Properties of *p*-Hydroxybenzoate Hydroxylase When Stabilized in Its *Open* Conformation†

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ABSTRACT: *p*-Hydroxybenzoate hydroxylase is extensively studied as a model for single-component flavoprotein monooxygenases. It catalyzes a reaction in two parts: (1) reduction of the FAD in the enzyme by NADPH in response to binding of *p*-hydroxybenzoate to the enzyme and (2) oxidation of reduced FAD with oxygen in an environment free from solvent to form a hydroperoxide, which then reacts with *p*-hydroxybenzoate to form an oxygenated product. These different reactions are coordinated through conformational rearrangements of the protein and the isoalloxazine ring during catalysis. Until recently, it has not been clear how *p*-hydroxybenzoate gains access to the buried active site. In 2002, a structure of a mutant form of the enzyme without substrate was published that showed an *open* conformation with solvent access to the active site [Wang, J., Ortiz-Maldonado, M., Entsch, B., Massey, V., Ballou, D., and Gatti, D. L. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 608−613]. The wild-type enzyme does not form high-resolution crystals without substrate. We hypothesized that the wild-type enzyme without substrate also forms an *open* conformation for binding *p*-hydroxybenzoate, but only transiently. To test this idea, we have studied the properties of two different mutant forms of the enzyme that are stabilized in the *open* conformation. These mutant enzymes bind *p*-hydroxybenzoate very fast, but with very low affinity, as expected from the *open* structure. The mutant enzymes are extremely inactive, but are capable of slowly forming small amounts of product by the normal catalytic pathway. The lack of activity results from the failure of the mutants to readily form the *out* conformation required for flavin reduction by NADPH. The mutants form a large fraction of an abnormal conformation of the reduced enzyme with *p*-hydroxybenzoate bound. This conformation of the enzyme is unreactive with oxygen. We conclude that transient formation of this *open* conformation is the mechanism for sequestering *p*-hydroxybenzoate to initiate catalysis. This overall study emphasizes the role that protein dynamics can play in enzymatic catalysis.

p-Hydroxybenzoate hydroxylase (PHBH)[1] from *Pseudomonas aeruginosa* and *Pseudomonas 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*). In addition, PHBH is a major example of the details of protein and flavin dynamics involved in enzymatic catalysis. This paper focuses upon the dynamics involved in substrate and product association. 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 NAD(P)H, followed by its reaction with oxygen to bring about hydroxylation of the substrate. The isoalloxazine of FAD is mobile in PHBH and can move some 7−8 Å from an *in* position to an *out* position (*3* and references therein). Before NADPH can reduce the flavin, the isoalloxazine of the FAD must move to the *out* position, where 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 *re* face of the exposed N5 of the isoalloxazine (*4*). Without pOHB bound, reduction of the FAD in the enzyme by NADPH is 10^5-fold slower because the protein does not readily adopt the *out* 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 connecting the phenolic proton to His72 at the surface of the enzyme (*3*, *5*). After it is reduced, the isoalloxazine moves back to the *in* conformation, where the reduced flavin reacts with oxygen in an environment in which solvent is excluded, to initiate the second half of the catalysis, oxygenation of the substrate. This gives PHBH two active sites to carry out two diverse

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[1] Abbreviations: PHBH, *p*-hydroxybenzoate hydroxylase; WT-PHBH, *p*-hydroxybenzoate hydroxylase derived from *P. aeruginosa*; Arg220Gln-PHBH and similar abbreviations, mutant forms of *p*-hydroxybenzoate hydroxylase; pOHB, 4-hydroxybenzoate; 2,4DOHB, 2,4-dihydroxybenzoate; 3,4DOHB, 3,4-dihydroxybenzoate.

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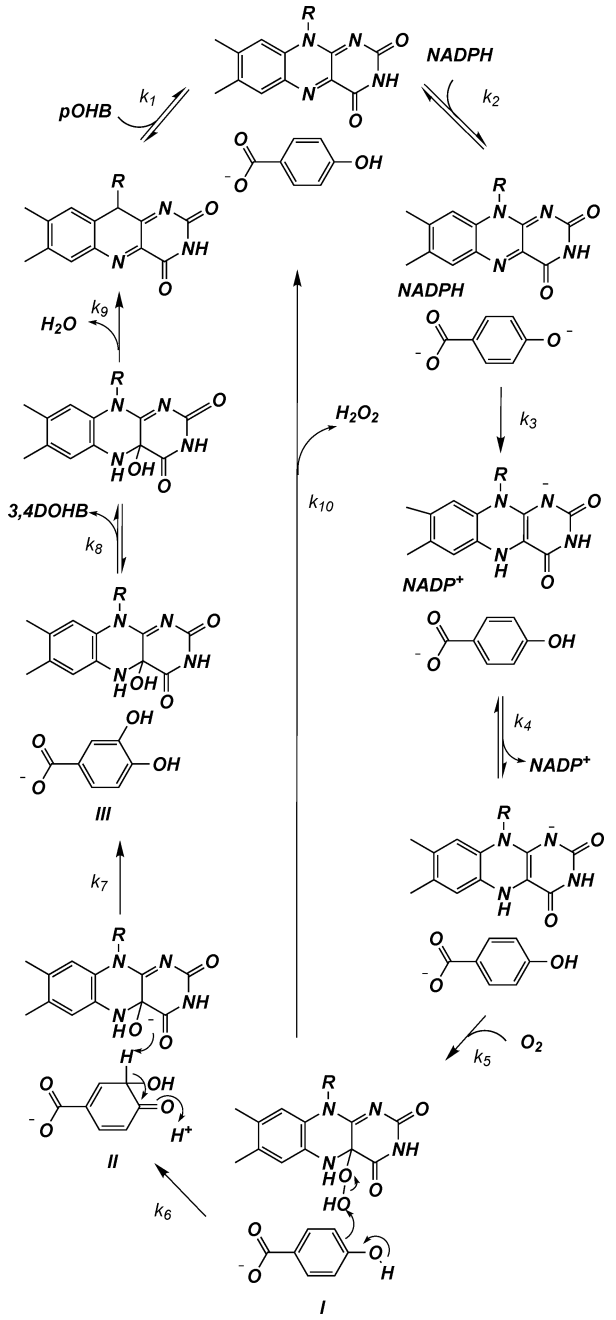


FIGURE 1: Catalytic cycle of PHBH. Three oxygen reaction intermediate states are labeled as intermediates **I**−**III**.

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 different reactions while exhibiting a ping-pong kinetic mechanism.

Our understanding of how substrates and products gain access to the active sites of this protein has been enhanced by a structure of the mutant enzyme, Arg220Gln-PHBH, with NADPH bound (*6*). 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 from the flavin. For the NADPH to transmit its hydride to N5 of the FAD, the isoalloxazine has to move to the *out* position 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 (*6*, *7*).

Arg220Gln-PHBH formed high-quality crystals without ligands, which enabled us to obtain the first high-resolution structure of PHBH without a bound ligand (*6*). 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 (*3* and references therein). Second, there is the movement between *open* and *closed* conformations (*open* and *in*, respectively) that is important for substrate and product exchange, and even for NADPH binding (*6*). PHBH thereby provides an outstanding demonstration of the role of dynamic conformational states in protein function.

