Evidence for Flavin Movement in the Function of *p*-Hydroxybenzoate Hydroxylase from Studies of the Mutant Arg220Lys†

Graham R. Moran,‡,§ Barrie Entsch,*,‡ Bruce A. Palfey,|| and David P. Ballou*,||

Department of Molecular and Cellular Biology, University of New England Armidale, New South Wales 2351, Australia, and Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109-0606

Received February 14, 1996; Revised Manuscript Received April 29, 1996⊗

ABSTRACT: The isoalloxazine ring system of the FAD cofactor of *p*-hydroxybenzoate hydroxylase must be secluded from solvent at specific stages of catalysis in order to form and stabilize a flavin C4a-hydroperoxide. This species may then react with the activated phenolate of *p*-hydroxybenzoate. A number of crystal structures of the enzyme with alterations to active site substituents or complexes with analogue benzoates have revealed an alternate position for the isoalloxazine (Gatti *et al.* (1994) *Science 266*, 110−114; Schreuder *et al.* (1994) *Biochemistry 33*, 10161−10170). This new flavin conformation is 7 Å "out" toward solvent and may open a passage for substrate entry to the active site. Arginine 220 is one of the few residues in the structure to demonstrate conformational changes when the flavin is "out". In this study we have made the Arg220Lys mutant to test the significance of this residue in flavin movement. The R220K mutation has brought about dramatic alterations to all aspects of catalysis. Stopped-flow kinetic characterization of the mutant has revealed that, while the effector role for the substrate is maintained, there exists an order of magnitude decrease in the limiting rate of reduction, even though there is 40-fold increase in association with NADPH. The mutant enzyme has only a fraction of its reductive half-reaction coupled to product formation, and the hydroxylation process is slow. This occurs despite a higher proportion of the more activated substrate phenolate in the active site. Many of the observed changes can be attributed to a decrease in the stability of the "in" conformation of the flavin during the catalysis and indicate a role for flavin conformational states in many of the catalytic processes of the enzyme.

Intramolecular movement can be a vital part of protein function, though it has always been difficult to detect and tends to be unique for each type of protein (McCammon & Harvey, 1987). We and others have recently reported a quite large movement of the flavin in the active site during the catalytic function of the flavoprotein, *p*-hydroxybenzoate hydroxylase (PHBH;[1] EC 1.14.13.2; Lah *et al.*, 1994b; Gatti *et al.*, 1994; Entsch *et al.*, 1994; van Berkel *et al.*, 1994a,b; Schreuder *et al.*, 1994). This work has suggested that movement of the flavin opens access to the substrate binding site and is linked to the substrate binding process. This detectable movement of the flavin in PHBH is particularly interesting because it involves the catalytic cofactor in the active site, rather than movement between domains or subunits.

PHBH has become a model example for the large number of flavoprotein hydroxylases that have been isolated (Entsch & van Berkel, 1995). The reaction catalyzed is illustrated in Figure 1. To carry out oxygenation of *p*-hydroxybenzoate (pOHB), the enzyme must be able to form a flavin hydroperoxide species and present it to the substrate while keeping it isolated from water. If water were to come in contact with the flavin hydroperoxide, the latter would rapidly lose H_2O_2, leaving oxidized flavin without hydroxylating the substrate. This is what occurs with flavin hydroperoxides in solution (Kemal & Bruice, 1977). The first X-ray crystallographic structures for the enzyme showed a conformation that had the flavin sufficiently proximal to the substrate to achieve hydroxylation (flavin "in") (Wierenga *et al.*, 1979; Schreuder *et al.*, 1989; and Figure 2). However, it has been known for many years (Entsch *et al.*, 1976) that PHBH exchanges substrate rapidly when the FAD is oxidized, but only very slowly (at a catalytically insignificant rate) when the FAD is reduced. This observation was difficult to explain until a second conformation of the active site was observed recently by crystallographic analysis (Lah *et al.*, 1994b; van Berkel *et al.*, 1994b). The flavin had moved from the "in" position to a solvent-accessible position in the protein (flavin "out" in Figure 2), where rapid exchange of pOHB might be possible with its binding position in the active site. It was proposed that the flavin is located in the "in" position when reduced, so that substrate exchange and access to the solvent are impeded. The clearest evidence in support of this proposal has been the covalent attachment of the protein to a photoactivated 6-azido flavin isoalloxazine ring while in the "in" position (Gatti *et al.*,

† Financial support for this research from the United States Public Health Service (GM 20877 to D.P.B., GM 11106 to Vincent Massey for B.A.P.) and an Australian Research Council Grant (A09330330 to B.E.).

