Flavin Conformational Changes in the Catalytic Cycle of *p*-Hydroxybenzoate Hydroxylase Substituted with 6-Azido- and 6-Aminoflavin Adenine Dinucleotide†

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ABSTRACT: Crystallographic studies have demonstrated two flavin conformations for *p*-hydroxybenzoate hydroxylase (PHBH) [Gatti, D. L., Palfey, B. A., Lah, M. S., Entsch, B., Massey, V., Ballou, D. P., & Ludwig, M. L. (1994) *Science 266*, 110−114. Schreuder, H. A., Mattevi, A., Obmolova, G., Kalk, K. H., Hol, W. G. J., van der Bolt, F. J. T., & van Berkel, W. J. H. (1994) *Biochemistry 33*, 10161−10170]. The isoalloxazine ring system of one conformation (the "out" conformation) is significantly more exposed to solvent and is not in position for necessary catalytic reactions, but when the natural substrate is bound to the enzyme, the isoalloxazine is in the correct position (the "in" conformation) for its chemical function. In this study, several aspects of the function of the conformational change in catalysis were explored using the wild-type and Tyr222Phe forms of PHBH substituted with 6-azido FAD. This flavin served as both a spectral probe and a photolabel. The enzyme containing 6-azido FAD was a relatively effective catalyst for the hydroxylation of *p*-hydroxybenzoate. However, the intermediate reduced 6-azido enzyme was chemically unstable, and a small fraction converted to 6-amino PHBH by the elimination of N_2 during each catalytic cycle. The reduction of 6-azido FAD PHBH by NADPH was almost as fast as the reduction of the natural enzyme. The characteristic spectral change caused by NADPH binding prior to hydride transfer strongly suggests that flavin movement from the "in" to the "out" conformation precedes flavin reduction. Irradiation of 6-azido PHBH with visible light covalently labeled proline 293, an active site residue, under conditions in which the flavin adopted the "in" conformation, while no protein labeling occurred under conditions in which the flavin was "out". The labeled protein exchanged substrate and was reduced by NADPH much more slowly than before photolysis. It is therefore concluded that isoalloxazine movement is required for pyridine nucleotide to gain access to the active site and for the exchange of aromatic ligands.

Conformational changes have frequently been postulated to play roles in the catalytic cycles of enzymes. Often, however, direct structural information is lacking to support such proposals. An unusual and interesting situation exists for the FAD-containing monooxygenase *p*-hydroxybenzoate hydroxylase (PHBH).[1] Recent crystallographic studies have revealed that the isoalloxazine moiety of the flavin can adopt a new, solvent-exposed conformation (referred to here as the "out" conformation; Gatti et al., 1994; Schreuder et al., 1994) significantly different from the relatively buried conformation observed in the first structural studies (the "in" conformation, Figure 1; Van der Laan et al., 1989; Schreuder et al., 1988, 1989, 1992; Lah et al., 1994). The "out" conformations observed thus far are the results of alterations in the structures of the enzyme, the flavin, or the aromatic ligand and have not been observed for the natural WT enzyme−*p*-hydroxybenzoate (pOHB) complex. Examples of crystallographically determined "out" structures include those of complexes with nonnatural substrates, such as 2,4-dihydroxybenzoate (2,4-diOHB) and 2-amino-4-hydroxybenzoate, where the 2-substituent of the ligand favors the "out" conformation by forming a hydrogen bond to the flavin N-3 (Gatti et al., 1994; Schreuder et al., 1994), site-directed mutants such as Tyr222Phe with pOHB bound (Gatti et al., 1994), and the WT−pOHB complex in which the normal FAD has been replaced with *arabino*-FAD, the ribityl-C2 epimer of normal FAD (Van Berkel et al., 1994). When the flavin adopts the "out" conformation, it is too far from the substrate to accomplish the hydroxylation reaction. Nevertheless, 2,4-diOHB is hydroxylated to a large extent (Entsch et al., 1976), which is only possible when the flavin is "in", making it clear that the flavin does move during turnover with this substrate. The observations that the same conformational change can be produced by several different relatively minor structural perturbations and that the flavin moves during the turnover of a nonnatural substrate raise the intriguing question: Is flavin movement a part of the natural catalytic cycle?

The catalytic cycle of PHBH (Scheme 1) can be divided in two halves that may be studied independently [see Entsch and van Berkel (1995) for a review]. In the reductive half-reaction, the aromatic substrate and NADPH bind to the enzyme, rapid reduction of the flavin occurs in the ternary complex, and $NADP^+$ is released. In the oxidative half-reaction, the reduced flavin reacts with molecular oxygen

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[1] Abbreviations: 2,4-diOHB, 2,4-dihydroxybenzoate; 6-N_3 FAD, 6-azido FAD; 6-N_3 FAD Tyr222Phe, Tyr222Phe PHBH substituted with 6-N_3 FAD; 6-N_3 FAD WT, WT PHBH substituted with 6-N_3 FAD; 6-NH_2 FAD, 6-amino FAD; 6-NH_2 FAD WT, WT PHBH substituted with 6-NH_2 FAD; PHBH, *p*-hydroxybenzoate hydroxylase; pOHB, *p*-hydroxybenzoate; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; WT, wild-type enzyme.

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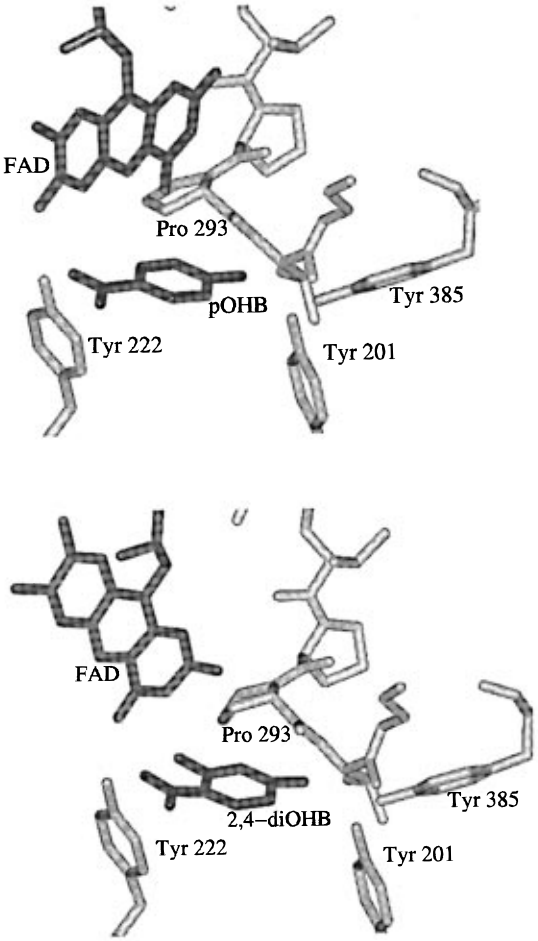


FIGURE 1: Active site of PHBH. The upper figure shows the active site of the WT·pOHB structure (PDB file 1pbe; Schreuder et al., 1989), with most of the protein omitted from the picture. This structure is representative of the several "in" structures available. The lower figure shows the active site of the WT·2,4-diOHB structure (PDB file 1dod; Gatti et al., 1994), which is representative of the many "out" structures available. Note that the ring of proline 293 is close to the isoalloxazine ring when it adopts the "in" conformation but not when it adopts the "out" conformation.

to form the key intermediate, the flavin C4a-hydroperoxide, a very reactive species. The flavin hydroperoxide hydroxylates the aromatic substrate, forming the aromatic product and the flavin C4a-hydroxide. Water elimination from the carbinolamine of the flavin hydroxide regenerates oxidized flavin, completing the catalytic cycle.

