Protein Dynamics Control Proton Transfers to the Substrate on the His72Asn Mutant of *p*-Hydroxybenzoate Hydroxylase†

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ABSTRACT: *p*-Hydroxybenzoate hydroxylase (PHBH) hydroxylates activated benzoates using NADPH as a reductant and O_2 as an oxygenating substrate. Because the flavin, when reduced, will quickly react with oxygen in either the presence or absence of a phenolic substrate, it is important to regulate flavin reduction to prevent the uncontrolled reaction of NADPH and oxygen to form H_2O_2. Reduction is controlled by the protonation state of the aromatic substrate *p*-hydroxybenzoate (pOHB), which when ionized to the phenolate facilitates the movement of flavin between two conformations, termed "in" and "out". When the hydrogen bond network that provides communication between the substrate and solvent is disrupted by changing its terminal residue, His72, to Asn, protons from solution no longer equilibrate rapidly with pOHB bound to the active site [Palfey, B. A., Moran, G. R., Entsch, B., Ballou, D. P., and Massey, V. (1999) *Biochemistry 38*, 1153−1158]. Thus, one population of the His72Asn enzyme reduces rapidly and has the phenolate form of pOHB bound at the active site and the flavin in the out conformation. The remaining population of the His72Asn enzyme reduces slowly and has the phenolic form of pOHB bound and the flavin in the in conformation. We have investigated the mechanisms of proton transfer between solvent and pOHB bound to the His72Asn form of the enzyme by double-mixing and single-mixing stopped-flow experiments. We find that, depending on the initial ionization state of bound pOHB and the new pH of the solution, the ionization/protonation of pOHB proceeds through the direct reaction of hydronium or hydroxide with the enzyme−ligand complex and leads to the conversion of one flavin conformation to the other. Our kinetic data indicate that the enzyme with the flavin in the in conformation reacts in two steps. Inspection of crystal structures suggests that the hydroxide ion would react at the *re*-face of the flavin, and its reaction with pOHB is limited by the movement of Pro293, a conserved residue in similar flavoprotein hydroxylases. We hypothesize that this type of breathing mode by the protein may have been used to compensate for the lack of an efficient proton-transfer network in ancestral hydroxylases, permitting useful catalysis prior to the emergence of specialized proton-transfer mechanisms.

The flavoprotein *p*-hydroxybenzoate hydroxylase (PHBH)[1] catalyzes the conversion of *p*-hydroxybenzoate (pOHB) to 3,4-dihydroxybenzoate. The catalytic cycle consists of a reductive half-reaction where the flavin is reduced by NADPH, and an oxidative half-reaction where reduced flavin reacts with oxygen and hydroxylates the aromatic substrate. Like other flavoprotein hydroxylases, PHBH activates molecular oxygen by forming a highly reactive C4a-hydroperoxyflavin intermediate that incorporates the peroxy oxygen into the aromatic substrate by an electrophilic aromatic substitution reaction (*1*). This flavin hydroperoxide intermediate must be isolated from water to ensure that it hydroxylates the substrate rather than eliminates H_2O_2. Crystal structures of PHBH have shown the flavin in a position that can protect the unstable flavin hydroperoxide from solvent and where it can readily oxygenate the substrate (*2*). Because the flavin is largely buried in the enzyme, this position is referred to as the "in" conformation. However, a number crystal structures of enzyme in complex with various substrates show an alternate flavin conformation in which the isoalloxazine has rotated by ∼30° around the C2′−C3′ bond of the ribityl chain to move to a solvent-exposed position (*3*, *4*), referred to as the "out" conformation (Figure 1). The proportion of out conformation can be affected by mutations (*3*, *4*), substituents on the aromatic ligand (*3*, *4*), or inversion of the stereochemistry of C2′ of FAD (*5*). In this out conformation, it is not possible for the flavin to hydroxylate aromatic substrates. Nevertheless, some of the enzyme−substrate complexes that are observed to adopt the out conformation effectively hydroxylate aromatic substrates, indicating that the flavin can move during catalysis. Since this movement is easily inducible, movement of the flavin cofactor is proposed to be a normal part of the catalytic cycle of PHBH (*6*).

PHBH has an internal proton transport network involving the 4-hydroxyl groups of pOHB, Tyr201, and Tyr385, as

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[1] Abbreviations: KPi, potassium phosphate buffer; PHBH, *p*-hydroxybenzoate hydroxylase; pL, the generalized equivalent of pH where L includes all isotopically different hydrogen ions; pOHB, *p*-hydroxybenzoate; SKIE, solvent kinetic isotope effect; Tris, tris(hydroxymethyl)aminomethane.

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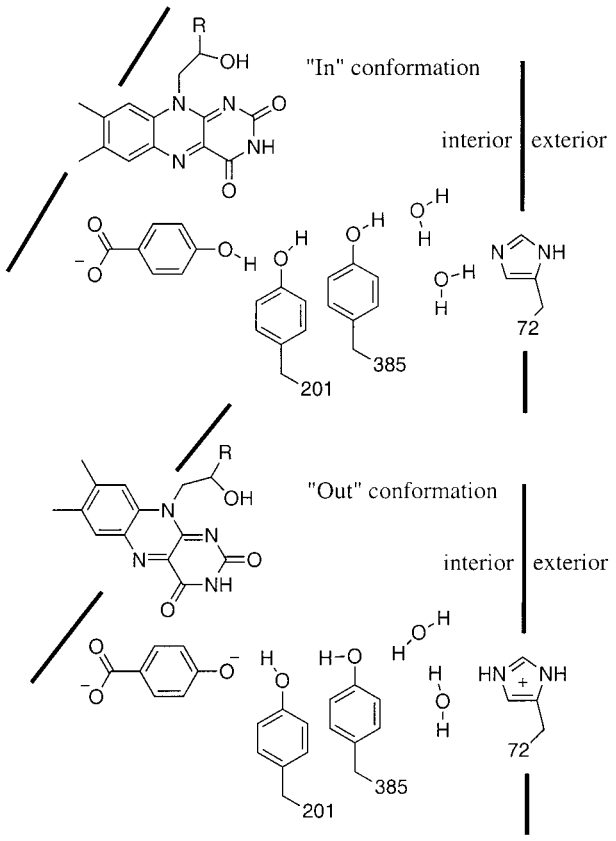


FIGURE 1: Cartoon of the active site of PHBH. There are two different positions of the flavin, in and out, and a proton-transfer network that includes the 4-OH of the aromatic substrate, Tyr201, Tyr385, two internal water molecules, and His72. Note that when pOHB is deprotonated, the flavin is in the out position and when the substrate is protonated, the flavin is in.