* To whom correspondence should be addressed.

‡ University of New England Armidale.

§ Present address: Department of Biochemistry and Biophysics, Texas A&M, College Station, TX 77843.

|| University of Michigan.

⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1996.

[1] Abbreviations: PHBH, *p*-hydroxybenzoate hydroxylase; *pobA*, the gene encoding *p*-hydroxybenzoate hydroxylase; 2,4DOHB, 2,4-dihydroxybenzoate; FAD, flavin adenine dinucleotide; WT, wild type; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

S0006-2960(96)00360-1 CCC: $12.00

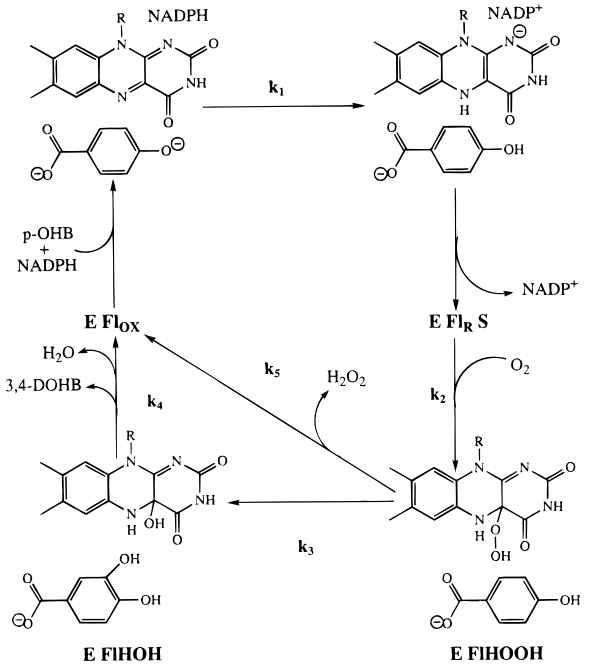


FIGURE 1: Catalytic cycle of *p*-hydroxybenzoate hydroxylase. Abbreviations used: E Fl$_R$S, enzyme−substrate complex with the flavin reduced; E FlHOOH, enzyme with C4a-flavin hydroperoxide; E FlHOH, enzyme with C4a-flavin hydroxide; E Fl$_{OX}$, enzyme with oxidized flavin.

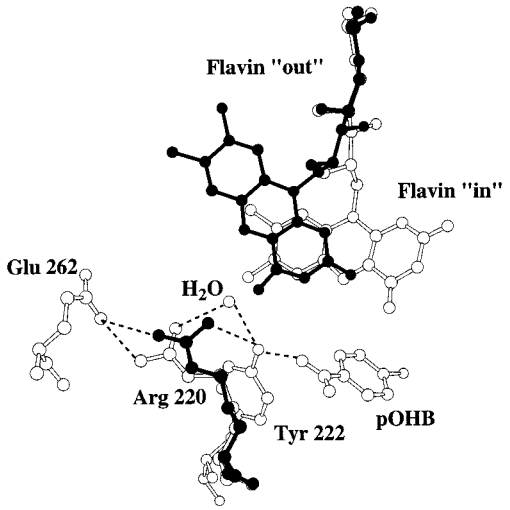


FIGURE 2: The shift in the R220 guanidinium during flavin movement. The "out" position of the flavin is overlaying the "in" position and is indicated by solid bonds. The shift of the R220 guanidinium is indicated in the same manner. Hydrogen bonding interactions between the pOHB carboxyl, Y222 hydroxyl, a water, R220 guanidinium, and the carboxylate oxygen of E262 are altered during the movement of the flavin to the "out" position. The water molecule is displaced and the R220 η guanidinium hydrogen bonds to Y222, while the hydrogen bond from R220 to E262 is maintained. Dashed lines denote sufficiently short distances for hydrogen bonding interaction.