The significance of the *open* conformation in PHBH has only been supported until now by the static crystal structure of the variant Arg220Gln-PHBH (*6*) and by clear kinetic evidence for a unique conformation required for binding of pOHB to the enzyme (*8*). Almost no evidence has been provided to show the difference in the properties of the enzyme in the proposed *open* conformation compared to being in the *out* conformation (required for reduction) or the *in* conformation (required for the oxygen reactions). In this paper, we present the properties of two variants of PHBH (Arg220Gln- and Ala45Val-PHBH) that are preferentially stabilized in the *open* conformation. Arg220 is a totally conserved residue in the sequences of many homologues of PHBH that have been identified in the sequence databases. However, Arg220 has no known direct function in the chemistry of catalysis. This residue is in the active site of PHBH and has multiple noncovalent interactions with other conserved residues, as seen in the *in* conformation of the enzyme (Figure 2). These interactions are required for the balance of conformational states in the function of the enzyme. For example, the Arg220Lys variant is preferentially stabilized in the *out* conformation (*9*), and the Arg220Gln form of the enzyme has the structure identified as the *open* conformation (*6*). Ala45 is a residue in the middle of the *si*-side loop of the protein in front of the isoalloxazine ring (Figure 2). This loop of residues is conserved in all the known sequences of the enzyme from different organisms. The methyl group of Ala45 is in van der Waals contact with the isoalloxazine, but has no known chemical function in catalysis. We now recognize that the *si*-side loop is involved in the conformational dynamics of the enzyme. For example, the mutant Ala45Gly-PHBH is preferentially stabilized in the *in* conformation (*10*), and the mutant Glu49Gln-PHBH has a large activation barrier to the binding of pOHB (*8*). Studies of Ala45Val-PHBH showed that it has properties similar to those of Arg220Gln-PHBH. Thus, it is clear that there is a connection between the function of the *si*-side loop and Arg220 (which is far from this loop in the sequence of the enzyme).

We demonstrate in these studies that Arg220Gln- and Ala45Val-PHBH bind substrates poorly, do not work as

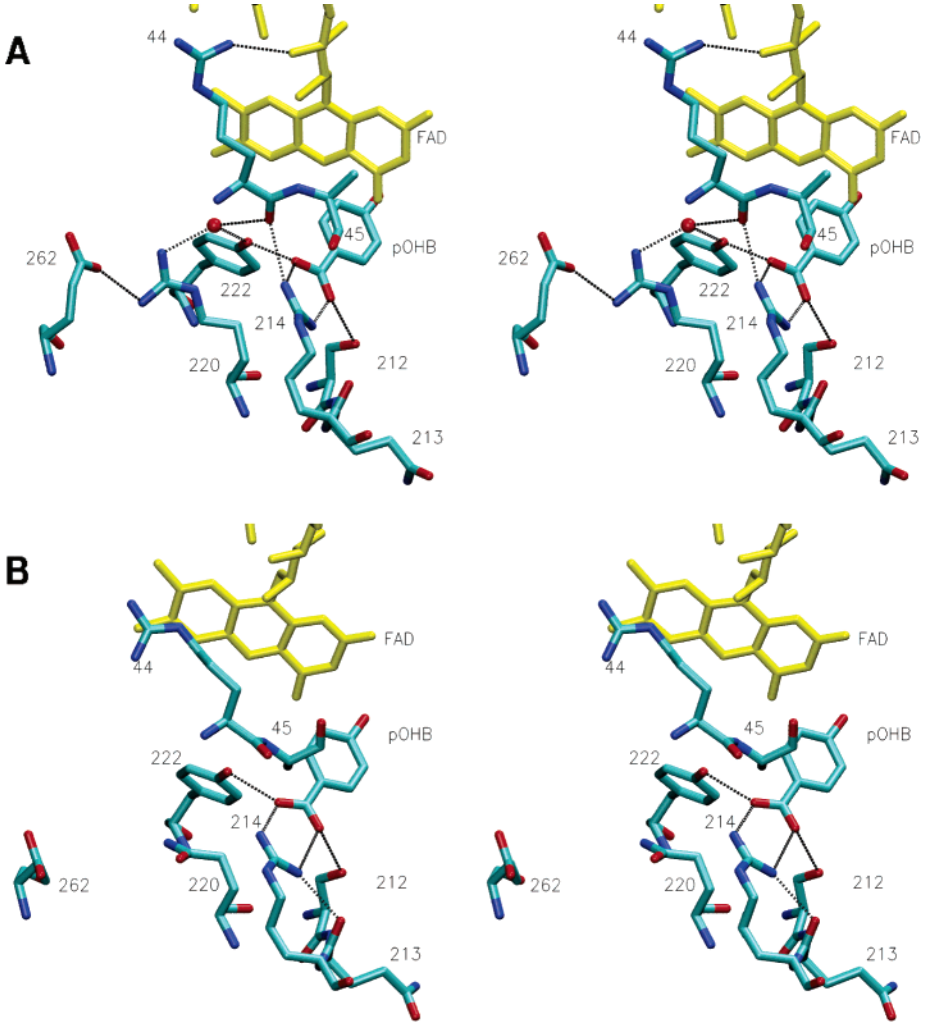


FIGURE 2: (A) Stereoview of WT-PHBH with pOHB bound, taken from PDB entry 1PBE (the *in* conformation). (B) Stereoview of Arg220Gln-PHBH with pOHB bound, taken from PDB entry 1K0I. In both panels, FAD is colored yellow and H-bonds are shown as dashed black lines.

effective catalysts in the reductive half-reaction (see Figure 1), substantially fail to produce product in the oxidative half-reaction, and form a conformation of the reduced enzyme that does not react rapidly with oxygen. These properties are a reflection of the preferential stabilization of the *open* conformation in these forms of the enzyme. Thus, these studies demonstrate the importance of dynamic flexibility and balance of the conformers of PHBH.

MATERIALS AND METHODS

All common reagents used in this work were analytical reagent grade. NADPH was at least 98% pure (purchased from Sigma). Other substrates for PHBH were purchased from commercial sources and were recrystallized before being used.

The construction of plasmids and the methods for expression of PHBH in *Escherichia coli* have been described previously (*11*, *12*). Mutagenesis of the expression plasmid to create 45Val was carried out using the QuikChange site-directed mutagenesis kit developed by Stratagene. The oligonucleotide primer pair sequence used to introduce the Ala45Val change in the mutagenesis reaction was GGC CGC ATC CGC GUC GGC GTG CTG G in the coding strand and its complement; underlined is the changed base pair. The Arg220Gln mutation was introduced into the expression plasmid by the method of Kunkel et al. (*13*). The oligonucleotide used for mutagenesis was GCC ACC CGC AGC CAG TAC TAC GTG CAG. Ala45Val and Arg220Gln forms of PHBH were isolated and purified as described previously (*14*, *15*).

Quantification of the product, 3,4-dihydroxybenzoate (3,4DOHB), from enzyme reactions for hydroxylation coupling measurements was achieved by separation of the reaction components on either a Synergi polar-RP HPLC column (Phenomenex) or a C18 HPLC column, using an isocratic mobile phase of 20% methanol and 1% acetic acid at a flow rate of 1 mL/min for 15 min. Elution of the product and substrate was monitored by absorbance at 254 nm, and they were analyzed by correlating the area of peaks with known standards of pOHB and 3,4DOHB.

