Altered Balance of Half-reactions in p-Hydroxybenzoate Hydroxylase Caused by Substituting the 2'-Carbon of FAD with Fluorine*

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Apo-p-hydroxybenzoate hydroxylase was reconstituted using 2'-fluoro-2'-deoxy-arabino-FAD, a synthetic flavin in which the hydroxyl of the 2'-center of the ribityl chain was replaced with fluorine in an inverted configuration. The absorbance spectral changes caused by the binding of either p-hydroxybenzoate (pOHB) or 2,4-dihydroxybenzoate (2,4-diOHB) indicated that the isoalloxazine of the artificial flavin adopts the more solvent-exposed “out” conformation rather than the partially buried “in” conformation near the aromatic substrate. In contrast, the flavin of the natural enzyme adopts the in conformation when pOHB is bound. Much of the behavior of the artificial enzyme can be rationalized in light of the preference for the flavin for the out conformation, including the weaker binding of pOHB, the tighter binding of 2,4-diOHB, and the slower reactions involved in the hydroxylation of pOHB and 2,4-diOHB. Particularly noteworthy is the enhancement of the reduction of the flavin by NADPH when pOHB is bound to the active site, consistent with the recent finding that the reaction occurs when the flavin adopts the out conformation (Palfey, B. A., Moran, G. R., Entsch, B., Ballou, D. P., and Massey, V. (1999) Biochemistry 38, 1153–1158). Thus, whereas the change that induces the out conformation is detrimental to the oxidative half-reaction, it improves the reductive half-reaction, showing that the control of the flavin position in p-hydroxybenzoate hydroxylase represents a compromise between the conflicting needs of two chemically disparate half-reactions, and demonstrating that the 2'-hydroxyl of FAD can serve as a critical control element in flavoenzyme catalysis.

Synthetic flavins have been used profitably as mechanistic and spectral probes in the study of flavoenzyme catalysis (1). Flavins substituted at the 8-position of the isoalloxazine have been used to study flavoproteins by 19F NMR (2), but the chemistry of the isoalloxazine. In an attempt to introduce a fluorine substituent close to the chemical center of activity yet leave the inherent chemical properties of the isoalloxazine unchanged, 2'-F arabinoflavins have been synthesized in which the 2'-hydroxyl of the flavin ribityl chain is substituted with fluorine and the configuration of the 2'-carbon is inverted for reasons of synthetic expediency. The redox properties of the free artificial flavin are virtually identical to those of natural flavin (3), suggesting that the introduction of a probe nucleus at the ribityl 2'-position has no significant effect on flavin solution chemistry. Surprisingly, although the fluorine probe is chemically isolated from the isoalloxazine reaction center, the catalytic and redox properties of several flavoenzymes are significantly different when the natural flavin is replaced with the 2'-F arabinoflavins or other 2'-derivatives (3–8). These important changes indicate that the 2'-hydroxyl of natural flavins plays an important chemical role as a hydrogen-bonding active site substituent much like a serine residue might. Thus rather than serving in their originally intended role as non-disruptive spectral probes, 2'-F flavins have proven to be best suited for elucidating enzyme chemistry by altering active site interactions.

This paper reports our studies on p-hydroxybenzoate hydroxylase (PHBH)1 in which the natural FAD has been substituted with 2'-F arabinoflavins (2'-F FAD). PHBH is an extensively studied aromatic hydroxylase that catalyzes the conversion of p-hydroxybenzoate (pOHB) to 3,4-dihydroxybenzoate (3,4-diOHB) using molecular oxygen and NADPH (9). The catalytic cycle may be conveniently divided into 2 halves (Fig. 1). In the reductive half-reaction, the oxidized enzyme-bound flavin is reduced by NADPH. When pOHB or certain analogs are bound to the enzyme, flavin reduction is accelerated by many orders of magnitude (10). After hydride transfer, NADP dissociates and molecular oxygen reacts with the reduced enzyme in the first step of the oxidative half-reaction. The reaction of molecular oxygen with the reduced enzyme produces the flavin C4a-hydroperoxide, a very reactive intermediate. This very electrophilic species transfers its distal oxygen atom to the aromatic substrate in an electrophilic aromatic substitution reaction. The immediate (enzyme-bound) products of the reaction are the flavin C4a-hydroxide and a non-aromatic tautomer of the ultimate phenolic product. In subsequent steps, the product tautomerizes to its more stable aromatic form and the hydroxyflavin eliminates water to form the oxidized flavin. For pOHB, the non-aromatic hydroxylation product and its tautomerization reaction are not observed, presumably for kinetic reasons, but the analogous intermediate has been observed with some unnatural substrates.

