Electrostatic Effects on Substrate Activation in *para*-Hydroxybenzoate Hydroxylase: Studies of the Mutant Lysine 297 Methionine[†]

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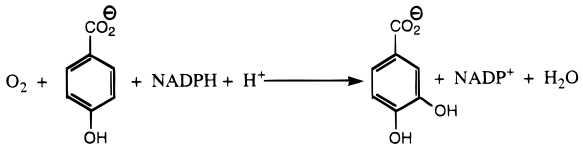
ABSTRACT: *p*-Hydroxybenzoate hydroxylase (EC 1.14.13.2) is a flavoprotein monooxygenase that catalyzes the incorporation of one atom of molecular oxygen into *p*-hydroxybenzoate to form 3,4-dihydroxybenzoate. The enzyme activates the substrate at the 3 position to electrophilic substitution by lowering the pK_a of the phenolic oxygen. The results presented here indicate that regions of positive potential in the active site facilitate this substrate activation, which is necessary for rapid hydroxylation. We have neutralized a positive point charge by mutating lysine 297 to methionine (K297M). This mutation changes an amino acid near the active site, but not directly in contact with the flavin or the substrate. A variety of transient state kinetic and static parameters have been determined with two substrates. The results indicate that the K297M mutant does not activate the substrate through phenolic ionization to the same extent as wild-type (WT) and yet remains a competent hydroxylase. However, catalysis by the mutant is slow compared to that of WT, particularly in the oxidative half-reaction. Thus, normally quite labile oxygenated flavin intermediates encountered in the hydroxylation pathway of WT *p*-hydroxybenzoate hydroxylase are stabilized and their decay is rate limiting in the K297M turnover. Electrostatic potential calculations offer an explanation for the lack of substrate activation. The stability of the oxidative reaction intermediates seems to be related to a lower degree of substrate activation.

Flavoprotein external monooxygenases incorporate one atom of molecular oxygen into organic compounds while using NAD(P)H as an external reductant. *p*-Hydroxybenzoate hydroxylase (PHBH,[1] EC 1.14.13.2) belongs to a subgroup of external monooxygenases that hydroxylates aromatic compounds; the large body of enzymological and crystallographic data assembled for PHBH makes it the paradigm for this family of enzymes (van Berkel & Müller, 1991; Entsch & van Berkel, 1995). PHBH catalyses the incorporation of one atom of molecular oxygen into *p*-hydroxybenzoate to form 3,4-dihydroxybenzoate (PCA) (Scheme 1). One feature of flavoprotein monooxygenases is that they form and stabilize flavin C4a-hydroperoxides as reactive oxygenating species (Entsch *et al.*, 1976; Entsch & van Berkel, 1995). Members of this family hydroxylate activated aromatic rings *via* electrophilic substitution of the distal oxygen of the hydroperoxyflavin. Although free flavins can form C4a-hydroperoxides, the hydroperoxides are unstable and the hydroxylation reaction does not occur;

Scheme 1



therefore, it can be concluded that the anisotropic environment provided by the active site residues of the enzyme stabilizes the intermediates and directs the course of the reaction. Directing the reactivity of the otherwise labile flavin hydroperoxide is thus a fundamental function of this enzyme. It has been demonstrated that the stability and the reactivity of this intermediate in PHBH (and other similar enzymes) depends on the presence of substrate. In the absence of substrate, the hydroperoxide decays quickly, eliminating hydrogen peroxide, while in the presence of substrate, the enzyme directs an efficient electrophilic aromatic substitution reaction.

Two recent advances from studies of PHBH have been the observation of alternate flavin conformations in the active site (Entsch *et al.*, 1994; van Berkel *et al.*, 1994; Schreuder *et al.*, 1994; Gatti *et al.*, 1994; Lah *et al.*, 1994a) and the identification of a network of hydrogen bonds connecting the substrate with the solvent (Palfey *et al.*, 1994; Schreuder *et al.*, 1994; Gatti *et al.*, 1996). These studies indicated that movement of the isoalloxazine of the flavin of PHBH is required in order for substrates to associate with the enzyme. Crystal structures of the WT enzyme in complex with various 4-hydroxy and 4-amino substrates indicate that a hydrogen bond network from the substrate to the protein surface serves in the deprotonation of the 4-hydroxyl group and the resultant activation of the substrate brings about an *ortho* hydroxylation reaction (Entsch *et al.*, 1991; Schreuder *et al.*, 1994;

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[1] Abbreviations: PHBH, *para*-hydroxybenzoate hydroxylase; *pobA*, the gene encoding *para*-hydroxybenzoate hydroxylase; *p*-OHB, *para*-hydroxybenzoate; PCA, 3,4-dihydroxybenzoate; 2,4-DOHB, 2,4-dihydroxybenzoate; 2,3,4-TOHB, 2,3,4-trihydroxybenzoate; WT, wild-type.

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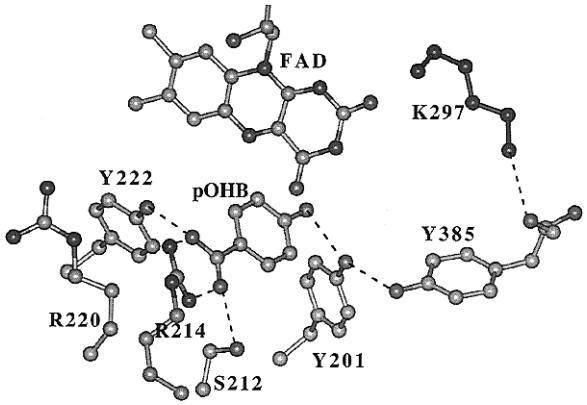

FIGURE 1: Active site residues of *para*-hydroxybenzoate hydroxylase. Dashed lines indicate potential hydrogen-bonding interactions to the substrate and lysine 297. Lysine 297 has been shaded in black.

