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Substrate Recognition by "Password" in *p*-Hydroxybenzoate Hydroxylase†

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ABSTRACT: The flavin of *p*-hydroxybenzoate hydroxylase (PHBH) adopts two conformations [Gatti, D. L., Palfey, B. A., Lah, M.-S., Entsch, B., Massey, V., Ballou, D. P., and Ludwig, M. L. (1994) *Science 266*, 110−114; Schreuder, H. A., Mattevi, A., Obmolova, G., Kalk, K. H., Hol, W. G. J., van der Bolt, F. J. T., and van Berkel, W. J. H. (1994) *Biochemistry 33*, 10161−10170]. Kinetic studies detected the movement of the flavin from the buried conformation to the exposed conformation caused by the binding of NADPH prior to its reaction with the flavin. The pH dependence of the rate constant for flavin reduction in wild-type PHBH and the His72Asn mutant indicates that the deprotonation of bound *p*-hydroxybenzoate is also required for flavin movement, and is accomplished by the same internal proton transport network previously found to be involved in substrate oxidation. The linkage of substrate deprotonation to flavin movement constitutes a novel mode of molecular recognition in which the enzyme tests the suitability of aromatic substrates before committing to the catalytic cycle.

Enzymes recognize their physiological substrates with a high degree of specificity, essential for maintaining the finely balanced network of metabolic pathways required for cell growth. Models explaining catalytic specificity, such as the Lock and Key model of Fischer (*1*) and the Induced Fit model of Koshland (*2*), have focused on the ability of an enzyme to discriminate between potential substrates by providing selective complementary binding and catalytic surfaces. If an alternate substrate is permitted to bind, it will be processed in accord with its chemical reactivity. Some enzymes, notably the aminoacyl-tRNA synthetases (*3*) and DNA polymerases (*4*), achieve further levels of control on fidelity through proof-reading mechanisms that detect errors and abort the normal catalytic cycle. Our studies of the flavin-containing enzyme *p*-hydroxybenzoate hydroxylase (PHBH)[1] reveal a novel form of substrate recognition in which the enzyme tests the suitability of a bound aromatic ligand before allowing further progress through the catalytic cycle. We show in this work that, for an effective reduction of the flavin by NADPH to occur, the enzyme must bind an ionizable phenolic substrate. In the presence of NADPH, ionization of the phenol constitutes a "password" that allows movement of the flavin ring system toward the surface of the enzyme, where reduction takes place.

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[1] Abbreviations: PHBH, *p*-hydroxybenzoate hydroxylase; pOHB, *p*-hydroxybenzoate; EFlHOOH, enzyme-bound flavin C4a-hydroperoxide; pAB, *p*-aminobenzoate; NADPD, (4*R*)-[4-^{2}H]NADPH.

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PHBH catalyzes the hydroxylation of *p*-hydroxybenzoate (pOHB) using O_2 and NADPH, giving 3,4-dihydroxybenzoate, water, and NADP. The reaction is one of the numerous bacterial oxidations that funnel the carbon of lignin into main-stream metabolism (*5*). A wealth of mechanistic and structural information about PHBH has accumulated (*6*), and this has served as a model for studies of a large number of flavoproteins having similar mechanisms (*7*). The tightly bound FAD prosthetic group remains bound during catalysis and is the key chemical participant in most of the catalytic steps. Catalysis may be divided into two half-reactions. In the reductive half-reaction, the free enzyme, with FAD in the oxidized form, rapidly and randomly binds pOHB and NADPH (*8*). The flavin gets reduced in this complex, and then NADP dissociates. In the oxidative half-reaction, O_2 reacts with the reduced enzyme−pOHB complex to form a flavin C4a−hydroperoxide intermediate (EFlHOOH), which is a potent electrophile. The distal oxygen atom of the hydroperoxide is transferred to the aromatic substrate by an electrophilic aromatic substitution to initially form a non-aromatic hydroxylation product that tautomerizes to 3,4-dihydroxybenzoate, and the resulting flavin C4a−hydroxide eliminates water to regenerate the oxidized enzyme.