1 The abbreviations used are: PHBH, p-hydroxybenzoate hydroxylase; pOHB, p-hydroxybenzoate; 2,4-diOHB, 2,4-dihydroxybenzoate; 2'-F FAD, 2'-fluoro-2'-deoxy-arabino-flavin adenine dinucleotide; Tricine, N-tris(hydroxymethyl)methylglycine.

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such as 2,4-dihydroxybenzoate (2,4-diOHB). The intermediate is characterized by a high UV-visible absorbance because of the conjugated product chromophore and the absorbance of the flavin hydroxide (11, 12). The non-aromatic product tautomer is also observed in phenol hydroxylase, a mechanistically related enzyme, and its properties have been characterized (13).

Structures of several PHBH-ligand complexes have been determined by x-ray diffraction (14–17). These studies have shown that the isoalloxazine moiety can adopt two conformations (Fig. 2), termed “in” and “out” (16, 17). The isoalloxazine moiety of structures that have the in conformation is largely shielded from solvent and lies close to the aromatic ligand in a position ideal for substrate hydroxylation. In contrast, when the isoalloxazine moves to the out conformation, it is much more exposed to solvent and is too far from the substrate for the oxygen transfer reaction. The out conformation has been observed under a number of conditions, for instance, in site-directed mutants or with aromatic ligands bearing a 2-substituent. The most significant difference between the in and out conformations is a $\sim 30^\circ$ rotation around the C1’-C2’ bond of the ribityl chain. Thus it might be expected that alterations at C2’ might also cause a change in the flavin conformation of PHBH. This has been demonstrated for wild-type PHBH substituted with arabino FAD when pOHB is bound (5). In that case, the flavin was found in the out conformation by crystallographic analysis. That study also reported some catalytic properties of the substituted enzyme implying that the chemistry of several steps was altered by the change in the flavin. Perhaps the most intriguing finding was that the rate constant for the reduction of the unnatural flavin was significantly higher than in the natural enzyme. Using site-directed mutants, pH, and kinetic isotope effects, it has recently been shown that the flavin of PHBH moves to the out position prior to reduction (18). The results of the studies reported here characterize in detail the changes brought about by altering the 2’-position of FAD and indicate that several steps of the catalytic cycle depend on the conformational balance afforded by the natural 2’-ribityl substituent. Therefore, the 2’-hydroxyl of FAD can be an important participant in catalysis by flavoenzymes indirectly by acting as a regulator of conformational changes.

**EXPERIMENTAL PROCEDURES**

PHBH was overexpressed in *Escherichia coli* containing the plasmid pNE130 (19) and purified as described previously (20). The natural FAD of the enzyme was replaced with 2’-F FAD by the Red-A method (16). 2’-F FAD was synthesized as reported (6). Absorbance spectra were recorded with a Hewlett-Packard 8452a diode array spectrophotometer, a Cary 3 spectrophotometer, or a Shimadzu UV-2501PC spectrophotometer. Reduction potentials were determined in 0.1 M phosphate buffer, pH 7.0, 25 °C using the xanthine/xanthine oxidase method (21). Rapid reactions were studied using a Hi-Tech SF-61 according to methods described previously (22). For studies of the oxidation of the enzyme-pOHB complex, single wavelength absorbance traces were collected from 350 to 500 nm, combined into a single data file, and fit using the program Specfit (Spectrum Software Associates). Single wavelength traces obtained for the reduction reaction were analyzed using Marquardt exponential fitting routines implemented in Program A developed in the laboratory of Professor D. P. Ballou, University of Michigan.

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**Fig. 1. The catalytic cycle of PHBH.** The reductive half-reaction is shown in the *lower half* of the cycle, and the oxidative half-reaction is shown in the *upper half* of the cycle. When $X = -H$, *i.e.* the substrate is pOHB, the non-aromatic hydroxylation product is not observed. When $X = -OH$, *i.e.* the substrate is 2,4-diOHB, the non-aromatic product has a significant lifetime and is observed as a high-extinction species.

