A Rate-Limiting Conformational Change of the Flavin in *p*-Hydroxybenzoate Hydroxylase Is Necessary for Ligand Exchange and Catalysis: Studies with 8-Mercapto- and 8-Hydroxy-Flavins†

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Received September 11, 2000; Revised Manuscript Received November 30, 2000

ABSTRACT: The FAD of *p*-hydroxybenzoate hydroxylase (PHBH) is known to exist in two conformations. The FAD must be in the in-position for hydroxylation of *p*-hydroxybenzoate (*p*OHB), whereas the out-position is essential for reduction of the flavin by NADPH. In these investigations, we have used 8-mercapto-FAD and 8-hydroxy-FAD to probe the movement of the flavin in catalysis. Under the conditions employed, 8-mercapto-FAD (pK_a = 3.8) and 8-hydroxy-FAD (pK_a = 4.8) are mainly anionic. The spectral characteristics of the anionic forms of these flavins are very sensitive to their environment, making them sensitive probes for detecting movement of the flavin during catalysis. With these flavin analogues, the enzyme hydroxylates *p*OHB efficiently, but at a rate much slower than that of enzyme with FAD. Reaction of oxygen with reduced forms of these modified enzymes in the absence of substrate appears to proceed through the formation of the flavin-C4a-hydroperoxide intermediate, as with normal enzyme, but the decay of this intermediate is so fast compared to its formation that very little accumulates during the reaction. However, after elimination of H_2O_2 from the flavin-C4a-hydroperoxide, a perturbed oxidized enzyme spectrum is observed (E_{ox}*), and this converts slowly to the spectrum of the resting oxidized form of the enzyme (E_{ox}). In the presence of *p*OHB, PHBH reconstituted with 8-mercapto-FAD also shows the additional oxidized intermediate (E_{ox}*) after the usual oxygenated C4a-intermediates have formed and decayed in the course of the hydroxylation reaction. This E_{ox}* to E_{ox} step is postulated to be due to flavin movement. Furthermore, binding of *p*OHB to resting E_{ox} follows a three-step equilibrium mechanism that is also consistent with flavin movement being the rate-limiting step. The rate for the slowest step during *p*OHB binding is similar to that observed for the conversion of E_{ox}* to E_{ox} during the oxygen reaction in the absence or presence of substrate. Steady-state kinetic analysis of PHBH substituted with 8-mercapto-FAD demonstrated that the apparent k_{cat} is also similar to the rate of E_{ox}* conversion to E_{ox}. Presumably, the protein environment surrounding the flavin in E_{ox}* differs slightly from that of the final resting form of the enzyme (E_{ox}).

Detailed structural data on active sites of enzymes can be obtained from both X-ray crystallographic and NMR studies. However, neither of these techniques permits the convenient study of dynamic events involved in catalysis. In this work, we present spectroscopic data that directly probe dynamic events important in catalysis and that complement the previously obtained structural data.

Reconstitution of apoflavoproteins with flavin analogues carrying substitutions with relevant novel characteristics (e.g., hydrogen-bonding capabilities) can often be used to study the environment of the flavin that affects its reactivity; these flavin analogues can also provide information about the solvent accessibility of protein-bound flavin (*1*−*4*). For example, the reaction, of the −SH group of 8-mercapto-flavin with iodoacetamide or with iodoacetic acid, which is accompanied by large spectral changes, has been used to probe the accessibility of solvent to the dimethylbenzene portion of the flavin in several enzymes (*1*). When the rate of this reaction with the enzyme is much slower than with the *free* flavin, the conclusion is that the flavin is not accessible to solvent. The rates of these changes can then be compared with the rates obtained with these flavins *free* in solution. Using this technique, the 8-position of the flavin in such proteins as melilotate hydroxylase, D-amino acid oxidase, old yellow enzyme, flavodoxin, and *p*-hydroxybenzoate hydroxylase, among others, was found to be accessible to solvent, while position 8 of the flavin in L-lactate monooxygenase, glucose oxidase, putrescine oxidase, and riboflavin binding protein appears to be inaccessible. Moreover, X-ray crystallographic studies have confirmed those conclusions in all cases so far examined (*5*−*8*).

The anionic state of 8-mercapto-flavin is a sensitive monitor of the dipole environment of the active site (*3*). In the protonated state (pK_a = 3.8), 8-mercapto-flavins can exist in two tautomeric forms [Scheme 1, (A and C)], while the

† Financial support was received from the U.S. Public Health Service (GM 20877 to D.P.B., GM 11106 to V.M.) and The University of Michigan Rackham Merit Fellowship (to M.O.-M.).

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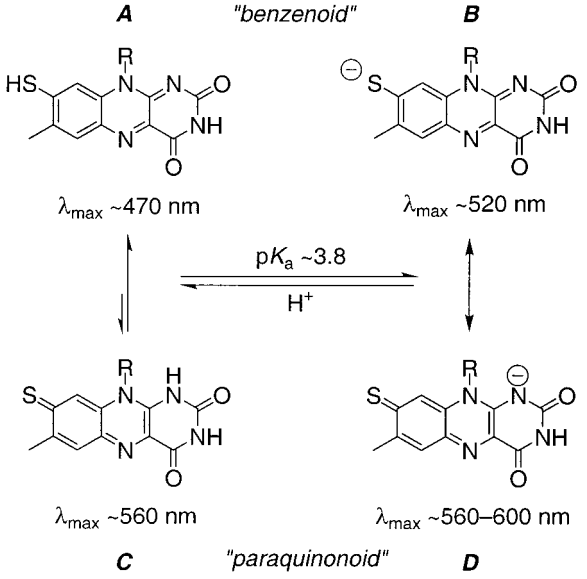
10.1021/bi002139s CCC: $20.00
Published on Web 12/30/2000

Scheme 1

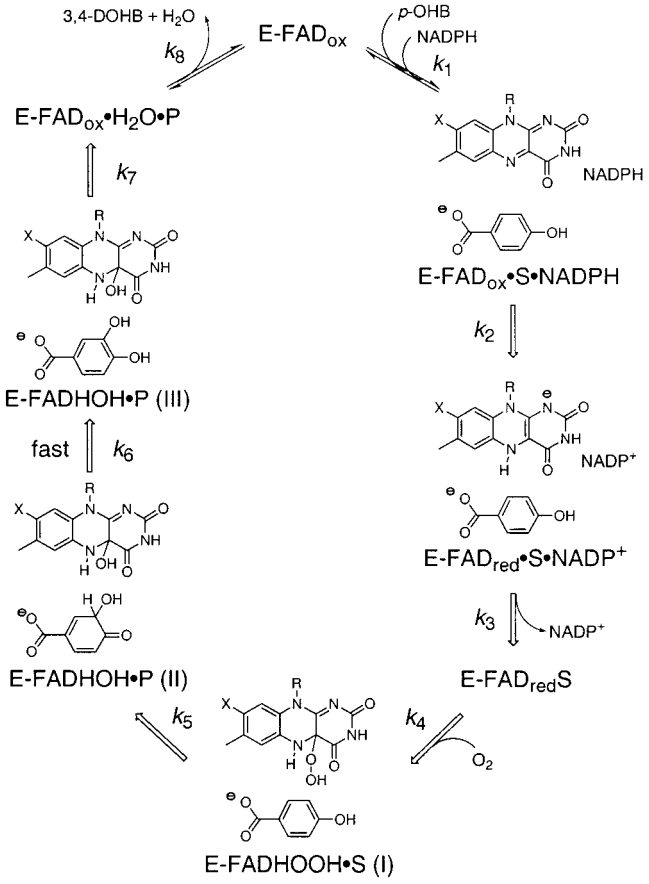


deprotonated state has thiolate and *p*-benzoquinonoid resonance structures [Scheme 1, (B and D)]. Both anionic species are in thermodynamic equilibrium, and influenced by the solvent environment. Because sulfur is less likely than oxygen to participate in conjugation in π systems, the thiolate form (B) is more favorable in solution (*3*). Each of the four species can be differentiated by their absorption spectra, which exhibit characteristic wavelengths of maximum absorbance (see Scheme 1 for λ_{max}). Specific H-bonding interactions or charges at the binding site of the enzyme could preferentially stabilize one form. Thus, the 8-mercapto-flavin can serve as a sensitive indicator of the flavin binding site environment. Flavoproteins that share similar catalytic functions have been found to also have similar spectral properties of bound 8-mercapto-flavin (*3*).