PHBH uses three mechanisms to avoid NADPH oxidase activity that might result from rapid solvent-catalyzed elimination of H_2O_2 from the EFlHOOH intermediate. (A) The reactants are sequestered from solvent when pOHB is present. By comparison, when substrate is absent, solvent has access to the active site, and any reduced PHBH that forms reacts with oxygen and quickly eliminates H_2O_2. (B) Reduction of the flavin by NADPH is as much as 5 orders of magnitude slower in the absence of aromatic substrate than in its presence, so that essentially no reduced flavin is formed without an aromatic substrate bound to the enzyme (*8*). However, the binding of pOHB affects neither the affinity for NADPH nor the redox potential of the flavin, suggesting that the effect must be conformational. (C) In contrast to oxidized PHBH, dissociation of pOHB from the reduced enzyme is slow compared to the subsequent reaction with O_2, ensuring that, once the flavin is reduced, PHBH is committed to attempt hydroxylation of whatever aromatic molecule is bound (*9*). The experiments reported here demonstrate that some of these control mechanisms have their basis in known PHBH conformational changes.

EXPERIMENTAL PROCEDURES

Mutants of PHBH were constructed from the gene *pobA* from *Pseudomonas aeruginosa* PAO1 as described (*10*). Mutant and wild-type PHBH were expressed and purified according to published procedures (*11*, *12*). NADPD was synthesized by an adaptation of a method (*13*) in which NADP was stereospecifically reduced by deutero-acetaldehyde in a reaction catalyzed by aldehyde dehydrogenase in 20 mM TAPS, pH 9.0, 0.1 mM DTT, 1 mM K_2SO_4. The reaction was stirred for 12 h and enzyme was added periodically. The pH was kept between 8.5 and 9.0 by the addition of NaOH. Enzyme was removed by ultrafiltration with a Centricon-30, and the NADPD was purified with a Mono-Q anion exchange column (*14*) using an NH_4HCO_3 gradient. Fractions containing NADPD were pooled and lyophilized.

In the absence of O_2, the reduction of the flavin of PHBH may be conveniently studied kinetically without the complications inherent in interpreting steady-state kinetics. Solutions of PHBH (∼20 μM) with saturating concentrations of aromatic ligand (∼1 mM) were reacted with solutions of NADPH anaerobically in a Hi-Tech SF-61 stopped-flow spectrophotometer as described previously (*12*). For pH values in the range 6−7.8, the buffer was 50 mM potassium phosphate; in the pH range 8−9, 100 mM Tris-sulfate was used, and 50 mM glycine was used at pH values higher than 9. In single-wavelength absorbance mode, reactions were observed by recording the flavin absorbance at 450 nm, and were analyzed by fitting them to sums of exponentials. Some reactions were also observed by fluorescence; excitation was at 450 nm and the emitted light was passed through a 515 nm cutoff filter. The spectrum of the PHBH−pOHB−NADPD complex was obtained by mixing the anaerobic PHBH−pOHB complex with an anaerobic solution of NADPD in a Hi-Tech SF-61 stopped-flow instrument configured for diode-array data collection. Spectra were collected every 1.25 ms for 120 ms. The reaction mixture contained 20 μM PHBH, 0.5 mM pOHB, and 1.5 mM NADPD after mixing. The spectrum of the unreacted PHBH−pOHB complex was obtained by mixing the enzyme with buffer free of NADPD.

RESULTS AND DISCUSSION

Not all aromatic ligands are effective stimulators of flavin reduction. *p*-Aminobenzoate (pAB), a vital precursor to folic acid, is an excellent structural analogue of pOHB and binds to PHBH with an affinity similar to that of pOHB; yet pAB binding causes little stimulation of the flavin reduction rate (*15*). We found that PHBH discriminates between pOHB and pAB by linking flavin reduction to the formation of a negative charge on the 4-substituent of the aromatic ligand, as hypothesized previously (*15*). Thus, when pAB was bound to wild-type PHBH, we measured saturating reduction rate constants of 0.081 s^{-1} at pH 6.5 and 0.084 s^{-1} at pH 9.5 at 4 °C. The reduction rate constant is independent of pH in the PHBH−pAB complex because the enzyme cannot deprotonate the amine ($ArNH_2$) to the amide anion ($ArNH^-$) at physiological pH. In contrast, the rate constant for reduction of the PHBH−pOHB complex is larger by 3 orders of magnitude, from 38 s^{-1} at pH 6.1 to a maximum value of 75 s^{-1} at pH > 8, exhibiting a pK_a of 6.2 ± 0.1 (Figure 1)[2]. It is known that the pK_a of the phenolic oxygen of pOHB is lowered from 9.3 (free) to 7.4 (bound) (*11*) by several structural features, including a network of hydrogen bonds extending ∼12 Å from the buried substrate to the protein surface, terminating with His 72 (Figure 2). The pK_a of 6.2 for the reduction rate constant differs significantly from that for bound pOHB, but is consistent with ionization of a