**Fig. 2. Active site environment of PHBH.** The *top panel* shows some of the active site residues surrounding the flavin and aromatic substrate in the natural PHBH-pOHB complex (Protein Data Bank code 1pbe (14)). The *bottom panel* shows the same residues in the arabino FAD-substituted PHBH-pOHB complex (Protein Data Bank code 1pdh (5)). In the natural complex, the flavin adopts the in conformation (top), whereas it assumes the out conformation when FAD is replaced with arabino FAD (bottom).
The artificial chromophore has maxima at 456 nm (10.5 mM liberated upon denaturation with SDS. When bound to PHBH, served when the 2\'-F FAD-substituted PHBH were obtained by titrating the enzyme (~20 \mu M) with ligand solutions at 4 °C, pH 6.5, and subtracting the spectrum of the free enzyme from the spectra recorded after ligand addition.

**RESULTS**

**Spectral Properties**—The flavin absorbance spectrum of native PHBH is only slightly perturbed compared with that of free FAD. In that system, the enzyme has a slightly lower extinction coefficient (10.3 m\text{-}M\text{-}1 cm\text{-}1) compared with that of free FAD (11.3 m\text{-}M\text{-}1 cm\text{-}1) at 450 nm, but the absorbance peak positions are essentially unchanged (450 and 374 nm). In contrast, a significant shift in the absorbance maxima was observed when the 2\'-F FAD of the substituted enzyme was liberated upon denaturation with SDS. When bound to PHBH, the artificial chromophore has maxima at 456 nm (10.5 m\text{-}M\text{-}1 cm\text{-}1) and 368 nm (7.98 m\text{-}M\text{-}1 cm\text{-}1), whereas the free 2\'-F FAD has maxima at 448 nm (10.5 m\text{-}M\text{-}1 cm\text{-}1) and 376 nm (8.18 m\text{-}M\text{-}1 cm\text{-}1). The flavin fluorescence intensity of native PHBH is essentially the same as that of free FAD, and is quenched to ~40% upon binding of pOHB. The fluorescence of the 2\'-F FAD-substituted enzyme in the absence of ligands was found to be 5.4 times lower, and the binding of pOHB caused a ~2-fold quenching of fluorescence.

**Ligand Binding**—The binding at 4 °C, pH 6.5, of ligands to the enzyme substituted with 2\'-F FAD caused changes in the absorbance maxima in the visible absorbance spectra (Fig. 3), which enabled the determination of dissociation constants (Table I). The natural substrate of the enzyme, pOHB, bound to the enzyme some 5-fold less tightly than it did to natural PHBH. An alternate substrate, 2,4-diOHB, was also studied. This ligand bound to the 2\'-F FAD-substituted enzyme more tightly than to the natural enzyme by about a factor of 4.

The isosaloxazine moiety of PHBH is conformationally mobile and its position can be influenced by the identity of the aromatic ligand. The position of the flavin in a particular enzyme-ligand complex correlates with the flavin difference spectrum observed in solution caused by ligand binding (16, 17). When 2,4-diOHB binds to the natural enzyme, a difference spectrum with large increases in absorbance at ~480 and ~390 nm is observed. A very similar change in the absorbance spectrum was observed when 2,4-diOHB bound to the 2\'-F FAD-substituted enzyme. Thus it can be concluded that when 2,4-diOHB is bound to the substituted enzyme, the artificial flavin probably adopts the out conformation. In contrast, the pOHB complex of natural PHBH is characterized by relatively small decreases throughout most of the region in which the flavin absorbs. However, the 2\'-F FAD-substituted enzyme does not exhibit this difference spectrum that is characteristic of the in conformation, nor does it exhibit a difference spectrum similar to that of the 2,4-diOHB complex. Instead, a difference spectrum characterized by a decrease in absorbance in the 490-nm region and an increase centered around 430 nm. Of the structurally characterized PHBH complexes, this spectral change most closely resembles that of the arabinose FAD-substituted enzyme with pOHB bound at the active site (5). The crystal structure of this complex shows that the flavin is in the out conformation, so that we conclude that the isosaloxazine is also out in the 2\'-F FAD-substituted PHBH.

\[ \text{pK}_a \text{ of Bound pOHB—Wild-type PHBH lowers the phenolic pK}_a \text{ of pOHB from its value of 9.3 in solution to a value of 7.4 (22). The pK}_a \text{ of pOHB bound to 2\'-F FAD-substituted PHBH was determined at 25 °C by titrating the artificial enzyme (10.5 \mu M) with pOHB; buffers ranged in pH from 6.0 to 8.5 (22). The absorbance of the phenolate extends to 300 nm, where it was observed with little interference from other chromophores. The variation of the saturating absorbance at 300 nm with pH allowed a pK}_a \text{ value of 6.7 to be calculated (Fig. 4). This value is 0.7 units lower than the value of the pK}_a \text{ in the natural enzyme, demonstrating that flavin position is linked to the ionization state of the ligand as proposed previously (18).} \]