8-Hydroxy-flavin can exist in analogous resonance and tautomeric structures as does 8-mercapto-flavin. The pK_a for 8-hydroxy-flavin is 4.8. The unusually low pK_a of the phenolic 8-OH group, as compared with 2,4-dinitrophenol (pK_a = 7.1), is the result of the electron deficiency of the adjacent isoalloxazine ring (*9*). 8-Hydroxy-flavin has a wavelength maximum at 472 nm (ϵ = 41 000 M^{-1} cm^{-1}). The higher extinction coefficients of the 8-mercapto-flavin and 8-hydroxy-flavin relative to native flavin, and the dependence on the state of ionization of the 8-mercapto and 8-hydroxy groups, indicate that the electronic distribution in the chromophore is strongly altered. These specific properties of 8-mercapto-flavin and 8-hydroxy-flavin, which are very sensitive to the environment of the flavin, can be useful for the study of flavin dynamics in the active sites of flavoproteins.

The flavoprotein *p*-hydroxybenzoate hydroxylase (PHBH,[1] EC 1.14.13.2) catalyzes the NADPH- and oxygen-dependent

Scheme 2



hydroxylation of *p*-hydroxybenzoate (*p*OHB), an important step in the degradation of many aromatic compounds. The overall mechanism is diagrammed in Scheme 2. Crystallographic studies have indicated that the flavin prosthetic group moves between two positions on the enzyme (Figure 1), depending on the ligand that is bound or which active-site residue is changed (*5*, *8*). Movement between these two flavin conformations is essential for catalysis. Evidence is accumulating that the out-conformation—where the isoalloxazine ring system of the flavin has swung out about 31° along the dihedral angle defined by C1′, C2′, and C3′ of the FAD, and is exposed to solvent—is essential for substrate binding dynamics (*5*) as well as for reduction of the FAD by NADPH (*10*, *11*). On the other hand, crystal structures of PHBH (*5*, *8*, *12*) strongly suggest that the flavin must be in the in-conformation for the subsequent hydroxylation reaction to occur. The in-conformation of the flavin sequesters the reactive flavin-C4a-hydroperoxide intermediate from solvent, thus preventing the nonproductive elimination of H_2O_2. The crystal structure of phenol hydroxylase, a flavoprotein monooxygenase from the same family as PHBH, also presents two analogous conformations, termed open and closed, of the flavin in the active site (*13*). It was suggested that these conformational extremes are similarly involved during catalysis by phenol hydroxylase. Indeed, such obligate flavin movements may be common to catalysis in several members of this family of enzymes.

In addition to reduction of the flavin and binding of the substrate to the enzyme, it was also postulated that flavin movement was involved in release of the product and dehydration of the flavin-C4a-hydroxide. To probe this

[1] Abbreviations: PHBH, *p*-hydroxybenzoate hydroxylase from *Pseudomonas aeruginosa*; *p*OHB, *p*-hydroxybenzoate; 2,4dOHB, 2,4-dihydroxybenzoate; 3,4dOHB, 3,4-dihydroxybenzoate; FAD, flavin adenine dinucleotide; apoPHBH, *p*-hydroxybenzoate hydroxylase that has been depleted of FAD; FADHOOH, flavin-C4a-hydroperoxide; FADHOH, flavin-C4a-hydroxide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; P_i, phosphate; E_{ox}, oxidized enzyme; E_{ox}*, intermediate oxidized enzyme; $E^{o\prime}$, redox potential at pH 7.0.

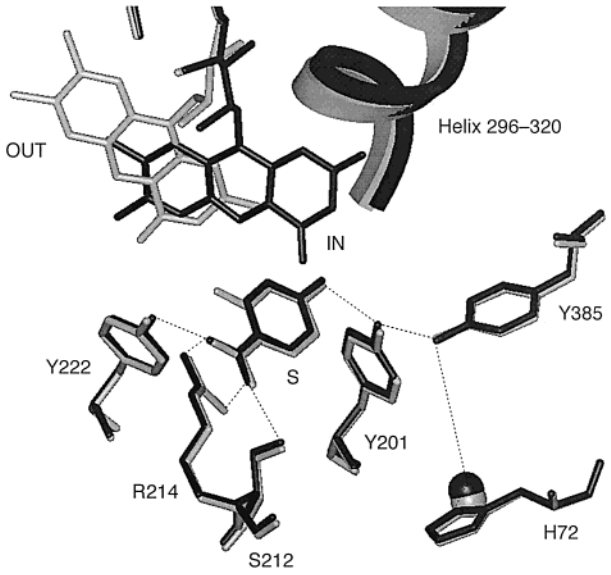


FIGURE 1: Flavin conformations in PHBH. The active site of PHBH contains a positive dipole from the α-helix comprising residues 296−320, three residues (Tyr201, Tyr385, and H72) that are part of the proton network that participates in the ionization of *p*OHB, and three residues in hydrogen bonding distance to the carboxylate group of the substrate (Ser212, Arg214, and Tyr222). The 8-position of the flavin is accessible to solvent when the conformation of the FAD is either in or out. The *si*-side of the flavin is shown. The structure of PHBH in the presence of *p*OHB is shown in black, whereas the structure of PHBH in the presence of 2,4dOHB is shown in gray.

possibility, we characterized the reaction of oxygen with PHBH substituted with reduced forms of 8-mercapto-FAD and 8-hydroxy-FAD (the 8-position is accessible to solvent in either conformation). After the flavin is oxidized, a slow spectral change occurs when PHBH is substituted with 8-mercapto-FAD or with 8-hydroxy-FAD either in the presence or in the absence of substrate. We postulate that this corresponds to the flavin moving from an in-conformation to the out-conformation. Similar spectral changes are observed upon *p*OHB binding to resting PHBH that is substituted with 8-mercapto-FAD. Our studies provide evidence that flavin movement is indeed essential for catalysis and is required for ligand binding and exchange.

EXPERIMENTAL PROCEDURES

Materials. 8-Cl-Riboflavin was obtained from Dr. J. P. Lamboy, University of Maryland, and 8-OH-riboflavin was from Dr. S. Ghisla, University of Konstanz (Germany). Conversion of riboflavin analogues into the corresponding FAD analogues was achieved using FAD synthetase and flavokinase isolated from *Brevibacterium ammoniagenes* as reported in (*14*). 8-Mercapto-FAD was prepared by reacting 8-Cl-FAD that was bound to PHBH with Na_2S (*1*) in 0.1 M sodium pyrophosphate buffer (pH 8.5) containing 0.1 mM EDTA; the extent of the reaction was monitored at 534 nm. The extinction coefficients used for the various free 8-substituted FAD analogues (M^{-1} cm^{-1}) were: ϵ_{448}(Cl) = 10 600, ϵ_{472}(hydroxy) = 41 000, and ϵ_{534}(mercapto) = 30 000.

*p*OHB, 2,4dOHB, and 3,4dOHB obtained from Sigma were recrystallized from water before use. Na_2S and benzyl viologen dichloride were from Aldrich. Other chemicals were obtained as described elsewhere (*15*). Solution concentrations were estimated spectrophotometrically using ϵ_{282} = 16 300 M^{-1} cm^{-1} for *p*OHB (in 1 N NaOH), ϵ_{258} = 13 600 M^{-1} cm^{-1} for 2,4dOHB (in 1 N HCl), and ϵ_{290} = 3890 M^{-1} cm^{-1} for 3,4dOHB (at pH 7.0).

PHBH Purification and Apoenzyme Preparation. PHBH was expressed and purified from extracts of *E. coli* JM105/pIE-130 using published procedures (*16*). ApoPHBH was prepared as reported by Müller and van Berkel (*17*), but with the modifications reported in (*15*) that were implemented to increase the yield of apoenzyme. The purified apoenzyme had no detectable activity in assay mixtures lacking FAD and no observable absorbance at wavelengths greater than 310 nm. The molar absorptivity at 280 nm of apoPHBH used was 73 000 M^{-1} cm^{-1} (*15*).

PHBH with 8-Substituted FAD Forms. ApoPHBH in 0.1 M Tris−sulfate buffer (pH 8.0) containing 1 mM EDTA was reconstituted by incubating it for 1 h at 25 °C with a 1.5−2-fold molar excess of 8-substituted-FAD as reported in (*15*). Each 8-substituted FAD binds tightly to PHBH, as shown by the lack of flavin dissociation from the enzyme during gel-filtration chromatography or ultrafiltration. The methods for determining extinction coefficients for the various types of oxidized 8-substituted-FAD PHBH, as well as the enzymatic hydroxylation and the dissociation constants for *p*OHB, have been described in detail (*16*) and the results reported in (*15*). The redox potentials of PHBH reconstituted with the 8-substituted flavins, in the presence or absence of *p*OHB, were determined as described in reference (*18*) and are reported in (*15*).

Transient State Kinetics. The instruments used during transient state kinetic experiments were described by Ortiz-Maldonado et al. (*15*). The stopped-flow apparatus was made anaerobic by using the protocatechuic acid (PCA)/protocatechuic acid dioxygenase (PCD) system as described in (*19*). All reactions and measurements, unless noted otherwise, were carried out at 4 °C in 50 mM KP_i buffer (pH 6.5).