1994), thus preventing its movement to the "out" position. This modified enzyme no longer exchanged pOHB with the active site at a significant rate.

We wanted to test by mutagenesis the wider significance of flavin movement in protein function. The crystal structure in which the flavin "in" conformation was first observed was the complex between wild type enzyme (WT) and its native substrate, pOHB (Wierenga *et al.*, 1979; Schreuder *et al.*, 1989), while the flavin "out" conformation was detected in the complex between mutant enzyme Y222F and pOHB, in the mutant Y222A complexed with 2-amino-4-hydroxybenzoate, in the WT enzyme complexed with 2,4-dihydroxybenzoate, and in the complex between enzyme containing arabino-FAD and pOHB (Lah *et al.*, 1994b; Gatti *et al.*, 1994; van Berkel *et al.*, 1994a,b; Schreuder *et al.*, 1994). All of the structures in which the flavin "out" positions have been detected include some change to active site substituents or substrates. The proposal that the flavin "out" conformation participates in catalysis would be more convincing if it could be shown to occur in enzyme with no modifications to the active site. A common feature of the observed flavin "out" conformations is a 1−2 Å tilt of the R220 guanidinium group toward the flavin (Figure 2 and Lah *et al.*, 1994b). The simplest crystallographic interpretation was that R220 may be involved in flavin conformational movements. Arginine 220 is well outside the immediate area thought to be essential to the chemical events in catalysis. The R220K mutant was therefore developed in an attempt to make a conservative change that might alter the balance of interactions of flavin in the active site. Other mutations of R220 would involve a change of charge in the enzyme, and earlier work had shown that charge changes in the area around the active site can cause major changes to enzyme function (van Berkel *et al.*, 1992; Lah *et al.*, 1994a; Palfey *et al.*, 1994; Eppink *et al.*, 1995).

This paper reports the properties of the mutant R220K relative to the WT enzyme. We found that R220K is an enzyme with substantially modified properties. This mutant stabilizes the conformation with flavin "out" more than does the WT, and this apparently small perturbation substantially decreases the catalytic efficiency of the enzyme. The results presented are consistent with the hypothesis that the "out" conformation is an integral part of the mechanism for exchange of substrate with the active site, and also that the balance of conformational states is an important component in many of the chemical processes in catalysis.

EXPERIMENTAL PROCEDURES

The bacterial strains and plasmids used in this work have been described in detail in previous publications (Palfey *et al.*, 1994; Entsch *et al.*, 1991; Moran & Entsch, 1995). Reagents and equipment used are described in the References (Palfey *et al.*, 1994; and Entsch *et al.*, 1991. The structural graphics shown in Figure 2 were generated from the coordinates for the enzyme using the WT·pOHB complex structure (Schreuder *et al.*, 1989) and the WT·2,4DOHB complex structure (Gatti *et al.*, 1994).

The Arg220Lys mutation was generated based on the procedures originally described by Kunkel *et al.* (1987) and presented in detail in Entsch *et al.* (1991). To generate R220K, the unusual step of a triple-base substitution was required (CGC to AAG). This was successfully carried out with a 27-mer synthetic oligonucleotide with a mutating codon in the middle of 12-base arms that were complementary to the *pobA* gene.

The mutant enzyme expressed at very high levels (greater than 10% of cell protein in the native folded state) in *Escherichia coli* when using the expression vector described by Moran and Entsch (1995). The protein was easily purified using a Procion Red column as described by Palfey *et al.* (1994). Preparations used in the experiments reported here

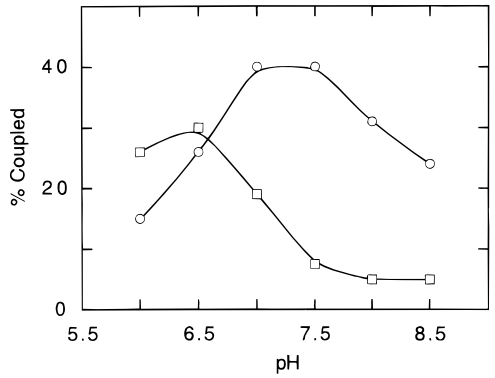


FIGURE 3: pH dependence of coupling of R220K. "% Coupled" represents the number of moles of substrate consumed as a percentage of the number of moles of NADPH oxidized. pOHB (□), 2,4DOHB (○).

had a ratio of absorbance at 280−450 nm of less than 10 with the enzyme FAD as the only species absorbing at wavelengths longer than 320 nm.