**Hydroxylation Stoichiometry**—The flavin C4a-hydroperoxide intermediate (Fig. 1) can either hydroxylate the aromatic substrate or eliminate \( \text{H}_2\text{O}_2 \). Natural PHBH efficiently hydroxylates pOHB so that \( \text{H}_2\text{O}_2 \) production is barely detected. A variety of factors can influence the relative rates of these two competing reactions, thus influencing the hydroxylation stoichiometry. We found that PHBH substituted with 2\'-F FAD-hydroxylated pOHB with slightly lower efficiency than the natural system. In assay mixtures in which pOHB was the limiting reagent, the ratio of pOHB consumed to NADPH consumed (as measured by the change in absorbance at 340 nm) allowed the calculation of the hydroxylation stoichiometry. At pH 6.5, 25 °C, a value of 90% was found. At pH 8.0, 25 °C, \( \text{pOHB hydroxylation was less effective, with 77% of the NADPH consumed resulting in the hydroxylated product. The natural enzyme hydroxylates 92% effectively under these conditions (23). The lower hydroxylation efficiency of the artificial enzyme at higher pH indicates that the elimination of \( \text{H}_2\text{O}_2 \) from the flavin hydroperoxide is faster, which we attribute to the base-catalyzed deprotonation of the flavin N5.} \]

**Reactions of Reduced Enzyme with O\(_2\) and Hydroxylation of pOHB—Free reduced native PHBH reacts with \( \text{O}_2 \) to form oxidized enzyme and \( \text{H}_2\text{O}_2 \) with a bimolecular rate constant of \( 2.6 \times 10^4 \text{ M}^{-1} \text{ s}^{-1} \) at pH 6.5, 4 °C. No intermediates are detectable when the ligand-free enzyme reacts (11), although it has been proposed that a flavin semiquinone-superoxide pair and a flavin hydroperoxide are intermediates (24) that are unobservable because of kinetic reasons. Free reduced 2\'-F FAD-substituted PHBH is also oxidized by \( \text{O}_2 \) without the formation of observable intermediates. However, the reaction is nearly an order of magnitude slower, with a rate constant of \( 3.4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1} \).

The oxidative half-reaction of PHBH in complex with a number of substrates has been extensively studied by stopped-flow methods. When the reduced enzyme-substrate complex is mixed with oxygen-containing buffer at 4 °C, pH 6.5, absorbance changes between 300 and 500 nm allow the observation of

| \( \lambda_{\text{max}} \) (nm)/c (mM\text{-}1 cm\text{-}1) | \( K_d \text{(pOHB)} \) (\mu M) | \( K_d \text{(2,4-diOHB)} \) (\mu M) | \( E_{\text{m7}} \text{(free)} \) (mV) | \( E_{\text{m7}} \) (pOHB) (mV) |
| --- | --- | --- | --- | --- |
| Natural PHBH | 450/10.3 | 9.5 | 21 | -163 | -165 |
| 2\'-F FAD PHBH | 456/10.5 | 49 | 5.5 | 160 | 113 |

*Values for the natural enzyme are from Ref. 22.
reaction intermediates and the determination of their absorbance spectra. We studied the reaction of the reduced 2′-F FAD-substituted PHBH-pOHB complex in the absence and presence of the inhibitor azide. The reactions of the oxidative sequence were slower than those of native PHBH. In the absence of azide, three reaction phases were observed. The first phase, readily observed as an absorbance increase between 340 and 420 nm, was the second-order reaction between reduced enzyme and O_2 to form the flavin hydroperoxide. The linear variation with O_2 concentration of the pseudo first-order rate constant for hydroperoxide formation, determined at 380 nm, gave a value of 1.2 × 10^7 M⁻¹ s⁻¹ for the second-order rate constant. In native PHBH, a value of 2.8 × 10^7 M⁻¹ s⁻¹ has been reported (25). Interestingly, this bimolecular rate constant is nearly 2 orders of magnitude larger than the rate constant for the formation of O_2 in the absence of substrate, so that pOHB binding to the artificial enzyme stimulates the reaction from an abnormally low rate to a normal rate.