A logical role for flavin movement during catalysis is suggested by the solvent inaccessibility of the active site (Schreuder et al., 1989, 1992). This inaccessibility is dictated by the chemical requirement of protecting the unstable flavin hydroperoxide from the solvent. By inhibiting solvent access, the protein prevents the elimination of H_2O_2 as a wasteful side reaction. However, this isolation is not without cost. The pOHB binding site is not accessible to the solvent, so that a transient conformational change is required for substrate exchange (Gatti et al., 1994). Furthermore, the elimination of water from the hydroxyflavin intermediate, a reaction analogous to H_2O_2 elimination, would be retarded by restricted solvent access. To compensate for these restrictions, flavin movement can easily be imagined as a component of both these processes.

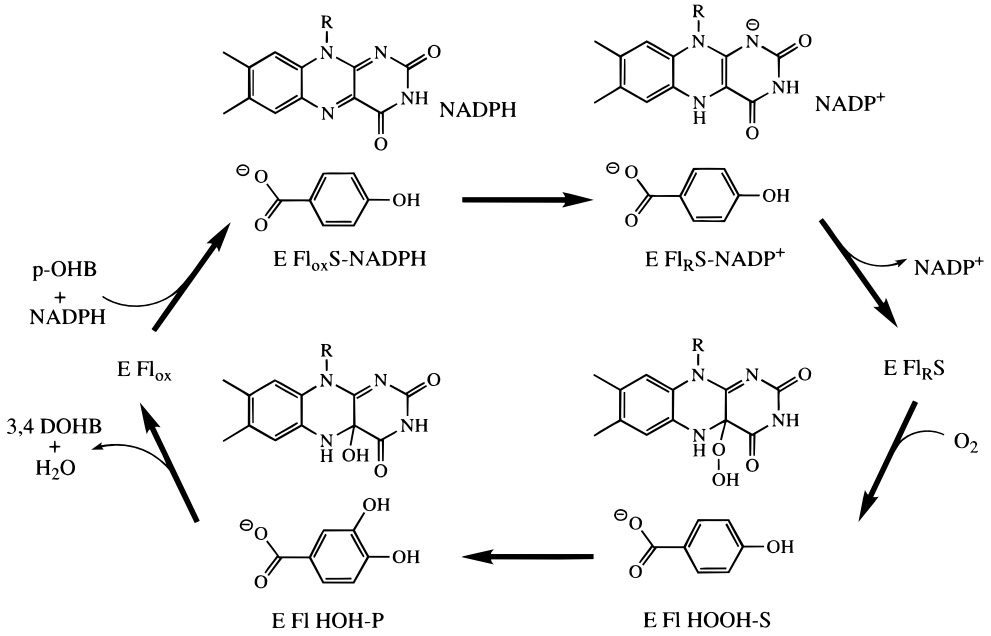
Another possible function of flavin movement is the control of the reduction reaction of enzyme-bound FAD by NADPH. The reduction rate of the flavin of PHBH by NADPH is highly dependent on the presence of pOHB in the active site (Spector & Massey, 1972; Husain & Massey, 1979; Shoun et al., 1979). Binding of pOHB has no effect on the flavin redox potential or the K_d for NADPH, yet it stimulates the rate of hydride transfer by a factor of 10^4−10^5. This catalytic control feature might be the result of a conformational change. However, no structures of a pyridine nucleotide−PHBH complex have been determined, leaving this an unresolved issue. In light of the newly discovered flavin conformational change, we wondered whether flavin movement might be involved in some way in the control of the reduction reaction.

Here we report new data that provide insight into the roles flavin movements play in catalysis, particularly in the reduction reaction, by using 6-azido FAD (6-N_3 FAD) as a spectral probe and a photolabel. We demonstrate that the artificial flavin alters the normal functioning of the enzyme only slightly, so that the conclusions obtained for the 6-N_3 FAD WT are directly applicable to the natural enzyme. The properties of both PHBH substituted with 6-N_3 FAD and the product of photoirradiation indicate that FAD movement is indeed involved in both the binding of the aromatic substrate and the reduction of the flavin by NADPH.

EXPERIMENTAL PROCEDURES

6-N_3 FAD was prepared from 6-NH_2 FAD by forming the diazonium salt and reacting this with NaN_3 as described (Ghisla et al., 1986). Mutant and WT PHBHs were expressed in *Escherichia coli* containing the plasmid pNE130 (Moran & Entsch, 1995) and purified as described previously (Palfey et al., 1994). PHBH was routinely assayed at 25 °C in Tris-sulfate, pH 8.0, with 0.3 mM pOHB and 230 μM NADPH (Entsch, 1990). Enzymes were substituted with artificial flavins using the Red-A chromatographic method, in which the Red-A matrix dye displaces the flavin and thus retains the protein (Gatti et al., 1994). The column is eluted with high-ionic strength buffer containing the artificial flavin, which binds the protein and displaces it from the dye. The extinction coefficient of 6-N_3 FAD-substituted PHBH was determined to be 15.4 mM^{-1} cm^{-1} at 428 nm using the method described by Entsch et al. (1991). Photolabeling of 6-N_3 FAD-substituted PHBH in 0.1 M potassium phosphate buffer, pH 7.5, or 0.05 M potassium phosphate buffer, pH 6.5, was accomplished by irradiating the enzyme solution at 25 °C with a tungsten−halogen lamp ("sun gun"). Reactions were monitored spectrally and were generally complete within 2 min. Enzyme that had not been photolabeled was separated from the covalently photolabeled enzyme by applying the mixture to a small column of Red-A, as previously described (Gatti et al., 1994). The nonlabeled enzyme bound to the column matrix, while the photolabeled enzyme passed directly through the column. The extent of covalent photolabeling was estimated by precipitating the protein with trichloroacetic acid (TCA) and determining the amount of flavin associated with the protein and that free in the supernatant as described previously (Gatti et al., 1994). Alternatively, noncovalently bound flavin was liberated from enzyme in 50 mM sodium phosphate buffer by the addition of SDS to 0.1% final concentration and separated by centrifugal ultrafiltration (Centricon 30). The spectra of the filtrate and the retentate (diluted to an appropriate volume) allowed the distribution of flavin to be estimated.

Scheme 1: Catalytic Cycle of PHBH[a]



[a] See the text for a description.

Electrospray mass spectra were obtained at the University of Michigan Protein and Carbohydrate Structure Core Facility. Labeled and unlabeled PHBH samples were extensively dialyzed against water before mass spectra were obtained, causing a significant amount of protein precipitation. However, enough protein remained in solution to obtain good mass spectra.

In order to identify the site of covalent labeling, labeled enzyme in 50 mM ammonium bicarbonate buffer, pH 7.9, was digested with trypsin (Sigma). Labeled peptides were separated from the mixture on a 0.21- × 25-cm microbore C_{18} HPLC column (4−48% acetonitrile, 0.1% trifluoroacetic acid, over 1 h at 0.1 mL min^{-1}). While numerous peptides were detected by absorbance at 220 nm, only a few peaks contained flavin label, as indicated by monitoring the absorbance at 430 nm. Peaks of flavin absorbance were collected and subjected to automated Edman sequencing on an Applied Biosystems Model 743A protein sequencer.

