in the reactions of oxygen with reduced PHBH. We now realize that some of the results in earlier studies were sometimes interpreted incorrectly, but can now be integrated into a more satisfying and coherent explanation of the oxygen reactions.

In the oxygen half-reaction of PHBH, the first step is the formation of the flavin-C4a-hydroperoxide in a second-order reaction (k_2 in Figure 1) between the reduced flavin anion and oxygen. This process probably involves three chemical events—the rate-determining exchange of an electron between reduced flavin and oxygen to form a flavin semiquinone and superoxide caged pair, followed by the much faster second electron exchange and protonation of the peroxide formed (*8*, *24*, *25*). The rate constant for the primary electron exchange reaction declines significantly when the FAD in the enzyme is replaced by different 8-substituted FADs where the redox potential of the flavin is substantially more positive

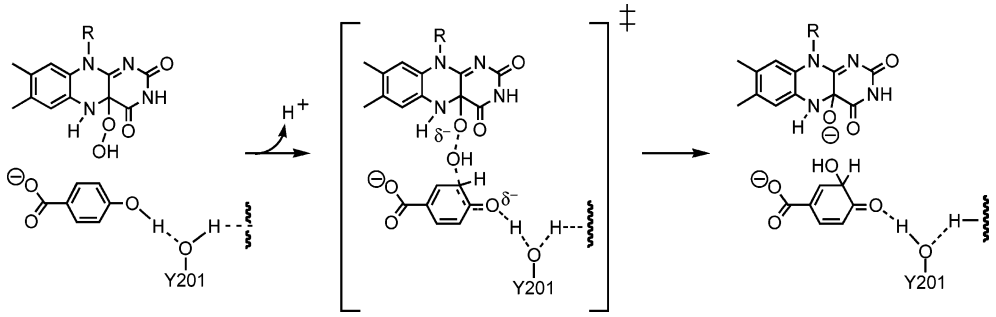

FIGURE 9: Proposed model of the transition state in the hydroxylation reaction (k_3 in Figure 1). This model is similar to that proposed in an earlier publication (*32*).

(*8*). There is no pH dependence in the rate of hydroperoxide formation over the range of pH studied in this work. Thus, the pK_a for reduced FAD in PHBH must be less than 5 in the strongly positive electrostatic field of the active site. In addition, the proton that forms the hydroperoxide is unlikely to come from the H-bond network since the value of k_2 is not changed (or the reaction is not split into more than one phase) by disruption of the H-bond network. The source of the proton for formation of the hydroperoxide could be directly from solvent on the *re* side of the flavin (*26*). A flavin protonation step kinetically separated from the initial electron exchange has been observed for phenol hydroxylase with resorcinol as substrate (*27*). We can now report that a similar protonation step can probably be observed in Lys297Met-PHBH. The partly oxygen-dependent phase in fluorescence between k_2 and k_3 (described in Results and shown in Figure 5A) appropriately declines in rate as pH rises, independently of the effects of the H-bond network on the remainder of the reaction. It should be noted that the mutation of Lys297 occurs in the protein strand on the *re* side of the flavin near the ribityl side chain. We currently have no structure of this form of the enzyme, but it is possible that the mutation has partly restricted solvent access to the flavin where oxygen must bind against the flavin to react as predicted from the structure of PHBH with flavin in (*28*). This structural water is connected to bulk water out past the ribityl side shain on the *re* side of the flavin. The structure of phenol hydroxylase (*14*) also shows water bound where oxygen would be expected to interact, and a channel to solvent in the structure similar to that in PHBH. Thus, it is difficult to understand why these two enzymes have a difference in the rate of peroxide protonation in the initial reaction with oxygen. PHBH can hydroxylate at high rates (see WT enzyme in Figure 3B) and this implies that protonation of the peroxide in WT enzyme is also very fast even above pH 8, because it must precede hydroxylation.