Substrate hydroxylation and H_2O_2 elimination by the flavin hydroperoxide was followed with an observed rate constant of 16.8 s⁻¹. The hydroxylation stoichiometry measurements (described above) of 90% product formation is a measure of the partitioning of the flavin hydroperoxide between the hydroxylation and elimination pathways. With that information, values of 15.1 s⁻¹ for the hydroxylation rate constant and 1.7 s⁻¹ for the elimination reaction were calculated. In the native system, the substrate is hydroxylated with a rate constant of 47 s⁻¹ and there is no detectable H_2O_2 formation (25), although an upper limit of 0.9 s⁻¹ has been estimated (22). Thus the modification of the ribityl chain has both slowed the hydroxylation reaction and accelerated the elimination of H_2O_2. The flavin hydroxide formed by the hydroxylation of pOHB eliminated water to form oxidized flavin with a rate constant of 2.1 s⁻¹. In native pOHB, this reaction occurs with a rate constant of 14 s⁻¹, faster by more than a factor of 6.

By collecting data at wavelengths from 330 to 500 nm, it was possible to calculate the absorbance spectra of the intermediates (Fig. 5). The flavin hydroperoxide of the 2′-F FAD-substituted enzyme had a slightly blue-shifted absorbance spectrum with a maximum at 385 nm, compared with 390 nm for native PHBH (11, 25). The spectrum of the flavin hydroxide was blue-shifted compared with the hydroperoxide, with an absorbance maximum at 380 nm, the same as the maximum in native PHBH.

Monovalent anions are inhibitory in many of the reactions in the PHBH catalytic cycle, allowing high concentrations of azide to be used to kinetically resolve the reactions of the oxidative half-reaction (11). In the presence of 0.1 mM azide, the reaction with molecular oxygen is virtually unaffected, as is the case with native PHBH; a rate constant of 1.1 × 10^8 M⁻¹ s⁻¹ was determined. The hydroxylation of pOHB was slowed more than a 10-fold and had a rate constant of 1.4 s⁻¹, an inhibitory effect that is somewhat greater than with the native enzyme. The elimination of water from the hydroxyflavin was also slowed by 0.1 mM azide, but only by a factor of ~3 to a value of 0.61 s⁻¹, in contrast to the 10-fold decrease with the native enzyme. Thus rather than kinetically resolving the conversion of the flavin hydroperoxide to the flavin hydroxide, 0.1 mM azide had the opposite effect. It seems likely that this difference is because of changes in the affinity of azide for the various enzyme forms caused by interactions with the altered ribityl chain.

**Hydroxylation of 2,4-diOHB—PHBH hydroxylates other substrates, although not as efficiently as the natural substrate pOHB. The most extensively studied alternate substrate is 2,4-diOHB (11, 26). An additional intermediate with a high extinction coefficient, called “intermediate II,” forms upon transferring the distal oxygen atom of the flavin hydroperoxide to the substrate. The high absorbance of the intermediate is thought to be the summation of the absorbance of the flavin hydroxide formed in the reaction and the absorbance of the initial non-aromatic hydroxylation product (12). Subsequent tautomerization to the aromatic product causes the loss of this chromophore leaving only the absorbance of the flavin hydroxide in the 300–500 nm region. The high extinction intermediate is not observed in pOHB hydroxylation, probably for kinetic reasons.

The same sequence of reactions in the hydroxylation of 2,4-diOHB that occur in native PHBH were also observed in stopped-flow experiments at pH 6.5, 4°C, with the 2′-F FAD-substituted enzyme. The bimolecular reaction between the reduced enzyme-2,4-diOHB complex with O_2 had a rate constant of 3.32 × 10^8 M⁻¹ s⁻¹ and formed a flavin C4a-hydroperoxide, similar to the native system. The substrate was hydroxylated less effectively by the artificial enzyme, with a rate constant of 2.7 s⁻¹ observed for the consumption of the flavin hydroperoxide. The elimination of H_2O_2 from the flavin hydroperoxide was clearly observed at long wavelengths such as 480 nm where only the oxidized enzyme produced by the elimination reaction absorbs significantly. This reaction phase accounted for 64% of the total oxidized enzyme that was ultimately produced, allowing rate constants of 1.0 s⁻¹ and 1.7 s⁻¹ to be calculated for the hydroxylation and elimination reactions, respectively. Native PHBH hydroxylates 2,4-diOHB with a rate constant of 16 s⁻¹ and H_2O_2 eliminates with a rate constant of 5 s⁻¹, forming only 24% oxidized enzyme in this reaction phase (11). The alteration of the ribityl chain has slowed both reactions of the hydroper-
oxide, with the hydroxylation reaction being more severely inhibited. After correcting for the large amount of oxidized enzyme present, a spectrum of intermediate II was calculated that had an absorbance maximum near 390 nm, similar to the native enzyme. Intermediate II decayed with a rate constant of 0.45 s⁻¹, only marginally slower than in the native enzyme (0.625 s⁻¹). The observed rate constant of this reaction in the natural enzyme is sensitive to the concentration of 2,4-diOHB, presumably because of secondary binding of 2,4-diOHB. It is conceivable that small changes in ligand affinity at this second binding site could be responsible for the kinetic difference. The last reaction of the oxidative sequence, the elimination of water from the flavin C4a-hydroxide, had a rate constant of 0.916 s⁻¹, about 10-fold slower than the reaction in the native system. Azide (0.1 M) had an inhibitory effect on the 2,4-diOHB oxidation sequence, as it did with pOHB as substrate (Table II).