[2] Owing to the insolubility of PHBH and the instability of NADPH at low pH, pH 6.1 was the lowest value used. Because the low-pH end of the k_{red} vs pH curve is poorly defined, the curve was fit initially in two ways: by fixing the low-pH limit of k_{red} to 0, giving a pK_a of 6.2 ± 0.1, or by allowing it to vary. When allowed to vary, values of 22 s^{-1} at the low-pH limit of k_{red} and 6.5 ± 0.2 for the pK_a were obtained. If pAB is taken as an appropriate model of pOHB on the enzyme at low pH, then a lower-pH limit of 0.081 s^{-1} should be used. The pK_a value determined using either assumption remains nearly constant and is indicative of histidine.

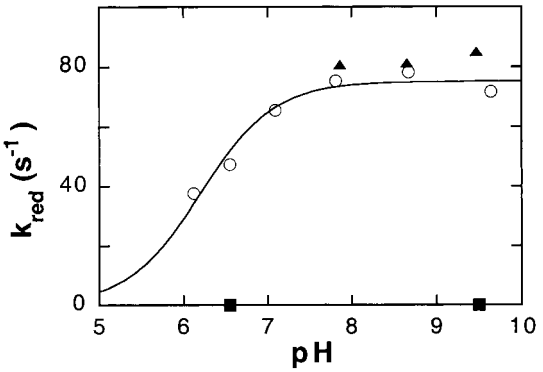

FIGURE 1: The pH dependence of the rate constant for reduction at 4 °C. The rate constants for hydride transfer from NADPH to the FAD of PHBH were obtained from fits to the hyperbolic dependence of the observed rate constant on NADPH concentration. The circles are the rate constants for the wild-type PHBH−pOHB complex, the squares are the rate constants obtained for the wild-type PHBH−pAB complex, and the triangles are the rate constants obtained for the form of the His72Asn−pOHB complex that reduced rapidly (see Figure 3).

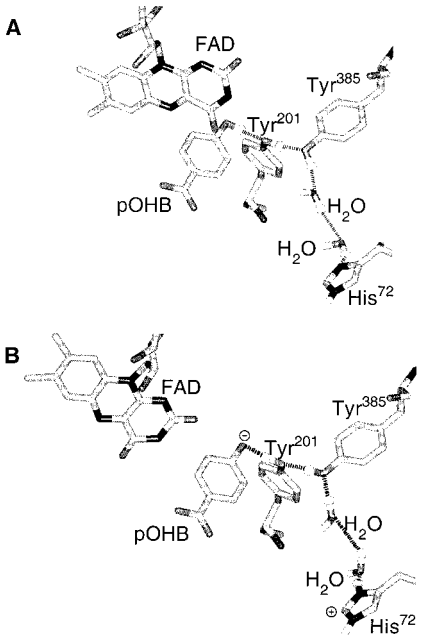

FIGURE 2: Flavin movement linked to proton movement. Two different FAD conformations have been observed in structures of PHBH. (A) The structure taken from 1pbe.pdb is typical of the "in" conformation, where the flavin is located near pOHB. An internal hydrogen-bonding network runs through the interior of the protein from pOHB to Tyr 201, Tyr 385, a structural water molecule (taken from 1ius.pdb in this illustration), a second internal water molecule visible in other structures, and finally to the surface at His 72 (*31*). The positions of the protons (balls) of the hydrogen-bonding network are not available from structural data, but have been built into the structure to indicate the configuration when pOHB is in the phenolic state and His 72 is neutral. This form is poised for deprotonation of the bound pOHB through protonation of His 72 and the hydrogen bond network (*31*). (B) The FAD in several PHBH structures is in the "out" conformation, as in 1dob.pdb shown here. Few other structural changes accompany the conformational change. The protons of the hydrogen-bonding network are shown in the charge-separated configuration necessary for flavin movement that allows rapid reduction by NADPH. This generates the phenolate of pOHB and protonates His 72 while reorienting the directions of the other hydrogen bonds.

histidine. Therefore, we ascribe the pK_a value observed for the reduction rate constant to His 72. Because the active site is sequestered from solvent, the hydrogen-bonding network plays an important role during the oxygenation reaction in the rapid deprotonation of the phenolic oxygen of pOHB necessary for the electrophilic substitution effected by EFlHOOH (*12*). Our data now indicate a previously unrecognized role for the proton-transfer network in the reductive half-reaction. For rapid reduction, the phenolate of pOHB must be transiently generated as a password via the hydrogen-bonding network, ultimately protonating His 72. This can only happen when His 72 is neutral, explaining why reduction is controlled by the pK_a of this distant residue.