Gatti *et al.*, 1996). Exactly how the enzyme controls the position of the flavin or promotes the activation of the substrate is not known. However, from earlier studies, it seems likely that charge plays a role in influencing substrate binding (flavin movement) and substrate activation (Shoun *et al.*, 1983; Palfey *et al.*, 1994).

Specific electrostatic effects are thought to play important roles in many enzyme systems. For example, although superoxide dismutase has a net negative charge, its charged residues are arranged in such a way that a positive electrostatic field directs the anionic substrate to the active site, and modeling studies have demonstrated the importance that particular charged residues can have in this role (Polticelli *et al.*, 1994). Swenson and Krey (1994) and Zhou and Swenson (1995) demonstrated that when acidic residues within 13 Å of the flavin of flavodoxin are mutated to their neutral amide counterparts, the redox potential is increased by about 15 mV per unit of charge. In PHBH, deprotonation of the substrate 4-hydroxyl (Schreuder *et al.*, 1994; Gatti *et al.*, 1996) and the localization of the reduced anionic flavin in the active site proximal to the substrate (Schreuder *et al.*, 1990) can be influenced by electrostatics. Lys297 of PHBH is a positively charged residue at the base of helix H10 adjacent to the pyrimidine end of the flavin. The side chain of this residue points away from the flavin and interacts with the backbone carbonyl of Tyr385, one of the residues participating in the proton network (see Figure 1). The charged side chain amine is approximately 8 Å from N-1 and N-3 of the pyrimidine ring of FAD. In this study, we have mutated Lys297 to the neutral methionine residue and determined many of the properties of the mutant enzyme. The results that we have obtained are consistent with current theories of flavin mobility and substrate activation and highlight the importance of charge in the function of PHBH.

METHODS

Expression and Purification. The lineage of plasmids used to overexpress the mutant enzyme has been described previously (Entsch *et al.*, 1991; Moran & Entsch, 1995). The expression plasmid, pIE130, was derived from an earlier construct, pNE130, by optimizing the region of the *lac* promoter using PCR loop deletion. The methods used to express the mutant enzyme in *Escherichia coli* JM105 are described in Moran and Entsch (1995) and are based on the methods of Entsch *et al.* (1991). The purification procedures are described by Entsch (1990).

Mutagenesis. The directed mutation was made using the Bio-Rad adaptation of the methods of Kunkel *et al.* (1987) and are described in Entsch *et al.* (1991). The K297M mutation was generated using a 21-mer oligonucleotide (5′-ACCGGCGCCATGGGACTGAAC-3′) that had nine bases that were complementary to the *pobA* gene on each side of the single base change in the mutated codon (Entsch *et al.*, 1988).

Experimental Measurements. Unless otherwise stated, the standard experimental conditions were 50 mM phosphate buffer (potassium salt) and 10 mM EDTA (sodium salt), pH 6.5, 4 °C. These conditions have been demonstrated to facilitate the detection of catalytic intermediates of PHBH (Entsch *et al.*, 1976). The procedures used in the measurement of ligand dissociation constants for the oxidized enzyme, reduction potentials, and extinction coefficients have been described (Entsch *et al.*, 1991). Ligand dissociation constants for the reduced enzyme were determined according to the methods described in Entsch *et al.* (1976). Enzyme-monitored turnover was carried out according to the methods of Gibson *et al.* (1964). Anaerobic conditions were achieved based on the procedures of Foust *et al.* (1969), using an inert atmosphere of purified argon. Redox potential measurements were carried out using the method described by Massey (1990).

Electrostatics Calculations. Electrostatic potentials within the protein were calculated using the DelPhi program (Gilson et al., 1988) on a Silicon Graphics Indigo workstation. Charges for the amino acids were taken from charge files compiled for AMBER (Cornell *et al.*, 1995). The charges of the adenosine, phosphate, and ribityl portions of FAD were taken from chemically similar examples in charge files for tRNA that had been compiled for AMBER. The charge of the isoalloxazine and ribityl C1′, as well as those of *p*-OHB, were derived by least-squares fitting of the electrostatic potential of the 6-31 G* optimized structures of lumiflavin and *p*-OHB and are available as Supporting Information. *Ab initio* calculations were done using TurboMole, implemented by Biosym/MSI in the Insight II modeling package. The DelPhi electrostatics calculations (Gilson *et al.*, 1988) were performed for the protein dimer defined by Schreuder *et al.* (1992). The potential was calculated for all points of a 2.3 Å/point cubic grid whose center was the center of the protein and whose boundaries were at least 20 Å from the protein surface. The charge at points on the faces of the grid was defined as zero. The radius of all atoms within the cube were defined from their van der Waals radii. A solvent dielectric constant of 80.00, solvent radius of 1.4 Å, and an ionic strength of 0.145 were used.

Stopped Flow Kinetic Measurements. All kinetic data were measured on a Hi-Tech Scientific Model SF-61 stopped-flow spectrophotometer (2 ms dead time) in single wavelength detection mode. Data acquisition and the instrument were controlled by a Macintosh IIcx computer running KISS software (Kinetic Instruments Inc.). Single wavelength reaction traces were fit within the KISS program using the Marquardt−Levenberg algorithm (Press et al., 1992) applied to a maximum of five irreversible steps.