All experimental measurements with PHBH were carried out at 4 °C, except where noted; this slowed reactions, facilitated quantitative analysis, and made comparisons to previously conducted experiments with WT-PHBH possible. For pH values of 6.5 and 7.0, 50 mM potassium phosphate buffer with 0.5 mM EDTA was used, and for pH values of 8.0 and 8.5, 50 mM Tris-SO_4 buffer was used. The measurement of ligand dissociation constants, pK_a values of

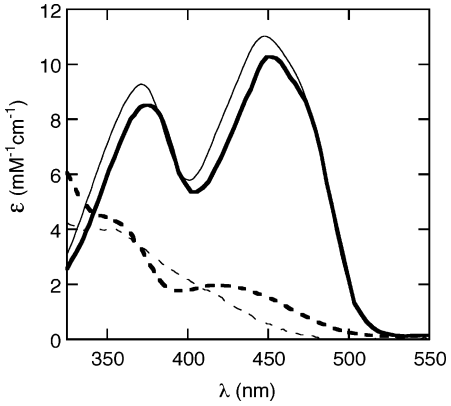


FIGURE 3: Overlay of the oxidized (solid lines) and reduced (dashed lines) spectra of Ala45Val-PHBH (thin lines) and WT-PHBH (thick lines, from ref *22*) in pH 6.5 buffer at 4 °C.

pOHB bound to the enzyme, extinction coefficients, and redox potentials of the enzyme was carried out as described by Entsch et al. (*11*) and Moran et al. (*16*). All kinetic data were collected with a Hi-Tech Scientific model SF-61 stopped-flow spectrophotometer in either absorbance or fluorescence modes. Rapid reaction kinetic traces were analyzed and simulated with Program A (an MS-DOS-based series of programs developed in our laboratory at the University of Michigan), KISS (Kinetic Instruments Stopped-flow System), or Berkeley Madonna. Analysis is based upon the Marquardt algorithm for fitting data to sums of exponentials (*17*).

Energy minimization to model the effect of Val45 on the structure of PHBH was carried out using the Gromacs program package and Gromacs force field (*18*). Topologies for FAD and pOHB were determined using the Dundee Prodrug server (*19*). The protein molecule was solvated in a periodic octahedral box of water that was at least 20 Å from the edge of the enzyme, with the addition of appropriately charged ions to neutralize the charge of the protein molecule. The system was then run through 5000 conjugated-gradient energy minimization steps.

RESULTS

Properties of Mutant Enzymes. Pure Ala45Val-PHBH has a blue-shifted flavin absorbance spectrum (Figure 3), and the degree of the blue shift is dependent on temperature (visible peak at 442 nm at pH 7.0 and 25 °C and 446 nm at pH 7.0 and 4 °C). Arg220Gln-PHBH has a similar blue-shifted visible spectrum (peak at 443 nm; see Table 1 and Figure 7B). The spectrum of reduced flavin in Ala45Val-PHBH lacks revolved bands and is similar to that of $FMNH^-$ in aqueous solution, unlike the spectrum of the WT enzyme (Figure 3).

Spectrophotometric titrations of the mutant enzymes with substrates to form substrate complexes were useful in probing the conformational state of the oxidized enzyme. It has been found that pOHB bound to the WT enzyme favors the *in* conformation of the protein, and the alternate substrate, 2,4-dihydroxybenzoate (2,4DOHB), bound to the WT enzyme favors the *out* conformation (*20*). The K_d values for binding of the substrate pOHB to both Ala45Val- and Arg220Gln-PHBH were remarkably high (Table 1) compared to those for the WT enzyme. The spectral changes caused by binding of pOHB to Ala45Val-PHBH are similar to those observed

Table 1: Some Properties of Arg220Gln- and Ala45Val-PHBH Compared to Those of WT-PHBH

	WT[a]	Arg220Gln	Ala45Val
% coupling with pOHB[b]	100 ± 1	25 ± 2	15 ± 5
K_d(pOHB) (μM)[b]	9.5 ± 0.5	6000 ± 300	1800 ± 200
K_d(2,4DOHB) (μM)[b]	22 ± 1.5	260 ± 20	800 ± 80
k_{cat} (s^{-1})[b]	5.7 ± 0.2	0.22	<0.007
$E^{o\prime}$(free enzyme) (mV)	−163 ± 1.5	−181 ± 1.5	−185 ± 1.5
$E^{o\prime}$(enzyme+pOHB) (mV)	−165 ± 1.5	ND[c]	−157 ± 1.5
visible maximum, oxidized enzyme, pH 7, 25 °C ($mM^{-1}\,cm^{-1}$)	ϵ_{450} = 10.3	ϵ_{443} = 10.1	ϵ_{442} = 10.8

[a] WT data from ref *11*. [b] At pH 6.5 and 4 °C. [c] This mutant enzyme was unstable during this analysis.

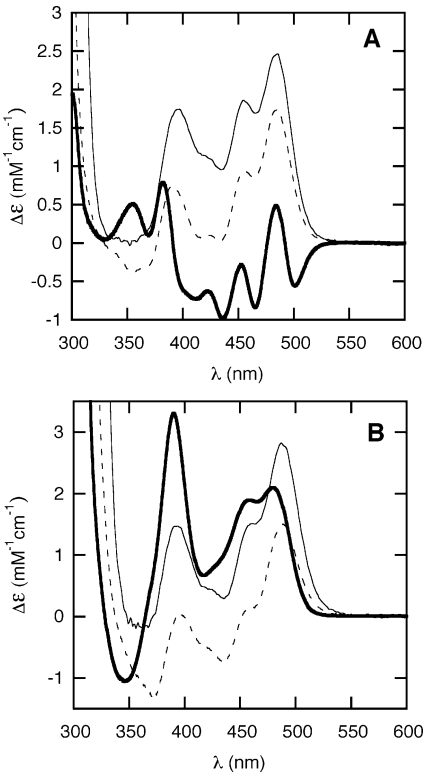


FIGURE 4: (A) Difference in extinction for binding saturating concentrations of pOHB to the following variants of PHBH at pH 6.5 and 4 °C: WT-PHBH (thick solid line), Ala45Val-PHBH (thin solid line), and Arg220Gln-PHBH (dashed line). (B) Difference in extinction upon binding saturating concentrations of 2,4DOHB to the following variants of PHBH at pH 6.5 and 4 °C: WT-PHBH (thick solid line), Ala45Val-PHBH (thin solid line), and Arg220Gln-PHBH (dashed line).

upon binding of 2,4DOHB to the WT enzyme (Figure 4B), but very different from those caused by binding of pOHB to the WT enzyme (Figure 4A). The spectrum of Arg220Gln-PHBH with pOHB bound is not uniquely representative of either the *in* or *out* conformation, and this is in agreement with the crystal structure of this complex (PDB entry 1K0I and Figure 2B), which shows that the *si*-side loop of the protein remains in a conformation similar to that found in the *open* conformation. The K_d values for the two mutant forms with 2,4DOHB are higher than that for the WT enzyme, but the magnitudes of the differences from WT are notably smaller than for pOHB (Table 1). Thus, the mutant forms of PHBH are in conformational states that are able to bind 2,4DOHB with a higher affinity than pOHB. This conclusion might be expected for Ala45Val-PHBH, which

has a spectrum with 2,4DOHB bound that is similar to that of the *out* conformation (Figure 4B). It has been shown that 2,4DOHB stabilizes the *out* conformation by forming an extra H-bond from the protein to the 2-hydroxyl of the substrate (*20*). There may be additional interactions of Arg220Gln-PHBH with the 2-hydroxyl of 2,4DOHB, but they are not the same interactions seen in the structure of the *out* conformation, because the absorption spectrum of this complex is more characteristic of the *in* conformation (Figure 4B).