well as two internal water molecules, and the distal His72 at the surface of the protein that shuttles protons between the solvent and the solvent-sequestered active site (Figure 1) (*7*). Because the phenolate form of the substrate is more reactive toward electrophilic aromatic substitution than is the protonated form (*8*), this network provides a means for deprotonating the solvent-sequestered pOHB that is vital for effective substrate activation. It is becoming apparent that the proton transfer network also controls the position of the oxidized flavin and that the flavin position controls reduction. Although NADPH and pOHB bind to the enzyme randomly (*9*), there is an absolute requirement that the aromatic substrate bind before rapid reduction of the flavin by NADPH can occur; binding of pOHB stimulates reduction by 10^5-fold. Because the aromatic substrate is not involved chemically in the reductive half-reaction, this stimulation must be regulatory and it prevents the wasteful consumption of NAPDH and production of H_2O_2 in the absence of pOHB. The proton-transfer network controls flavin reduction by controlling the position of the flavin; the binding of pOHB does not change the K_d of the enzyme for NADPH nor alter the redox potential of the flavin (*8*). Spectral changes that occur prior to reduction (*6*, *10*, *11*, *12*), as well as the behavior of enzyme with the FAD covalently fixed in the in conformation by chemical modification (*10*), indicate that hydride transfer occurs in the out conformation. The movement of the flavin from the in to the out conformation that is necessary for reduction appears to require the development of a negative charge on the 4-substituent of the aromatic ligand (*11*). The deprotonation of pOHB (or other substrate-like effectors) is accomplished by the proton-transfer network, and an intact network is required for rapid reduction. This is demonstrated by the up to 100-fold decrease in rate with the Tyr201Phe and Tyr385Phe mutant forms of the enzyme, which have disrupted proton-transfer networks (*8*).

The rate constant for reduction of the flavin in the wild-type enzyme is controlled by the ionization state of His72 at the end of the proton transfer network (*11*). The His72Asn mutation retards the equilibration of protons between pOHB and solvent that is mediated by the proton transfer network. Thus, two species of this mutant enzyme can be observed, one that reduces quickly ($\sim$80 s^{-1} at all pH values) and one that reduces slowly (1.6$-$4 s^{-1} depending on the pH value). The proportions of these forms of the enzyme can be determined from the amplitudes of the fast and slow phases in stopped-flow studies of the reductive half-reaction. As the pH of the reduction experiment was increased, the proportion of the fast form of the mutant enzyme increased to a maximum value of $\sim$80% of the total enzyme. The fast form of the enzyme corresponds to enzyme with the phenolate form of pOHB bound, which triggers the flavin to adopt the out conformation, enabling rapid reduction. The slowly reducing form of His72Asn corresponds to enzyme with protonated substrate bound that has the flavin in the in conformation. The latter reduces more slowly because the proton of pOHB cannot be removed by the proton transfer network; thus an alternate means of removing the proton must enable reduction (*11*). The fact that proton equilibration between the solution and the bound pOHB is slow was demonstrated in pH-jump/reduction experiments, in which enzyme at one pH was mixed with NADPH solutions at another pH (*11*). In these experiments, the proportions of fast and slow forms of the enzyme did not change to values appropriate for the new pH, indicating that proton equilibration was slow compared to the rapid phase of reduction. In this paper, we elucidate the mechanism of isomerization of the fast and slow forms of the His72Asn enzyme by single-mixing and double-mixing stopped-flow experiments and propose a likely pathway for proton equilibration. Our results suggest that in the absence of the optimized proton-transfer network, slower protein dynamic modes may allow the necessary catalytic proton transfers, and such modes might have represented a vital property of an ancestral hydroxylase. We propose that such protein breathing could facilitate the evolution of novel enzyme activities prior to the development of specialized proton-transfer mechanisms.

MATERIALS AND METHODS

The His72Asn mutant was expressed in *Escherichia coli* (*13*) and purified as described (*7*). Solutions were made anaerobic using adaptations of described techniques (*14*, *15*), with argon as the atmosphere for exchange. Rapid reactions at 4 °C were observed in a stopped-flow spectrophotometer. For single-mixing experiments, either a Hi-Tech SF-61 stopped-flow spectrophotometer (Hi-Tech Limited) operated by the KISS (Kinetic Instruments Stopped-flow Systems) software or a Kinetic Instruments stopped-flow spectrophotometer controlled by Program A (C.-J. Chiu, R. Chang, J. Diverno, and D. P. Ballou, unpublished material) was used. The Hi-Tech SF-61 instrument was reconfigured for fluo-

rescence measurements for some experiments. Enzyme-bound FAD was excited at 450 nm with a xenon lamp and a 530 nm emission cutoff filter was used on the emission detector. For double-mixing experiments, a Hi-Tech SF-61 DX2 stopped-flow spectrophotometer with KinetAsyst 2 software (Hi-Tech Limited) was used. Rate constants were obtained from exponential fits based on the Marquardt−Levenberg algorithm using Program A, KISS, Scientist (MicroMath Inc.), or pro Fit (Quantum Soft).

In pH-jump experiments, the buffer for pH values in the range 6−7.8 was potassium phosphate, in the range of 7.8−9, the buffer was Tris-sulfate, and for values of 9 and above, the buffer was glycine. To make deuterated buffers, concentrated stock solutions of the desired buffers were evaporated to dryness in a Speed-Vac centrifugal evaporator (Savant) and then redissolved with D_2O. The process was repeated three times. All pL values were checked at 4 °C. pL was determined using a glass pH electrode and then adding to the meter reading a constant determined by the fractions of D_2O and H_2O as described (*16*). The final pH values after double-mixing stopped-flow spectrophotometry experiments were measured from independent mixtures of 3 parts of 200 mM buffer with 1 part of 5 mM buffer.

A psi-BLAST search of the NCBI database using phenol hydroxylase (g464369) as a query sequence was performed on the "3D-PSSM Protein Fold recognition (Threading) Server" (http://www.bmm.inet.uk/~3dpssm/). The program MOLSCRIPT (*17*) was used to generate the active site figure.