Rapid reactions at 4 °C were observed using a Hi-Tech SF-61 apparatus as described previously (Entsch et al., 1991). Enzyme solutions in 50 mM potassium phosphate, pH 6.5, were made anaerobic and reduced with NADPH when necessary. The absorbance of reaction mixtures was monitored at single wavelengths using a tungsten light source and a photomultiplier tube for detection, or entire spectra were acquired (1.2 ms/spectrum) using a Hi-Tech diode array detector and a xenon light source. Single-wavelength data were acquired using a Macintosh computer and the program KISS (Kinetic Instruments, Inc.) or a Dell computer using Program A, written in this laboratory. Diode array data were acquired using the program supplied with the Hi-Tech instrument. In addition to these two programs, data were also analyzed using SpecFit 2.09Y (Spectrum Software Associates), Excel 5.0 (Microsoft Inc.), and HopKINSIM [a Macintosh implementation of KINSIM (Barshop et al., 1983) by Daniel Wachsstock, Department of Cell Biology and Anatomy, Johns Hopkins University] for various fitting and simulating purposes.

RESULTS

Photoreactions of 6-N_3 FAD WT and 6-N_3 FAD Tyr222Phe. Visible light causes the loss of N_2 and the generation of a 6-nitrene flavin from 6-N_3 FAD. Nitrenes are quite reactive, so the flavin intermediate can be expected to react with protein groups or solvent molecules in its local environment (Ghisla et al., 1986; Massey et al., 1986). In this way, changes in covalent labeling of the enzyme by 6-N_3 FAD could serve to indicate changes in the position of the flavin and how it responds to ligand binding. Irradiation of PHBH substituted with 6-N_3 FAD resulted in the conversion of the flavin to a new chromophore, as followed by UV/vis spectra (Figure 2). The nature of the absorbance change depended on the presence and identity of ligands in the reaction mixture. In general, the 6-N_3 FAD WT PHBH was characterized by an absorbance maximum near 420 nm and a shoulder at 480 nm. Upon irradiation, the peak at 420 nm shifted to about 430 nm, the shoulder disappeared, and new peaks near 320 and 600 nm developed. The formation of the broad absorbance at 600 nm correlated with the crystallographically derived flavin position. Thus, in cases where the flavin was observed to be "in" (free WT and WT·pOHB), the absorbance increase in this region is relatively high. In the case where the flavin is "out" (WT·2,4-diOHB), a significantly smaller absorbance increase was observed at 600 nm. Absorbance in this region was also found to be indicative of covalent protein modification. The amount of covalently labeled protein, estimated spectrally after denaturing photoreacted samples, indicated that ligands known to favor the "out" conformation prevented photolabeling (Table 1). Flavin covalently bound to denatured PHBH showed absorbance peaks at ∼435 and ∼600 nm, while the unbound flavin photoproducts exhibited a variety of spectral properties (depending on the ligand present during irradiation), a common feature being the lack of absorbance at 600 nm. The absorbance spectra of the covalently labeled enzymes were very similar to the spectrum of 6-aminoflavin, an expected result if the intermediate nitrene inserts into a

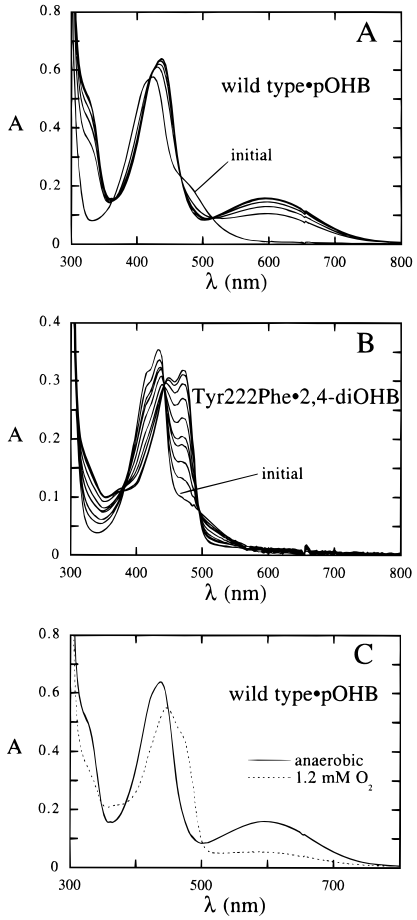


FIGURE 2: Photoreactions of 6-N_3 FAD WT and 6-N_3 FAD Tyr222Phe. Representative spectra are shown for the reactions of 6-N_3 FAD WT·pOHB (A) and 6-N_3 FAD Tyr222Phe·2,4-diOHB (B). The effect of O_2 on the products of the photoreaction of 6-N_3 FAD WT·pOHB is illustrated in panel C, which compares the final spectrum obtained upon irradiating in the presence or absence of O_2. Reactions were at 25 °C in 0.1 M sodium phosphate buffer, pH 7.0. For reactions with O_2 present, the enzyme solution was equilibrated under an atmosphere of 100% O_2 in a cuvette usually used for anaerobic experiments. Reactions were 35 μM in 6-N_3 FAD WT or 16 μM in 6-N_3 FAD Tyr222Phe and were monitored spectrally until complete (<2 min in all cases).

protein C−H bond during labeling to produce a 6-(alkylamino)flavin.

The estimated yield of labeled protein varied with the ligand present in the reaction mixture (Table 1) and did not vary with either the TCA or SDS denaturation methods, indicating that the photoproducts were acid-stable. In cases where the flavin is expected to be "in" from crystallographic results, relatively large amounts of covalent labeling were obtained, while a low level of labeling was obtained for the 6-N_3 FAD WT·2,4-diOHB complex, where the flavin is expected to be "out". Furthermore, Br^- has been observed to partly shift the flavin back "in" in the WT·2,4-diOHB complex (Gatti et al., 1994), and an increase in labeling was observed when the photoreaction was conducted in the presence of both 2,4-diOHB and Br^-. These data indicate that the conformational response of PHBH to ligands in solution parallels its behavior in the crystalline state, a finding that is not necessarily general in all protein−ligand systems. Mutation of Tyr222 to Phe has been shown to cause natural FAD to adopt the "out" conformation (Gatti et al., 1994). The Tyr222Phe enzyme thus provides an example of a PHBH form that will generally favor the "out" conformation of the flavin. When substituted with 6-N_3 FAD, no photolabeling and no absorbance increase at 600 nm were obtained for the Tyr222Phe mutant enzyme, probably as a consequence of the ease of flavin movement to the "out" position and increased solvent accessibility. It is interesting to note that while the spectrum of the irradiated mutant enzyme varied with the ligand present in the reaction mixture, the spectrum of the irradiated flavin freed by denaturation was the same in all cases.

Table 1: Percentage of Covalently Labeled PHBH[a]

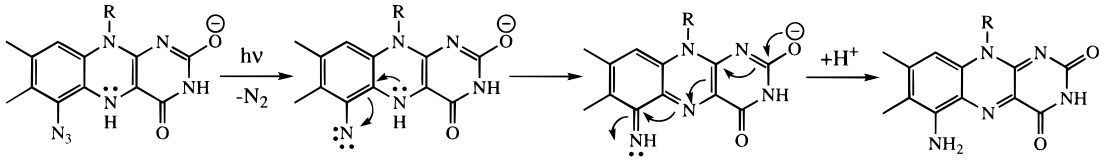
	anaerobic		air	~1.2 mM O_2	
	TCA	SDS	TCA	TCA	SDS
6-N_3 FAD WTox					
free	81	80	81[b]	46	45
+pOHB	58	63	66[b]	20	26
+2,4-diOHB			17[b]		
+Br^-			78[b]		
+2,4-diOHB + Br^-			35[b]		
6-N_3 FAD WTred					
+pOHB	0				
Tyr222Phe					
free			0		
+pOHB			0		
+2,4-diOHB			0		

[a] The amount of covalent labeling was estimated by measuring the absorbance associated with the protein fraction upon denaturing the enzyme, with either TCA or SDS, as described in Experimental Procedures. A blank entry for a particular combination of enzyme, ligand, and denaturant indicates that the experiment was not done. [b] Values from Gatti et al. (1994).