**Flavin Reduction**—The flavin of native PHBH is reduced by NADPH with a rate constant of 50 s⁻¹ at 4 °C, pH 6.5, when pOHB is bound to the enzyme. This reaction is accompanied by charge transfer absorbance of the isoalloxazine-pyridine nucleotide pair that are thought to indicate that the reactants are aligned optimally (22). Substitution of FAD with 2-F FAD-PHBH and the pOHB complex were measured at 4 °C, pH 6.5, the anaerobic oxidase-pOHB, and various concentrations of NADPH. The oxidized flavin absorbance at 450 nm was bleached after a slight lag in a reaction whose observed rate constant approached a value of 187 s⁻¹ at saturating NADPH concentrations, more than 3-fold faster than the value observed in the native system (Fig. 6). A dissociation constant of 160 μM was determined from the half-saturating concentration of NADPH, a value that is slightly lower than that for native PHBH (210 μM). When reactions were observed at 720 nm, charge-transfer absorbance was clearly evident (Fig. 6, inset). The increase in absorbance at 720 nm corresponded to the hydride transfer reaction and was fit with an observed rate constant matching that obtained at 450 nm. The decrease at 720 nm was satisfactorily fit for all NADPH concentrations with a value in the range of 140 to 200 s⁻¹ that was insensitive to NADPH concentration, leading us to ascribe this phase to NADP⁺ dissociation, which is 20 s⁻¹ in the natural enzyme.

The two-electron reduction potentials of the free 2-F FAD-substituted PHBH and the pOHB complex were measured at pH 7.0, 25 °C, using the xanthine oxidase reduction method with indigo disulfonate (Eₚₒ₋ₗ = −116 mV) as an indicator dye. The modification of the ribityl chain of the flavin caused large increases in redox potentials, to −113 mV for the free enzyme and −119 mV for the enzyme-pOHB complex, compared with the corresponding values of −163 and −165 mV for the natural enzyme (22). Thus the alteration of the ribityl chain enhanced the reductive half-reaction in a number of ways: reduction was more favorable thermodynamically, the kinetic barrier to hydride transfer was lower, and the strength of NADPH binding was increased.

**DISCUSSION**

The isoalloxazine moiety of FAD or FMN has traditionally been thought to be the only catalytically important portion of the prosthetic group. However, several enzymes have been identified (3, 5–8) in which the 2'-hydroxyl of the ribityl chain appears to play an important role augmenting the redox chemistry. PHBH clearly belongs to this group of enzymes. In PHBH, the alteration at the 2'-ribityl position changes the conformation of the flavin. Inspection of the structure of natural PHBH with pOHB bound (Protein Data Bank code 1pbe (4)) shows that inverting the configuration of the 2'-ribityl carbon would place a substituent in van der Waals contact with the side chain of Gln-102. The crystal structure of the arabino FAD-substituted PHBH (5) shows that the 2'-OH of that flavin moves to a position that instead enables it to form a hydrogen bond with Gln-102. This altered binding mode of the ribityl chain is responsible for causing the arabino FAD to move to the out conformation; analogous forces would also favor the out conformation of 2'-F FAD, and this appears to be responsible for many of the effects on catalysis. As described below, the position of the flavin influences the ionization state of bound pOHB, the reactivity of NADPH, the strength of ligand binding, and steps in the oxidative half-reaction.