RESULTS

Double-Mixing pH-Jump Experiments. Two reactions were studied by double-mixing pH-jump stopped-flow experiments: the reaction of the rapidly reducing conformation of the enzyme with H^+ to form the slowly reducing conformation and the reaction of the slowly reducing form with OH^- to give the rapidly reducing form. The conversion between the fast and slow reducing forms of the His72Asn mutant was investigated by rapidly changing the pH of the solution and then measuring the fraction of the fast and slow forms as a function of time. Enzyme solution (80−100 μM before mixing) in 5 mM glycine at pH 10.19 was rapidly mixed with various 200 mM buffers at lower pH values in the first mix. The enzyme at the new pH was allowed to age for periods varying from 10 ms up to 5 s, and then it was mixed with a saturating concentration of NADPH (3 mM after mixing), also in the more concentrated buffer. All solutions contained 0.5 mM pOHB. Because the reduction of flavin by NADPH was fast compared to the conversion between forms (~80 vs ~ 3 s^{-1}), this reaction could be used as a probe to determine the fractions of fast and slow forms of the enzyme, as indicated by the amplitudes of the slow and fast phases of reduction. For these high-pH to low-pH jumps, the amplitude of the first phase decreased and the amplitude of the second phase increased as the solutions were allowed to age (Figure 2A).

Similar double-mixing pH jump experiments, where His72Asn was initially at a low pH value and was mixed with strong buffers of higher pH values, were performed to observe the conversion from the slow to the fast reducing form of the enzyme. For these low-pH to high-pH jumps, the amplitude of the first phase increased and the amplitude of the second phase decreased as the solutions were allowed to age (data not shown), behaving exactly opposite to the high-pH to low-pH jumps.

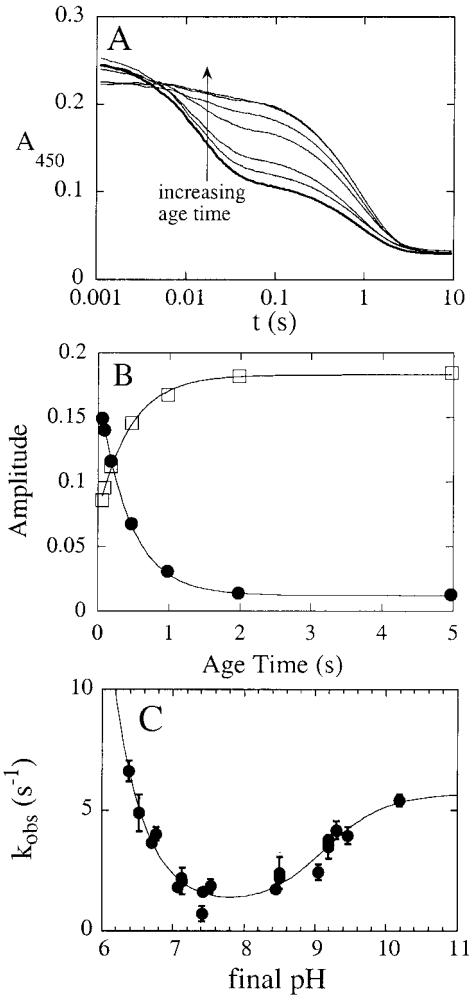


FIGURE 2: (A) Reaction traces for a high pH to low pH jump. A solution of His72Asn (100 μM) and 0.5 mM pOHB in 5 mM glycine, pH 10.19, was rapidly mixed at 4 °C with 200 mM KPi, pH 7.0, containing 0.5 mM pOHB, and allowed to age for various times. The aged solution was then rapidly mixed with 6 mM NADPH and 0.5 mM pOHB in 200 mM KPi at pH 7.0, and flavin reduction was monitored at 450 nm. These traces are representative of all pH jumps. Note that a logarithmic time scale was used in order to clearly display the two phases of reduction. Also note the small variations in the initial absorbance values at different pH values. (B) Amplitudes as a function of age time for His72Asn in a high pH to low pH jump. The traces in panel A were fit as described in Materials and Methods. The amplitudes from these fits representing the rapidly (circles) and slowly (squares) reducing enzyme forms are plotted as a function of the age time. The data presented here are from the analysis of the experiment shown in panel A (pH 10.19 to 7.0) and result in one point for panel C. (C) Observed rate constant of isomerization of the slow and fast forms of His72Asn as a function of final pH. Each point represents a k_{obs} as determined in panel B and the curve represents a fit of the data to eq 1. Experiments for pH jumps in both directions are represented.

Reaction traces for the reduction of flavin were fit to a sum of two exponentials; occasionally a third small phase was added to the fit to account for a slight contamination with oxygen. The observed rate constants for reduction (both phases) were the same regardless of the aging time (80 s^{-1} for the fast phase and between 1 and 4 s^{-1} for the slow phase, depending on the pH after mixing). At a given pH, only the

amplitudes of the two phases varied with aging time (Figure 2B), and they varied in a process that could be described by a single-exponential function. For each pH jump, both the decay of the amplitude of one phase and the formation of amplitude of the other had the same apparent rate constant.

The dependence of the isomerization reaction on buffer concentration was determined. His72Asn (80 μM before mixing) and 0.5 mM pOHB in 5 mM glycine buffer at pH 9.41 was rapidly mixed with either 200 or 400 mM KPi at pH 7.1, each containing 0.5 mM pOHB, and allowed to age for varying lengths of time before rapidly mixing with 6 mM NADPH (before mixing) and 0.5 mM pOHB in either 200 or 400 mM KPi at pH 7.1. The observed rate constant for the isomerization was 2.1 ± 0.6 s^{-1} for the 100 mM buffer and was 1.8 ± 0.1 s^{-1} for the 200 mM buffer (buffer concentrations are after the first mix). Table 1 shows that there is essentially no dependence on buffer concentration, which rules out buffer catalysis.

The dependence of the observed isomerization rate constant on pOHB concentration was investigated by rapidly mixing a solution of His72Asn (100 μM before mixing) and 0.5 mM pOHB in 5 mM KPi, pH 6.34, with 200 mM glycine buffer at pH 9.19 containing different concentrations of pOHB (0, 0.5, or 10 mM), and the mixture was allowed to age. The aging solution was then rapidly mixed with a solution of 6 mM NADPH (before mixing) and pOHB (0, 0.5, or 10 mM before mixing) in 200 mM glycine at pH 9.19, and the reduction of the flavin was monitored by the absorbance at 450 nm. The observed rate constants for isomerization were 3.8 ± 0.2 s^{-1} for 0.25 mM pOHB, 3.5 ± 0.8 s^{-1} for 0.5 mM pOHB, and 3.6 ± 0.2 s^{-1} for 5.2 mM pOHB (pOHB concentrations are those after the first mix; Table 1). Thus, there is no significant effect of substrate on the rate of isomerization.