RESULTS

General Properties. The expression of active K297M is at least equal to that of the wild-type (WT) enzyme using

Table 1: Selected Properties of PHBH WT and Mutant K297M

property	WT	K297M
TN at pH 6.5, 4 °C (s^{-1})[a]	6.2	0.5
% coupled pH 6.5, 4 °C[b]	100	87
K_d *p*-OHB (μM)[c]	9.5	7.5
K_d 2,4-DOHB (μM)[c]	20	49
K_d *p*-OHB-E_{red} (μM)	21	20
K_d 2,4-DOHB-E_{red} (μM)[c]	180	91
K_d NADPH E-*p*-OHB (μM)[d]	210	373
K_d NADPH E-2,4-DOHB (μM)[d]	1500	951
E_{m7} − free enzyme (mV)[e]	−163	−176
E_{m7} − enzyme + *p*-OHB (mV)[e]	−165	−176
E_{m7} − enzyme + 2,4-DOHB (mV)[e]	−172	−174
$E_{ox}\epsilon_{450}$ (mM^{-1} cm^{-1})	10.3	10.4

[a] TN (turnover number) refers to the maximum rate of reaction measured in enzyme monitored turnover experiments with limiting oxygen and initially saturating concentrations of *p*-OHB and NADPH extrapolated to infinity. [b] Hydroxylation stoichiometry determined from single turnover experiments and subsequent quantitative product analysis. [c] Dissociation constants for *p*-OHB and for 2,4-DOHB were determined from static titrations at pH 6.5, 4 °C. The plot of absorbance increments at wavelengths that demonstrate maximal perturbation against free substrate concentration could be fit to a hyperbola for the determination of dissociation constants [WT dissociation constants from Entsch *et al.* (1976)]. [d] The dissociation constants for NADPH were determined by fitting the dependence of the rate of reduction by NADPH to an hyperbola [WT K_d for NADPH from Husain and Massey (1979)]. [e] Redox potentials were determined in 0.05 M phosphate pH 7.0, 25 °C [WT potentials from Entsch *et al.* (1991)].

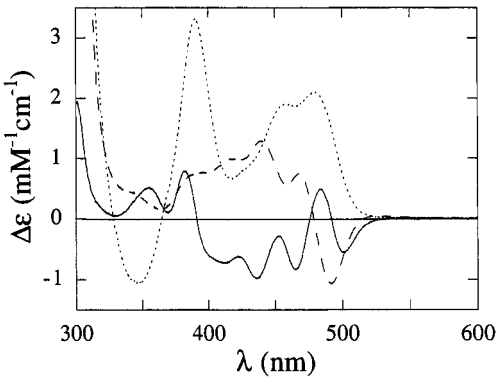

FIGURE 2: Difference spectra obtained in static titrations of the PHBH mutant K297M with ligands; (—) *p*-OHB, (•••) 2,4-DOHB, and (- - -) PCA at pH 6.5, 4 °C. Very similar difference spectra are obtained for WT under the same conditions [*e.g.*, Moran *et al.* (1996)].

the pIE130 expression system (20% cell protein; Moran & Entsch, 1995). Furthermore, the enzyme can be purified with no changes to the procedure used to purify WT (Entsch, 1990). The visible spectral properties are identical to those of the native enzyme, with a maximum at 449 nm and an extinction coefficient of 10.3 $mM^{-1}cm^{-1}$. FAD bound to the mutant is 70% as fluorescent as the WT enzyme, and like the WT enzyme, 70% of this fluorescence is quenched in the presence of saturating *p*-OHB or 2,4-dihydroxybenzoate (2,4-DOHB). According to the crystal structure of the WT·*p*-OHB complex, lysine 297 does not interact directly with the substrate binding pocket (see Figure 1). Consistent with this, the affinities of the mutant enzyme for *p*-OHB, 2,4-DOHB, and the product PCA are very similar to those measured for the WT (Table 1) and the spectral perturbations of the flavin upon binding these ligands indicate that the flavin of K297M resides in a very similar environment to that of the WT enzyme (Figure 2). The K297M mutant binds the substrate with essentially the same affinity in both the

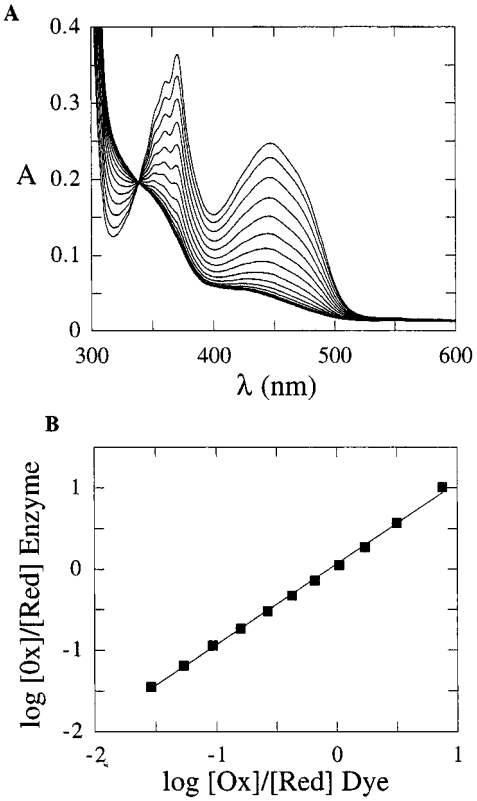

FIGURE 3: Determination of the redox potential of K297M in the absence of substrate. Procedures were carried out according to Massey (1990). (A) Spectra recorded during the anaerobic reduction of the enzyme (24 μM) and the indicator dye, 1-hydroxyphenazine (20 μM). (B) Determination of the redox potential of the enzyme-bound flavin from the known midpoint potential of the dye.

oxidized and reduced forms as is the case for the WT (Entsch *et al.*, 1976). Accordingly, the redox potential of the mutant enzyme is unaltered by the substrate (see Table 1). The redox potential of the ligand-free enzyme is 13 mV more negative than that of the WT (see Figure 3), a value that is in good agreement with the change in redox potential caused by a 1 unit charge alteration for flavodoxin (Zhou & Swenson, 1995).

Kinetics of Ligand Binding. The similarity of the spectral perturbations observed when *p*-OHB binds to the WT and to K297M is evidence that the flavin environment is very similar in both proteins. The binding of the substrate to the WT enzyme is complete within the 2 ms dead time of the stopped flow instrument (Entsch *et al.*, 1976). The K297M mutant, in contrast, binds to *p*-OHB at observable rates. The binding process was observed by stopped-flow fluorescence (Figure 4) and was found to comprise three phases, with the rates of each of these phases varying with the concentration of substrate. The rate of the fastest process increased hyperbolically with *p*-OHB concentration, but the rate of the second and third phases increased and then decreased as *p*-OHB concentration increased (Figure 4). The K_d for *p*-OHB, measured by static titration, is 7.5 μM, so the kinetic binding experiment employed sufficient substrate concentrations to ensure saturation of the enzyme with *p*-OHB. A consistent kinetic model decribing the binding process could not be obtained from the available data. Thus, we do not fully understand the molecular events giving rise to the three phases at this time.