Transient kinetic studies of the oxidative half-reaction were initiated by mixing reduced enzyme with a buffered solution containing O_2 (final concentrations of 0.062−0.98 mM) as described (*16, 20*). Anaerobic enzyme (∼30 μM) in a tonometer (alone or with substrate present) was reduced by one of two methods, depending on which FAD derivative was used. PHBH reconstituted with 8-mercapto-FAD in anaerobic solutions containing xanthine (0.18 mM) and benzyl viologen (20 μM) was reduced by adding xanthine oxidase (∼40 nM final) from a sidearm of the tonometer. PHBH reconstituted with 8-hydroxy-FAD was reduced by titrating the enzyme with small aliquots of 6 mM sodium dithionite in 60 mM KP_i buffer (pH 7.0). Rate constants for the formation and decay of the transient intermediates were calculated from absorbance traces at various wavelengths (*16*) and from fluorescence emission traces (515 nm cutoff filter) resulting from excitation at 400 nm. Rate constants were calculated from exponential fits of traces recorded at appropriate wavelengths using either the KISS software or Program A, each of which uses the Marquardt algorithm (*21*).

Effect of pH on 8-Hydroxy-FAD Bound to PHBH. Measurement of the apparent pK_a value of the 8-hydroxy-FAD that was bound to PHBH was performed by recording the spectra with a Hi-Tech M300 diode array detector (using a xenon lamp) in 1.25 ms intervals out to several seconds or within the first 3 s after mixing with a Hi-Tech SF-61

spectrophotometer equipped with a tungsten lamp. Enzyme in 10 mM KP_i buffer (pH 6.5) was rapidly mixed with an equal volume of 100 mM buffer at the desired pH. Citrate/phosphate buffer was used for solutions with pH values of 2.65 and 3.25, sodium acetate/acetic acid was used for pH values of 3.60, 4.01, 4.50, 5.00, and 5.49, and KP_i buffer was used for pH values of 6.03, 6.55, and 7.07. The spectra obtained either at 10 ms or within 3 s after mixing were similar and used for these analyses because the enzyme is unstable below pH 5.0. Spectral changes were completed by 10 ms.

Binding Kinetics and Dissociation Constants of pOHB, 2,4dOHB, and 3,4dOHB for PHBH Reconstituted with 8-Mercapto-FAD. The dissociation and kinetic rate constants for ligand binding to PHBH reconstituted with 8-mercapto-FAD were determined in 50 mM KP_i buffer (pH 6.5) at 4 °C using a stopped-flow spectrophotometer. Transient kinetic studies of ligand binding were initiated by mixing the oxidized enzyme (12 μM) with a buffered solution containing various concentrations of ligand (final concentrations between 0.05 and 8.00 mM). Rate constants for ligand binding were calculated from absorbance traces recorded at the wavelength at which the greatest perturbation of the enzyme-bound flavin spectrum occurred upon ligand binding. Plotting the observed rate constants of each of the binding processes as a function of ligand concentration permitted the derivation of the binding model.

Competition Effect of pOHB on Binding 2,4dOHB to 8-Mercapto-FAD PHBH. PHBH substituted with 8-mercapto-FAD (40 μM) was first incubated with 58 μM *p*OHB. Transient kinetic studies of 2,4dOHB binding were initiated by mixing the enzyme that was partially bound with *p*OHB with a buffered solution of various concentrations of 2,-4dOHB (final concentrations of 0.13−4.00 mM). Rate constants for 2,4dOHB binding and *p*OHB release were calculated from absorbance traces recorded at 525 nm as previously described.

RESULTS

General Properties of PHBH Reconstituted with 8-Mercapto-FAD and 8-Hydroxy-FAD. In aqueous solution at neutral pH, because of their low pK_a values (8-mercapto-FAD = 3.8, 8-hydroxy-FAD = 4.8), 8-mercapto-FAD and 8-hydroxy-FAD exist predominantly in the ionized form (Scheme 1). Each has a significantly higher molar extinction coefficient than FAD at the λ_{max} (Figure 2). The molar extinction coefficients at λ_{max} of the UV−visible peaks of PHBH that had been reconstituted with 8-mercapto-FAD and 8-hydroxy-FAD are about 1000 M^{-1} cm^{-1} less than those for the corresponding free flavins (Figure 2). Binding of 8-mercapto-FAD to apoPHBH causes a shift of the λ_{max} of the free flavin from 534 to 546 nm (Figure 2A), and binding of 8-hydroxy-FAD to apoPHBH causes a shift of the λ_{max} from 480 to 488 nm (Figure 2B). On the basis of their spectral properties, the enzyme-bound 8-mercapto-FAD and 8-hydroxy-FAD analogues can be considered predominantly in the thiolate and phenolate forms, like those observed for the *free* flavin analogues [species B in Scheme 1 for 8-mercapto-flavin (*3*)].

We have shown previously (*15*) that binding of *p*OHB to enzyme that has been reconstituted with either 8-mercapto-FAD or 8-hydroxy-FAD is similar to that with native enzyme, and it does not affect the redox potential of the enzyme-bound flavin (Table 1). The dissociation constants of *p*OHB for PHBH range from 9.5 μM (native FAD) to 31 μM (8-hydroxy-FAD) [Table 1, (*15*)], indicating that no major changes in binding properties occur. This is evidence that the substituents on the flavin do not greatly affect the interaction of the enzyme with substrate, as previously reported (*15*). This is also consistent with the crystal structure of PHBH, which shows that the substrate is not bound near the 8-position of the flavin. Binding of *p*OHB to PHBH reconstituted with 8-mercapto-FAD causes a further red-shift of the λ_{max} of the flavin from 546 to 564 nm and is accompanied by a decrease in the molar extinction coefficient of the visible absorption peak (Figure 3). Upon binding of 3,4dOHB (product) to PHBH substituted with 8-mercapto-FAD, the spectrum of the free modified enzyme is perturbed similarly to that observed upon *p*OHB binding, but the changes in the molar extinction coefficient are only about 65% of those with *p*OHB (data not shown). On the other hand, binding of 2,4dOHB to PHBH reconstituted with 8-mercapto-FAD perturbs the flavin spectrum differently (Figure 3), as observed in studies with PHBH substituted with native FAD (*5*). Interestingly, binding of *p*OHB to PHBH reconstituted with 8-hydroxy-FAD causes the peak at λ_{max} (488 nm, ϵ = 40 400 M^{-1} cm^{-1}) to split, giving peaks at 474 nm (ϵ = 28 900 M^{-1} cm^{-1}) and 492 nm (ϵ = 27 700 M^{-1} cm^{-1}) (Figure 3).

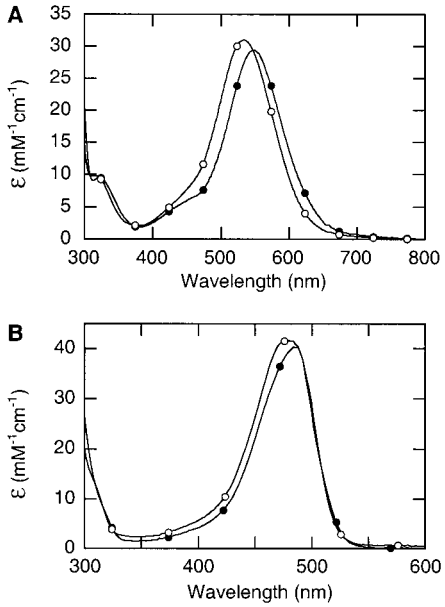


FIGURE 2: Spectra of the free and enzyme-bound flavin analogues. Spectra of PHBH substituted with (A) 8-mercapto-FAD and (B) 8-hydroxy-FAD were recorded in 100 mM NaPi buffer (pH 7.2) at 25 °C. Oxidized form of the enzyme-bound flavin (●) and the *free* flavin analogue (○). *Free* flavin was released from the enzyme by adding 0.1% SDS to the enzyme solution.