The dependence of isomerization on pH was investigated. The observed rate constant of isomerization between the fast and slow forms of the enzyme was dependent only upon the final pH of the jump and was independent of the direction of the jump itself. When jumping to the acidic extreme, the dependence of the observed rate constant on hydronium ion concentration was linear, implying a simple bimolecular reversible reaction mechanism. On the basic pH extreme, the dependence of the observed rate constant for isomerization on hydroxide ion concentration reached a limiting value, which implies that the hydroxide ion reacts in at least two steps to convert the slow form of the enzyme to the fast form of the enzyme (*18*). At intermediate pH values, both processes will be operational (Scheme 1), leading to a combined rate equation (eq 1)

$$k_{obs} = k_a[H^+] + k_b + k_4 + \frac{k_3[OH\text{-}]}{[OH\text{-}] + K_1} \qquad (1)$$

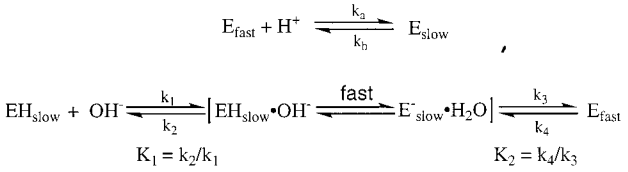
that describes the dependence of the observed rate of isomerization on final pH (Figure 2C). Data were fit to eq 1 using a p$K_w = 14.741$ at 4° (*19*), giving k_a equal to $1.4 \times 10^7 \pm 0.1 \times 10^7$ M^{-1} s^{-1}, k_3 equal to 4.7 ± 0.5 s^{-1}, and K_1 for the reaction of hydroxide equal to 2.4 ± 0.7 μM. While the sum of k_b and k_4 can be accurately determined, k_b and k_4 cannot be determined independently by eq 1. The sum of k_b and k_4 is 1.0 ± 0.2 s^{-1}. However, k_b could be determined from consideration of the pK_a of the ionizable group. The

Table 1: Effect of Solute Concentration on Isomerization

condition[a]	k_{obs} (s^{-1})
pH 9.4 to 7.1	
100 mM buffer	2.1 ± 0.6
200 mM buffer	1.8 ± 0.1
pH 6.34 to 9.19	
0.25 mM pOHB	3.8 ± 0.2
0.5 mM pOHB	3.5 ± 0.8
5.2 mM pOHB	3.6 ± 0.2

[a] Concentrations are those during the aging phase of the experiment.

Scheme 1



proton equilibrium shown in Scheme 1 defines the pK_a of pOHB bound to the enzyme. Previously, a value of 8.0 was reported for this pK_a (*11*). Our double-mixing experiments provided an independent means for measuring this pK_a. The variation with pH of the amplitudes obtained at very long age times consistently gave pK_a values between 7.9 and 8.1 (data not shown). The value of k_b was calculated from the pK_a and the from k_a obtained from the fit to eq 1. The value of k_b was then used to calculate the value of k_4. Values for k_b of 0.12 ± 0.01 s^{-1} and k_4 of 0.9 ± 0.2 s^{-1} were obtained.

Single-Mixing pH-Jump Experiments. In the double-mixing experiments described above, a small change in the absorbance of the oxidized enzyme was observed prior to its reduction by NADPH (Figure 2A). This variation in the starting absorbance depended on the final pH and the aging time, suggesting that the spectral change might report whether the enzyme was initially in the fast or slow reducing form. To determine the rate of isomerization of these two forms of His72Asn, the spectral change associated with a change in pH was monitored in a single-mixing stopped-flow spectrophotometer. Lightly buffered enzyme in the presence of 0.5 mM pOHB at one pH extreme was rapidly mixed with 0.5 mM pOHB and 200 mM buffer at a different pH value. Rates of isomerization were determined at 463 nm, which is a peak in the difference spectrum generated by subtracting the spectra of enzyme at low pH from enzyme at high pH (Figure 3A). Monophasic traces were obtained (data not shown) and the variation of the observed rate constant with the final pH value agreed with data obtained from double-mixing experiments. Data were fit to eq 1 and are summarized in Table 2 (absorbance, single-mixing). Because the signal change obtained by absorbance was small, the same experiment was repeated using fluorescence detection to obtain independent and more reliable values. Like the traces obtained by absorbance, the traces obtained by fluorescence were monophasic and depended upon the final pH of the jump (Figure 4A). The dependence of the observed rate of isomerization upon the final pH was independent of the direction of the jump and could be described by eq 1 (Figure 4B). The kinetic contants for isomerization obtained from all methods were identical, demonstrating that fluorescence is an excellent method for observing the intercon-

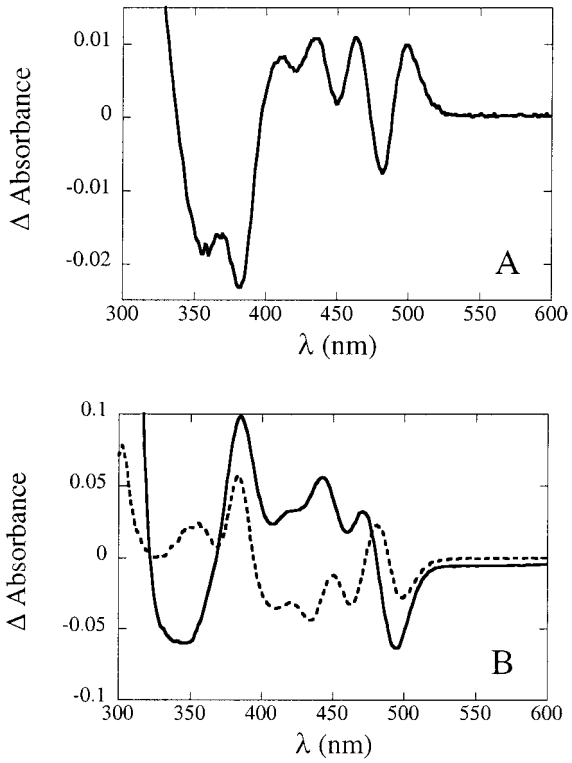


FIGURE 3: (A) Difference spectrum for His72Asn at high pH−low pH. The spectrum of His72Asn (20 μM) with 0.5 mM pOHB at pH 10.22 at 4 °C was subtracted from the spectrum of His72Asn (20 μM) with 0.5 mM pOHB at pH 6.26 at 4 °C. (B) Difference spectra indicating the in and out positions of the flavin. The spectrum of His72Asn (60 μM) in the presence of 0.5 mM pOHB and either 50 mM KPi and 0.5 mM EDTA, pH 6.60, at 4 °C (dashes) or 50 mM glycine, pH 9.60, and 5% glycerol at 4 °C (solid line) was subtracted from the spectra of the same enzyme solutions before the addition of pOHB.