No covalent labeling of reduced WT PHBH substituted with 6-N_3 FAD was detected, probably because of the altered chemistry of the reduced 6-N_3 isoalloxazine. The 6-N_3 FAD WT·pOHB complex (11 μM) in 50 mM phosphate buffer, pH 6.5, was made anaerobic in a cuvette and rapidly reduced by adding an aliquot of NADPH (47 μM final). Immediately afterward, the solution was irradiated by intense light at 25 °C. Analysis of the protein revealed no covalently labeled enzyme. The lack of labeling can be attributed to a fundamental difference in reactivity between the initial photoproducts of the oxidized and the reduced 6-N_3 FAD. In the case in which the nitrene is formed from the oxidized flavin, a reactive electron-deficient group connected to an electron-deficient ring system is formed. When the flavin is reduced, however, the electron-deficient group is formed on an electron-rich ring system, and rapid electronic redistribution forms oxidized 6-NH_2 FAD much more quickly than nitrene insertion (Scheme 2). Thus, covalent labeling by reduced 6-N_3 FAD is chemically disfavored. An alternative explanation for the lack of labeling is that the reduced 6-N_3 FAD adopts the "out" conformation. However, the reduced flavin of the natural enzyme-pOHB complex adopts the "in" conformation (Schreuder et al., 1992). In view of our current results suggesting that the 6-N_3 FAD-substituted enzyme behaves similarly to the natural enzyme, it seems less likely that the lack of labeling is due to the conformation of the reduced azidoflavin.

The covalent labeling of the oxidized enzyme was specific and not the result of free 6-N_3 FAD. This was demonstrated by the electrospray mass spectrum of irradiated 6-N_3 FAD WT that was dialyzed against distilled water (Figure 3). The mass spectrum of native PHBH (Figure 3a) gave a mass of 44 314 ± 16 Da, in agreement with the expected mass, and

Scheme 2



also showed a small amount of enzyme with FAD still noncovalently bound to the enzyme after the harsh dialysis and electrospray treatments giving a mass of 45 106 ± 23 Da. The mass spectrum of irradiated 6-N_3 FAD WT (Figure 3B) shows a mixture of two enzyme populations with masses of 44 315 ± 16 and 45 122 ± 20 Da. The difference in mass of 807 ± 36 Da is the mass of a single covalently attached flavin. There was no sign of multiply labeled enzyme, which would be expected if the photolabeling were not occurring specifically in the active site. By passing the irradiated 6-N_3 FAD WT over a Red-A column, most of the noncovalently labeled enzyme was removed (Figure 3C).

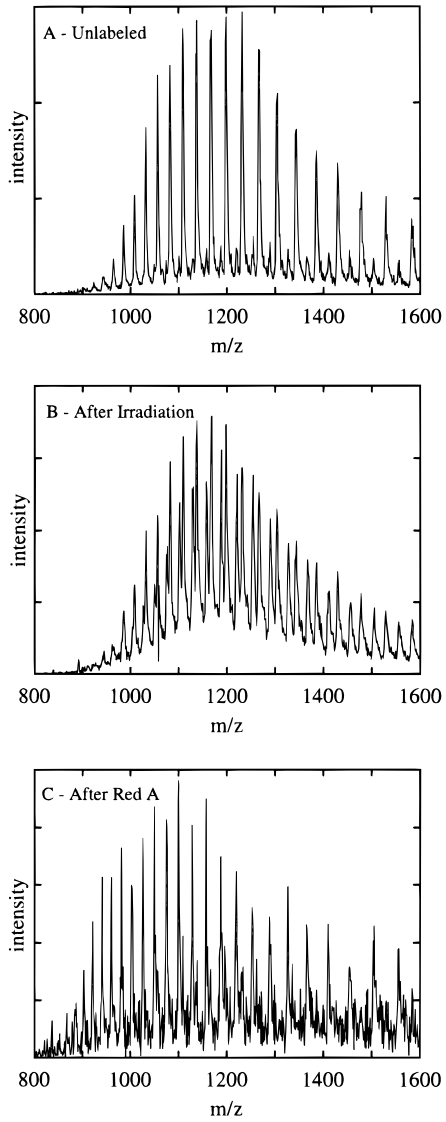


FIGURE 3: Electrospray mass spectra of PHBH. Enzyme samples were dialyzed against distilled H_2O, which caused significant protein precipitation. Spectrum A shows native WT PHBH. The spectrum of a photoreacted sample of the free 6-N_3 FAD WT is shown in B, while C shows the spectrum that results from removing unlabeled enzyme by Red-A as described in Experimental Procedures. Note that in A a small amount of enzyme-bound FAD remained after dialysis (short peaks falling between the large peaks) and in B similar amounts of covalently labeled and flavin-free enzyme were present, while in C only a small amount of flavin-free enzyme was present. The peaks in B occur in pairs separated by 807 ± 36 Da, the mass of one covalently bound FAD.

Covalent labeling was at the active site, as expected. This was demonstrated by identifying the labeled residue. First, the unlabeled enzyme in a photoreaction mixture was removed by passing over a Red-A column. The covalently labeled enzyme (which did not bind to the column) was digested with trypsin, and peptides were separated by reverse-phase microbore HPLC. Three major peaks bearing a flavin label (as observed by the absorbance at 430 nm) were collected from the eluent (Figure 4) and were submitted for sequence analysis. One fraction, eluting at 30 min, had a very high ratio of absorbance at 430 to 220 nm, yielded no sequence data, and was presumed to be flavin freed during the processing of the samples. The other two flavin-containing fractions eluted at 34 and 36 min and accounted for roughly 60% of the flavin absorbance. The fraction collected at 34 min was a mixture of two peptides; the fraction collected at 36 min was a mixture of four peptides. Sequencing these mixtures allowed the identification of the peptides within each fraction based on the known sequence of PHBH (Entsch et al, 1988). Both the 34- and 36-min fractions contained a peptide extending from Leu281 to Lys297, with an unknown residue at position 293 where proline was expected, indicating that this was the labeled residue. The fact that there were two labeled peptides of the same sequence, both modified at the same residue yet eluting at slightly different times, may indicate that the nitrene is capable of inserting at either the β- or γ-carbons of Pro293. Proline 293 is an active site residue situated (see Figure 1) near the 6-position of the isoalloxazine moiety when the flavin is in the "in" conformation (Schreuder et al., 1989), whereas there are no protein atoms near the 6-position of the isoalloxazine moiety when it is in the "out"

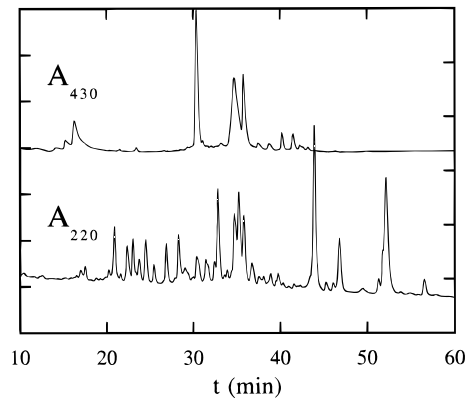


FIGURE 4: Chromatogram of trypsin-digested labeled WT. Photo-labeled WT (300 μL of ~6 μM) in 20 mM NH_4HCO_3, 2 M urea, pH 7.9, was digested with trypsin (20 μg) for 17 h, and the mixture of peptides was separated by HPLC as described in Experimental Procedures. The three peaks of flavin absorbance (430 nm) eluting at 30−35 min were collected and sequenced.