The His72Asn mutant of PHBH dramatically illustrates the importance of the hydrogen-bonding network in controlling the reductive half-reaction. At higher pH values, two forms of the mutant enzyme were observed in stopped-flow experiments. One population of His72Asn reacted quickly with NADPH ($\sim$80 s^{-1} at all pH values), while the other population of enzyme reduced slowly (1.6−4 s^{-1} depending upon pH), resulting in biphasic reaction traces (Figure 3A). The fraction of the quickly reducing mutant enzyme increased with pH and exhibited a pK_a of 8.1 (Figure 3B), nearly the same value (8.0) determined independently for the pK_a of pOHB bound to this mutant enzyme. Therefore, we conclude that, in the phenolate form, the His72Asn−pOHB complex is reduced by NADPH as rapidly as the maximum rate attained by the wild-type enzyme. A qualitatively similar result was obtained for another PHBH mutant in the proton-transfer network, Tyr385Phe (*11*). Tyr385 is closer to pOHB than His 72, so the mutation has a larger effect on the ability of the enzyme to lower the pK_a of bound pOHB. In this case, the pK_a is too high to be determined (*16*). Slow reduction of Tyr385Phe predominates, but at pH 9.5 a biphasic reduction was observed, with only 18% of the Tyr385Phe−pOHB complex reacting at the 80 s^{-1} characteristic of the fast form (results not shown). Our model—that the phenolate of pOHB enables rapid reduction, and that the two reaction phases observed in the proton-transfer mutants are due to the inability of the phenolate and phenolic forms of pOHB to rapidly equilibrate—predicts that, when a nonionizable ligand such as pAB is bound to PHBH, the proton-transfer network will be irrelevant and mutants should behave like the wild-type enzyme. Indeed, the His72Asn−pAB complex is reduced by NADPH slowly in a single phase at pH 8.65 with a rate constant (0.03 s^{-1}) similar to that observed for wild type.

Disruption of the proton-transfer network in His72Asn prevents pOHB from equilibrating rapidly with the solution pH, as demonstrated by pH-jump stopped-flow experiments (Figure 3C). When anaerobic oxidized His72Asn−pOHB complex at pH 8.65 was rapidly mixed with NADPH in buffers of lower pH, the proportion of mutant enzyme reducing in the fast phase, and the rate constant for the fast phase, were the same as those observed when the pH of the NADPH solution matched the pH of the enzyme solution. However, the rate of reduction of the enzyme in the slowly reducing population responded to the pH-jump. This result illustrates that, in the His72Asn−pOHB complex, the ionizable group that allows rapid reduction is unable to respond quickly to external solution conditions, consistent with its assignment to the buried phenolic oxygen of pOHB. In contrast, flavin reduction in wild-type PHBH responds to pH-jumps, giving the same k_{obs} obtained for enzyme equili-