Table 2: Kinetic Parameters for Isomerization

	absorbance double-mixing	absorbance single-mixing	fluorescence single-mixing	SKIE[a]
k_a (M^{-1} s^{-1})	$1.4 \times 10^7 \pm 0.1 \times 10^7$	$1.2 \times 10^7 \pm 0.1 \times 10^7$	$1.4 \times 10^7 \pm 0.1 \times 10^7$	1.3 ± 0.2
k_3 (s^{-1})	4.7 ± 0.5	4.7 ± 0.2	4.9 ± 0.3	2.3 ± 0.2
K_1 (μM)	2.4 ± 0.7	1.1 ± 0.3	0.8 ± 0.2	1.9 ± 0.6
$k_b + k_4$ (s^{-1})	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	1.5 ± 0.3
k_b (s^{-1})	0.12 ± 0.01	0.13 ± 0.05	0.08 ± 0.04	5 ± 4
k_4 (s^{-1})	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	1.4 ± 0.4

[a] SKIE, solvent kinetic isotope effects were calculated from fluorescence data.

version of the rapidly and slowly reducing enzyme forms (Table 2).

Flavin Conformation at High and Low pH. Crystallographic and spectroscopic studies have shown that the two flavin conformations are correlated by characteristic changes in the flavin visible spectrum (*3*, *4*, *10*). When an aromatic substrate forms a complex with the flavin in the in position, only small extinction changes occur between 370 and 500 nm. This was also observed for the His72Asn mutant at pH 6.60 (Figure 3B), indicating that the flavin is in the in conformation at this pH. In contrast, the substantial change in the flavin environment when ligand binding causes the isoalloxazine moiety to adopt the out conformation is reflected by large extinction changes. A difference spectrum

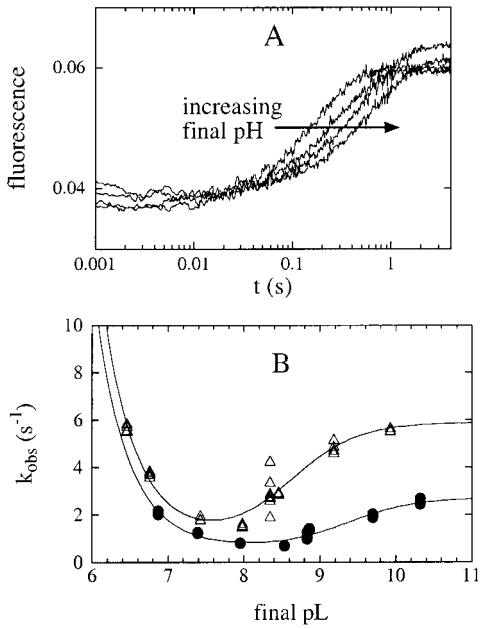


FIGURE 4: Reaction traces for a high to low single-mixing pH jump. His72Asn (20 μM before mixing) in 5 mM buffer with 0.5 mM pOHB was rapidly mixed with a 200 mM buffer containing 0.5 mM pOHB at a low pH value at 4 °C in a single-mixing stopped-flow spectrophotometer. The changes in fluorescence were observed by exciting at 450 nm and using a 530 nm emission cutoff filter. Panel A shows traces for a high pH to low pH jump. Final pH values were 6.45, 6.75, 7.42, and 7.97. Panel B shows the solvent kinetic isotope effects on the observed rate constant of isomerization of flavin in and flavin out positions as a function of pL. The curves represent fits of the data in panel B to eq 1.

that was nearly identical to that observed when pOHB binds to *arabino*-FAD-substituted wild-type PHBH, whose structure shows an out conformation (*5*), was observed for the His72Asn mutant at pH 9.60 (Figure 3B), indicating that the flavin is in the out position at this pH.

Proton Inventories. To determine the number of protons involved in the isomerization reaction, a proton inventory was determined by single-mixing stopped-flow pH jump experiments using fluorescence detection. Lightly buffered enzyme in the presence of 0.5 mM pOHB at a pL extreme was rapidly mixed with 200 mM buffer containing 0.5 mM pOHB at a different pL value. The kinetics of isomerization were observed at 0, 25, 50, 75, and 100% D_2O. Reaction traces were fit to one exponential phase, and the dependence of the observed rate constant upon the final pL values was fit to eq 1. To fit to eq 1, the pK_w of water (14.741) and of D_2O (15.739) at 5 °C were used to calculate the concentration of hydroxide (eq 1) (*19*). To calculate the concentration of hydroxide in mixtures of H_2O and D_2O, the autoprotolysis constant, pK_{Wn}, was calculated using eq 2

$$K_{Wn} = K_{Wh}\left[1 - n + n\left(\frac{K_{Wd}}{K_{Wh}}\right)^{1/3}\right]^3 \qquad (2)$$

where pK_{Wh} is the autoprotolysis constant in H_2O, pK_{Wd} is the autoprotolysis constant in D_2O, and n is the mole fraction of deuterium (R. Schowen, personal communication). Significant solvent isotope effects were obtained for all pL values (Figure 4B, Table 2), with most values ranging from 1.3 to 2.3. The solvent kinetic isotope effect on k_b was significantly larger (5 ± 4), but it is also the least accurately

determined rate constant. Proton inventories were obtained by plotting the rate constants from the fit to eq 1 against the fraction of deuterium in solution. The values of k_a and k_3, and the sum of k_b and k_4 all decreased linearly with increasing deuterium content, consistent with one proton being involved in each of these reactions. The pK_a of pOHB on the mutant enzyme was 8.8 ± 0.1 in D_2O, a shift of 0.8 units from the pK_a in H_2O. We attribute this relatively large SKIE on the pK_a to the strong hydrogen bond between pOHB and Tyr201. The hydrogen bond between the phenolic oxygen of these two groups is modeled by 2,2′-dihydroxybiphenyl, which also has a pK_a that is 0.80 units higher in D_2O (*20*) than the H_2O value of 7.56. Thus, the 0.8 shift is a reasonable value for a hydrogen bond between two phenolic groups.