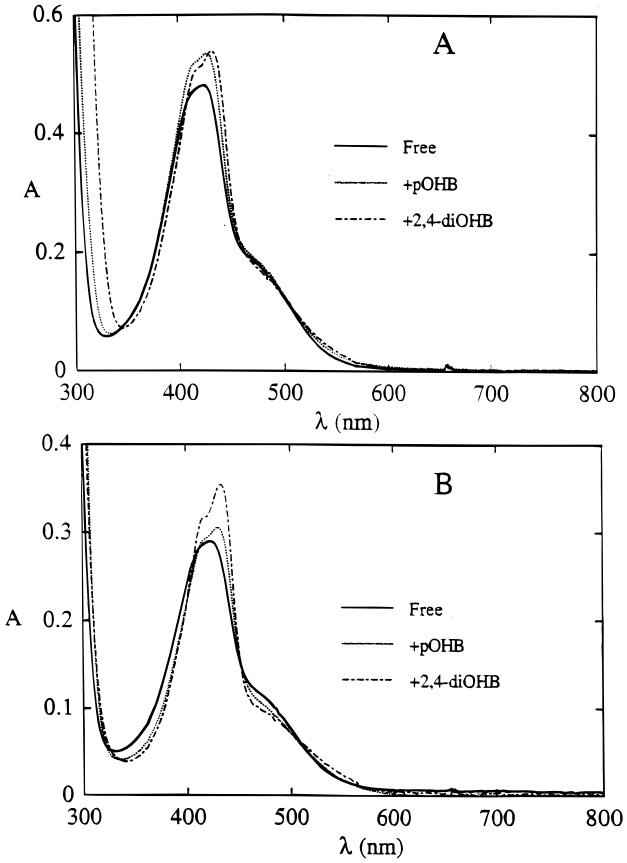


FIGURE 5: Spectra of substrate complexes of 6-N_3 FAD WT and 6-N_3 FAD Tyr222Phe. Ligand binding caused changes in the flavin absorbance spectrum of 6-N_3 FAD WT (A) and 6-N_3 FAD Tyr222Phe (B) that were characteristic of the flavin conformation. Enzymes (30 μM 6-N_3 FAD WT and 20 μM 6-N_3 FAD Tyr222Phe) in 50 mM KP_i, pH 6.5, were titrated with ligands at 4 °C.

position (Gatti et al., 1994; Schreuder et al., 1994). Thus the peptide labeling result concurs with crystallographic results.

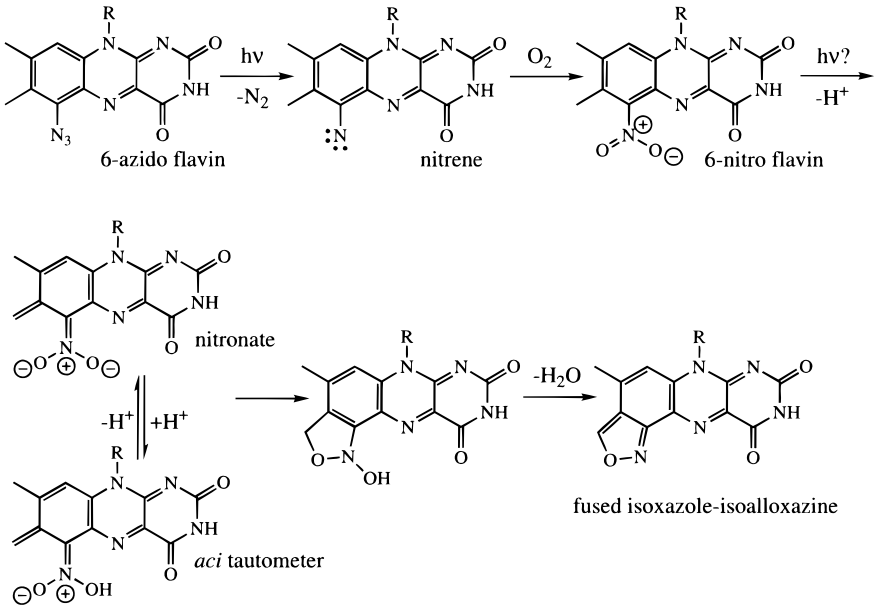
6-N_3 FAD PHBH Spectral Changes due to Ligand Binding. The spectrum of 6-N_3 FAD-substituted PHBH with ligands bound indicated the flavin position in the complexes. The analysis of the covalently labeled enzyme, described in previous sections, established the position of the azidoflavin in the presence of various ligands. The flavin in these positions exhibited distinct absorbance spectra. When the artificial flavin was in the "out" position, as with the 6-N_3 FAD Tyr222Phe·2,4-diOHB complex (Figure 5B), the flavin absorbance increased and the peaks sharpened, becoming almost resolved into transitions centered at 418 and 432 nm. In contrast, when the flavin was "in" as with pOHB bound to 6-N_3 FAD WT, an increase in extinction without significant resolution was observed (Figure 5A). The spectrum of the 6-N_3 FAD Tyr222Phe·pOHB complex appears to be intermediate between these extremes, perhaps indicating some population of the "in" conformation. Distinctive spectral changes caused by the binding of aromatic ligands to native PHBH have been shown to indicate the flavin conformation observed in crystal structures of these complexes (Gatti et al., 1994; Schreuder et al., 1994). Thus the difference in flavin−protein and flavin−solvent interactions in the two conformations causes observable changes in the spectrum of the isoalloxazine.

Effects of Molecular Oxygen on the Photoreaction. It was found that the presence or absence of O_2 altered the spectral changes of the photoreaction of 6-N_3 FAD WT, as well as the yield of labeled enzyme. As illustrated in Figure 2, 1.2 mM O_2 caused a substantial decrease in the amount of the photoproduct of the 6-N_3 FAD WT·pOHB complex absorbing near 600 nm and a red shift to the absorbance in the ~430-nm region. This was also the case in the absence of pOHB. Table 1 summarizes the effect that 1.2 mM O_2 had on the yield of covalently labeled PHBH. It can be seen that molecular oxygen partially traps the reactive nitrene intermediate, leading to roughly one-half the yield of the labeled enzyme, in either the presence or absence of pOHB. The noncovalently bound flavin produced in the presence of oxygen was highly fluorescent and had excitation, emission, and absorbance spectra that closely resembled the spectra previously reported for the photoproduct formed by 6-N_3 FAD-substituted xanthine oxidase. The latter photoproduct was identified as an isoxazole fused to the isoalloxazine system 6- and 7-positions (Saito et al., 1992). Thus it appears that in the presence of high concentrations of O_2, the same photoproduct is being formed with PHBH, in competition with covalent labeling.

The interception of the nitrene by O_2 argues against the previously proposed O_2-independent mechanism for isoxazole formation, in which it was suggested that the nitrene reacted with water and the resulting hydroxylamine species was further photochemically oxidized and cyclized, yielding the product. If this were the case, then high concentrations of O_2 could not compete with the protein for the nitrene, as was observed in the present study. Instead, an alternate mechanism can explain the observed isoxazole production and the reaction with O_2 (Scheme 3). In this proposal, O_2 reacts with the photoproduced nitrene, forming the 6-nitroflavin as the initial product. Cyclization then takes places, probably via the deprotonated nitronate form or the protonated *aci*-tautomer, similar to an isoxazole-forming reaction reported for *o*-nitrotoluene (Fukunaga et al., 1982). The required deprotonation or tautomerization may be a photochemical process, though the presence of the strongly electron-withdrawing nitro group on the already electron-withdrawing isoalloxazine system may make the 7-methyl protons sufficiently acidic to react in the ground state. Subsequent dehydration of the isoxazoline *N*-oxide (Fukunaga et al., 1982) results in the observed fused flavin−isoxazole product. As a test of this mechanism, it was found that 6-azido-3-methyllumiflavin reacted upon irradiation in the presence of oxygen in anhydrous dioxane to form a similar product. Isoxazole formation in the absence of water is inconsistent with the previously proposed mechanism and is consistent with the current proposal.