The redox potentials of both free mutant enzymes compared to that of the WT enzyme are ~20 mV more negative (Table 1). The *open* conformation as seen in the structure of Arg220Gln-PHBH (*5*) allows solvent access to the isoalloxazine. The presence of solvent contacting the FAD may be all that is required to shift the redox potential closer to that of flavin in aqueous solution (free FAD; $E^{\circ\prime} = -208$ mV). In the case of the Arg220Gln variant, a positive charge near the flavin has been removed. This change should make the electrostatic field around the isoalloxazine less positive, thus making the reduction of the flavin less favorable. However, in the *open* conformation, solvent largely surrounds the residue at position 220, thus masking any electrostatic effect. Addition of a saturating concentration of pOHB to Ala45Val-PHBH results in a 32 mV increase in redox potential (Table 1), indicative of a 14-fold increase in affinity for pOHB when the enzyme is reduced. Unfortunately, the method of measuring the redox potential of PHBH, using the xanthine−xanthine oxidase system (*21*) with 1-hydroxyphenazine as the redox indicator dye, results in denaturation of a significant portion of the Arg220Gln enzyme when bound with saturating concentrations of pOHB (100 mM). Instead, the K_d for binding of pOHB to reduced Arg220Gln-PHBH (approximately 7 mM at pH 6.5 and 20 °C, similar to the value of 6 mM for the oxidized enzyme; Table 1) was determined directly from measurements of spectral perturbations of the reduced enzyme upon addition of pOHB, similar to that described in ref *22*. This K_d value may be slightly higher than the value at 4 °C, but it is clear that Arg220Gln-PHBH does not bind pOHB tightly in either the oxidized or reduced state.

An important indicator of the effectiveness of an oxygenase is the percentage of hydroxylated product formed from a known amount of NADPH (reducing donor) in enzymatic turnovers (coupling percentage, Table 1). A perfect enzyme (like WT-PHBH at pH 6.5) achieves 100% coupling (Table 1). Ala45Val-PHBH was so inactive that steady-sate methods were not suitable for measuring coupling efficiency in hydroxylation. Therefore, its hydroxylation efficiency was measured from single oxidative half-reactions, starting with a known amount of dithionite-reduced enzyme in complex with pOHB, and exposing this to air to oxidize the enzyme. Then the enzyme was denatured and the product stabilized by acidification of the reaction mixture to a pH of 1.0 with phosphoric acid. The sample was analyzed by HPLC (see Materials and Methods), and the concentration of the product was correlated with the amount of enzyme used. This gave a hydroxylation efficiency of 15% (Table 1). Arg220Gln-PHBH could be analyzed in normal turnover for hydroxylation efficiency at 25 °C; at 4 °C, the reaction was too slow to measure by this method. The enzyme was allowed to react in steady-state turnover with excess pOHB, NADPH, and O_2. The amount of NADPH consumption was measured using the absorbance change at 340 nm. After a known amount of NADPH had been consumed, the reaction was stopped by acidification with phosphoric acid. The amount of product (3,4DOHB) produced was determined by HPLC analysis and correlated with the amount of NADPH consumed. This gave a hydroxylation efficiency of 25% (Table 1). Thus, both of these mutant enzymes were almost inactive in catalyzing NADPH oxidation and were poor hydroxylases when reduced.

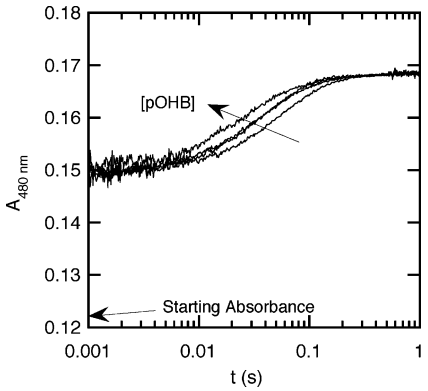
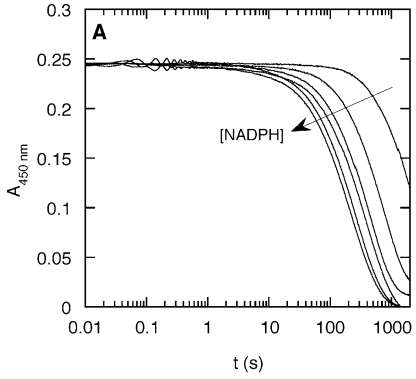


FIGURE 5: Kinetics of binding of pOHB to Ala45Val-PHBH. The binding of pOHB to oxidized Ala45Val-PHBH (18.5 μM) at pH 6.5 and 4 °C was monitored by absorbance changes at 480 nm. Final concentrations of pOHB were 10 mM ($k_{obs} = 16$ s^{-1}), 25 mM ($k_{obs} = 23$ s^{-1}), and 50 mM ($k_{obs} = 33.8$ s^{-1}). The absorbance change observed (0.018) is 40% of the expected absorbance change (0.046) from static titrations (Figure 4A), implying that ~60% occurred within the dead time of the stopped-flow instrument (~3 ms).

pOHB Binding Kinetics. Oxidized Ala45Val-PHBH was mixed with various concentrations of pOHB in a stopped-flow spectrophotometer, and absorbance changes were measured at wavelengths where large changes in absorbance are observed in titration of enzyme with substrate (Figure 4A). A single phase was observed at all wavelengths (Figure 5), and the reaction exhibited a partial dependence upon pOHB concentration. This change only accounted for 40% of the absorbance change observed in static titrations (Figure 4A), with the remainder of the absorbance change occurring in the dead time of the stopped-flow instrument (<3 ms). Similar results were obtained with Arg220Gln-PHBH when binding pOHB. These observations imply that a fast interaction occurs between enzyme and substrate, which is followed by a slower conformational change in the enzyme. WT-PHBH binds pOHB within the dead time of a stopped-flow spectrophotometer (<3 ms) (*22*) without any observable slower secondary changes. A two- or three-step substrate binding process is a common observation in binding of pOHB to many mutant forms of PHBH, including Glu49Gln (*8*), Lys297Met (*16*), and Asp300Asn (*14*).