Unless stated otherwise, all reactions and measurements with enzyme were carried out in 50 mM phosphate buffer (K salt) and 10 mM EDTA (Na salt), pH 6.5 at 3−4 °C, conditions favorable for the detection of catalytic intermediates (Entsch *et al.*, 1976). The following methods for measurements have been described in detail previously (Entsch *et al.*, 1991): enzyme extinction coefficients, ligand dissociation constants, reduction potentials, product analysis, enzyme monitored turnover, dissociation constants for ligand bound to enzyme, and kinetic analysis of transient states in catalysis. The procedure to measure the ligand dissociation constants with the reduced enzyme was a modified version of that described by Entsch *et al.* (1976). Purified argon was used to degas solutions and provided an inert atmosphere for titrating the enzyme.

RESULTS

Reaction Catalyzed. The catalytic cycle of the WT enzyme is shown in Figure 1. The WT enzyme consumes 1 mol of NADPH to produce 1 mol of 3,4-dihydroxybenzoate (3,4-DOHB) through the chemical steps identified by k_1, k_2, k_3, and k_4 in Figure 1. Measurements of product formation by HPLC indicate that the mutant R220K produces only a fraction of a mole of 3,4-DOHB per mole of NADPH consumed (see Figure 3), with the remainder resulting in formation of H_2O_2 via k_5. The relative balance of pathways is dependent upon pH (Figure 3). We observe in Figure 3 the deprotonation of two ionizable groups within the protein. The first ionization results in a greater proportion of hydroxylation and the second in a decrease in hydroxylation, with apparent pK_a's of 5.5 and 7.0 for when pOHB was substrate and 6.5 and 7.8 for when 2,4DOHB was substrate. A similar result was obtained with the mutant, Y222F (Entsch *et al.*, 1994), which has been shown by crystallography to have the flavin "out" conformation (Lah *et al.*, 1994b). The residue Y222 is involved in substrate binding in the active site (through a hydrogen bond to the carboxyl group of the substrate), whereas the residue R220 has no direct interaction with the substrate.

Substrate Binding. The formation of a complex between pOHB and enzyme causes spectral perturbations of the FAD in the active site. Recent work has established that there is a characteristic difference spectrum associated with formation

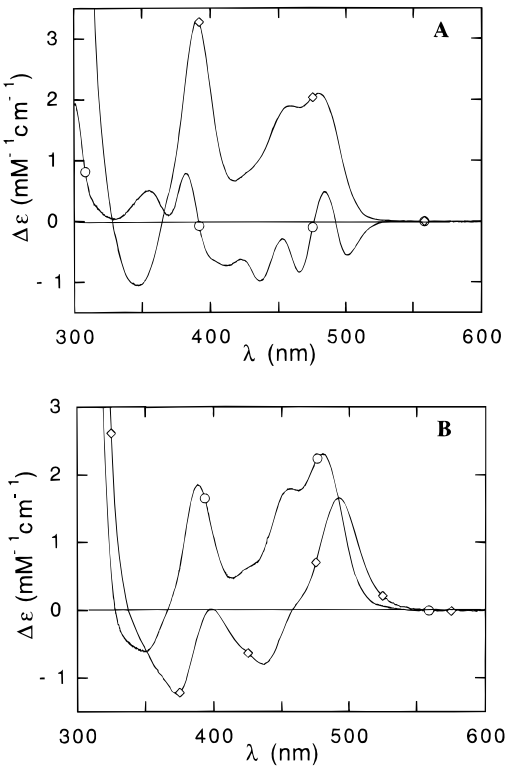


FIGURE 4: Comparison of the difference spectra observed for the WT and R220K enzymes. Saturated difference spectra of (A) WT and (B) R220K titrated with (○) pOHB or (◊) 2,4DOHB. Dissociation constants (see Table 1) were determined by titrating the enzyme (∼30 μM) in 50 mM KP_i and 10 mM EDTA, pH 6.5, 4 °C, with substrate. Absorbance spectra were recorded and the difference spectra obtained by subtracting the free enzyme spectrum from all subsequent spectra.