Although the initial reaction with oxygen to form the flavin hydroperoxide showed no response to pH, the hydroxylation process (k_3 in Figure 1) responded to pH in all the forms of the enzyme that were studied, provided that the substrate was a phenol—pOHB in this paper and 2,4-DOHB in an earlier study (*12*). With pAB as substrate (no readily removable substrate proton), the hydroxylation rate was independent of pH, with or without an intact H-bond network. These results show that at least part of the hydroxylation reaction involves the ionization of the substrate phenol. Moreover, the mutant enzymes with partial disruption of the H-bond network were clearly different from those with an intact network. With phenolic substrates, rates of hydroxylation in the forms of the enzyme with an intact H-bond network increased by approximately an order of magnitude from pH 6 to 9: a single deprotonation with a pK_a between 6.8 and 7.4 fitted the results. In the case of Tyr385Phe-PHBH (disrupted network), this apparent pK_a is shifted to much higher pH, while in the case of His72Asn-PHBH (disrupted network), hydroxylation splits into two separate reactions (see Figure 8). The results show that the H-bond network is specifically responsible for about 100-fold acceleration in the rate of hydroxylation. This can be seen by comparing the rate of hydroxylation at pH 6 by Tyr385Phe-PHBH (1.2 s^{-1}, Figure 7) to the rate by WT-PHBH at pH 8.5 (120 s^{-1}, Figure 3B). Since the oxygen reaction with pOHB begins with the phenol protonated (*29*) and it has not been possible to observe deprotonation of the substrate before hydroxylation in PHBH or in a similar enzyme, melilotate hydroxylase (ref *30* and unpublished data), it is reasonable to propose that the proton is removed in forming the transition state of the oxygen transfer (Figure 9). A proton can be removed from pOHB by the expulsion of a proton to solvent from His72 at the protein surface [ref *4* and Figure 2]. The measured pK_a (7.1) is a function of the surface His72. A similar function for the proton network has been described for control of flavin reduction in the catalytic cycle (*3*, *23*). In reduction, expulsion of a proton at the solvent interface deprotonates pOHB, and the resulting phenolate triggers conformational changes in the protein that favor the structure required for reduction of the FAD by NADPH.

Why should mutant enzymes with disrupted H-bond networks hydroxylate and partly respond to pH? It has been shown that protons can be exchanged from the active site of His72Asn-PHBH without the normal operation of the H-bond network but at much slower rates of 1−3 s^{-1} (*23*). When reduced enzyme is prepared for reaction with oxygen, some portion of the H-bond network at equilibrium may be oriented to accept the substrate proton and the remainder in the reverse configuration where the substrate proton is isolated by H-bonding to Pro293 (Figure 2). This proposal can explain the biphasic hydroxylation by His72Asn-PHBH (Figure 8). The enzyme molecules with the substrate proton oriented to the H-bond network could perform like WT enzyme and hydroxylate rapidly, while those in the opposite orientation (the substrate proton oriented toward the carbonyl of Pro293) would have to rearrange the H-bond network slowly before hydroxylation could occur. The Tyr385Phe mutant enzyme retains the structure of the WT enzyme, except for the H-bond to residue 385 (*31*). Proton exchange with the active site should be slow as illustrated by the slow reductive half-reactions of this mutant enzyme (*3*). Results

suggest that a fraction of substrate bound in reduced Tyr385Phe-PHBH must be in the phenolate form upon reaction with oxygen. This fraction (approximately 20%) immediately eliminates H_2O_2 by triggering movement of the flavin to the out conformation, a process demonstrated in the control of reduction (*3*). The bulk of the enzyme molecules (with substrate protonated) are capable of hydroxylation but less efficiently than WT enzyme, except at high pH (Figure 7). There must be some alternative mechanism (different from WT enzyme) for a proton loss from the active site, particularly at high pH. Protein breathing has been proposed to substitute for a disrupted H-bond network in PHBH (*23*).