Reductive Half-Reaction. Reductive half-reactions of Ala45Val-PHBH were carried out in a stopped-flow spectrophotometer by mixing the anaerobic oxidized enzyme in complex with pOHB (20 mM) with anaerobic solutions of NADPH over a range of concentrations. The reaction at pH 6.5 and 4 °C was monitored by the change in absorbance of the flavin at 450 nm. A single very slow phase of flavin reduction was observed (Figure 6). Analysis of the results as shown in ref *7* gave a calculated limiting rate constant for reduction of 0.007 s^{-1} and a high K_d for NADPH of 4.0



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	WT	*Arg220Gln*	*Ala45Val*
k_{red} (s^{-1}), pH 6.5 and 4 °C	[a] 50 ± 1.5	[b] 0.37	0.007 ± 0.0005
$K_{d\ (NADPH)}$ (mM), pH 6.5 and 4 °C	[a] 0.210 ± 0.01	[b] 44	4 ± 0.4
k_{red} (s^{-1}), pH 8.0 and 4 °C	[a] 75	4 ± 1	0.07 ± 0.02
$K_{d\ (NADPH)}$ (mM), pH 8.0 and 4 °C	0.025	20 ± 5	15 ± 3

[a] Value from Palfey *et al*. (*3*).

[b] Value from Wang *et al*. (*6*).

FIGURE 6: Reduction of Ala45Val- and Arg220Gln-PHBH in complex with pOHB. (A) Stopped-flow reaction traces of the reduction of the Ala45Val mutant monitored at 450 nm, pH 6.5, and 4 °C under anaerobic conditions, with 23 μM enzyme, 20 mM pOHB, and NADPH concentrations of 0.250, 1, 2, 3.3, 5.7, and 7.9 mM. The kinetics were analyzed as described by Ortiz-Maldonado et al. (*7*). The traces could be fitted with one exponential function. The measured k_{obs} values were plotted against NADPH concentration and fitted to a hyperbola to provide the K_d of NADPH and k_{red}. (B) Summary of kinetic parameters for the reductive half-reaction for WT-, Arg220Gln-, and Ala45Val-PHBH.

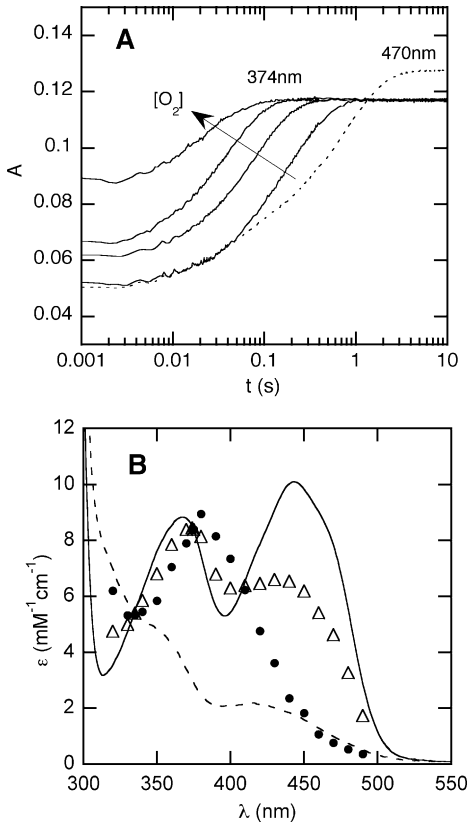


FIGURE 7: Oxidative half-reaction of substrate-free Arg220Gln-PHBH. (A) Reaction traces of absorbance changes from the reaction of the reduced enzyme (30 μM at pH 6.5 and 4 °C) with various concentrations of oxygen (125, 310, 620, and 980 μM). The single trace at 470 nm is for the reduced enzyme reacting with 620 μM O_2. (B) Spectra of the enzyme from the reaction in panel A using 620 μM O_2. The reaction started with the reduced enzyme (- - -), formed the spectrum (△) 150 ms after mixing, and formed the final oxidized species (—) after 10 s. The black dots represent the calculated spectrum of the enzyme forming 100% of the transient intermediate in the reaction (rather than only 75% formed in the actual reaction), typical of the flavin-C4a-hydroperoxide formed by PHBH.

mM. The rate of reduction is approximately 10^4-fold slower than that for WT-PHBH, and the K_d for NADPH is 22-fold higher under the same conditions. This extremely slow rate of reduction accounts for the negligible activity of this mutant enzyme (Table 1). When the experiment was repeated at a higher pH (8.0), the K_d for NADPH increased to 15 mM and the rate constant for reduction increased by a factor of 10 (to 0.07 s^{-1}). An increase in the reduction rate constant with pH is also observed in WT-PHBH in response to deprotonation of the phenolic oxygen of pOHB (*3*), and these results suggest that Ala45Val-PHBH is responding in a similar manner.

The reductive half-reaction of Arg220Gln-PHBH in complex with pOHB was studied in a manner similar to that for Ala45Val-PHBH described above, at both pH 6.5 and 8.0. At pH 6.5, the observed reaction traces fitted a monophasic decrease in absorbance at 450 nm, with a limiting rate constant of 0.37 s^{-1}, with a saturating level of NADPH and a K_d for binding of NADPH to the enzyme of 44 mM, 200-fold higher than that of the WT enzyme under these conditions (see Figure 6 and ref *6*). At pH 8.0, the rate constant for reduction with a saturating level of NADPH has increased 10-fold from the rate constant at pH 6.5 to 4 s^{-1}, but still with a very high K_d for NADPH (20 mM). Thus, Arg220Gln-PHBH also responds to deprotonation of the phenolic oxygen of pOHB.

At very high concentrations of pOHB and NADPH (much higher than that which occurs in cells), a small fraction of Arg220Gln-PHBH forms the conformation that permits NADPH to approach the flavin for hydride transfer. This ineffective reduction step is responsible for the measurable turnover of this form of the enzyme (Table 1).

Oxidative Half-Reaction. The oxygen reactions (from k_5 to k_9 in Figure 1) were studied by stopped-flow techniques that have been used extensively to study PHBH (*22*). This enzyme (like all one-component flavoprotein oxygenases) reacts with oxygen very differently without substrate bound compared to with substrate bound. Thus, the reaction with oxygen should be measured with and without substrate to assess the effects of a mutation. Anaerobic, substrate-free Arg220Gln-PHBH was reduced by dithionite and then reacted in a stopped-flow spectrophotometer at pH 6.5 and 4 °C with buffer saturated with known mixtures of oxygen. Absorbance changes were recorded from 340 to 600 nm. Sample traces at 374 and 470 nm are shown in Figure 7A. The reaction was biphasic at most wavelengths. The first phase in the reaction depended strongly on the concentrations of O_2 as can be seen by the changes at 374 nm. A plot of the observed rate against oxygen concentration was linear

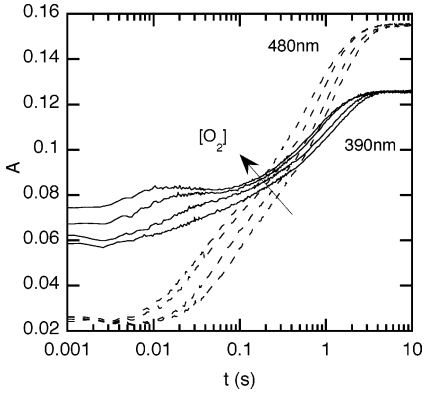


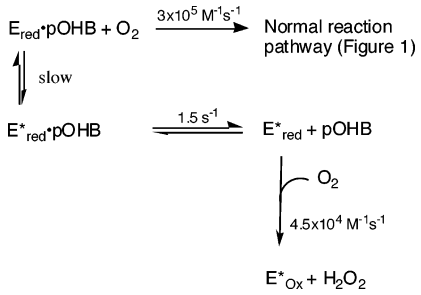
FIGURE 8: Oxidative half-reaction of Arg220Gln-PHBH in complex with pOHB. Kinetic traces at 390 (—) and 480 nm (- - -) of absorbance changes from the reaction of the reduced enzyme (40 μM at pH 6.5 and 4 °C in complex with 80 mM pOHB) with various concentrations of O_2 (62, 125, 310, and 620 μM).