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**TABLE II**

Rate constants determined by stopped-flow experiments for 2'-F FAD PHBH

| Rate constant | Aromatic substrate | pOHB | pOHB + 0.1 mM NaNO₂ | 2,4-diOHB | 2,4-diOHB + 0.1 mM NaNO₂ |
|---------------|--------------------|------|---------------------|----------|------------------------|
| k₁ (s⁻¹)      | 187                | ND   | ND                  | ND       | ND                     |
| k₂ (s⁻¹)      | 170                | 1.2 × 10⁵ | 1.1 × 10⁵       | 3.32 × 10⁵ | 10⁵                    |
| k₃ (m⁻¹ s⁻¹)  | 1.2 × 10⁵          | 1.1 × 10⁵       | 3.32 × 10⁵         | 1.0           | 1.0                     |
| k₄ (s⁻¹)      | 15.1               | 1.0            | 1.0                | 0.51       | 0.03                   |
| k₅ (s⁻¹)      | NO⁺                | 0.45           | 0.45               | 0.016      | 0.0039                 |
| k₆ (s⁻¹)      | 2.1                | 0.61           | 0.61               | 1.7        | 0.91                   |
| k₇ (s⁻¹)      | 1.7                | 0.4            | 0.4                | 1.7        | 0.91                   |

* ND, not determined. Reactions were in 0.1 M potassium phosphate, pH 6.5, 4 °C.  
* NO, reaction not observed.

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Fig. 6. Reduction of 2'-F FAD-substituted PHBH by NADPH.

The reduction of the enzyme-bound 2'-F FAD was observed by mixing the anaerobic enzyme-pOHB complex with anaerobic solutions of pOHB and NADPH (0.054–1.09 mM) in a stopped-flow apparatus at pH 6.5, 4 °C. The enzyme concentration was 15 μM after mixing, and the pOHB concentration remained constant at 2 mM. The bleaching of the flavin chromophore upon hydride transfer was observed at 450 nm; the formation and decay of a charge-transfer complex was evident at 720 nm. The reaction traces in the figure were obtained for 1.09 mM NADPH (after mixing). A fit to the 720 nm trace is also shown, with the absorbance increasing at 163 s⁻¹, the same value derived from the decrease at 450 nm, and decreasing with an observed rate constant of 146 s⁻¹. The NADPH concentration dependence of the observed rate constant for flavin reduction is shown in the inset.
Flavin movement is linked to the ionization state of the phenolic oxygen of pOHB through interactions between the flavin, the side chain of Asn-300, the peptide backbone to the carbonyl of Pro-293, and the phenolic oxygen of pOHB (18, 23, 27). Mutating Pro-293 to the more flexible Ser removes this linkage, allowing the carbonyl oxygen of residue 293 to move freely away from the phenolate of pOHB without driving the conformational change. Relieving the dipole-charge repulsion in the P293S mutant lowers the phenolic \( pK_a \) of pOHB by 0.7 units. In this work, we have shown that substituting FAD in the wild-type enzyme with 2'-F FAD also lowers the phenolic \( pK_a \) of pOHB by 0.7 units. However, instead of breaking the linkage between the flavin position and the protonation state of pOHB, inverting the stereocenter forces the isoalloxazine to the out position. With the linkage between conformation and ionization state (described above) retained, the out conformation lowers the phenolic \( pK_a \) of pOHB. This state of the enzyme-pOHB complex, with pOHB ionized to the phenolate and the flavin in the out conformation, is thought to represent the form that is reactive toward NADPH. In agreement with this, the 2'-F FAD-substituted enzyme reacted almost 4-fold faster than the natural enzyme, and the binding of NADPH was tighter. A similar result has been reported for \textit{arabino} FAD-substituted PHBH (5). These effects are because of the preorganization of the enzyme in the reactive conformation, so that the energy derived from NADPH binding no longer drives the flavin conformational change. Moving the flavin from the in to the out conformation might increase the thermodynamic driving force in the reaction with NADPH while the enzyme is properly configured for reduction, as evidenced by the \( \sim 50 \) mV increase in the flavin potential of the artificial enzyme compared with the natural system. This difference cannot be explained simply by inherent chemical differences between the flavins because the redox potentials of the two free flavins are essentially identical (\( \sim 207 \) mV for FAD and \( \sim 206 \) mV for 2'-F FAD (3)). However, it is also possible that the high redox potential is the result of improved protein-flavin interactions once the artificial flavin is reduced. These possibilities cannot be differentiated at present.