Substrate Activation. Activation of the substrate is thought to be crucial in the hydroxylation process of PHBH (Entsch

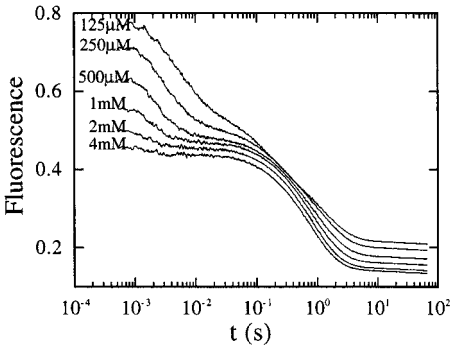

FIGURE 4: Binding kinetics of *p*-OHB to 10 μM PHBH mutant K297M measured by fluorescence. The sample was excited at 450 nm, and emission beyond 515 nm was selected using a short wavelenth cutoff filter. Concentrations of *p*-OHB are indicated.

et al., 1991). WT PHBH provides a network of hydrogen bonds that extends from the substrate 4-hydroxyl group to histidine 72 at the surface of the protein (Palfey *et al.*, 1994; Schreuder *et al.*, 1994, Gatti *et al.*, 1996). One purpose of this network is to facilitate the deprotonation of the substrate and activate it to *ortho* substitution. In solution, the pK_a of the *p*-OHB 4-hydroxyl proton is 9.3, and when bound to the hydrogen bond network of the WT enzyme, it is lowered to 7.4 (Entsch *et al.*, 1991; Eschrich *et al.*, 1993; Schreuder *et al.*, 1994). The extinction change between anionic and dianionic forms of the substrate is 16.9 mM^{-1} cm^{-1} at 290 nm. Measurements of the extinction changes of K297M at pH values of 6−8.5 indicated that only 25% of the possible degree of deprotonation occurred at pH 8.5 (data not shown). The enzyme is not sufficiently stable above pH 8.5 to permit accurate measurements, but extrapolation of the available data suggests a pK_a of approximately 9.

Calculation of Electrostatic Potential. The program DelPhi was used to calculate the electrostatic potential within the active site to ascertain the charge that might direct the hydroxylation reaction path. The calculations were performed using the WT•*p*-OHB crystal structure for three conditions. First, bonds in the hydrogen bond network (described above) were oriented with *p*-OHB donating its 4-hydroxyl proton to Tyr201 and Tyr201 donating to Tyr385. In the second orientation, the protons of Tyr385, Tyr201, and *p*-OHB were reversed so that the hydrogen of *p*-OHB pointed toward the carbonyl of Pro293, which lies behind the pyrimidine end of the isoalloxazine moiety in Figure 1. Both of these orientations are thought to occur upon binding *p*-OHB when the pH is lower than the phenolic pK_a (Gatti *et al.*, 1996; Schreuder *et al.*, 1994). The electrostatic potential was also calculated with *p*-OHB in the phenolate form and the protons of Tyr201 and Tyr385 in the same orientation as in the first condition above. These calculations indicate that the flavin, substrate, and the majority of active site amino acids of the WT enzyme lie in a field of positive potential that does not change greatly with the orientation of the hydrogens within the proton network or with the protonation state of the substrate. There are two regions of higher positive potential within the active site (≥50 kT/e). One of these is located on the *si* face of the isoalloxazine, adjacent to the xylene moiety, and it engulfs the carboxylate of the substrate and the guanidinium of Arg214. The second region is centered on the hydroxyls of Tyr201 and Tyr385 (see Figure 5A). Calculations of the electrostatic field for the K297M mutant (based on the coordinates for the WT•*p*-OHB crystal structure, see Figure 5B) indicate that the overall distribution of positive potential in the mutant is very similar to that calculated for the WT, except in a region near the side chains of Tyr201 and Tyr385, where there is a significantly less positive character than in the WT (decreases of 16 kT/e at Tyr201 and 23 kT/e at Tyr385; see Figure 5C). This calculation suggests that the high pK_a of *p*-OHB bound to the mutant enzyme is the result of a perturbation in the electrostatic field near *p*-OHB.