and indicated a second-order reaction with a rate constant of 4.2×10^4 M^{-1} s^{-1}, very similar to the reaction of the WT enzyme with oxygen in the absence of substrate (*22*). With the WT enzyme, at the conclusion of the initial reaction with oxygen, the enzyme is found to be fully oxidized; no flavin-C4a-hydroperoxide is observed unless high concentrations of anions such as azide are present (*22*). However, Arg220Gln-PHBH is only ~55% oxidized at the completion of the first phase in Figure 7B. The other half of the enzyme was present as the flavin-C4a-hydroperoxide (Figure 7B). The flavin-C4a-hydroperoxide then loses H_2O_2 to form oxidized enzyme in a first-order reaction with a rate constant of 1.3 s^{-1} (as can be seen in the reaction trace for 470 nm beyond 0.1 s in Figure 7A). Application of nonlinear least-squares fitting for a two-step consecutive reaction to this experiment showed that 25% of reduced Arg220Gln-PHBH behaved like the WT enzyme and formed H_2O_2 without accumulating any observable intermediate, and 75% reacted to form a transiently stable hydroperoxide. Thus, it is clear that when this mutant enzyme is reduced, it exists in two conformations that do not rapidly interconvert.

The oxidative half-reaction of substrate-free Ala45Val-PHBH was studied in the same manner as that described above for Arg220Gln-PHBH. Absorbance changes in the reduced enzyme upon reaction with oxygen were recorded from 340 to 600 nm (not shown). The observed changes occurred in a single phase that was second-order with respect to oxygen, with a rate constant of 3.7×10^4 M^{-1} s^{-1}. The behavior of Ala45Val-PHBH in this reaction was similar to that of the WT enzyme. Thus, it is clear that there are significant differences in the balance of conformational states formed in the reduced state by each of the mutant enzymes being studied.

Anaerobic, dithionite-reduced Arg220Gln-PHBH had to be mixed with pOHB at a final concentration of 80 mM to convert most of the enzyme to a complex. This concentration of pOHB is ~200-fold higher than that required to saturate WT-PHBH with pOHB. The reaction with oxygen was carried out by reacting a solution of the reduced enzyme and pOHB with buffer and known mixtures of oxygen and nitrogen in a stopped-flow spectrophotometer using the standard conditions of pH 6.5 and 4 °C. Absorbance changes were observed from 330 to 600 nm (examples shown in Figure 8). The reaction was unlike the oxidative half-

Scheme 1: Model for the Oxidative Half-Reaction of Arg220Gln-PHBH



reactions observed for a wide range of mutant forms of PHBH. The early stage of the reaction (up to ~20 ms at higher oxygen concentrations) was dominated by a fast, oxygen-dependent increase in absorbance in the range of 350−420 nm (see 390 nm changes in Figure 8) with a second-order rate constant of 3×10^5 M^{-1} s^{-1}, similar to that seen with the WT enzyme. This first phase is consistent with the formation of a flavin-C4a-hydroperoxide, but only by a fraction of the enzyme in the reaction. This initial reaction was followed by two more phases that can be detected by absorbance, as shown in the traces at 480 nm in Figure 8. The second phase of the reaction (between 10 and 100 ms at 480 nm) also appears to be oxygen-dependent, but its observed rate saturates at high oxygen concentrations at approximately 30 s^{-1}. The absorbance changes in this phase occur mostly in the range of 420−500 nm, indicating that it involves the formation of the oxidized enzyme. These first two phases in the reaction are consistent with approximately 30% of the enzyme reacting by a pathway like that of the WT enzyme, forming a flavin-C4a-hydroperoxide that converts to the oxidized enzyme as product is formed. This conclusion is substantiated by the formation of 25% of the product in the turnover of this mutant enzyme (Table 1). Finally, the third phase in the reaction (see the absorbance change at 480 nm between 0.1 and 10 s in Figure 8) converts the remaining 70% of the enzyme from a reduced state to the oxidized state. This phase shows only a weak dependence on oxygen concentration. The observed kinetic traces described above could be simulated effectively by the reaction shown in Scheme 1. The reduced Arg220Gln enzyme in complex with pOHB (just as without substrate above) is in two distinct conformational states that interconvert very slowly. One form accounts for 30% of the total enzyme and reacts with oxygen with a second-order rate constant of 3×10^5 M^{-1} s^{-1} to form a flavin-C4a-hydroperoxide intermediate (Figure 1, intermediate I). The flavin-C4a-hydroperoxide hydroxylates bound pOHB to form the product 3,4DOHB with subsequent elimination of water, observed as an increase in the absorbance of the flavin at 470 nm with a first-order rate constant of 30 s^{-1}. The second form of the reduced enzyme that makes up the remaining 70% of the enzyme (E^*_{red}•pOHB in Scheme 1) does not react with oxygen directly, but instead converts to a third form of the enzyme (at a rate of 1.5 s^{-1}) that is capable of reacting with O_2 with a second-order rate constant of 4.5×10^4 M^{-1} s^{-1} to form the oxidized enzyme without forming the product. The simulations are consistent with the reaction with oxygen having the same rate as the reaction of the enzyme without pOHB described above. Since no product is formed in association with this fraction of the enzyme reacting with

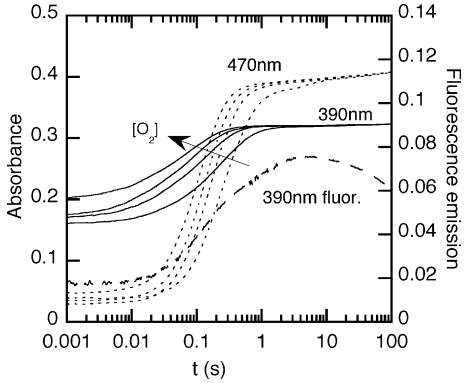


FIGURE 9: Oxidative half-reaction of Ala45Val-PHBH in complex with pOHB. Reaction traces showing changes in absorbance and in fluorescence with excitation at 390 nm, and emission at ≥510 nm, from the reaction of reduced Ala45Val-PHBH (40 μM in buffer containing 20 mM pOHB at pH 6.5 and 4 °C) with varying concentrations of oxygen (125, 310, 620, and 980 μM). The 390 nm fluorescence trace is for the reaction of the reduced enzyme with 980 μM O_2. Concentrations given are after mixing in the reaction.

oxygen, the third form of the enzyme (above) required to describe the reaction is consistent with the slow step (1.5 s^{-1}) being dissociation of pOHB from the enzyme.