Sequence Alignment. We were interested to find out if other flavin monooxygenases have proton transfer networks similar to that of PHBH. Therefore, we did a psi-BLAST search (*21*) using PHBH as the query sequence; this identified numerous homologous sequences. The quality of the alignments was evaluated by considering phenol hydroxylase, the only other aromatic flavoprotein hydroxylase whose structure has been determined. The psi-BLAST alignment did not agree with the alignment of the structures performed by Enroth et al. (*22*). Therefore, we used phenol hydroxylase as the query sequence and found that the psi-BLAST alignment to PHBH was ∼75% in agreement with the structural alignment. The psi-BLAST alignment based on phenol hydroxylase also showed conservation with other flavin monooxygenase sequences in the alignment of motifs associated with the binding site for FAD and a presumed NADPH binding site (*23*, *24*). Close examination of the alignment showed that neither the residues of the proton transfer network of PHBH (Tyr210, Tyr385, His72), nor the putative basic residue in phenol hydroxylase (Asp 54; Dong Xu, University of Michigan, personal communication) were conserved. However, other active site residues, such as Pro293 in PHBH, were generally conserved.

DISCUSSION

It has been shown that flavin reduction in PHBH cannot proceed rapidly without an anionic substituent *para* to the carboxylate moiety of the aromatic ligand (*11*, *25*). In the His72Asn mutant, the normal rapid pathway for deprotonating pOHB has been disrupted, resulting in two populations of slowly interconverting enzyme. Therefore, the kinetics of flavin reduction are biphasic, with enzyme carrying the deprotonated form of pOHB reducing rapidly, and enzyme carrying the protonated form reducing slowly at a rate that depends on the final pH of the solution. Because the two forms can interconvert by a pH-dependent mechanism, there must be an alternate pathway for substrate protonation and deprotonation in the His72Asn mutant. The nature of the two enzyme forms, the mechanisms for their interconversion, and the structural interpretation of these mechanisms are described below, followed by implications for the evolution of the flavoprotein hydroxylases.

Correspondence between the Reduction Rates of His72Asn and the Flavin Conformation. Rapidly and slowly reducing forms of the His72Asn mutant enzyme were detected in double-mixing experiments. In single-mixing experiments, two distinct enzyme forms were also detected. Identical kinetics were obtained for the isomerization of the rapidly and slowly reducing forms of the mutant enzyme and for the interconversion of the two spectral forms. Flavin absorbance spectra of PHBH have been reliably correlated with the crystallographically observed flavin conformation (*3*, *4*), and this correlation allowed us to assign the flavin conformation of the slowly reducing form of the enzyme as in, and the rapidly reducing form as out. This assignment agrees with our earlier assignment based on the kinetics of quenching of the fluorescence of FAD by NADPH (*11*). We have also shown that the rapidly reducing enzyme form has the phenolate of pOHB bound, while the slowly reducing enzyme has the phenolic form of pOHB bound. Therefore, our single-mixing stopped-flow experiments monitored the movement of the flavin between the in and the out conformations, and this movement was caused by proton transfers.

Mechanisms of Interconversion. Several mechanisms were considered for protonation/deprotonation of pOHB bound to the enzyme. One was buffer catalysis, in which protons are donated from the conjugate acid of the buffer to the phenolate of pOHB to convert rapidly reducing enzyme to slowly reducing enzyme, or removed from the phenolic oxygen of pOHB by the conjugate base of the buffer to convert slowly reducing enzyme to rapidly reducing enzyme. If buffer catalysis were the mechanism for pOHB deprotonation, the observed rate constant for isomerization should have increased with the increase in buffer concentration in our experiments. However, we found no significant difference in the kinetics of isomerization determined at different buffer concentrations. Therefore, buffer catalysis can be eliminated as a mechanism for protonating or deprotonating pOHB.

Another mechanism considered for isomerization involved ligand exchange, where pOHB is the carrier of a proton or of a negative charge. For example, if the rapidly reducing enzyme form could not protonate the bound phenolate of pOHB to form slowly reducing enzyme, it is possible that protonated pOHB from solution could exchange with bound deprotonated pOHB to generate the slow enzyme form. In this case, the kinetics of isomerization should exhibit a dependence on pOHB concentration. No concentration dependence was observed, and pOHB exchange was eliminated as a mechanism for isomerization.

With the elimination of buffer and pOHB as possible reactants, the only other species in solution that could protonate or deprotonate the enzyme are hydronium and hydroxide. Our experiments addressed two distinct but related reactions: in acidic solution, the protonation of the deprotonated form of the enzyme having the flavin in the out conformation, and in basic solution, the deprotonation of the protonated form of the enzyme having the flavin in the in conformation. A simple bimolecular reaction between the deprotonated out form of the enzyme and hydronium ion was suggested by the linear dependence of the observed rate constant on hydronium ion concentration at the lower pH values.[2] The observed rate constant of the reaction of the protonated (in) conformation with hydroxide approached a limiting value at high hydroxide concentrations, suggesting that hydroxide ion reacts with the enzyme in two steps. At intermediate pH values, both pathways contribute to the relaxation of the system, requiring a rate equation that combines the terms from the rate equations describing both pH extremes (eq 1).

Distribution of Enzyme Species at High pH. Our results explain the previously observed limit to the maximum amount of rapidly reducing His72Asn that could be formed by increasing the pH. At low pH, when the pOHB bound to the enzyme is in the phenolic form, the enzyme is almost completely in the slowly reducing form. However, at the high pH extreme, only a maximum of about 80% of the enzyme is in the fast form; the remaining 20% of the enzyme reduces slowly (*11*). The two-step reaction of hydroxide with the enzyme that is illustrated in Scheme 1 can account for this phenomenon. According to this scheme, an increase in the hydroxide concentration can only shift the equilibrium distribution of enzyme in the first reaction while the equilibrium position of the unimolecular second step cannot be altered by a change in hydroxide concentration. Our kinetic analysis, combined with the known pK_a of 8.0 controlling the overall isomerization, gave values of $k_3 = 4.8\ s^{-1}$ and $k_4 = 0.9\ s^{-1}$. An equilibrium constant (K_2) of 5.6 can be calculated for the second reaction step of pOHB by hydroxide. Our estimate of the equilibrium constant predicts that there will be a maximum of ~85% of the enzyme in the fast form, in close agreement with the experimental observation.