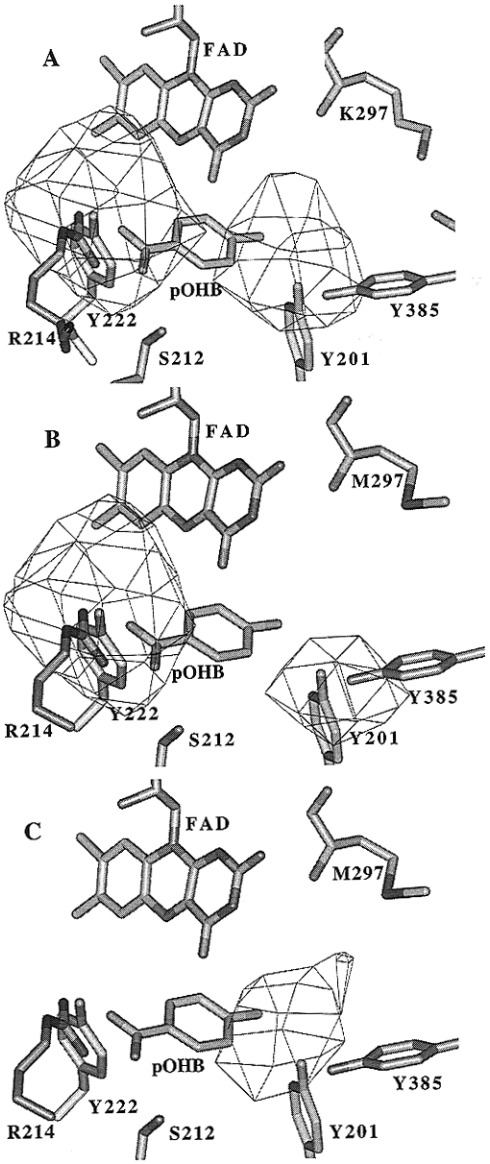

FIGURE 5: DelPhi electrostatic calculations for the WT and K297M forms of PHBH. All coordinates files were based upon the WT•*p*-OHB crystal structure determined by Schreuder et al. (1989). Part A represents regions of high positive potential (×b3≥50 kT/e) in the WT active site by shaded areas. Part B represents a similar determination as in part A, except that lysine 297 has been replaced with nonoptimized coordinates for a methionine residue. Part C has the coordinates for part B overlayed with the difference of the potential grids of parts B and A. The shaded area shown in part C represents a region of less positive potential (≤−20 kT/e) than the average of the protein.

Catalytic Properties. The catalytic cycle of PHBH consists of a reductive half-reaction, in which the flavin is reduced by NADPH, and an oxidative half-reaction, in which O_2 reacts with the reduced flavin and the substrate is hydroxylated. Each half-reaction can be studied by stopped-

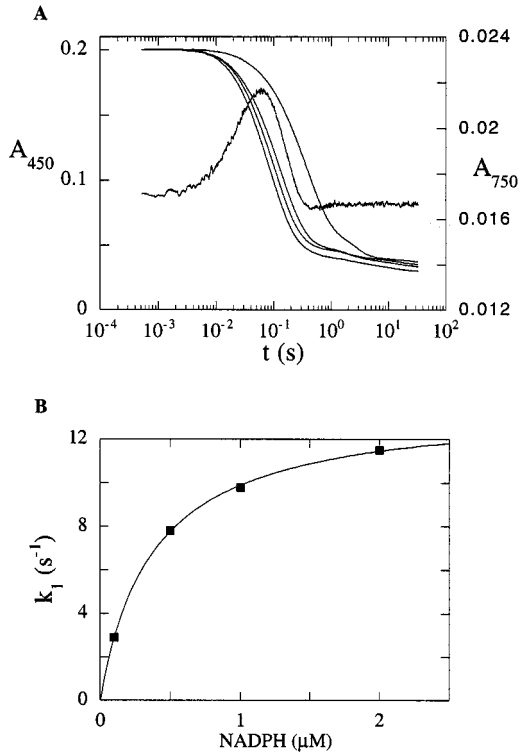

FIGURE 6: Kinetics of the reductive half-reaction of the PHBH mutant K297M·*p*-OHB complex. (A) Stopped-flow reaction traces of the reduction process monitored at 450 nm. NADPH concentrations were 100, 500, 1000, and 2000 μM (right to left). Charge-transfer interaction measured at 750 nm and 2000 μM NADPH overlays the 450 nm traces and shows a rise and fall (noisier trace). The concentration of enzyme used in the reaction was 20 μM. (B) Dependence of the rate of reduction of K297M·*p*-OHB complex on NADPH concentration.

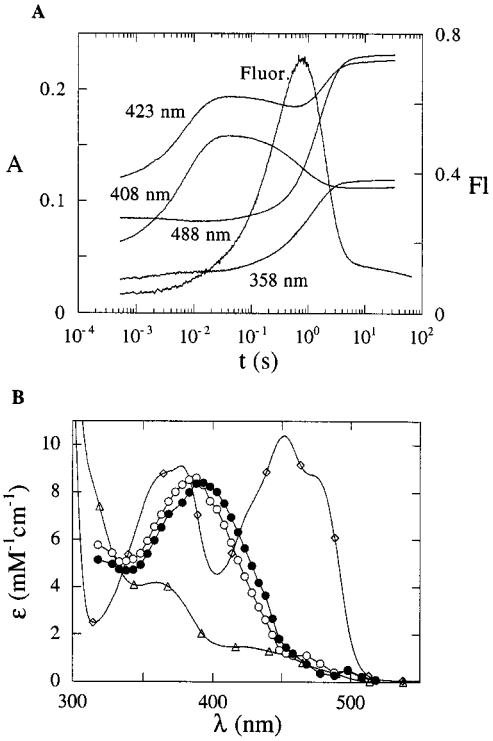

FIGURE 7: Kinetics of the oxidative half-reaction of the PHBH mutant K297M in the presence of the native substrate, *p*-OHB. (A) Absorbance (27.1 μM K297M, 200 μM *p*-OHB) and fluorescence (8.7 μM K297M, 400 μM *p*-OHB) changes observed in the oxidative half-reaction (fluorescence excitation at 400 nm). (B) Spectra of all species detected in the oxidative half-reaction; (△) reduced K297M·*p*-OHB complex, (●) C4a-hydroperoxyflavin, (○) C4a-hydroxyflavin, (◇) oxidized K297M·*p*-OHB complex.

flow methods, allowing a detailed picture of catalysis to be obtained.

Reductive Half-Reaction. In the stopped-flow spectrophotometer, anaerobic oxidized K297M complexed with *p*-OHB was mixed with anaerobic solutions of NADPH and *p*-OHB. At 450 nm, the traces showed a short lag followed by a monophasic process (see Figure 6). The observed rate constant for the reduction of the flavin demonstrated a hyperbolic dependence on NADPH concentration, with a limiting reduction rate constant of 13.6 s^{-1} (Figure 6B), about one-quarter that of WT, and a K_d for NADPH of 373 μM, almost twice that of WT. Long wavelength charge-transfer absorbance was observed in the reduction reaction (750 nm trace in Figure 6A); its formation and decay could be fit to two phases. One phase was dependent on NADPH and corresponded to the reduction observed at 450 nm, while the other (25 s^{-1}) was independent of NADPH concentration and probably represents the dissociation of NADP from the reduced enzyme.