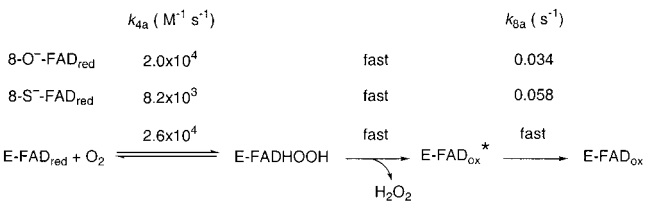
Oxidative Half-Reaction. The reactions of oxygen with PHBH containing the reduced 8-mercapto-FAD or 8-hydroxy-FAD were measured in the absence of substrate. Both of these substituted reduced enzymes convert to the final oxidized forms in biphasic reactions. At the end of the first phase, in a reaction first-order with oxygen (8.2×10^3 M^{-1} s^{-1} for 8-mercapto-FAD; 2.0×10^4 M^{-1} s^{-1} for 8-hydroxy-FAD), the spectrum indicates that an oxidized form of the

Table 1: Thermodynamic and Kinetic Constants[a] for PHBH Reconstituted with 8-Mercapto-FAD and 8-Hydroxy-FAD[b]

property	units	8-substituent CH_3[c]	S^-	O^-
E°′ free	mV	−207	−290	−334
E°′ bound	mV	−163	−259	−304
E°′ bound + *p*OHB	mV	−165	−267	−308
hydroxylation	%	100	100	99
K_d *p*OHB (E_{ox})	μM	9.5	15	31
K_d *p*OHB (E_{red})	μM	21	nd[d]	77
K_d 3,4DOHB (E_{ox})	μM	230	410	nd[d]
K_d 2,4DOHB (E_{ox})	μM	22	290	nd[d]
reductive half-reaction				
K_d NADPH	μM	210	1500	1170
k_2	s^{-1}	52	0.026	0.048
k_3	s^{-1}	25	nd[d]	nd[d]
oxidative half-reaction				
k_{4a}	$M^{-1}\ s^{-1}$	2.6×10^4	8.2×10^3	2.0×10^4
k_{8a}[e]	s^{-1}	nd[d]	0.058	0.034
hydroxylation reaction				
k_4	s^{-1}	2.6×10^5	1.2×10^5	4.2×10^5
k_5	s^{-1}	48	14.3	7.6
k_7	s^{-1}	14.5	5.7	nd
k_8[e]	s^{-1}	nd[d]	∼0.07[f]	∼0.029[g]

[a] Rate constants are from Scheme 2. [b] Most of the thermodynamic and kinetic rate constants for PHBH substituted with 8-mercapto-FAD and 8-hydroxy-FAD, unless otherwise noted, were reported in references *15* and *30*. These values are tabulated here for the convenience of the reader. [c] Values are from reference *16*. [d] nd = not determined. [e] Kinetic rate constant for the isomerization of oxidized enzyme from the in-conformation (E_{ox}*) to the out-conformation (E_{ox}). Values of rate constants were the average of at least four determinations. Rate constants varied ≤5% from one another. [f] The value of k_8 is an estimate based on the complex kinetics observed after 300 ms (Figure 5). The multistep process is due to binding of *p*OHB after release of 3,4dOHB formed during the hydroxylation reaction. Rate constants after the chemical steps are ∼1.9, ∼0.14, ∼0.07, and ∼0.0067 s^{-1}. [g] The value of k_8 is assumed to be small. An accurate estimate of the rate constant is not possible because there are only small changes in absorbance in the wavelengths studied and because of the complex kinetics observed after 300 ms. Rate constants after the chemical steps are ∼2.0 and ∼0.029 s^{-1}.

Scheme 3



flavin is the product. Any putative flavin-C4a-hydroperoxide intermediate that forms without substrate present (*22*) must decay so quickly that none is detected in these experiments. This step is represented by the first phase observed before 1 s at indicated wavelengths in Figure 4A for 8-mercapto-FAD PHBH. The second-order rate constants (k_{4a} in Scheme 3) for the formation of the flavin-C4a-hydroperoxide of the modified enzymes are somewhat affected by the nature of the substituent at the 8-position of the FAD (Table 1). After the formation of the initial substituted oxidized enzyme (E_{ox}*), the final E_{ox} is formed slowly in a process that is independent of oxygen concentration (Figure 4B for PHBH substituted with 8-mercapto-FAD and Figure 4C for PHBH reconstituted with 8-hydroxy-FAD). The rate of the interconversion step (E_{ox}* to E_{ox}) is 0.034 s^{-1} for PHBH containing 8-hydroxy-FAD and 0.058 s^{-1} for enzyme substituted with 8-mercapto-FAD. The spectra of this additional intermediate (E_{ox}*) for the two flavins are shown in Figure 4B,C. These data are consistent with the reaction in Scheme 3.[2] The simplest explanation of the data is that the conformation of the first observed oxidized enzyme (E_{ox}*), detected after the oxygen reaction with reduced enzyme, is somehow different from the final resting oxidized enzyme; thus, with the substituted flavins, a slow conformational change occurs to form the final oxidized species. With FAD, this change is presumably rapid or spectrally silent, so that no E_{ox}* intermediate is observed.

In the presence of *p*OHB, the reaction of oxygen with PHBH substituted with reduced 8-mercapto-FAD or with reduced 8-hydroxy-FAD proceeds about 10-fold faster than when substrate is not present (analogously to WT) (Table 1). For both FAD analogues, this reaction of oxygen with substrate-bound enzyme results in the formation of observable C4a-hydroperoxides, consistent with previous studies of native PHBH (*22*). In the presence of *p*OHB, the kinetics of the reaction of oxygen with PHBH substituted with 8-hydroxy-FAD can be described by two phases and one observable intermediate (8-hydroxy-FADHOOH),[3] whereas the traces for PHBH reconstituted with 8-mercapto-FAD can be described by three phases (Figure 5A) and two observable flavin-C4a-intermediates within the first 300 ms of the reaction [Scheme 2 and (*15*)]. Native PHBH similarly exhibits three phases (*25*). With 8-mercapto-FAD bound to PHBH, an additional prominent slow multistep process is observed after 250 ms (Figure 5A). These extra phases occur after the flavin has been oxidized. The trace at 567 nm shows primarily the formation of oxidized flavin. The first phase in the reaction with oxygen can be seen at 416 nm as an increase in the first 30 ms to presumably form the flavin-C4a-hydroperoxide. The rate of this step is linearly dependent on oxygen concentration, and a biphasic decrease at 416 nm and a biphasic increase at 567 nm follow this. The spectrum of this additional E_{ox} intermediate was calculated from kinetic traces at wavelengths between 390 and 650 nm and is shown in Figure 5B. The intermediate is presumably oxidized enzyme with bound 3,4dOHB, the product of the hydroxylation reaction. Its spectrum is quite similar to that of E_{ox}*, discussed above, presumably with perturbations from the bound product. This intermediate slowly converts to the final oxidized form (E_{ox}) in a multistep process that is independent of oxygen concentration (particularly notable by the increase in absorbance at 416 nm between 200 ms and 20 s). This slow multistep process is probably due to displacement of 3,4dOHB and binding of *p*OHB to the enzyme. In the case of enzyme substituted with 8-hydroxy-FAD, this extra phase is not as prominent between 360 and 600 nm because with this flavin the spectrum of the oxidized enzyme (E_{ox}) with *p*OHB bound is similar to that observed when the oxidized intermediate (E_{ox}*) is formed after the hydroxylation reaction has occurred and 3,4dOHB is bound (not shown). However, notable changes are observed at wavelengths below 400 nm. The slow phase due to the conversion of E_{ox}* to E_{ox} observed for PHBH substituted with either flavin analogue is insensi-

[2] We cannot distinguish whether H_2O_2 is released from the enzyme in step 2 or in step 3.

[3] Supporting Information for the World Wide Web edition.

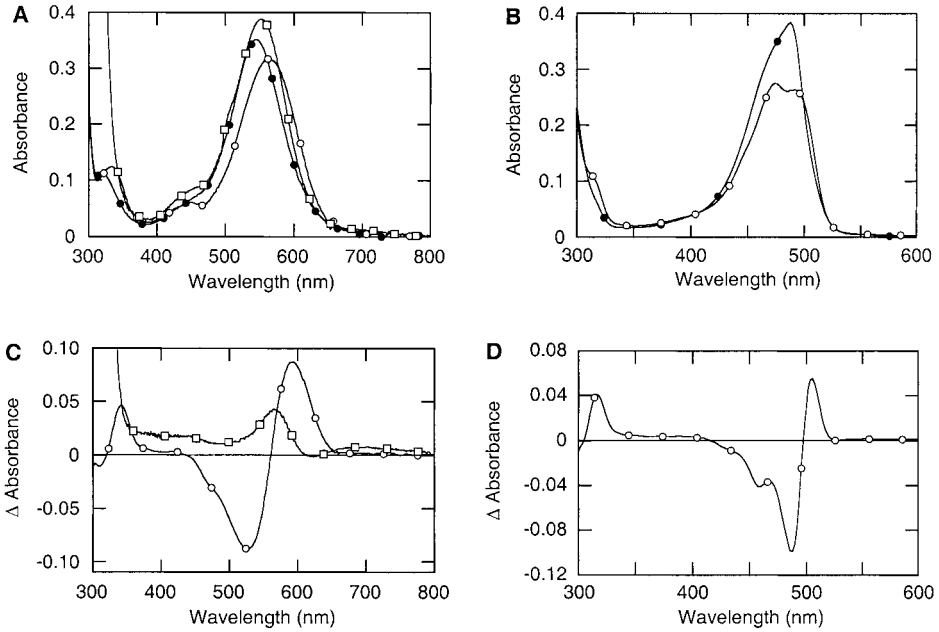


FIGURE 3: UV−visible spectral perturbations of PHBH reconstituted with 8-mercapto-FAD and 8-hydroxy-FAD upon ligand binding. Absorbance spectra for the substrate-free (●), *p*OHB-saturated (○), and 2,4dOHB-saturated (□) PHBH reconstituted with (A) 8-mercapto-FAD and (B) 8-hydroxy-FAD. Difference spectra obtained with the maximum concentrations of ligand used are shown for PHBH substituted with (C) 8-mercapto-FAD and (D) 8-hydroxy-FAD. Dissociation constants were determined by titrating enzyme (7−9.5 μM) with ligand in 50 mM KPi and 1 mM EDTA, pH 6.5, at 4 °C and are reported in Table 1. After absorbance spectra were recorded and corrected for dilution, the difference spectra were obtained by subtracting the free enzyme spectrum from each successive spectrum.

tive to changes in either substrate concentration or pH (data not shown).