Interpretation of the Reaction of Hydroxide with the In Conformation. The observation that the isomerization rate constant approaches a saturating value as the hydroxide concentration increased indicates a two-step reaction. The site of this reaction is suggested by the documented behavior of hydroxylases and by crystal structures. Monovalent anions such as bromide and azide have long been known to inhibit flavoprotein aromatic hydroxylases (*26*, *27*) and bind at three sites identified crystallographically in PHBH. It is reasonable to suppose that the reactive hydroxide ion that was detected kinetically reacts with the enzyme at one of these sites. Because the proton inventory for the reaction of hydroxide is linear, only one reaction site is likely to be involved in the isomerization reaction. There are two crystal structures of wild-type PHBH that each show three bromide binding sites (*3*). One site is near the backbone of residues Ile43 and Arg44. Another site is near the peptide backbone at Tyr385. The third site is on the *re*-side of the flavin, near N10. The anion binding sites near Ile43, and at the backbone of Tyr385, appear to be too distant from the 4-hydroxyl of pOHB to facilitate the deprotonation reaction without a considerable conformational change and, therefore, are considered unlikely to be the sites of action of the critical hydroxide. The most likely chemically relevant site for the reaction of hydroxide ion is on the *re*-face of the flavin. Not only is this the site nearest to the substrate, but crystal structures of PHBH with the flavin in the in conformation (in the absence of monovalent anions) show electron density from a water molecule at this site. The water molecule found over the face of the flavin [717H in 1pbe (*28*)] is in a region of high electrostatic potential (*29*) and the positive dipoles of the peptide backbones of residues 291, 295, 297, and 298 point toward this molecule. It makes a hydrogen bond with Gly 298, and also makes a hydrogen bond to another water (597H in 1pbe) that is in contact with bulk solvent.

Hydroxide is an extremely strong base and reacts in solution via proton transfers through its intervening shell of solvation and the extended network of hydrogen bonds in water. Hydroxide would react with the in form of the His72Asn−pOHB complex by deprotonating the network of hydrogen-bonded water molecules that extends from the solvent into the anion binding site, transiently generating a negative charge on water 717H bound over the *re*-face of the isoalloxazine. Due to the extreme basicity of hydroxide, the short-lived hydroxide generated at the anion binding site would react with the amide group of Gly 298, and the negative charge would be dispersed between these two rapidly equilibrating centers (Scheme 1, intermediates in brackets). As described above, the protein environment of the anion binding site provides electrostatic interactions that could partially stabilize the short-lived negative charge. This equivalent of strong base located over the isoalloxazine would subsequently be quenched when the residue separating it from the phenolic oxygen of pOHB (Pro293, see below) moves, allowing the rapid transfer of a proton from pOHB.

The distance from the center of the phenolic oxygen atom of pOHB to the center of the water molecule bound over the face of the flavin is ~6 Å. The water/hydroxide must move ~3.5 Å to achieve a center-to-center distance of ~2.5 Å to remove a proton from pOHB. To make contact with pOHB, water/hydroxide must move from the face of the flavin past Pro293 and Ala296 (Figure 5). This requires the movement of the peptide carbonyl of Pro293, which has been proposed to trigger flavin movement (*30*). Our value of k_3 suggests that this conformational change occurs with a rate constant of $4.8\ s^{-1}$. The substantial solvent kinetic isotope effect on this reaction, and the linear proton inventory imply that the conformational change is activated by breaking a single hydrogen bond with an isotope effect of 2.3. This would be most consistent with breaking a hydrogen bond between two groups of similar pK_a values. Such a condition might be met by the phenolic oxygens of pOHB and Tyr201.

Interpretation of the Reaction of H^+ with the Out Conformation. The mutant enzyme with the phenolate of pOHB bound appears to have the flavin in the out conformation. In the out conformation, crystal structures of the wild-type enzyme show that three water molecules derived from solvent occupy the cavity vacated by the isoalloxazine and could have access to pOHB. This represents the likely pathway for protonating the phenolate of pOHB in the high-to-low pH jump experiments with His72Asn. The rate constant for this reaction was $1.4 \times 10^7\ M^{-1}\ s^{-1}$, which is 3 orders of magnitude lower than the rate constant for typical solution proton transfers (*31*). This implies that the reaction cross section for the bimolecular protonation/deprotonation reaction is small, consistent with the small area that would be presented by the phenolate oxygen of pOHB. The solvent isotope effect of the proton transfer reaction is small (1.2), which would be expected for a transfer of a proton between

[2] A reviewer suggested that an unspecified aspartate or glutamate could be the primary proton acceptor and that the linear dependence of k_{obs} with hydronium concentration was due to the pH being well above the pK_a of the acid side chain. However, this mechanism requires a subsequent proton transfer to pOHB, because it is the pK_a of pOHB that controls the position of the final equilibrium (*11*). The crystal structure shows that there are no acid residues that could perform a direct proton transfer near pOHB. The only alternative is that the hypothetical acid side chain is at a remote site and a more global protein conformational change allows protonation of pOHB by the solvent. Since there is no evidence for a critical aspartate or glutamate, and there is no evidence for a long-range protein conformational change, we feel that this mechanism is less appealing than our proposal of the direct reaction of hydronium ion.