The substitution of 2'-F FAD for FAD alters some of the reactions on the pathway from reduced to oxidized enzyme. In the absence of an aromatic substrate, the reduced artificial enzyme reacts with O\(_2\) an order of magnitude more slowly than the natural enzyme. Because it is thought that the rate-determining step in the oxidation process is the formation of the semiquinone-superoxide pair, the lower reaction rate indicates that this intermediate is less stable because of the alteration to the ribityl chain. Two possibilities should be considered for the origin of this instability: either the inverted stereocenter causes the flavin to adopt a less reactive conformation, or specific stabilizing interactions to the 2'-hydroxyl of the ribityl chain are absent. In the former case, it is expected that the reduced flavin in the out conformation will be less reactive than the in conformation because the environment of the flavin in the out conformation more closely resembles aqueous solution, where flavins react comparatively slowly with O\(_2\) (28–30). Apparently, the active site of the enzyme stabilizes the requisite semiquinone-superoxide intermediate and the rate-determining transition state forming this intermediate, and this stabilizing influence would be unavailable in the out conformation. The change in environment might be expected to lower the potential of the semiquinone/hydroquinone couple. Unfortunately, the semiquinone of PHBH is too unstable to be observed under normal circumstances, so changes to its stability cannot be discerned. Alternatively, it might be imagined that the 2'-hydroxyl of the flavin normally has a direct role in stabilizing the intermediate, perhaps by forming a hydrogen bond to superoxide. However, the structures of the natural PHBH-pOHB complex (Protein Data Bank code 1pe) and the \textit{arabino} FAD-substituted PHBH-pOHB complex (which has the same stereochemistry as 2'-F FAD) show that the 2'-substituents are too far from the si-face of the isoalloxazine, the presumed site of O\(_2\) reaction, to directly interact with nascent superoxide. Therefore, we conclude that the change in the rate of reaction of the free reduced artificial enzyme with O\(_2\) is the result of flavin conformational effects. Interestingly, normal rates were observed for the reaction of O\(_2\) with either the pOHB complex or the 2,4-diOHB complex suggesting that in the presence of these substrates, when the artificial flavin is reduced, the flavin adopts the in conformation.

The flavin hydroperoxide, presumably formed in the in conformation, is less reactive in the artificial enzyme than in the natural enzyme when either pOHB or 2,4-diOHB are used as substrate. Because the isoalloxazine moiety is unaltered in the artificial enzyme, the lower hydroxylation rates cannot be because of changes in the inherent reactivity of the intermediate. Instead, the inversion and substitution at the 2'-ribityl carbon must alter reactivity in a more subtle manner. The propensity of the 2'-F FAD to adopt alternate conformations on PHBH could cause the flavin hydroperoxide to adopt a less-than-optimal position to hydroxylate the substrate. Moving the distal oxygen of the flavin hydroperoxide by only a tenth of an Ångstrom is likely to cause significant changes to the reaction rate. Alternatively, a water molecule bound to the re-face of the flavin in the oxidized wild-type enzyme has been invoked as an active site acid (31), protonating the proximal oxygen during hydroxylation. Small changes in the position of the isoalloxazine could cause chemically important changes in the position or orientation of this putative acid, slowing the hydroxylation reaction. Following substrate hydroxylation, the non-aromatic product tautomerizes. When 2,4-diOHB is the substrate, this reaction is slow enough to observe. The rate of this reaction was not very different between the artificial and natural enzymes, consistent with the notion that it is a tautomerization reaction.

Our results show that the position of the flavin in PHBH has important effects in catalysis. Interestingly, significant rate increases are possible in the reductive half-reaction, as evidenced by the changes caused by substituting 2'-F FAD or \textit{arabino} FAD (5) for natural FAD. Presumably, it should be possible to alter the protein to cause similar changes. However, improving the reductive half-reaction of PHBH has only been achieved at the expense of the efficiency of the oxidative half-reaction. Factors that improve the reductive half-reaction increase the likelihood that the flavin will be in the out conformation, whereas the oxidative reactions require the in conformation to generate the flavin hydroperoxide and shield it from solvent so that it can hydroxylate the substrate. Therefore, the control of the flavin position in PHBH represents a compromise between the conflicting needs of two chemically disparate half-reactions that have been optimized by evolution to maximize the overall hydroxylation of pOHB.

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Altered Balance of Half-reactions in p-Hydroxybenzoate Hydroxylase Caused by Substituting the 2'-Carbon of FAD with Fluorine
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