Some Properties of Covalently Labeled WT PHBH. WT enzyme, photolabeled and enriched as described above, was studied further as an example of PHBH where the isoalloxazine moiety is unable to swing to the "out" conformation. We have previously reported that labeled enzyme binds pOHB very slowly in a bimolecular fashion ($k_{on} = 0.4\ M^{-1}\ s^{-1}$, $k_{off} = 1.7 \times 10^{-3}\ s^{-1}$; Gatti et al., 1994). It is significant that the binding of ligands is so slow. The binding of ligands to the natural WT enzyme is too fast to be detected by stopped-flow methods, but with the isoalloxazine moiety covalently fixed in place substrate binding is at least 10^7-fold slower. Most of this effect is not due to the steric hindrance at the 6-position. The kinetics of pOHB binding to the 6-N_3 FAD WT and 6-NH_2 FAD WT enzymes were

Scheme 3



investigated, and binding was found to be much faster than for the covalently labeled enzyme, with rate constants in the range of 700 s^{-1} (6-NH_2 FAD WT) and 0.6 s^{-1} (6-N_3 FAD WT) at 2 mM pOHB, still much faster than substrate binding by the labeled enzyme.

The covalently labeled enzyme is dysfunctional not only in the binding of phenolic substrates but also in the reduction of the flavin by NADPH. The anaerobic labeled WT·pOHB complex was reduced by NADPH, but on a time scale of days. Using 20 mM NADPH, a reduction rate constant of $\sim 7 \times 10^{-6}$ s^{-1}, or a $t_{1/2} = \sim 27$ h, was estimated at 4 °C, roughly 7 million-fold slower than the reduction of the natural WT enzyme, which is reduced with a rate constant of 47 s^{-1} (Entsch et al., 1991). This extremely slow reduction rate is not the consequence of the chemical nature of the flavin, since the labeled enzyme was readily reduced by titrating with sodium dithionite. Furthermore, the spectral properties of the labeled enzyme and the known chemistry of nitrenes are consistent with the covalently attached flavin being a 6-alkylamino FAD. As a model, 6-NH2 FAD WT was reacted with NADPH under anaerobic conditions at pH 6.5, 4°. With this chemically similar but mobile flavin, relatively fast reduction (4.8 s^{-1}) was observed, implying that flavin movement is required for rapid reduction. Interestingly, the Kd for NADPH determined by the concentration dependence of the observed rate constant for the reduction of the 6-NH_2 FAD WT was 43 μM, lower than that for native PHBH by ~5-fold, indicating that the presence of a substituent at the 6-position of the isoalloxazine is not inherently detrimental to NADPH binding.

Turnover and Instability of Reduced 6-N_3 FAD WT. In order to be assured that the presence of a 6-substituent was not altering the properties of the enzyme significantly, the 6-N_3 FAD WT PHBH was studied in some detail. The enzyme turns over in standard assay mixtures (pH 8.0) at a reasonably fast rate (turnover number of 5.5 s^{-1}, compared to 43 s^{-1} for WT). However, over the course of turnover, the reaction eventually slowed to a new steady-state rate as the enzyme-bound flavin converted from the 6-N_3 form to the 6-NH_2 form. By extrapolating the linear portion of the new slower steady state back to time zero, the amount of NADPH consumed (and therefore the total number of turnovers) by the 6-N_3 form of the enzyme could be calculated, allowing a partition ratio for the conversion of the 6-N_3 form to the 6-NH_2 form to be estimated as 1 in every 1900 turnovers. This reaction was directly observed in a stopped-flow experiment in which the anaerobic 6-N_3 FAD WT·pOHB complex was mixed with slightly less than 1 equiv of NADPH in anaerobic buffer. The azidoflavin was reduced within 1 s. Over the course of 0.5 h, the reduced 6-N_3 flavin converted to the oxidized 6-NH_2 form as N_2 gas was eliminated. The conversion was a biphasic process, with about 20% of the enzyme reacting within 1 min. This reaction is a result of the previously reported chemical instability of reduced 6-N_3 FAD (Ghisla et al., 1986), which eliminates N_2 and converts to oxidized 6-NH_2 FAD, as represented in Scheme 2 except for the photochemical initiation. Thus, 6-N_3 FAD WT turns over at a rate that is not very much lower than the turnover number of regular WT, but during each turnover, a fraction of the enzyme converts to 6-NH_2 FAD WT.

Oxidative Half-Reaction of 6-N_3 FAD WT. The oxidative half-reaction of PHBH is typically studied at 4 °C by mixing in a stopped-flow spectrophotometer the reduced enzyme−substrate complex with buffer containing O_2 (Entsch et al., 1976). The chemical instability of the reduced 6-N_3 FAD WT enzyme described above made this type of experiment technically difficult and prevented high-quality data from being collected. However, by rapidly titrating cold anaerobic enzyme in the presence of pOHB with NADPH in the stopped-flow spectrophotometer, followed immediately by reaction with O_2, triphasic reaction traces were obtained at 370 nm demonstrating the reoxidation of the flavin through two intermediates, in analogy with regular PHBH (data not shown). Reaction traces obtained at 425 nm, near the peak of oxidized 6-N_3 FAD WT absorbance, consisted of a lag followed by two phases of increasing absorbance. A pseudo-first-order rate constant at pH 6.5, 4 °C, for the reaction of O_2 with reduced enzyme of 81 s^{-1} (corresponding to a second-order rate constant of 1.3×10^5 M^{-1} s^{-1}) was observed when mixing with buffer saturated with pure O_2. The intermediate formed by this reaction, presumably the