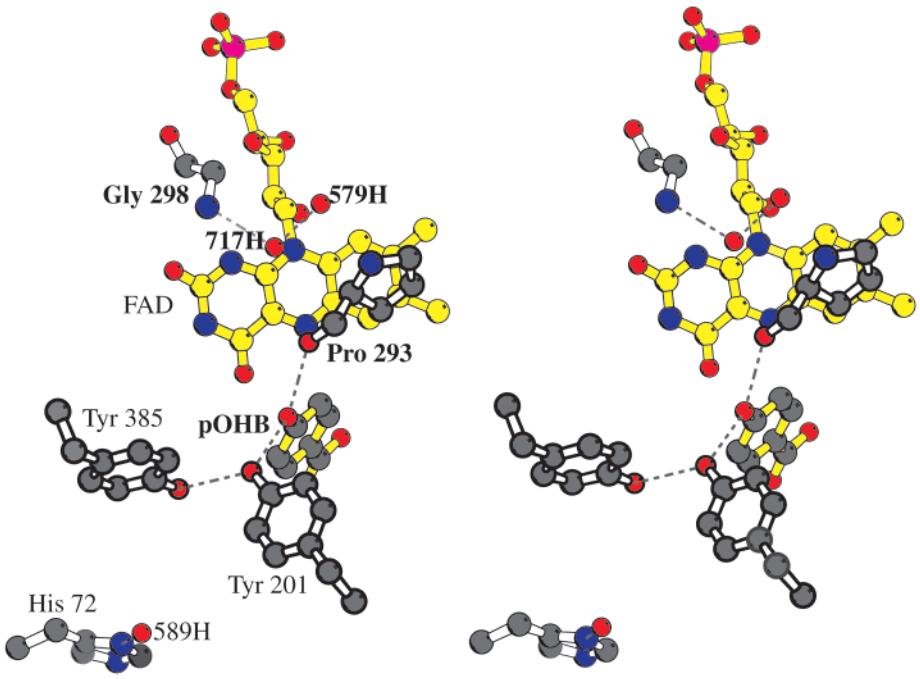


FIGURE 5: Stereoview of the postulated pathway for the hydroxide reaction. Coordinates for the wild-type PHBH structure (1pbe) at 1.9 Å resolution (*28*) are used to illustrate the possible hydroxide reaction pathway. The flavin is in the in position and is viewed from the *re*-face. Hydroxide would deprotonate water molecule 717H by removing a proton via the network of hydrogen bonds that extends to bulk solvent (right side of the isoalloxazine) through water 579H. The short-lived hydroxide would be in excellent position to deprotonate the amide of Gly 298, so it is expected that the negative charge would be distributed across at least these two centers before Pro293 moves, allowing the phenolic proton of pOHB to be removed.

two groups of vastly different pK_a values, such as hydronium ion and the phenoxide of pOHB.

Implications for the Evolution of Flavoprotein Hydroxylases. PHBH has evolved an intricate system that permits the rapid reduction of the flavin by NADPH only when an aromatic ligand that ionizes at the 4-position is present (*11*). This system allows the enzyme to discriminate between pOHB and the close analogue *p*-aminobenzoate (a vital metabolite) in the catalytic cycle at a stage prior to their direct chemical involvement. The chemical reactivity of the aromatic substrate is not relevant until the oxidative half-reaction, which occurs after NADPH has been consumed. The enzyme accomplishes this recognition by linking the flavin conformation to the charge of the 4-substituent of the bound benzoate that can be deprotonated by the proton-transfer network. Like PHBH, the crystal structure of phenol hydroxylase shows that the flavin can adopt two positions, in and out (*22*). Thus, it appears that flavin movement could be a general feature of flavoprotein aromatic hydroxylases.

While all aromatic hydroxylases incorporate oxygen via electrophilic aromatic substitution reactions (*32*) and, therefore, are likely to activate phenolic substrates by deprotonation, the specific elaborate means for linking flavin reduction to the proton transfer network found in PHBH may not be a general feature of hydroxylases. Nevertheless, for all flavoprotein aromatic hydroxylases so far studied, reduction by reduced pyridine nucleotide is stimulated by the presence of substrate. Phenol hydroxylase does not have a proton transfer network analogous to that of PHBH and probably uses Asp 54 as a proton acceptor/donor during the oxidative half-reaction (Dong Xu, University of Michigan, personal communication). Our sequence alignment of flavoprotein monooxygenases shows that none of the residues known to be critical for proton transfers in PHBH (Tyr201, Tyr385, and His72) or phenol hydroxylase (Asp 54) are conserved, suggesting that different methods for activating the aromatic substrate have evolved for individual flavoprotein hydroxylases. Crystallography has demonstrated the structural similarity of PHBH and phenol hydroxylase. The appearance of conserved motifs and numerous other residues in sequence alignments among other hydroxylases strongly suggests that the aromatic hydroxylases are structurally similar and constitute a family that has evolved from a common ancestor. The different strategies for dealing with substrate protons thus represent divergent evolutionary pathways. Presumably, both PHBH and phenol hydroxylase evolved from an ancestral hydroxylase that lacked a specialized mechanism for dealing with protons. The differences in proton handling reflect the different needs for substrate specificity. Whereas PHBH probably requires high substrate specificity because the substrate analogue *p*-aminobenzoate is a folate precursor, phenol hydroxylase does not require similar specificity. The analogous substrate for phenol hydroxylase is aniline, which is not an important precursor to any major metabolites. Therefore, phenol hydroxylase does not need and did not develop a specialized substrate recognition mechanism like that of PHBH. Instead, the presence of phenol controls the reduction of the flavin by an as yet unknown mechanism.

A primordial hydroxylase might have been able to effectively hydroxylate substrates without a complex recognition mechanism for aromatic substrates. The His72Asn mutation may mimic this pre-evolved state of PHBH. In this circumstance, protons would still need to equilibrate with the solvent in the oxidative half-reaction. A mode of protein breathing, such as the movement of Pro293 in the His72Asn mutant, could open a channel for solvent access. This could

explain why the His72Asn mutant is able to hydroxylate pOHB effectively (~80% coupled) with a rate constant of ~2 s^{-1} (Palfey and Dauber, unpublished material), even though the protein can no longer activate pOHB to the phenolate via the proton-transfer network. Interestingly, Pro293 is one of the residues that is conserved throughout the hydroxylase family. Thus, the possibility exists that a similar breathing mode involving the conserved proline could assist proton transfers in other hydroxylases. It is also worth noting that the binding of monovalent anions is a general property of the flavin aromatic hydroxylases. Binding of azide, iodide, bromide, or chloride perturb the kinetics of all these hydroxylases. In fact, these effects have been useful in better characterizing the steps involved in oxygenation (see, for example, ref *26*). Though the sites of anion binding are known only for PHBH, it is probable that other hydroxylases bind anions in a fashion similar to PHBH, including a site separated from the active site by the conserved proline. Thus, the fortuitous proximity of an anion binding site to the conserved proline confers a useful function to a particular protein breathing mode and facilitates proton exchange. Although hydronium and hydroxide have the potential to transiently penetrate the interior of the protein via its normal breathing modes, the reaction of hydroxide with pOHB would clearly be enhanced at sites that transiently favor its presence. Analogous protein breathing modes (often occurring with rate constants between 0.5 and 10 s^{-1}), especially in the vicinity of ion-binding sites, may also constitute an unrecognized factor in the proton-transfer reactions of other enzymes. Such proton-transfer mechanisms may enable biological activities before more specialized mechanisms for proton transfer have evolved.

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SUPPORTING INFORMATION AVAILABLE

Proton inventories of k_a, $k_b + k_4$, k_3, and K_1 as well as graphs of the observed rate of isomerization vs final pL values at different mole fractions of D_2O for the single-mixing pH jumps. This material is available free of charge via the Internet at http://pubs.acs.org.

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