Only in the case of the Tyr201Phe mutant of PHBH is the substrate phenol in the active site of the reduced enzyme completely isolated from any H-bond partners as has been shown by the structure of the mutant (*31*). Tyrosine 201 is the residue that directly H-bonds to the substrate phenol. In this mutant enzyme, the rate of hydroxylation is 10^3-fold slower than in WT enzyme (*17*). This factor is probably a true reflection of the effect of deprotonation of the substrate on the hydroxylation reaction. Even in mutant enzymes without a fully functional H-bond network, protons or solvent can access the active site but at a much slower rate than with an intact H-bond network (mentioned above for His72Asn-PHBH) to allow a slightly functional enzyme. It should be noted that phenol hydroxylase, which hydroxylates phenol an order of magnitude more slowly than the analogous reaction in PHBH, shows no significant pH effect on hydroxylation (*13*) and has no H-bond network to the phenolic group of the substrate (*14*) that could connect the substrate to the pH of the solvent.

Deprotonation of the substrate phenol is not the only mechanism to stimulate the hydroxylation reaction. We have established that stabilization of the flavin alkoxide leaving group in hydroxylation stimulates the rate of reaction (*6*, *8*). This is achieved in WT-PHBH by the presence of a positive electrostatic field around the flavin when in the position for hydroxylation. Deprotonation of the substrate phenol is a separate but synergistic effect upon the reaction as shown by the results in Figure 5C compared to Figure 3B and discussed in detail in a separate publication (*32*). The mutation Lys297Met (Figure 5C), by reducing the electrostatic field of the active site (*20*), slows the rate of hydroxylation 25-fold but still responds to the removal of the phenolic proton in the transition state. A contrasting stimulation of hydroxylation has been shown to occur by increasing the positive field (*6*). It is impossible to say what is the total effect of the electrostatic environment of the flavin on hydroxylation in PHBH, but it is likely to be at least a 100-fold stimulus, based upon the earlier work on Lys297Met and Glu49Gln-PHBH. Putting the electrostatic effect together with proton removal in the reaction can lead to a rate enhancement on the order of 10^5-fold. When this factor is multiplied by the typical entropy effects of the enzyme and possible enhancement of hydroperoxide reactivity by protein interactions with O4 of the isoalloxazine (*33*), the rate enhancement becomes enormous. Thus, it is not surprising, in retrospect, that the efficient and fast hydroxylation of an aromatic compound by a flavin hydroperoxide, as carried out by an enzyme like PHBH has not been effectively modeled chemically (*34*).

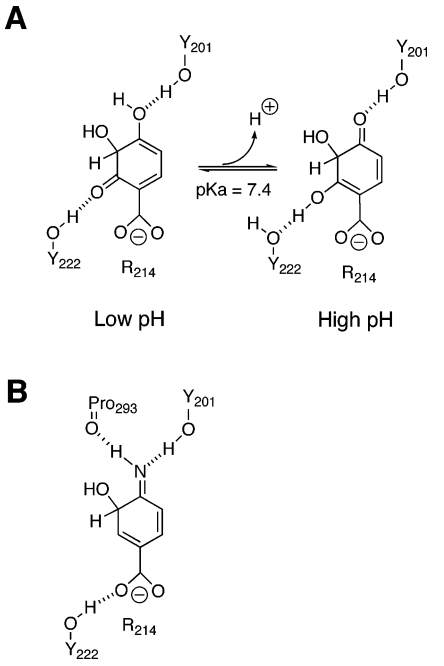

FIGURE 10: Models for dienone or enimine (nonaromatic) products formed in the active site of PHBH upon reactions with 2,4-DOHB and pAB. In panel A, the structures shown for the dienone form of 2,3,4-trihydroxybenzoate account for the experimental observation that the structure of the product must change with pH (*12*) through the mediation of the H-bond network. In panel B, this model accounts for experimental observations with pAB as substrate. A proton is rapidly removed through the H-bond network upon hydroxylation, the spectrum of the enimine does not change with pH, and the rate of rearomatization of product does not change with pH.