The oxidative half-reaction of Ala45Val-PHBH was studied in a manner similar to that for Arg220Gln-PHBH described above. On the basis of the redox potentials shown in Table 1, less pOHB is required to completely complex the reduced Ala45Val-PHBH than for the Arg220Gln enzyme. Absorbance changes were followed from 340 to 600 nm, and fluorescence changes were followed with excitation at 390 or 450 nm and with emission beyond 510 nm (Figure 9). The reaction had characteristics similar to those of the reaction of Arg220Gln-PHBH, and thus different from those of any other previous oxygen reactions observed with PHBH.

The observed kinetic traces could be explained effectively by the following reaction sequence. The reduced Ala45Val enzyme in the complex with pOHB is in two distinct forms (like reduced Arg220Gln-PHBH above). One form accounts for approximately 20% of the total enzyme and is discussed first. This minor form reacts with oxygen with a second-order rate constant of 1.5×10^4 M^{-1} s^{-1} to form a flavin-C4a-hydroperoxide intermediate (Figure 1, intermediate I) as illustrated by the increase in absorbance at 390 nm out to ~30 ms in Figure 9, and the lag in absorbance at 470 nm. For this mutant enzyme, this reaction with oxygen is ~10-fold slower than that observed with the WT enzyme and Arg220Gln-PHBH under the same conditions.

The flavin-C4a-hydroperoxide hydroxylates bound pOHB to form product (3,4DOHB) with a first-order rate constant of 1.2 s^{-1}. This reaction is uniquely observed as an increase in fluorescence at 390 nm (Figure 9) between 0.1 and 3 s that is not observed in fluorescence excited at 450 nm. Compared to those of WT- and Arg220Gln-PHBH, this rate of hydroxylation is slow, indicating that this productive form of the enzyme cannot be structurally the same as the former two. Table 1 shows that 15% of Ala45Val-PHBH forms product, consistent with the hypothesis that the conformer forming product is the approximately 20% of enzyme reacting with O_2 in the faster phase (Figure 9). Some fraction of this minor conformer is trapped in a complex that converts to oxidized enzyme very slowly [as illustrated by the slow decrease in fluorescence at 390 nm and the slow increase in absorbance at 470 nm occurring between 3 and 100 s (Figure 9)]. The second form of reduced enzyme that makes up the remaining 80% of the enzyme reacts with oxygen to yield the large increase in absorbance at 470 nm between 30 and 300 ms. The rate of this observed increase in absorbance at 470 nm saturates with respect to oxygen concentration and is biphasic, because the increase is preceded by a lag. Thus, like Arg220Gln-PHBH in Scheme 1, this unproductive form of the enzyme does not react with oxygen directly, but instead likely converts to a third form of enzyme at a rate of ~13 s^{-1}, based upon simulations of this reaction sequence. The third form is capable of reacting with O_2 with a second-order rate constant of approximately 4×10^4 M^{-1} s^{-1} to form the oxidized enzyme. This simulated rate of reaction (like the reaction of Arg220Gln-PHBH above) is consistent with the free enzyme reacting with oxygen after dissociation of pOHB from the species that does not react with oxygen.

DISCUSSION

It is striking that the two specific mutant forms of PHBH described here have rather similar properties that set them apart from other forms of PHBH that have been studied in the past. It was found earlier that Arg220 had no function in the catalytic chemistry, but its guanidinium group is important in the dynamics of conformational interactions (*9*). The structure of Arg220Gln-PHBH (Figure 2) demonstrated the potential importance of the protein *si*-side loop in the dynamics of catalysis. The importance of this loop was confirmed by specific mutations of Ala45 within the loop. Ala45Gly-PHBH is thermodynamically stabilized in the *in* conformation (*10*), and it has properties very different from those of Ala45Val-PHBH, a variant in which the larger valine residue most likely changes the packing interactions between the isoalloxazine and the protein. Although we were unable to obtain suitable crystals of Ala45Val-PHBH for X-ray crystallography, the properties of this form of the enzyme strongly suggest that the enzyme is stabilized in the *open* conformation seen in crystals of Arg220Gln-PHBH (Figure 2). By modeling Val45 in the *in* structure of the WT enzyme (as described in Materials and Methods), we found that the extra methyl groups added by valine are not accommodated without changes to the structure. Alternative solutions to this packing problem can be to favor the *open* or *out* conformation, where the isoalloxazine ring and Val45 can exist without modifications to the protein conformation. The experimental results with Ala45Val-PHBH are consistent with this conclusion. For example, when pOHB is bound to this enzyme (Figure 4A), the absorption spectrum of the enzyme is very similar to that of the WT enzyme with 2,4DOHB bound (Figure 4B), which is characteristic of the *out* conformation (*20*). Without a ligand bound in the active site of Ala45Val-PHBH, the absorption spectrum is notably blue-shifted (Figure 3), which is probably a signature of the *open* conformation as shown by the similar blue-shifted spectrum of Arg220Gln-PHBH (Figure 7B).

We have proposed that oxidized WT-PHBH in solution without substrate ligands is finely balanced between conformational states that are in rapid equilibrium (*1*). By contrast, the mutant enzymes studied here are unusually stabilized in the *open* conformation, and thus provide tools for investigating the properties of this state of the enzyme.

Although there are no changes in the functional groups responsible for the chemistry catalyzed by PHBH, Arg220Gln- and Ala45Val-PHBH variants are almost totally inactive (see Table 1). Arg220Gln-PHBH has theoretically ~3% of WT activity, but only at substrate concentrations more than 100-fold higher than with WT. The mutant enzymes are ineffective at properly binding pOHB and NADPH (Table 1) in the reductive half-reaction. The changes in affinity for pOHB are readily understood from the structure of Arg220Gln-PHBH (*6*). It has been established that Arg214 is responsible for most of the binding energy for pOHB in the active site (*23*). In the crystal structure of the substrate-free Arg220Gln-PHBH, Arg214 is rotated out of the active site. This change is a logical requirement for the entry of pOHB into the active site. The fact that Ala45Val-PHBH is poor at binding pOHB, as is Arg220Gln-PHBH (Table 1), suggests that rotation of the *si*-side loop (with Ala45 in the middle) is linked to movement of Arg214. If the *open* conformation is required for pOHB to access the active site, then the mutant forms under study should bind pOHB rapidly. In both cases, the initial binding of pOHB occurred during the dead time of a stopped-flow instrument at 4 °C as described in Results, as for the WT enzyme (*22*). However, unlike the WT enzyme after the initial interaction, a relatively slow conformational change in the enzyme occurs, as illustrated by Figure 5. Perhaps this slow change represents the rearrangement of some enzyme into the *in* or *out* conformation against a significant activation energy barrier.