Effect of pH on the Spectrum of 8-Hydroxy-FAD Bound to PHBH. To test whether this change in absorbance after formation of the initial oxidized enzyme is due to ionization of the 8-substituent on the FAD, the effect of pH on the spectrum of 8-hydroxy-FAD bound to PHBH was measured (Figure 6). The spectrum of *free* 8-hydroxy-FAD is pH-dependent (pK_a = 4.8) with extensive differences between the protonated (λ_{max} 454 nm, ϵ_{454} = 26 900 M^{-1} cm^{-1}) and anionic forms (λ_{max} 481 nm, ϵ_{481} = 41 600 M^{-1} cm^{-1}) (*9*). The enzyme-bound flavin at pH 7.07 has a λ_{max} at 488 nm (ϵ_{488} = 40 400 M^{-1} cm^{-1}), and the spectrum is pH-dependent, with an apparent pK_a of ca. 3.7. This was determined in rapid mixing experiments, so that the spectrum at low pH could be recorded within 3 s after mixing, which is before denaturation of the protein occurred (Figure 6). The low-pH form has a λ_{max} at 480 nm (ϵ_{480} = 31 700 M^{-1} cm^{-1}). Thus, the pH-dependent spectral changes of the *free* and enzyme-bound 8-hydroxy-FAD are very different. At neutral pH, the flavin is clearly bound in its anionic form. The changes in the spectrum of the enzyme-bound form at low pH are due to protonation of the flavin and/or of protein groups interacting with the flavin. The active site of PHBH is electrostatically positive (*23*) and would be expected to stabilize anionic flavin forms, in keeping with the lowered pK_a of ca. 3.7 for the 8-hydroxy-FAD when bound to the enzyme. If this pK_a is due to protonation of the flavin, the spectral properties at low pH would indicate that the protonated form is predominantly the quinonoid tautomer with the proton located at N1 rather than at O8 (analogous to structure C of Scheme 1). Whatever the explanation of the observed pH effects, it is clear that the observed absorbance changes during the oxidative half-reaction of PHBH reconstituted with 8-hydroxy-FAD (and probably also 8-mercapto-FAD) and the spectral characteristics of the intermediate E_{ox}* cannot be attributed to protonation/deprotonation of the substituent at the 8-position of the flavin.

Binding Kinetics and Dissociation Constants of pOHB, 2,4dOHB, and 3,4dOHB for PHBH Reconstituted with 8-Mercapto-FAD. Binding of *p*OHB, 2,4dOHB, and 3,4dOHB to PHBH containing native oxidized FAD occurs very rapidly and cannot be measured by stopped-flow methods (*24*). Although replacement of the flavin in PHBH by 8-mercapto-FAD causes a dramatic decrease in the rate of ligand binding, the dissociation constants for these substrates and the product are similar for PHBH with either FAD or 8-mercapto-FAD, indicating that no major changes in binding properties occur. Studies with 8-mercapto-FAD enzyme show that the mechanism for ligand binding to the modified enzyme follows a complex, three-step process.

Absorbance traces at 525 and 568 nm monitoring the binding of *p*OHB to PHBH substituted with 8-mercapto-FAD (Figure 7A,B) indicate two observable intermediates (E·L and E*·L in Scheme 4). The first phase seen as a small increase in absorbance at 568 nm, completed by 100 ms, represents the initial binding of *p*OHB to the enzyme and is directly related to the concentration of *p*OHB. A plot of k_{obs} vs [*p*OHB] can be analyzed for a bimolecular rate constant of 1.5 × 10^5 M^{-1} s^{-1} (k_1, Scheme 4) and an off rate of 10.3 s^{-1} (k_2, Scheme 4). The second phase (∼2.0 s^{-1}), k_3 in Scheme 4 and in Figure 7A (small decrease in absorbance at 525 nm, see inset), is independent of *p*OHB concentration, whereas the third phase at 525 nm and at 568 nm between 1 and 200 s (conversion of E*·L to E**·L, Scheme 4) is hyperbolically dependent on the concentration of *p*OHB [with calculated rate constants of 0.040 s^{-1} (k_7 + k_8) at infinite concentration of *p*OHB and 0.022 s^{-1} (k_8, Scheme 4) as the concentration of *p*OHB approaches zero, Figure 7C]. Although the kinetics can be fit with a two-step binding process,

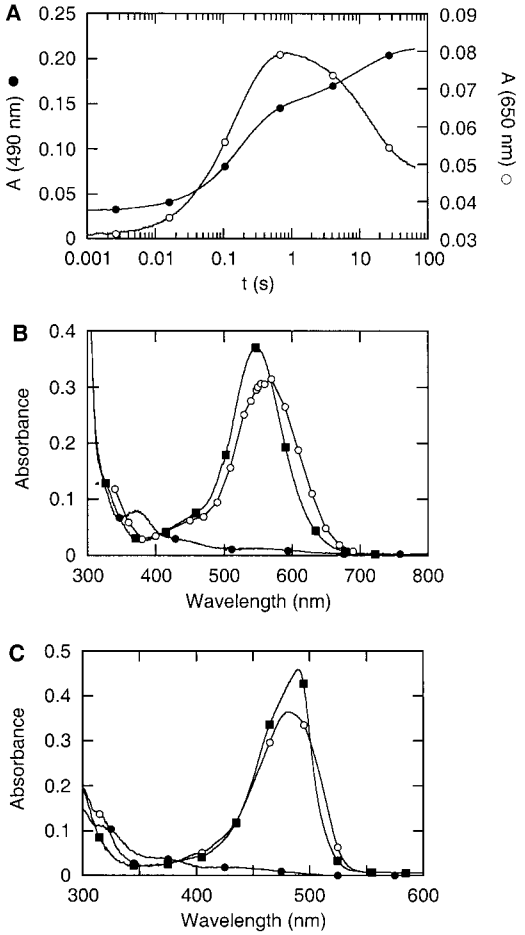


FIGURE 4: Reaction of oxygen with PHBH substituted with reduced forms of 8-mercapto-FAD or 8-hydroxy-FAD in the absence of *p*OHB. Reduced enzyme (15 μM) was reacted with oxygen (0.62 mM) using a stopped-flow spectrophotometer (all concentrations given are after mixing). (A) Absorbance traces at (●) 490 nm and (○) 650 nm are shown. (B) Spectra for observed species of PHBH reconstituted with 8-mercapto-FAD. (●) Reduced 8-mercapto-FAD PHBH, (○) intermediate oxidized 8-mercapto-FAD calculated from kinetic traces at 1 s, and (■) final oxidized 8-mercapto-FAD detected at the end of the reaction trace (80 s). (C) Spectra for observed species of PHBH reconstituted with 8-hydroxy-FAD. (●) Reduced 8-hydroxy-FAD PHBH, (○) intermediate oxidized 8-hydroxy-FAD detected at 5 s, and (■) final oxidized 8-hydroxy-FAD detected at the end of the reaction trace (5 min).

the K_d calculated from the kinetic rate constants does not agree with the thermodynamic K_d obtained from binding titrations. However, the three-step mechanism shown in Scheme 4 does fit the data. Rate constants for all of the individual steps in the binding mechanism were obtained from simulations of the data for the different concentrations of *p*OHB studied (Table 2). Binding of the product, 3,4dOHB, to PHBH reconstituted with 8-mercapto-FAD follows a similar mechanism to that observed for *p*OHB binding to this modified enzyme (Scheme 4). Absorbance traces at 600 nm due to the binding of 3,4dOHB to PHBH substituted with 8-mercapto-FAD (not shown) indicate three phases and two observable intermediates. Analogous to studies (above) with *p*OHB, the third phase in the binding kinetics observed at 600 nm is hyperbolically dependent on the concentration of 3,4dOHB [with calculated *k* values of 0.223 s^{-1} ($k_7 + k_8$) at infinite concentration of 3,4dOHB and 0.050 s^{-1} (k_8) as the concentration of 3,4dOHB approaches zero, Table 2].