After pOHB is hydroxylated in PHBH, the nonaromatic product immediately rearranges to the aromatic product with a rate constant much larger than that of oxygen transfer, presumably by donating the proton at position 3 of the product to the flavin as shown in Figure 1, without any need for exchanges with the environment. The H-bond network might assist in this reaction by donating a proton to the carbonyl oxygen of the nonaromatic product. However, this rearrangement is not so effective when 2,4-DOHB and pAB are substrates. These substrates form relatively stable nonaromatic products within the enzyme (k_4 in Figure 1 is small compared to k_3) and have characteristic absorption spectra in the range between 350 and 450 nm. In the case of 2,4-DOHB, the value for k_4 decreases with increasing pH (pK_a about 7.4), and the absorption spectrum of the product changes with the same pK_a as the pH effect on the rate constant (*12*). These results suggest that the H-bond network is involved in trapping the nonaromatic structure in a stabilized form. Based upon the structure of WT-PHBH with 2,4-DOHB bound (*35*), one likely structural model of the pH effect is shown in Figure 10A. When pAB is hydroxylated, there is a rapid subsequent pH-dependent quenching of fluorescence (Figure 4) that coincides with the formation of the stable intermediate containing the nonaromatic product. These results suggest that the proton generated by hydroxylation at position 4 of the substrate is removed from the nonaromatic product by the H-bond network forming a structure that exhibits a constant spectrum at each pH studied (Figure 10B). It is not clear why the nonaromatic structure formed from pAB on the enzyme is so much more stable

than that formed from pOHB. The enzyme is trapped at this stage in the reaction with the flavin as a C4a-adduct and can only return to oxidized enzyme by release of the nonaromatic product (described in Results) with a rate of 0.06 s^{-1}. By contrast, the nonaromatic product formed from pAB in the Tyr201Phe mutant enzyme rearranges to the aromatic product at a higher rate (0.6 s^{-1}) on the enzyme because, due to the disrupted H-bond network, the proton generated in hydroxylation remains in the active site and stimulates the formation of the aromatic product.

The final process in the oxygen half-reaction is step k_5 in Figure 1. This process consists of the essential chemical step of water loss from the flavin and the dissociation of product. For the enzyme to be an effective catalyst, these processes need to be fast. It was found early in the study of this enzyme that catalysis was inhibited by excess substrate, and this was due to a dead-end complex between substrate and the flavin hydroxide form of the enzyme (*36*). Thus, in the k_5 step of enzyme function, product release (under some conditions) occurs slightly faster than the loss of water from the flavin. Recently, it was found that the reverse could also be true. The mutant enzyme Glu49Gln-PHBH eliminates water from the flavin faster than it releases product (*6*). Upon the basis of the results with Glu49Gln-PHBH (*6*) and the structure of Arg220Gln-PHBH (*5*), it was concluded that there must be at least three interactive conformations of the enzyme: the in form (hydroxylation arrangement), the out form (reduction arrangement), and the open form (product/substrate exchange) (*6*).

The results presented in Figures 3B and 6 show that the k_5 step responds to pH. For WT enzyme and pOHB, the response is similar to that of hydroxylation: the rate increases with increasing pH and exhibits a pK_a of approximately 7.1. The magnitude of the pH effect is about 10-fold in the rate constant. This effect of pH holds for all forms of the enzyme with a functional H-bond network and pOHB as the substrate. A similar result was found previously with 2,4-DOHB as substrate (*12*); there was a large increase in the magnitude of k_5 with increasing pH. It should be noted that k_5 cannot be measured in the reactions catalyzed by Tyr385Phe- and His72Asn-PHBH because k_4 and k_5 are considerably larger than k_3. The results again support a function for the H-bond network. To eliminate water from the C4a-OH-flavin, it must be exposed to solvent. This can occur by the protein adopting the out or open conformations, but to eliminate the product from the active site, the protein must adopt the open conformation (*5*).