Another striking feature of the *open* conformation is the weak binding of NADPH to the mutant enzymes compared to that to the WT enzyme (Table 1). This weak interaction provided the first direct structural information about the binding of NADPH to PHBH (*6*), probably because the *open* conformation does not allow NADPH to approach N5 of FAD for reduction. In the crystal structure of Arg220Gln-PHBH, only the adenine portion of NADPH is constrained in the enzyme, with the nicotinamide portion showing only weak electron density (*6*). It has been established that the nicotinamide ring can approach the isoalloxazine only transiently from the *out* conformation. This occurs when the isoalloxazine is initially *in* and the substrate is deprotonated to form the dianion. This causes a conformational rearrangement to move the isoalloxazine *out* where it reacts with the nicotinamide (*1*). This response is the primary control point in catalysis by PHBH and related enzymes because reduction does not occur rapidly in the absences of substrate. Arg220Gln-PHBH probably fails to function in the reductive reaction because its most stable conformation with pOHB bound is not the completely closed active site of the *in* conformation where formation of the pOHB dianion triggers formation of the *out* conformation. In the structure of PDB entry 1K0I (Figure 2), when Arg220Gln-PHBH binds pOHB, the strained configuration of the *si*-side loop (formed by the WT enzyme) is not formed, even though all the known interactions found in WT are formed with pOHB. Thus, only with very high concentrations of NADPH can some small fraction of the mutant enzyme develop the conformation competent for reduction. In the case of Ala45Val-PHBH, the correct conformation for reduction is even less likely than with Arg220Gln-PHBH, based upon the rates of reduction (Table 1). Thus, Ala45Val-PHBH may also not form the strained *si*-side loop upon binding pOHB. Ala45Val-PHBH forms the *out* conformation of the flavin (as shown in Figure 4A), but rotating the isoalloxazine ring is not by itself sufficient to cause the nicotinamide of NADPH to come into position close to N5 of the isoalloxazine to achieve hydride transfer (*7*).

The very slow reductive half-reactions for the mutants under study (described above) are attributed to the dominance of abnormal conformations of the enzyme with pOHB bound (Figure 2B). The oxidative half-reactions of these mutant enzymes in the absence of pOHB provide direct evidence for these abnormal conformations. In the reaction of reduced Arg220Gln-PHBH with oxygen, two separate reactions occur as illustrated in Figure 7. Approximately 25% of the enzyme reacts with oxygen to form oxidized flavin at nearly the same rate as the WT substrate-free enzyme. The remaining 75% forms a transiently stable flavin-C4a-hydroperoxide (Figure 7B); in contrast, the WT enzyme does not form any detectable hydroperoxide without substrate bound, except in the presence of high concentrations of the anion, azide (*22*). Two forms of the reduced enzyme were not detected in the reaction of reduced Ala45Val-PHBH with oxygen. This enzyme appeared to react more like the WT enzyme (results not shown). For 75% of Arg220Gln-PHBH to form a hydroperoxide, the conformation of the reduced enzyme must be stabilized in a closed conformation where the C4a−N5 portion of the flavin is isolated from solvent. It has been shown in the crystal structure of PHBH with anions bound that the *in* conformation is favored (*20*). Thus, it is likely that an abnormal *in* conformation occurs with reduced Arg220Gln-PHBH. In the case of Ala45Val-PHBH, the reduced flavin spectrum (Figure 3) lacks the resolution of the reduced WT spectrum, and is similar to the spectrum of reduced flavin in water. This observation suggests a more exposed reduced flavin that could not form transient C4a oxygen adducts, as was found in the experimental analysis.

When pOHB is bound to the mutant enzymes, the reactions with oxygen become very interesting. In the case of Arg220Gln-PHBH, two different reactions are observed. First, approximately 30% of the enzyme reacts fast with oxygen in a second-order process, as described in Results. This ~30% of the enzyme behaves like the WT enzyme, and indeed, 25% of the enzyme yields product in turnover by the mutant enzyme (see Table 1). The other 70% of the mutant enzyme reacts slowly with oxygen (see traces at 390 and 480 nm between 0.2 and 3 s in Figure 8), and the reaction is not second-order, but is only slightly dependent upon oxygen concentration. The model shown in Scheme 1 accounted for the results when appropriate rate constants were used. This suggests that this abnormal form of the enzyme can only react with oxygen by first converting slowly to an oxygen-reactive conformation, which is not the functional enzyme, because no product is formed. The conformation of enzyme forming product appears to release product considerably faster than does the WT enzyme, based upon its rate of formation of the oxidized enzyme (25−30 s^{-1}), which is governed by the rate of hydroxylation. This observation is consistent with faster formation of a more stable *open* conformation than with the WT enzyme for release of 3,4DOHB and water (see k_8 and k_9 in Figure 1). The remarkable reactions of Arg220Gln-PHBH when in a complex with pOHB were duplicated with subtle variations by Ala45Val-PHBH. The latter mutant enzyme also behaves

as two forms of the enzyme upon reaction with oxygen, but this is not immediately seen by inspection of the data in Figure 9. The analysis presented in Results shows that ~20% of the enzyme functions as a hydroxylase, with 80% reacting by a pathway similar to that of Arg220Gln-PHBH. The large, unproductive fraction of Ala45Val-PHBH rearranges into a conformation that reacts with oxygen 10-fold faster than does Arg220Gln-PHBH. The implication from our analysis of the reactions of Arg220Gln- and Ala45Val-PHBH is that there is at least one reduced conformation of these forms of the enzyme with pOHB bound that reacts slowly (or not at all) with oxygen. Only once before has similar behavior of PHBH been observed, with Arg214Lys-PHBH (G. R. Moran, Doctoral Dissertation, University of New England, NSW, Australia). This conclusion is unprecedented for PHBH and other flavoprotein oxygenases. The oxygen reactions under consideration can be explained by Scheme 1, in which an oxygen-insensitive form of PHBH converts to a form that reacts with oxygen at the same rate as the enzyme without pOHB. This is a reasonable proposal if pOHB can dissociate from the reduced enzyme at a rate higher than that observed with the WT enzyme (*22*). Again, this would be possible for forms of PHBH that prefer forming the *open* conformation.

Previous studies have documented the importance of an *out* conformation of the FAD to enable reduction, as well as an *in* conformation that is important for the reaction of flavin with oxygen and the hydroxylation reaction. Here we have studied the properties of two different mutant forms of PHBH that are stabilized in the *open* conformation. The mutant enzymes bind pOHB very fast, but with very low affinity. The mutant enzymes are extremely inactive, but are capable of slowly forming small amounts of product by the normal catalytic pathway, although inefficiently. The lack of activity results from the stability of the *open* form preventing the mutant forms from readily adopting either the *in* or *out* conformation. Thus, NADPH cannot reduce the flavin effectively. Moreover, the mutant forms hydroxylate inefficiently because the *in* conformation necessary for hydroxylation is strongly disfavored. We conclude that transient formation of this *open* conformation is the mechanism for access to the active site for the substrate and egress of the product from the active site with simultaneous elimination of water from the C4a-flavin hydroxide after hydroxylation, and this form has mutually exclusive catalytic roles with respect to the other two conformations of PHBH. The conformational changes involved in substrate binding and hydride transfer are similar to the conformational model that has been proposed for dihydrofolate reductase (*24*) from using the results of crystallographic (*25*), NMR (*26*), transient kinetic, and single-molecule fluorescence studies (*27*). This model has dihydrofolate and NADPH binding to different conformations of the enzyme, and when both are bound, the enzyme forms a third conformation where hydride transfer can occur. In PHBH, this model is extended by a fourth state of the enzyme where $NADP^+$ diffuses away and the reduced flavin moves back to the *in* conformation, which promotes the reaction with O_2 to form the C4a-flavin hydroperoxide and subsequently, the hydroxylation of the substrate. Similar dynamics are likely to be involved with other multisubstrate enzymes; however, the natural flavin reporter group in PHBH provides a unique handle for monitoring such events.

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