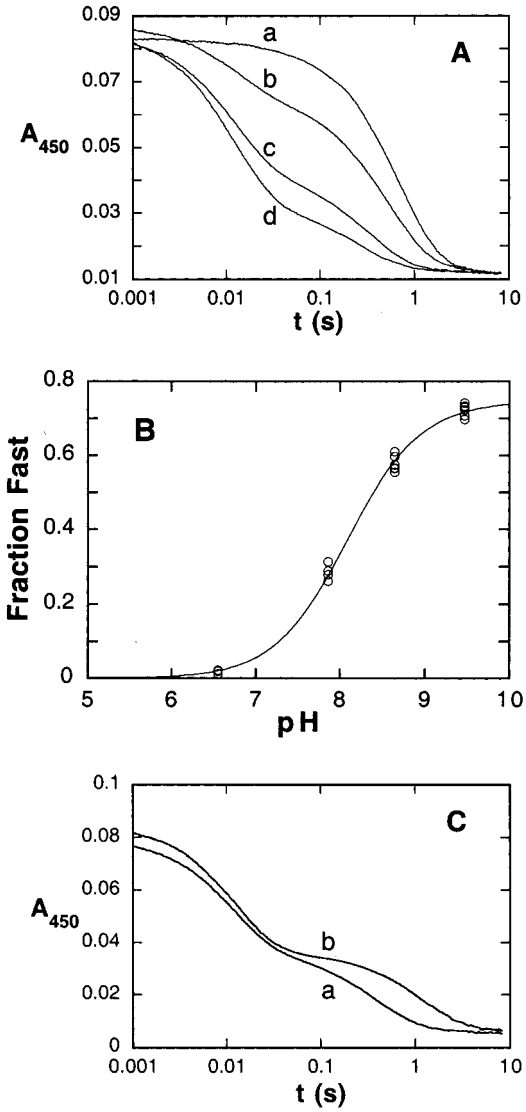

FIGURE 3: Two forms of the His72Asn−pOHB complex. (A) The anaerobic His72Asn−pOHB complex was mixed in the stopped-flow instrument with anaerobic solutions of NADPH at 4 °C. The reactions were observed by monitoring the absorbance of the oxidized flavin at 450 nm over time; note the logarithmic time scale. Reactions were at pH 6.55 (a), 7.86 (b), 8.65 (c), and 9.47 (d). (B) The fraction of His72Asn−pOHB that reacted rapidly was obtained by dividing the amplitude of the rapid phase of reduction by the total change for the fast and slow phases. (C) The rapidly reducing form of the His72Asn−pOHB complex did not respond to pH-jumps, while the slowly reducing form did. Trace (a) shows the reaction of the His72Asn−pOHB complex in pH 8.65 buffer with an NADPH solution in the same buffer. Trace (b) shows the reaction of the same enzyme solution in (a) with NADPH so that the final pH was 6.86. Note that the amount of enzyme reacting rapidly did not decrease to the amount expected for the lower pH, nor did the observed rate constant decrease, indicating that the pH-sensitive group cannot equilibrate with solution rapidly. The reaction of the slowly reacting form, however, slows in response to the pH change.

brated at the final pH (results not shown), which is expected for an enzyme with its proton-transfer network intact.

The structural basis for the control of flavin reduction in PHBH is not completely clear, since high-resolution structures have not been obtained for ligand-free PHBH or a PHBH−pyridine nucleotide complex, and the pyridine nucleotide binding site of PHBH has yet to be located. The enzyme lacks a known signature sequence for NADPH binding, although sequence comparisons among hydroxylases (*17*, *18*) and a recent study of several site-directed mutants suggest a novel motif (*19*). The only direct data available to date on the interaction of PHBH with NADPH come from stereochemical studies demonstrating that the *pro-S* hydride of NADPH is transferred to N5 on the *re*-face of the flavin (*20*, *21*).

Crystallographic studies have shown that the flavin of PHBH can adopt two conformations (*22*, *23*). In one conformation, referred to as the "in" conformation, the isoalloxazine moiety of the flavin is largely buried by the protein and is positioned next to pOHB optimally for the hydroxylation reaction (*24*). In the other conformation, the flavin is in a solvent-exposed position too distant from the aromatic ligand for hydroxylation. Flavin conformations of the PHBH-aromatic ligand complexes observed crystallographically are accompanied by characteristic changes in the flavin visible absorbance spectrum (*22*). When an aromatic substrate forms a complex with the flavin in the "in" position, only small extinction changes occur between 370 and 500 nm. In contrast, the substantial change in the flavin environment when ligand binding causes the isoalloxazine moiety to adopt the "out" conformation is reflected in a large extinction increase over the 370−500 nm range. This spectral correlation allows the flavin position to be monitored by difference spectroscopy. We detected a spectral change consistent with flavin movement (Figure 4A) ∼5 ms after mixing the wild-type enzyme−pOHB complex with NADPD. The 8.9-fold primary kinetic isotope effect for hydride transfer enabled the spectral observation of the PHBH−pOHB−NADPD complex immediately before hydride transfer. The increase in absorbance between 400 and 500 nm is consistent with the adoption of the "out" conformation. Thus, the spectral data strongly suggest that the hydride-transfer reaction occurs after movement of the flavin to the "out" conformation. However, this is modulated by spectral changes from formation of an NADPH−FAD charge-transfer complex, characterized by a broad long-wavelength absorbance (*25*), resulting from the stacking of the electron-rich dihydronicotinamide moiety of NADPH with the electron-deficient isoalloxazine moiety of FAD.