The observed dependence of the k_5 step on pH suggests that the dianion of the product must be formed in the active site to trigger rapid conformational change or to shift the equilibrium between conformations away from the flavin in the structure required for the hydroxylation reaction and stabilization of the flavin-C4a adducts. The H-bond network, displaying a pK_a of 7.1, can achieve deprotonation of the 4-OH of the aromatic product to form the dianion. If the protein responded in the same way as with substrate pOHB (*3*), then it should shift the conformational equilibrium to the out form. However, at pH values near 7, it has been shown that the product dissociates slightly faster than water is lost from the flavin (above). This evidence implies a preference for formation of the open conformation (rather than the out conformation) with product bound to the flavin-C4a-hydroxide form of the enzyme. The structure of oxidized WT-PHBH with product bound shows a subtle difference from the complex with pOHB bound (*33*). The plane of the ring of the product is rotated so that the 3-hydroxyl can form a strong H-bond with the backbone carbonyl of Pro293. This interaction probably prevents the product, which should be more reactive than the substrate, from exposing its 5-carbon to attack in subsequent cycles of catalysis and inhibits formation of the out conformation for enzyme reduction in the presence of product. Pro293 is known to be fundamental to the conformational response of the protein to signals from the substrate pOHB (*11*). Rotation of the product in the active site shifts the position of Arg214, disrupting the H-bond between this residue and the strained backbone carbonyl of Arg44 (*33*). Perhaps this change permits rotation of the peptide backbone around Arg44 to an unstrained configuration, a process essential to the formation of the open conformation and release of product (*5*). The preferential formation of the open conformation may be enhanced by deprotonation of the 4-hydroxyl of the product by the H-bond network, rather than the effect of phenolate formation with the substrate, pOHB, to stimulate the out conformation (*3*). This hypothesis can explain the results with the enzyme at all pH values studied. In the mutant enzymes with a disrupted H-bond network, the hydroxylation and rearomatization processes should finish with the product in the dianionic state that cannot be protonated rapidly from solvent. This would explain why these mutants eliminate water from flavin rapidly at all pH values so that hydroxylation is rate-determining in the oxygen half-reactions around pH 7.

The results and discussion presented in this paper demonstrate that PHBH makes extensive use of the H-bond network to optimize the oxygen reactions in catalysis. The illustration of the network in Figure 2 provides a simple model to explain the observations. At pH values below 6, His72 is mostly protonated, and as shown by the model, the phenol of pOHB is protonated and isolated in the active site. Thus, below pH 6, deprotonation of pOHB is an infrequent event and the rate of hydroxylation is slow. In addition, the subsequent release of product is slow. As pH is raised, His72 deprotonates and allows the phenolic proton of pOHB to reorient to the H-bond network. Then, with neutral His72, reversal of the orientation of the network of H-bonds results in either protonation of pOHB with uptake of a proton from solvent or deprotonation of pOHB with liberation of a proton to solvent as shown in Figure 2. The coordinated removal of the phenolic proton from pOHB in association with oxygen transfer enables fast and complete hydroxylation around pH 7−8 without loss of H_2O_2 from the unstable flavin hydroperoxide. Hydroxylation is followed by conformational rearrangement by PHBH stimulated by deprotonation of the 4-OH of the aromatic product that is linked to the H-bond network, while the 3-OH of the product forms a H-bond to Pro293. This rearrangement forms the open conformation, and the rate of this change governs the last phase in catalysis, in which product and water dissociate rapidly from the open active site. The model in Figure 2 is probably one of several variations possible for the operation of the H-bond network in conducting protons to and from pOHB in the active site.

Perhaps the H-bond network is an extra refinement in the catalytic function of PHBH compared to other flavoprotein

hydroxylases. Only extensive research with other enzymes will answer this question.

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