The reduction reaction of this mutant in the presence of the substrate analog 2,4-DOHB was also found to be similar to the reduction of the WT·2,4-DOHB complex; the reaction was monophasic with a maximum rate constant (0.29 s^{-1}) one-third the rate constant for the reduction of the WT with this ligand (0.82 s^{-1}). The K_d for NADPH (951 μM) was half that of the WT (K_d, WT = 1.5 mM) and no charge-transfer interactions were observed in the reduction reaction.

Oxidative Half-Reaction. The reduced K297M·*p*-OHB complex was mixed in the stopped-flow spectrophotometer with solutions containing substrate and various concentrations of oxygen; three reaction phases were observed at wavelengths between 320 and 520 nm. The first of these phases was oxygen dependent and second-order, occurring with a rate constant of 2.4 × 10^5 M^{-1} s^{-1}, similar to the WT; this phase led to the formation of a C4a-hydroperoxyflavin (Figure 7) (Entsch *et al.*, 1976) that was stabilized relative to the WT. This hydroperoxyflavin intermediate converted to the C4a-hydroxyflavin in analogy to the conversions in the WT oxidative half-reaction. The deconvoluted spectra of the oxygenated flavin intermediates are shown in Figure 7B. The hydroperoxyflavin spectrum is essentially the same as that of WT (λ_{max} = 393 nm, ϵ = 8.6 $mM^{-1}cm^{-1}$). However, the hydroxyflavin spectrum is significantly red shifted compared to WT to the point that it is almost identical to that of the hydroperoxyflavin (λ_{max} = 388 nm, ϵ = 8.4 mM^{-1} cm^{-1}).

Due to the similarity in absorbance spectra of the hydroperoxy- and hydroxyflavins, there was minimal change in amplitude available to quantify the rate of hydroxylation (see Figure 7A, 423 nm trace). However, measurement of the hydroxylation rate was readily accomplished from observation of the fluorescence emission beyond 520 nm when the enzyme was excited at 400 nm. As observed in absorbance experiments, three phases were also detected by fluorescence (see Figure 7A). The first phase was rapid (complete within the first 50 ms) and corresponded to the reaction of reduced FAD with molecular oxygen to form the weakly fluorescent flavin hydroperoxide. The next phase occurred at 1.8 s^{-1} and was due to the formation of the highly fluorescent hydroxyflavin, accurately defining the hydroxylation rate

Table 2: Oxygen Half-Reaction Rate Constants for WT, N300D, and K297M, with and without Azide[a]

enzyme	k_2 (M^{-1} s^{-1})	k_3 (s^{-1})	k_4 (s^{-1})
WT	2.8×10^5	47	14
WT + N_3^-	2.7×10^5	6.5	1.5
N300D	3.9×10^5	1.1	0.12
N300D + N_3^-	2.8×10^5	0.8	0.09
K297M	2.4×10^5	1.8	0.9
K297M + N_3^-	2.4×10^5	0.8	0.3

[a] Values for N300D are reproduced from Palfey *et al.* (1994). Values for WT are reproduced from Entsch *et al.* (1976) and Entsch and Ballou (1989). The reaction shown below indicates the participation of the rate constants.

$$\text{EFlH}\cdot p\text{-OHB} + O_2 \xrightarrow{k_2} \text{EFlHOOH}\cdot p\text{-OHB} \xrightarrow{k_3} \text{EFlHOH}\cdot\text{PCA} \xrightarrow{k_4} \text{EFl}\cdot p\text{-OHB}$$

(which was 25-fold slower than that of WT). The final phase of 0.9 s^{-1} coincided with the decay of the hydroxyflavin (16-fold slower than WT) and regeneration of the oxidized enzyme.

The effect of 100 mM azide on the oxidative half-reaction of K297M was investigated. Crystal structures show three anion binding sites in PHBH (Lah *et al.*, 1994a; Gatti *et al*., 1994). In the WT enzyme, bound anions are known to reduce the affinity of the enzyme for *p*-OHB and for NADPH (Steenis *et al*., 1973), as well as the rates of hydroxylation and of dehydration of the flavin hydroxide (Entsch *et al.*, 1976; Shoun *et al.*, 1983). Azide has been used to resolve processes of the oxygen half-reaction. In 100 mM azide, the WT enzyme demonstrates a 7-fold lower rate of both hydroxylation and decay of the hydroxyflavin, while the initial formation of the flavin hydroperoxide remains unaffected. Azide has a minimal effect upon the oxidative half-reaction of the PHBH mutant N300D (Palfey *et al.*, 1994), slowing the hydroxylation and reoxidation steps by factors of only 1.2 and 1.4, respectively. With the K297M mutant, similar minor decreases in the rates of these processes were observed. In the presence of azide, hydroxylation was slower by a factor of 2 and the decay of the hydroxyflavin was slower by a factor of 3 (Table 2).

The mutant enzyme carries out catalysis at 0.50 s^{-1} at pH 6.5 and 4 °C when observed by enzyme-monitored turnover (WT is 6.2 s^{-1} under these conditions) and produces 0.87 mol of PCA for each mole of NADPH consumed. This turnover rate is completely consistent with the measured transient state reaction rates. During enzyme-monitored turnover, only 15% of the 450 nm flavin absorbance due to oxidized enzyme is observed. This implies that steps in the oxidative half-reaction are primarily responsible for the low turnover rate. Overall, the K297M mutant is a competent hydroxylase that is very similar to WT except for the lower turnover number observed.