This interpretation is consistent with several previous reports. In studies of PHBH in which the natural FAD was replaced by 6-azido FAD, spectral changes in this system prior to reduction also indicated that the reduction of the flavin by NADPH occurs when the flavin is in the "out" conformation (*26*). When the isoalloxazine moiety of this artificial enzyme was covalently fixed in the "in" conformation by photolabeling, reduction by NADPH was slower by 6 orders of magnitude, underscoring the necessity of flavin mobility for high reactivity. Spectral changes upon NADPH binding to the Ser212Ala-2,4-dihydroxybenzoate complex also indicate that the flavin moves to the "out" conformation prior to reduction (*27*, *28*). In several other modified PHBH systems (e.g., the Tyr222Phe mutant (*29*) and wild-type PHBH in which FAD has been replaced with *arabino*FAD (*30*) or 2′-fluoro-*arabino*FAD (Palfey et al., unpublished)), the flavin adopts the "out" conformation even in the presence of pOHB, resulting in a rate constant for reduction at pH 6.5 nearly as high or higher than the natural system. It is apparent from crystal structures of PHBH that the "in" conformation does not allow enough space for the dihydronicotinamide moiety of NADPH to approach the isoal-

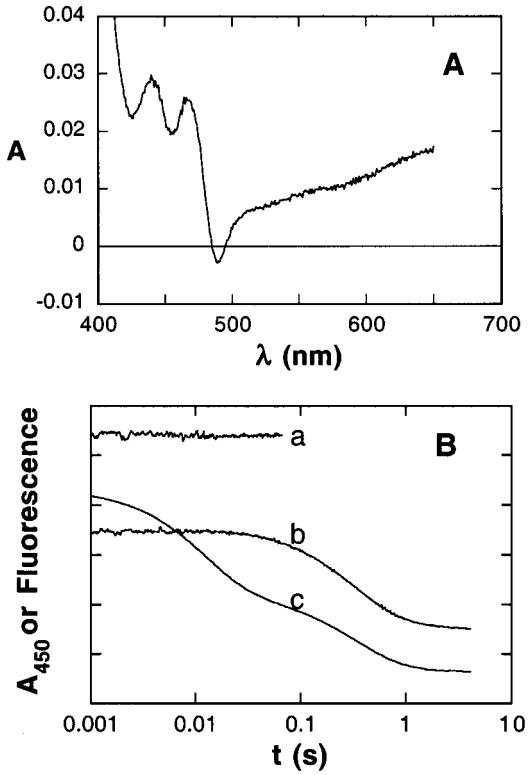

FIGURE 4: The flavin moves to the "out" conformation prior to reaction with NADPH. (A) The wild-type PHBH−pOHB complex at pH 6.5, 4 °C was mixed with NADPD (20 μM and 1.5 mM after mixing, respectively) in a stopped-flow instrument equipped with diode-array detection, and spectra were collected every 1.25 ms for 0.12 s. The figure shows the result of subtracting the enzyme spectrum prior to reaction from the spectrum 5 ms after mixing. The large absorbance increase at $\lambda < 410$ nm is due to the NADPD in the solution. The absorbance increase at $\lambda > 500$ nm is due to an oxidized flavin−NADPD charge-transfer complex. The absorbance increase in the 410−490 nm range is consistent with movement of the flavin from the "in" to the "out" conformation. (B) The isoalloxazine of the rapidly reducing form of His72Asn−pOHB associates with NADPH in the dead-time of the stopped-flow instrument, consistent with the interpretation that it is in the "out" conformation. The His72Asn−pOHB complex at pH 8.65 was reacted with NADPH in a stopped-flow instrument and observed by either fluorescence ($\lambda_{ex} = 450$ nm, $\lambda_{em} > 515$ nm) or absorbance at 450 nm. Trace (a) shows the fluorescence level in the absence of NADPH. Trace (b) shows the dead-time loss of fluorescence associated with NADPH binding, followed by the fluorescence decrease due to the reaction of the slowly reducing enzyme population. Trace (c) shows the same reaction monitored by absorbance, demonstrating that during the reaction of the rapidly reducing form ($t < \sim 30$ ms), there is no fluorescence change.

loxazine moiety of FAD with the proper stereochemistry, and accordingly an NADPH−PHBH complex in the "out" conformation has recently been modeled (*19*).