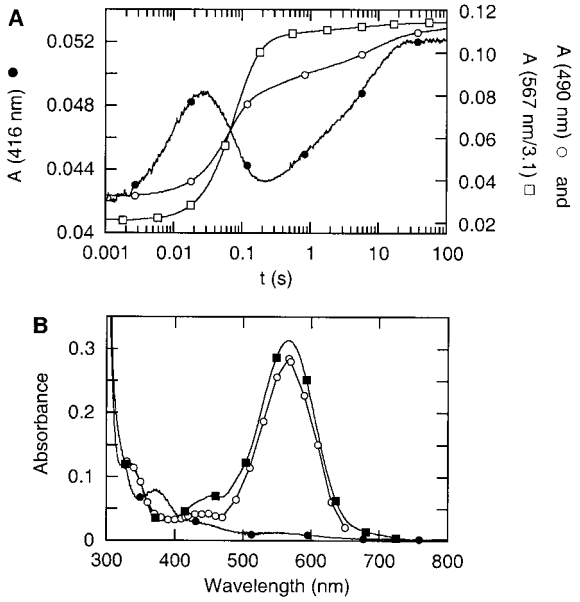


FIGURE 5: Kinetic traces of the oxidative half-reaction of 8-mercapto-FAD PHBH in the presence of *p*OHB. Reduced enzyme (15 μM) with bound *p*OHB (1.0 mM) was reacted with oxygen (0.62 mM) using a stopped-flow spectrophotometer (all concentrations given are after mixing). (A) Absorbance traces at (●) 416 nm, (○) 490 nm, and (□) 567 nm are shown. The absorbance trace recorded at 567 nm was divided by 3.1 to fit on the graph. The concentration of oxygen was 0.62 mM. (B) Spectra for the intermediate and oxidized forms of PHBH reconstituted with 8-mercapto-FAD. (●) Reduced 8-mercapto-FAD PHBH·*p*-OHB, (○) intermediate oxidized 8-mercapto-FAD determined from kinetic traces at 0.261 s, and (■) final oxidized 8-mercapto-FAD·*p*-OHB detected at the end of the reaction trace (256 s). Reaction conditions are the same as in Figure 3.

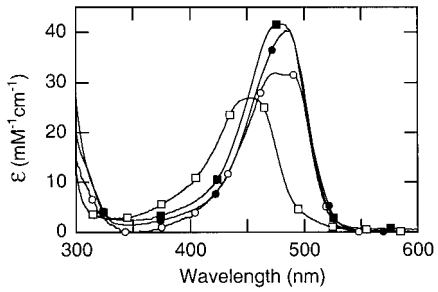


FIGURE 6: Effect of pH on the spectra of PHBH reconstituted with 8-hydroxy-FAD. Enzyme (~6.5 μM) in 10 mM KPi buffer (pH 6.5) was rapidly mixed with an equal volume of 100 mM buffer at the desired pH in a stopped-flow spectrophotometer. The data are the average of the observed spectral measurements collected in the first 3 s after mixing. Indicated are the spectra of bound 8-hydroxy-FAD to PHBH at pH 7.07 (●) and pH 3.60 (○). The spectra of *free* 8-hydroxy-FAD at pH 7.20 (■) and pH 3.25 (□) are shown for comparison. Spectral changes obtained where the ionic strength was kept constant at 0.15 M by addition of Na_2SO_4 were the same as shown here.

The overall binding of 2,4dOHB to PHBH reconstituted with 8-mercapto-FAD proceeds much faster than the binding of *p*OHB. Traces at 567 nm show one phase being completed before 40 ms (Figure 8). A plot of k_{obs} vs 2,4dOHB concentration shows that k_{obs} reaches a limiting value of 800 s^{-1} ($k_7 + k_8$, Scheme 4) with an intercept of 35 s^{-1} (k_8, Scheme 4), and a $K_{d,app}$ of 2.0 mM. The initial binding of 2,4dOHB to the modified enzyme is estimated to occur at $\geq 4 \times 10^5$ M^{-1} s^{-1} (k_1, Scheme 4) on the basis of the initial slope of a plot of the observed rate constants vs 2,4dOHB

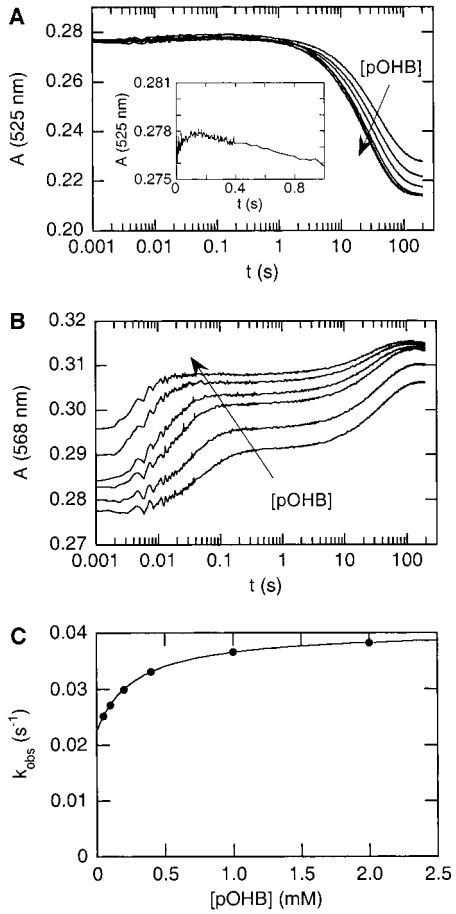
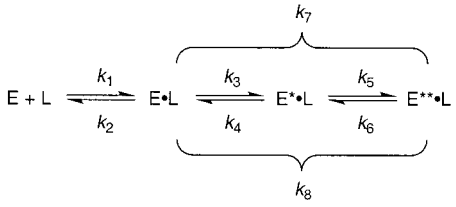


FIGURE 7: Kinetic traces from the binding of *p*OHB to PHBH reconstituted with 8-mercapto-FAD. Oxidized enzyme (12 μM) was rapidly mixed with 0.05−2.00 mM *p*OHB using a stopped-flow spectrophotometer (concentrations indicated are those after mixing). Absorbance at (A) 525 nm (the inset shows an expanded linear scale of the first 1 s for the reaction with 2 mM *p*OHB) and (B) 568 nm can be described with three phases. The first phase in the trace shows a first-order dependence on *p*OHB concentration ($k = 1 \times 10^5$ M^{-1} s^{-1}), and the second phase is independent of the concentration of substrate ($k = \sim 2.0$ s^{-1}). (C) The magnitude of the k_{obs} of the third phase in the kinetic traces has a hyperbolic dependence on *p*OHB concentration. Concentrations of *p*OHB were 0.05, 0.10, 0.20, 0.40, 1.00, and 2.00 mM.

Scheme 4



concentration. Simulation experiments predicted an off rate for 2,4dOHB of 1300 s^{-1} (k_2, Scheme 4). Fitting to the mechanism shown in Scheme 4 yields the rate constants shown in Table 2.