DISCUSSION

The active site of PHBH is surrounded by positively charged residues, and four are in relatively close proximity (<10 Å) to the isoalloxazine or aromatic substrate. The roles of three of these residues have already been investigated by site-directed mutagenesis (van Berkel *et al.*, 1992; Eppink *et al.*, 1995; Moran *et al.*, 1996). Arginine 214 forms a salt bridge with the carboxylate of *p*-OHB and other aromatic ligands. When it is mutated to an uncharged residue, ligand binding is much weaker and catalysis is severly disrupted. Arginines 220 and 44 are close to the active site. Arg 44 hydrogen bonds with the phosphate of the flavin, while Arg 220 interacts with the 4-carbonyl of the isoalloxazine when the flavin adopts the "out" conformation. Mutagenesis of both of these residues also severly disrupts catalysis. Impaired catalysis in these three cases is caused by changing a residue that is in direct contact with components of the reaction. Only the fourth positively charged residue within 10 Å of the active site, Lys 297, does not interact directly with the substrate or flavin. Thus, residue 297 is an ideal candidate for investigating electrostatic effects by mutagenesis.

The electrostatics calculations demonstrate that the active site of PHBH has a significant positive potential relative to the rest of the protein, such that it would stabilize negative charges generated during catalysis. For example, after reacting with NADPH, an anionic flavin is formed (Vervoort *et al.*, 1991). It has been proposed (Entsch *et al.*, 1994) that a positive electrostatic field localizes the anionic reduced flavin in the active site adjacent to the substrate. Since flavin movement appears to be required for substrate association and dissociation (Schreuder *et al.*, 1994; Gatti *et al.*, 1994), specific localization of the reduced form of flavin (the form that reacts with oxygen) into the hydroxylation site would prevent significant substrate dissociation before hydroxylation can occur. Consistent with this notion of electrostatic inhibition of ligand exchange from the reduced enzyme is the finding that the rate at which *p*-OHB associates with the reduced enzyme is at least 10^4-fold slower than the rate of association with the oxidized enzyme (Entsch *et al.*, 1976), even though the overall binding constant is essentially unaffected. It is known that when oxygen reacts with substrate-free reduced enzyme, H_2O_2 is formed rapidly, which in principle, could give rise to a wasteful consumption of NADPH. However, the enzyme ensures that productive reactions occur by at least two mechanisms. (a) Although the redox potential is barely affected, the rate of reduction of the flavin by NADPH is increased about 10^5-fold by the presence of substrate (Husain & Massey, 1979; Shoun *et al.* 1979). Thus, in the absence of substrate, very little reduced flavin is formed that would permit production of H_2O_2. (b) Once the flavin is reduced (in the presence of substrate), the enzyme maximizes hydroxylation by not easily releasing the substrate until the hydroxylation is complete.

WT PHBH facilitates deprotonation of the substrate by lowering the pK_a of the substrate 4-hydroxyl group (in a hydrophobic environment) by about 2 units below its value in water. The substrate 4-hydroxyl proton is part of a chain of five hydrogen bonds extending from the backbone carbonyl of proline 293 to histidine 72 at the surface of the protein (Schreuder *et al.*, 1994; Gatti *et al.*, 1996). The formation of the substrate phenolate would be promoted in a positive electrostatic field and our calculations indicate a region of high positive potential near the hydroxyls of tyrosines 201 and 385 that may accomplish this. Frontier orbital calculations (Vervoort *et al.*, 1992; Perakyla & Pakkanen, 1993) indicate that the highest electron density resides *para* and *ortho* to the 4-hydroxyl group in the phenolate form of *p*-OHB and is higher than in the phenolic form. Thus, the deprotonated substrate is more susceptible to an electrophilic substitution reaction. The heterolytic cleavage of the flavin hydroperoxide oxygen−oxygen bond

generates, at least transiently, a negative charge on the nascent flavin hydroxide. The ability to stabilize such a negative charge has been shown to be an important determinant in the oxygen transferring ability of model hydroperoxides (Bruice *et al.*, 1983). A positive electrostatic field provided by the protein may thereby facilitate the reactivity of the enzymatic flavin hydroperoxide.

In principle, selectively altering the charge of the protein by site-directed mutagenesis offers a means to elucidate the importance of electrostatics in various catalytic steps. However, mutagenesis can also alter the structure of the protein, causing changes in catalysis that are not purely electrostatic. Therefore, some means of assessing the structural integrity of a mutant protein is desirable. Unfortunately, attempts to crystallize the K297M mutant in complex with *p*-OHB produced only microcrystals that were unsuitable for diffraction analysis. However, our data indicates a flavin structural environment in the mutant that is essentially unchanged compared to that in the WT. This similarity is evident in the nearly identical ligand affinities and flavin spectral properties, which are known to be sensitive to changes in structure (Gatti et al, 1994). Both methionine and lysine are unbranched and of similar van der Waals volume and therefore they have similar packing requirements. It has been shown that the methionine to lysine mutations have a significant destabilizing effect in studies of helices of T4 lysozyme (Dao-pin *et al*., 1991). The reverse mutation (as in the present work) would therefore be expected to stabilize the helices of PHBH. It has also been shown that substitutions of amino acid residues (including lysine) by methionine have minimal effects on the structure of proteins (Gassner *et al.*, 1996), reflecting the flexibility of the methionine side-chain. For this reason, one might rationalize that the mutation in K297M would result in no significant structural alterations. If there are no significant structural changes, as seems likely, then the changes caused by the mutation are primarily the result of changes in the electrostatic environment of the active site. The decrease in the redox potential of the mutant by 12 mV compared to that of WT is very similar to the change suggested by the studies of Swenson and Krey (1994) and Zhoa and Swenson (1995), who found that an additional negative charge near the flavin of flavodoxin decreases the redox potential by about 15 mV.

The calculation of the electrostatic field of K297M based on the structure of the WT enzyme (see Figure 5B) indicates that the methionine substitution diminishes the positive potential adjacent to the tyrosines of the proton network (see Figure 5C); therefore, there would be less stabilization of the substrate phenolate resulting in a higher pK_a for the 4-hydroxyl group. This was confirmed experimentally. The apparent lack of substrate activation could account for our observation that the hydroxylation step is 25-fold slower than that of the WT.