The results described above demonstrate that the phenolate form of pOHB is required for rapid flavin reduction by NADPH, and that the flavin must move to the "out" conformation in order to react. Experiments with the His72Asn−pOHB complex indicate that the fast-reducing form of that enzyme, in which pOHB is bound as the phenolate, also has the flavin in the "out" conformation. During reaction with NADPH, the fluorescence and absorbance of the wild-type PHBH−pOHB complex decrease in a single phase with identical observed rate constants. Hydride transfer with the His72Asn mutant occurs in two phases, as observed by the decrease in flavin absorbance described above. The fluorescence decay of the slowly reacting form of His72Asn parallels its absorbance decay (Figure 4B). However, the fluorescence of the quickly reducing population of His72Asn is quenched in the dead-time of the stopped-flow instrument, prior to hydride transfer, indicating that fluorescence quenching is caused by the extremely rapid direct binding interaction of NADPH with the flavin. This strongly implies that the flavin of the His72Asn that has the phenolate of pOHB bound has already adopted the "out" conformation. The pOHB in the slowly reacting fraction becomes deprotonated slowly in the mutant enzyme, and this is rate-determining in reduction and fluorescence quenching.

Our data suggest that flavin reduction is controlled in PHBH in the following manner (Figure 2). When NADPH binds to the PHBH−pOHB complex, pOHB is transiently deprotonated by the proton-transfer network. The phenolate of pOHB and bound NADPH stimulate the movement of the flavin from the "in" to the "out" position, where the hydride-transfer reaction occurs. After the hydride transfer, NADP dissociates and the resultant anionic reduced flavin is drawn back to the "in" position by a region of high positive electrostatic potential (*10*), and is thus poised for the oxidative half-reaction. The mechanism whereby the phenolate of pOHB and the presence of NADPH cause the flavin to assume the "out" conformation is not yet clear. However, the phenolic oxygen of pOHB is within hydrogen-bonding distance to the carbonyl oxygen of Pro 293. It is likely that the negative charge of the pOHB phenolate would repel the negative dipole of this carbonyl, transmitting the stress along the local peptide backbone and causing a conformational change allowing the flavin to move to the "out" position. Evidence of this possibility can be seen in the crystal structure of the wild-type PHBH−pOHB complex at pH 9.5, where the peptide backbone around residue 294 can assume two conformations (*31*). Bound NADPH, absent in the structure, must also be necessary to elicit the full conformational change, since the flavin is "in" in the pH 9.5 structure.

Flavin movement prior to reaction with NADPH is very likely to be a general property of the enzyme family. The structure of phenol hydroxylase, another flavoprotein aromatic hydroxylase, has recently been determined (*32*). Different conformations were observed for the flavin of each subunit of the dimer, an "in" conformation and an "out" conformation, and it was noted that NADPH could not approach the flavin to react with the "in" conformation. The necessity for the conformational change can be understood by considering the diversity of the mechanistically distinct reactions promoted by flavoprotein aromatic hydroxylases in their catalytic cycles. An important determinant of enzyme catalysis is the preferential binding of transition states. There are four very different transition states in the catalytic cycles of PHBH and other hydroxylases: those of the hydride transfer, the formation of the flavin hydroperoxide from O_2 and reduced FAD, the electrophilic oxygenation of pOHB, and the elimination of water from the flavin hydroxide. Each of these transition states is likely to have different requirements from the protein for stabilization. It appears that the flavoprotein hydroxylases achieve the necessary catalytic diversity by utilizing two conformationally different active sites of the same protein molecule. This highly efficient organization contrasts with the mammalian pterin-utilizing

amino acid hydroxylation systems, which divide the analogous reactions among distinct pterin reductases, hydroxylases, and hydroxypterin dehydratases (*33*, *34*).

While the flavoprotein hydroxylases seem to share the characteristic of flavin movement, the linkage of the conformational change to proton movement is probably unique to PHBH. PHBH has evolved a mechanism to test the suitability of the aromatic ligand prior to committing to catalysis by requiring a "password", facile deprotonation of the potential substrate, for a catalytic step (reduction) that does not chemically involve the aromatic molecule. There is no analogous proton-transfer network in the phenol hydroxylase structure (*32*), and it is likely that this unique means of enforcing substrate specificity in PHBH is an evolutionary response to avoid the hydroxylation of pAB, which would inhibit folic acid synthesis and produce a toxic aminophenol (*35*).

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