Competition of pOHB on the Binding of 2,4dOHB to 8-Mercapto-FAD PHBH. Crystal structures show that *p*OHB and 2,4dOHB each bind to the same site in PHBH (*5*, *8*). Therefore, if one ligand is bound to the enzyme, it must be expelled before the second ligand can bind. Thus, if the modified enzyme in complex with *p*OHB were mixed with 2,4dOHB, the observed rate constant for 2,4dOHB binding would be expected to be controlled by any slow conformational changes that precede the release of *p*OHB. To test this hypothesis, PHBH substituted with 8-mercapto-FAD was first incubated with *p*OHB (66% saturating concentration), and the rate of 2,4dOHB binding was measured. The overall observations can be described by Scheme 5. Traces at 525 nm show five phases (Figure 9A). The first phase was completed before 40 ms and showed a hyperbolic dependence on 2,4dOHB concentration with rates that correspond to those in Figure 8 for the binding of 2,4dOHB to *free* modified enzyme ($k_1 = 4 \times 10^5$ M^{-1} s^{-1}, $k_2 = 1300$ s^{-1}, $k_7 = 760$ s^{-1}, and $k_8 = 35$ s^{-1}; see Scheme 4 and Table 2). We know the reaction started at $A_{525} = 0.39$; thus, at high concentrations of 2,4dOHB, part of this phase could not be monitored (refer to Figure 8). Moreover, the amplitude of

Table 2: Kinetic Constants[a] for *p*OHB, 2,4dOHB, and 3,4dOHB Binding to PHBH Reconstituted with 8-Mercapto-FAD

	ligand (L)			
rate constant	*p*OHB[b]	*p*OHB[c]	2,4dOHB	3,4dOHB
k_1 (M^{-1} s^{-1})	1×10^5	1×10^5	$\geq 4 \times 10^5$	$\sim 10^5$
k_2 (s^{-1})	10.3	10.3	1300[c]	nd[d]
k_3 (s^{-1})	~2.0	2.6 (2.4)[e]	nd[d]	~2.0
k_4 (s^{-1})	nd[d]	1.4 (0.67)[e]	nd[d]	nd[d]
k_5 (s^{-1})	nd[d]	0.037	nd[d]	nd[d]
k_6 (s^{-1})	nd[d]	0.014	nd[d]	nd[d]
k_7 (s^{-1})	0.018	(0.032)[e]	760	0.173
k_8 (s^{-1})	0.022	(0.094)[e]	35	0.050

[a] Rate constants are from Scheme 4. [b] Experimental rate constants. Values of rate constants were the average of at least four determinations. Rate constants varied ≤5% from one another. [c] Rate constants obtained from simulation experiments. [d] nd = not detected. [e] Values in parentheses are from the competition of *p*OHB on the binding of 2,4dOHB to 8-mercapto-FAD PHBH experiment.

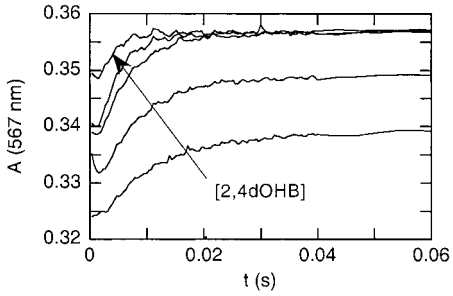
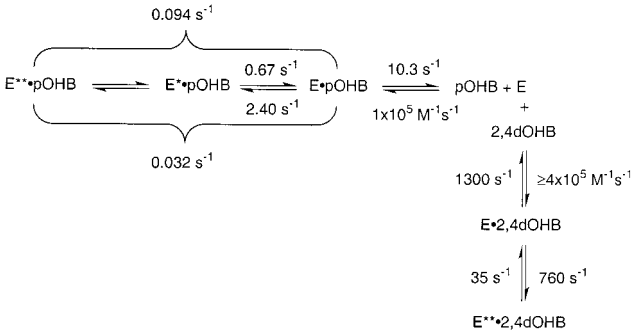


FIGURE 8: Kinetic traces from the binding of 2,4dOHB to PHBH reconstituted with 8-mercapto-FAD. Oxidized enzyme (12 μM) was rapidly mixed with 0.15−4.00 mM 2,4dOHB using a stopped-flow spectrophotometer (concentrations indicated are those after mixing). Absorbance at 567 nm can be described with one phase that saturates with increasing levels of 2,4dOHB concentration (not shown). Concentrations of 2,4dOHB were 0.15, 0.30, 0.60, 1.20, and 4.00 mM (lower to upper curves).

Scheme 5



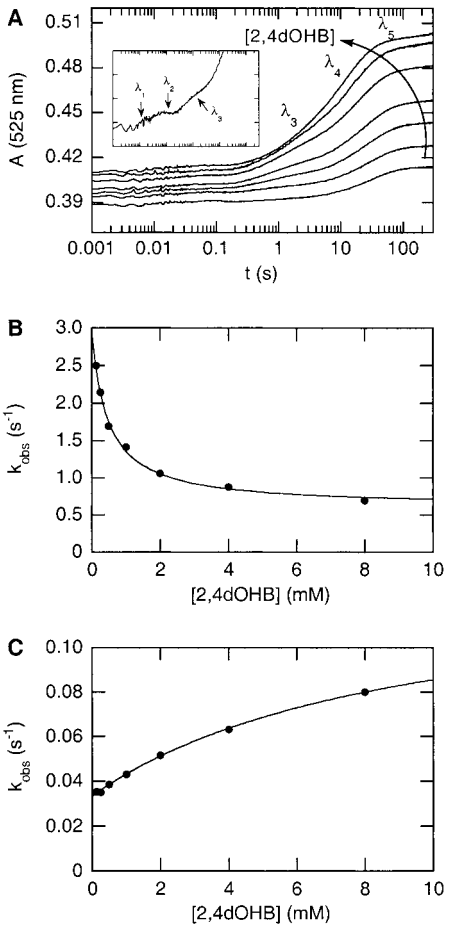
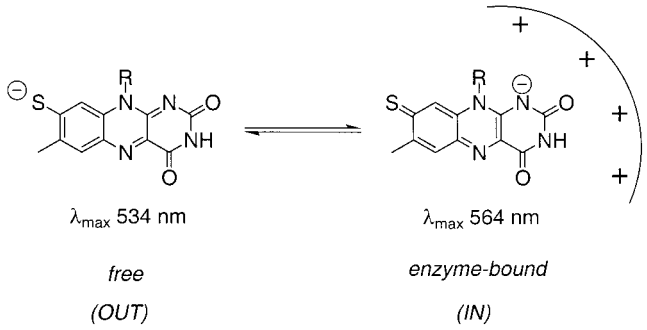


FIGURE 9: Experiments showing the competition of 2,4dOHB to the binding of *p*OHB to PHBH reconstituted with 8-mercapto-FAD. Oxidized enzyme (20 μM) with 66% bound *p*OHB (29 μM) was rapidly mixed with 2,4dOHB at concentrations from 0.13 to 8.00 mM (after mixing) using a stopped-flow spectrophotometer. (A) Absorbance traces at 525 nm can be described with five phases (λ_1, λ_2, λ_3, λ_4, and λ_5). The end of flow is 3 ms. Inset: Expanded absorbance trace (*y*-axis is 7.5-fold more sensitive) for the shot at 0.25 mM 2,4dOHB concentration. (B) The rate of the third phase in the binding kinetics decreases with increasing 2,4dOHB concentration and saturates to a minimum value of 0.67 s^{-1}. (C) The rate of the fourth phase in the kinetic traces saturates with increasing levels of 2,4dOHB concentration. Concentrations of 2,4dOHB were 0.13, 0.25, 0.50, 1.00, 2.00, 4.00, and 8.00 mM.

this phase corresponded to ~34% of free enzyme binding 2,4dOHB (Figure 3C shows the maximum absorbance change expected upon 2,4dOHB binding to PHBH reconstituted with 8-mercapto-FAD), indicating that this phase is indeed due to enzyme not in complex with *p*OHB. The second phase in the competition experiment is characterized by a small absorbance decrease at 525 nm and is independent of 2,4dOHB concentration; it can be ascribed to the off rate of *p*OHB (10.3 s^{-1}) from the E·*p*OHB complex that exists at equilibrium, as shown in the previous section. The k_{obs} for the third phase (between ca. 0.3 and 4 s) decreases, but the amplitude of this phase at 525 nm increases with increasing 2,4dOHB concentration. This is characteristic of a slow isomerization step being required for exchange of ligands, so that at low concentrations of 2,4dOHB, only a small fraction isomerizes, whereas at high concentrations of 2,4dOHB, all of the enzyme isomerizes from E*·*p*OHB to E·*p*OHB as most of the enzyme becomes bound to 2,4dOHB. Thus, one calculates rate constants of 0.67 s^{-1} at saturating

Scheme 6



concentrations of 2,4dOHB and 3.07 s^{-1} as the concentration of 2,4dOHB approaches zero (Figure 9B). When the concentration of 2,4dOHB approaches zero, the observed rate approaches the sum of the forward and reverse rate constants of the isomerization (3.07 s^{-1}) as shown in Figure 9B. Thus, the reverse rate constant can be calculated to be 2.4 s^{-1} as shown in Scheme 5 and Table 2 (Scheme 5 is an expansion of Scheme 4 to accommodate two ligands). This type of analysis has been described by Fersht and Requena (*25*) and by Halford (*26*). The dependence of the kinetics of the fourth phase (between ca. 8 and 30 s) on 2,4dOHB concentration is described by a hyperbola (Figure 9C) with a calculated maximal rate constant of 0.126 s^{-1} and an intercept of 0.032 s^{-1}. This step can be ascribed to the conversion between the form of the enzyme in the E**·*p*OHB to the E*·*p*OHB state (Scheme 5). The fifth phase (0.00515 s^{-1}), of unknown nature, is independent of 2,4dOHB concentration. However, the mechanism in Scheme 5 does not completely account for the observed data. Simulations of the experiments in Figure 9 according to Scheme 5 for the competition of *p*OHB to the binding of 2,4DOHB show that the k_{obs} for the fourth phase should have no dependence on 2,4dOHB concentration. This slight dependence on 2,4dOHB concentration of the fourth phase in the experimental data may be associated with the secondary weak binding site observed in WT enzyme for this ligand (~5 mM) because the ligand concentrations used were very high, in the millimolar range (see Supporting Information).