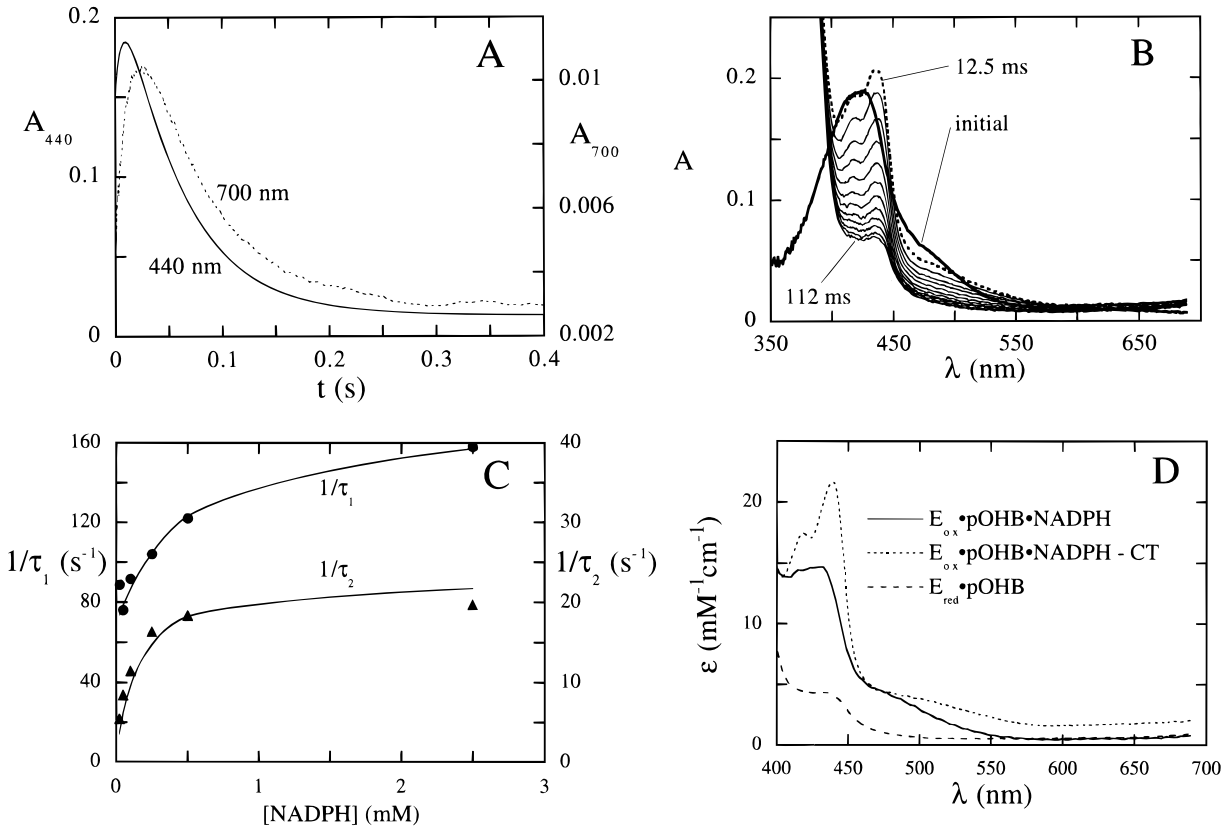


FIGURE 6: Reduction of 6-N_3 FAD WT•pOHB by NADPH. The anaerobic 6-N_3 FAD WT•pOHB complex (13 μM after mixing) was mixed with anaerobic solutions of NADPH at various concentrations in a stopped-flow spectrophotometer at 4 °C, pH 6.5. (A) Representative single-wavelength reaction traces (500 μM NADPH after mixing) are shown. The initial increase in absorbance at 440 nm is indicative of the movement of the flavin from the "in" conformation to the "out" conformation and occurs as a weakly absorbing charge-transfer complex forms as observed at 700 nm. The loss of absorbance at both wavelengths in the second reaction phase occurs upon hydride transfer. (B) The same reaction was observed using diode array data collection. The spectrum of the oxidized enzyme mixed with buffer is shown with a thick line, while the other spectra were obtained from a reaction with NADPH. Immediately after mixing the flavin spectrum becomes sharpened, and a peak forms at 440 nm by 12.5 ms (dashed spectrum). From 12.5 to 112 ms, the oxidized 6-N_3 FAD spectrum bleaches due to reduction. Note that the high concentration of NADPH obscures the flavin spectral changes for $\lambda < \sim$400 nm. (C) The observed reciprocal relaxation times for the two reaction phases, obtained by fitting the data to a two-exponential equation, are shown as a function of NADPH concentration. The smooth curves fitting the data represent the best fit to the scheme for reduction described in the text (Scheme 4) using the K_d for NADPH and the rate constants shown in Scheme 4. (D) Using these parameters, the spectra of the reactants, intermediates, and products were calculated as described in Experimental Procedures. Note that there is almost no spectral change caused by the initial binding of NADPH so that only one of these spectra is shown (solid line). The charge-transfer complex, with absorbance in the region of 425−450 nm resembling the spectral change caused by the movement of the flavin to the "out" conformation (see Figure 5), forms next (designated 6-N_3 FAD WT_{ox}•pOHB•NADPH - CT). This intermediate reacts to form the reduced enzyme (6-N_3 FAD WT_{red}•pOHB).

6-azido-4a-hydroperoxyflavin, hydroxylated pOHB with a rate constant of 6.6 s^{-1}. The 6-azido-4a-hydroxyflavin formed in the stopped-flow experiments by pOHB hydroxylation reaction eliminated water to form oxidized enzyme with a rate constant of 0.71 s^{-1}. The three rate constants are approximately 2-, 7-, and 20-fold slower than the WT reaction with natural FAD, respectively, and indicate that the 6-azido substituent has not drastically altered this half of the catalytic pathway.

Reductive Half-Reaction of 6-N_3 FAD WT. The reduction of the 6-N_3 FAD WT enzyme by NADPH was nearly unimpaired when compared to the corresponding reaction with natural PHBH. The anaerobic 6-N_3 FAD WT•pOHB complex was mixed with various concentrations of anaerobic NADPH in a stopped-flow apparatus. Reaction time courses were observed at several individual wavelengths and also by recording complete spectra (1.2 ms/spectrum) with a diode array detector. Identical kinetics were obtained by both methods for times earlier than ~0.1 s, long enough for most of the reduction reaction to be complete. However, at later times, significant differences attributable to photochemistry from the high-intensity xenon lamp used for the diode array experiments were observed between the data collected by the two methods.

Two phases were observed at single wavelengths upon mixing 6-N_3 FAD WT with NADPH (Figure 6A). At the peak of oxidized flavin absorbance (424 nm), a brief lag was followed by a large loss in absorbance as the hydride transfer occurred. At 440 nm, the absorbance first increased to a maximum by about 12 ms and then decreased as the enzyme flavin was reduced. Analogous to the case with natural WT, charge-transfer absorbance was observed at 700 nm. The charge-transfer absorbance appeared on the same time scale as the increase in absorbance at 440 nm and the lag at 424 nm and decayed during the hydride-transfer reaction.

The spectra obtained by the diode array instrument were particularly useful for interpreting the events in the reduction reaction (Figure 6B). Upon mixing with NADPH, the smooth spectrum of the oxidized 6-N_3 FAD WT•pOHB complex shifted somewhat and became more resolved at the peak flavin absorbance. The shoulder near 470 nm characteristic of 6-N_3 FAD WT decreased slightly, and long-

wavelength absorbance indicative of a charge-transfer complex appeared. The extent of these spectral changes increased over the course of the next ~12 ms, and these were followed by the loss of the absorbance of oxidized enzyme as NADPH reduced the flavin. The character of the spectral change in the first ~12 ms is especially noteworthy (Figure 6). The sharpening and resolution of the broad flavin peak into two peaks in the 420−440-nm region and the decrease in the shoulder near 470 nm are strikingly similar to the spectral changes observed when 2,4-diOHB binds to the oxidized enzyme (see Figure 5).

In order to obtain rate and equilibrium constants for these processes, the biphasic absorbance traces were fit to two exponentials. Both reciprocal relaxation times obtained from fitting the data increased to saturating values as the NADPH concentration was increased (Figure 6C), implying that the first observed reaction step was preceded by another event, presumably NADPH binding. The kinetic observations suggested a minimal model for the overall reductive sequence (summarized in Scheme 4) consisting of the rapid equilibration of NADPH with the enzyme to form a 6-N_3 FAD WT•NADPH complex, followed by the reversible isomerization to a charge-transfer complex in which the environment of the flavin is similar to that of the flavin when 2,4-diOHB is bound, and finally the irreversible transfer of hydride from NADPH to 6-N_3 FAD. Because of the reversibility of the isomerization step, the two observed rate constants for the reduction sequence must be described by equations for coupled reactions (eqs 1a,b), modified to take the initial rapid equilibration with NADPH into account by multiplying the forward isomerization rate constant (k_1) by the fraction of enzyme that has NADPH bound (given in eq 1c).

$$1/\tau_{fast} = \frac{1}{2}\left[fk_1 + k_2 + k_3 + \sqrt{(fk_1 + k_2 + k_3)^2 - 4k_1k_3}\right] \quad (1a)$$

$$1/\tau_{slow} = \frac{1}{2}\left[fk_1 + k_2 + k_3 - \sqrt{(fk_1 + k_2 + k_3)^2 - 4k_1k_3}\right] \quad (1b)$$

$$f = \frac{[NADPH]}{[NADPH] + K_d} \quad (1c)$$

Values for the rate and equilibrium constants were obtained according to the following procedure. First, the K_d for NADPH was estimated by averaging the results from eq 2 (which was derived from eq 1 for two different concentrations of NADPH designated "a" and "b") for each possible pair of NADPH concentrations.