The reductive half-reaction of K297M is not significantly different from that of WT. We observed a 4-fold decrease in the extrapolated maximum reduction rate, and only a 2-fold increase in the dissociation constant for NADPH. Flavin-pyridine nucleotide charge-transfer absorbance was observed at long wavelengths prior to and after hydride transfer, indicating orbital overlap of the pyridine nucleotide with the flavin, and a geometry between the ring systems favorable for reduction (Massey & Ghisla, 1974; Husain & Massey, 1979; Entsch *et al.*, 1991). The rate of decay of the long wavelength charge-transfer bands indicates that the NADP dissociation rate (25 s^{-1}) is the same as for WT. It can therefore be concluded that the mutation caused no important structural changes in the reduction reaction, and the positive electrostatic field at the active site is not of major importance in the hydride transfer reaction. A negative charge is produced upon isoalloxazine reduction (Vervoort *et al.*, 1991), and the flavin in the transition state should bear a partial negative charge. In accord with this, studies of model dihydropyridine hydride transfers indicate that significant charge develops in the transition state (Kurz & Frieden, 1975). Furthermore, a semiempirical quantum mechanical study of hydride transfer from dihydronicotinamide to isoalloxazine (Mestres *et al.*, 1996) found, using either the AM1 or PM3 Hamiltonians, that the flavin in the transition state develops a charge of about −0.5. Therefore, it is reasonable to expect the active site electrostatic field to provide significant stabilization of the partial charge of the transition state, but this is not our experimental finding with PHBH. It is not clear, however, that the hydride reaction takes place in the currently recognized active site. The finding that the flavin of PHBH is mobile (Gatti *et al.*, 1994; Schreuder *et al.*, 1994) introduces the possibility that NADPH and FAD react when the flavin adopts the "out" position, which is removed from the core of the positive electrostatic potential. Preliminary evidence consistent with this possibility has been obtained (Palfey *et al.*, 1997). If this is true, mutations altering the electrostatic potential near the "in" position would likely have little effect on any reaction occurring at the "out" position.

Many of the effects of the K297M mutation are reminiscent of those observed in the N300D mutation of PHBH (Palfey *et al*, 1994). The net positive charge of the active site was decreased in the N300D enzyme by the mutation of a neutral side chain to a negatively charged side chain. Like N300D, the K297M mutation also reduces the net positive charge near the active site by 1 unit. In the WT enzyme, the side chain of Asn300 is in direct contact with the flavin isoalloxazine. The mutation to the negatively charged aspartate residue caused several relatively long range but small structural changes, as determined crystallographically (Lah *et al.*, 1994b). This was consistent with the observation of only small changes in the flavin spectral properties and ligand affinities. The numerous small structural shifts observed in the crystal structure of the N300D mutant made it difficult to definitively attribute the catalytic alterations to charge alone.

A comparison of the results obtained from studies of the K297M mutant and the N300D mutant indicates that many of the altered catalytic properties of the N300D mutant, particularly those of the oxidative half-reaction, are due to electrostatic changes in the active site caused by the mutation. The pK_a of bound *p*-OHB is higher in both mutants than in the WT enzyme, an effect that is attributable to electrostatics. The oxidative half-reactions of both mutants are quite similar. The N300D mutant also hydroxylates slowly (1.1 s^{-1}) without elimination of H_2O_2 from the flavin hydroperoxide. The spectrum of the hydroxyflavin intermediate formed in the hydroxylation reaction of both mutants are very similar and are red shifted compared to that of the WT enzyme. It is not clear what causes this shift, although it appears to be a consequence of the change in the electrostatic field at the

active site. One possibility is that in the mutants, the C4a-oxygen of the hydroxyflavin is protonated, but in the WT enzyme, with a higher electrostatic potential near the flavin, the oxygen is partially deprotonated. It should be noted that the pK_a of this proton has been estimated to be about 9 for model flavin hydroxides in solution (Bruice et al, 1983) and when bound to the enzyme in a field of positive potential, the pK_a will likely decrease.

Although the mutants behave similarly in the oxidative half-reaction, the results obtained for the reduction of the two mutants are quite different. Hydride transfer occurred in the N300D mutant at a rate 330-fold slower than with the WT and no charge-transfer intermediates were observed, but the K297M mutant reacted only about 4-fold slower than the WT and charge-transfer intermediates did form. It was reasoned that redox potential alone could not account for the slow reduction in N300D, and it was assumed that much of the effect was a consequence of less-than-ideal orientations of the flavin and pyridine nucleotide rings in the active site (Palfey *et al.*, 1994) caused by the mutation. The presence of charge-transfer bands during the reduction of K297M and the lack of them in the N300D mutant supports the notion that active site alterations observed in the crystal structure of the N300D mutant impede hydride transfer due to an incorrect geometry for NADPH binding (on the *re*-side of the flavin; Manstein *et al.*, 1986).

The comparatively small inhibitory effects of azide on the oxidative half-reaction of the K297M mutant enzyme are similar to the small effects due to azide observed with the N300D mutant (Palfey et al, 1994) and may be rationalized in a similar manner. Azide apparently inhibits these enzymes by decreasing the electrostatic potential at the active site, resulting in deactivation of *p*-OHB by increasing the pK_a of the bound substrate 4-hydroxyl (Shoun *et al.*, 1983). In the case of the WT enzyme, this shift is significant. In the case of the two mutant enzymes, however, the electrostatic potential of the active site is already lowered, and the pK_a of bound *p*-OHB is already high, so that there is little additional effect upon azide binding.

CONCLUSION

The positive electrostatic field at the active site of PHBH appears to decrease the phenolic pK_a of bound *p*-OHB and enhance the rates of the hydroxylation reaction and the elimination of water from the flavin hydroxide. These features are perturbed significantly by the K297M mutation, which decreases the active site electrostatic potential. In contrast, the hydride transfer reaction from NADPH to FAD is not highly sensitive to alterations of the active site potential.

SUPPORTING INFORMATION AVAILABLE

Calculated charges of the isoalloxazine and ribityl C1′ as well as those of *p*-OHB (2 pages). Ordering information is given on any current masthead page.

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