DISCUSSION

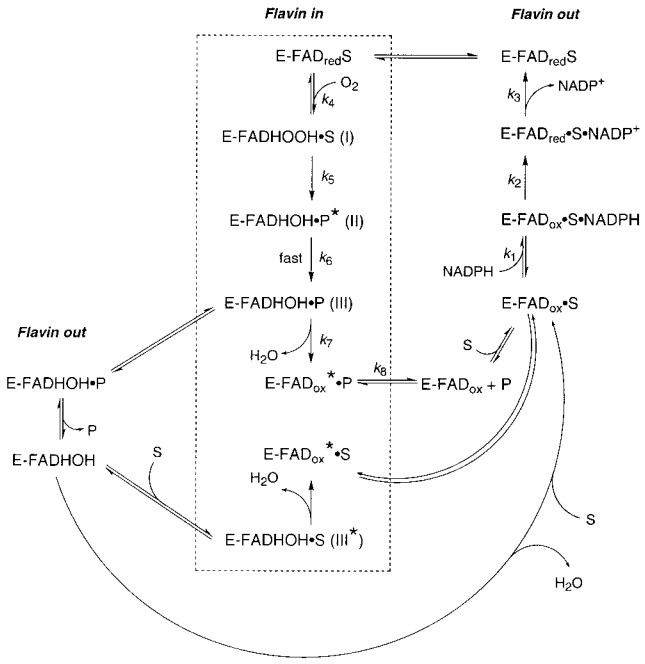
The two resonance structures of the anionic forms of 8-mercapto- and 8-hydroxy-flavins have the negative charge localized either on the 8-substituent or at the N(1) of the flavin (Scheme 6). In solution, the predominant form is the benzenoid structure with the negative charge on the 8-substituent (Scheme 1, B, with spectrum shown in Figure 2). Upon binding to apoPHBH, the λ_{max} of 8-mercapto-FAD is red-shifted to 546 nm, indicating weak stabilization of the paraquinonoid resonance form of the flavin [negative charge on N(1)], probably due to interactions of a positive electrostatic field from the protein (α-helix dipole) with the negative charge of the flavin anion (*5*, *8*) near N(1), C(2)O when the flavin is in the "in" conformation (Scheme 6). The active site is known to have a positive electrostatic character, especially near the N1, C2(O) position of the flavin when it is in the "in" conformation (*23*). Similar spectral perturbations are observed in PHBH reconstituted with 8-hydroxy-FAD. These spectral characteristics suggest that once the flavin analogue is bound to PHBH, the enzyme can stabilize

the paraquinonoid form to some degree, although the benzenoid form is still favored. Binding of *p*OHB to the oxidized form of the substituted enzymes causes the equilibrium to be further perturbed; there is more stabilization of the paraquinonoid species, and in the case of PHBH reconstituted with 8-mercapto-FAD, the paraquinonoid form is much more stable in the presence of *p*OHB.

In addition to the in- and out-conformations of the flavin in PHBH (*5*, *8*), it has recently been reported that the flavin in PHBH can also exist in an alternative middle position when the enzyme is crystallized in the absence of ligands (*27*). The movement of flavin among these conformations in native enzyme is fast; on mixing with substrate, the change is complete within the dead-time of stopped-flow measurements. However, binding of ligands to PHBH that has been reconstituted with 8-mercapto-FAD is much slower, presumably because the movement of 8-mercapto-FAD is slower than that of FAD. The data presented here for PHBH that has been reconstituted with 8-mercapto-FAD or with 8-hydroxy-FAD provide evidence for a conformational change that is necessary for ligand binding and exchange. Presumably, the flavin must move from the middle (or in) position to the out-conformation (E) to allow the ligand to gain access to the active-site channel and bind in the correct orientation. After the ligand is oriented in the active site, the flavin can move to the middle position (E*) and then back in (E**, in the case of *p*OHB). This hypothesis for flavin movement can account for the three steps in the binding of *p*OHB as shown in Scheme 4 (E + L ↔ E·L ↔ E*·L ↔ E**·L). In the case of 2,4dOHB, ligand binding is fit satisfactorily by a two-step process. In all of the cases examined, ligand binding to these modified forms of the enzyme is followed by a slow isomerization of the initial complex formed. Thus, in contrast to native enzyme, the oxidation of the reduced modified flavins by oxygen is followed by a slow absorbance change, either with or without substrate bound. This change in absorbance cannot be attributed to a proton transfer involving the 8-substituent of the flavin analogue, because the spectral changes from the anionic to the protonated states of the enzyme-bound 8-mercapto-FAD or 8-hydroxy-FAD are clearly different from those observed during these conversions to the final oxidized forms of the enzyme. However, the observed spectral changes could be due to a slow partial interconversion between the two mesomeric structures of the anionic form of these flavins that is caused by flavin movement. Moreover, these spectral changes are consistent with the flavin moving to a different position after the oxidation reaction, either in the absence or in the presence of *p*OHB. Flavin movement may also be relevant for the release of products (H_2O_2 in the absence of the aromatic substrate, or H_2O and aromatic product when the substrate is present).

In Scheme 7 we propose an expanded catalytic cycle for PHBH that includes conformational movements of the flavin. Beginning with E−FAD_{ox} in the out position (lower right of the scheme), substrate can bind and the flavin is in equilibrium between the in and the out (and the middle) positions. When substrate and NADPH are bound, the substrate becomes deprotonated, and the flavin is shifted to the out-position where it is reduced to form E−FAD_{red}·S·$NADP^+$ (*11*). When $NADP^+$ is released, the anionic FAD_{red}·S moves back into the electrostatically positive "in"

Scheme 7



site and reacts with oxygen (k_4) to form E−FADHOOH·S. This sequesters the flavin-C4a-hydroperoxide intermediate from solvent and allows hydroxylation to occur without significant nonproductive elimination of H_2O_2. Hydroxylation results in the nonaromatic product (E−FADHOH·P*), which quickly rearomatizes to form the final product (E−FADHOH·P). Two alternative pathways are then available for release of product and dehydration of the flavin-C4a-hydroxide. The hydroxyflavin can lose water to form E−FAD_{ox}*·P, and move to the out position to lose product and continue a second round of catalysis (k_7, k_8). Alternatively, if the hydroxyflavin moves to the out position (E−FADHOH·P), product may dissociate and, when the substrate concentration is high, substrate will rebind before dehydration of the flavin-C4a-hydroxide (E−FADHOH) occurs. This allows the flavin-C4a-hydroxide to move back "in" where it is quite stable as the E−FADHOH·S (III*) complex. In this case, loss of water is slow, and it is not known whether it occurs in the out or the in position. When the flavin is substituted with the 8-hydroxy or 8-mercapto groups, the rates of all of the in−out movements are substantially decreased and can be observed by kinetic spectrophotometry.

This study has provided evidence that when the native FAD is replaced by 8-mercapto or 8-hydroxy-FAD, a rate-limiting conformational change in PHBH is necessary for exchange of ligands during catalysis. Because all of the other observed steps of the reaction with the flavin analogues are analogous to those with FAD, it can be predicted that similar conformational changes are also important for ligand exchange when the enzyme has FAD bound to it; however, in that case they are too rapid to observe. Indeed, the crystal structure shows a suitable channel for substrate to enter the active site only when the flavin is out. Furthermore, this type of conformational change might be a common characteristic of the family of flavin-containing aromatic hydroxylases. Limiting-conformational changes in anthranilate hydroxylase (*28*) as well as in melilotate hydroxylase and in phenol hydroxylase substituted with 1-deaza-FAD (*29*) have been

reported. It is logical to conclude that conformational changes in these enzymes also involve flavin movement that is requisite to catalysis. The rates of flavin dynamics for different members of this family of enzymes may be somewhat different; in some cases, the rate of flavin movement may be fast, as in PHBH, whereas in other cases, such as in anthranilate hydroxylase (*28*), this movement may be much slower so that it can be detected without the use of flavin analogues.

ACKNOWLEDGMENT

We thank Drs. Barrie Entsch and Graham R. Moran for providing the pIE-130 plasmid.

SUPPORTING INFORMATION AVAILABLE

Spectra for the two observable oxidized forms of PHBH reconstituted with 8-hydroxy-FAD in the presence of *p*OHB and binding of 2,4dOHB to native PHBH (3 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

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BI002139S