$$K_d = \frac{\left(\frac{(1/\tau^b_{fast})(1/\tau^b_{slow})}{(1/\tau^a_{fast})(1/\tau^a_{slow})} - 1\right)[NADPH]_b}{\frac{[NADPH]_b}{[NADPH]_a} - \frac{(1/\tau^b_{fast})(1/\tau^b_{slow})}{(1/\tau^a_{fast})(1/\tau^a_{slow})}} \quad (2)$$

Then, the "Solver" function of Microsoft Excel was used to minimize (via a conjugate gradient algorithm) the sum of the squares of the difference between the predicted and observed reciprocal relaxation times by first varying the values of the rate constants with the K_d of NADPH held fixed and then letting all parameters vary. The rate constants and equilibrium constant obtained in this way were then used to calculate the spectra of the species in the reaction (Figure 6D) by defining the model of Scheme 4 in the program Specfit and fitting the differential equations to diode array data, keeping the rate and equilibrium constants fixed. The quality of the parameters obtained by this procedure was confirmed by simulating single-wavelength stopped-flow traces at each NADPH concentration with the program HopKINSIM. Excellent agreement was obtained.

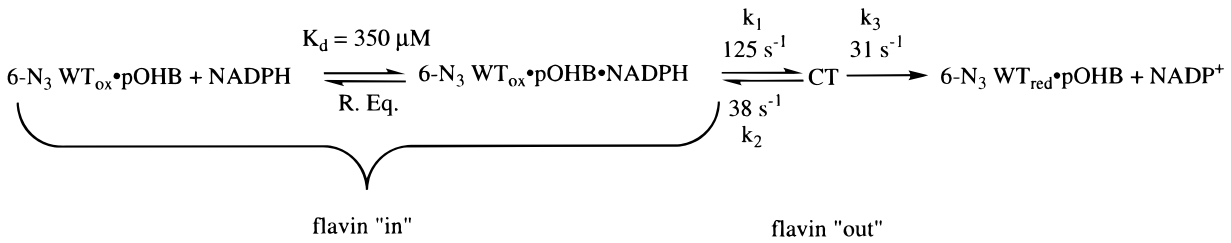
By this procedure, a K_d for NADPH of 350 μM was obtained, similar to the value of 210 μM reported for native PHBH, indicating that the azido substituent did not impede the binding of the pyridine nucleotide. The subsequent isomerization reaction slightly favors the reaction of the initial Michaelis complex to the charge-transfer complex in the "out" conformation, with a forward rate constant of 125 s^{-1} and a reverse rate constant of 38 s^{-1}. A rate constant of 31 s^{-1} was found for the actual hydride-transfer reaction, compared to 47 s^{-1} reported for the native enzyme (Entsch et al., 1991).

DISCUSSION

The role for the movement of the isoalloxazine ring system in PHBH has been studied by replacing the natural flavin with 6-N_3 FAD, which served as both a spectral probe and a photolabel. The 6-N_3 substituent apparently does not significantly alter the efficiency of the enzyme, as evidenced by the observation of the usual catalytic intermediates, which form and decay at rates that are not very different from those of the natural enzyme. The major change caused by the introduction of the 6-N_3 group is its relatively slow conversion of the enzyme in the reduced state to oxidized 6-NH_2 FAD PHBH, a perturbation due to the inherent instability of reduced 6-N_3 FAD and not to changes in flavin−protein interactions. Thus, this artificial flavin does not alter the behavior of the isoalloxazine of PHBH greatly, making it a useful mechanistic probe of the enzyme.

Our results point to an involvement of isoalloxazine movement in normal enzyme turnover in at least two ways:

Scheme 4



the binding of aromatic substrate and the reduction of the flavin by NADPH. The requirement for flavin mobility in these processes is clearly evident from the extremely low rates observed for pOHB binding to the covalently labeled enzyme (Gatti et al., 1994) and from the slow reduction of the enzyme by NADPH. These very large rate decreases (at least 7 orders of magnitude in both cases) are not merely the result of increased steric bulk caused by the flavin azido substituent, nor are they the result of changes in the chemical reactivity of the flavin, since other 6-substituted flavins used in this work (6-N_3 FAD and 6-NH_2 FAD) or in the past [6-OH FAD (Entsch et al, 1987)] did not cause dramatic effects such as those obtained by covalent labeling.

The influence of different ligands on the flavin position of 6-N_3 FAD was determined by the ability to form a photoadduct with the protein. The response by 6-N_3 FAD enzyme to ligand binding was quite similar to that of the native enzyme, indicating that the 6-N_3 FAD WT is a good model for the native enzyme. The crystallographically established correlation of conformation and absorbance spectrum has proven useful in the study of conformational changes of the native flavin in different PHBH forms. A similar correlation was established here for 6-N_3 FAD PHBH, based on protein chemistry rather than X-ray diffraction. Thus the distinctive spectral signature associated with 2,4-diOHB binding to 6-N_3 FAD PHBH is associated with the "out" conformation, as illustrated by the lack of photochemical labeling. This correlation enabled us to detect flavin movement during the reduction of 6-N_3 FAD WT by NADPH.

The spectral changes caused by NADPH binding to 6-N_3 FAD WT prior to hydride transfer are particularly interesting. The binding of NADPH to the 6-N_3 FAD WT·pOHB complex induces a spectral change similar to that produced by 2,4-diOHB binding, indicating that pyridine nucleotide binding causes the flavin to adopt the "out" conformation. At the same time, an NADPH−flavin charge−transfer complex is formed. The next detectable event is the flavin reduction reaction. Thus, in the simplest model that fully accommodates our kinetic data (shown in Scheme 4), the flavin reacts with the pyridine nucleotide while in the "out" conformation. Because the 6-N_3 FAD-substituted enzyme does hydroxylate pOHB, the flavin must move back to the "in" position after reduction, presumably at a rate much faster than its reaction with O_2.

The fact that the conformational change was observed in the present case is the result of the favorable rate constants and spectral properties of the system, which allow their detection. It is worth noting that in the Ser212Ala mutant of PHBH (bearing natural FAD), spectral changes have been observed prior to hydride transfer that are suggestive of flavin movement from the "in" position to the "out" position (Moran, 1995; Palfey et al., 1997). It therefore appears that flavin movement from "in" to "out" is a necessary part of the competent binding of NADPH. Evidently, part of the energy of NADPH binding is used to move the flavin from "in" to "out". Consistent with this, in several mutant forms of PHBH that favor the "out" conformation when pOHB is bound [Tyr222Phe (Entsch et al., 1994), Arg220Lys (Moran et al., 1996)], the K_d for NADPH is lower than it is with WT PHBH.

It is fortuitous that the conformational mobility of the flavin is observable in PHBH. A few other instances of coenzyme movement on enzymes have been documented, including the motion of acylated dihydrolipoamide in the transacetylase of α-keto acid dehydrogenase multienzyme complexes (Reed, 1974) and the rotation of the pyridine ring of NAD(H) of UDP-galactose 4-epimerase (Thoden et al., 1996). In the former example, coenzyme movement serves to shuttle the product from one active site to another active site for subsequent reaction, and in the latter a role probably is played in accessing opposite faces of the reaction intermediate. In PHBH, the conformational movement functions to make the active site accessible to reactants but inaccessible to solvent during the hydroxylation step. The generality of coenzyme movement in the flavoprotein hydroxylases and of movements of other prosthetic groups in other types of enzymes remains to be seen, but conformational changes may constitute an important aspect of coenzyme chemistry that is relatively unexplored.

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