Table 1: Selected Properties of PHBH Mutant R220K Compared to WT

	WT	R220K
TN (s^{-1}) at pH 6.5, 4 °C[a]	6.2	2.1
% coupled pH 6.5, 4 °C[b]	100	16
K_d(pOHB)[c] (μM)	9.5	28
K_d(pOHB-E_{red})[c] (μM)	21	250
K_d(NADPH)[d] (μM)	210	5
E_{m7}, free enzyme[e] (mV)	−163	−178
E_{m7}, enzyme + pOHB[e] (mV)	−165	−212
E_{ox}, extinction 450 nm (mM^{-1} cm^{-1})	10.3	9.7

[a] Turnover number (TN) refers to the maximum rate of turnover measured in enzyme monitored turnover experiments with NADPH concentration at 50 μM and the concentrations of pOHB and O_2 extrapolated to infinity. [b] Hydroxylation stoichiometry determined from single turnover experiments and subsequent quantitative product analysis. [c] Dissociation constants for pOHB were determined in static titrations at pH 6.5 and 4 °C. Plotting the absorbance increments at wavelengths that demonstrate maximal perturbation against free substrate concentration gave a hyperbolic relationship that could be fit for the determination of dissociation constants (WT dissociation constants from Entsch *et al.*, 1991). [d] The dissociation constant for NADPH was determined by fitting the dependence of the rate of reduction by NADPH to a hyperbola (WT K_d for NADPH from Husain & Massey, 1979). [e] Redox potentials were determined in 0.05 M phosphate, pH 7.0, 25 °C (WT potentials from Entsch *et al.*, 1991).

of each of the two known conformational states of the flavin in oxidized enzyme (Gatti *et al.*, 1994; Entsch *et al.*, 1994; Schreuder *et al.*, 1994). When R220K was titrated with pOHB (Figure 4B and Table 1), the difference absorption spectrum generated correlates to the flavin shifting to the "out" conformation (Figure 4A).

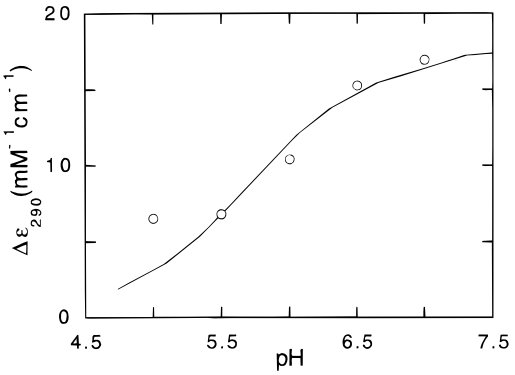


FIGURE 5: Determination of the pK_a of the 4-hydroxyl of pOHB bound to R220K. Deprotonation of the 4-hydroxyl of pOHB is associated with a 16.9 mM^{-1} cm^{-1} extinction increase at 290 nm. Titrating the mutant enzyme (15 μM) to saturation with pOHB at various pH values produces a range of $\Delta\epsilon_{290nm}$ measurements that permit the determination of the pK_a from the characteristic sigmoid curve. The curve (—) for a theoretical pK_a of 5.8 is overlaying the actual data (○). The measurement for pH 5.0 was included in the plot but excluded from the theoretical fit due to enzyme instability at this pH.

The oxidized WT enzyme in complex with pOHB has a characteristic pK_a of 7.4 due to deprotonation of the phenolic group on the substrate. This shift depends on both the interaction of Y201 with this group (Eschrich *et al.*, 1993; Entsch *et al.*, 1991) and the distribution of charge in the area of the active site (Palfey *et al.*, 1994). In the mutant under study (R220K), the pK_a of the substrate bound to the enzyme was lowered even further, to a value of approximately 5.8 (Figure 5), even though there is no obvious interaction of R220 with the substrate in the WT enzyme.

The crystal structure of the reduced WT enzyme in complex with pOHB has the flavin "in" (Schreuder *et al.*, 1992). The K_d for the substrate in this complex is very similar to that observed in the oxidized enzyme (Entsch *et al.*, 1976). When pOHB binds to the reduced R220K mutant, we observe a perturbation of the flavin spectrum similar to that seen for the reduced WT·pOHB complex, which suggests that flavin may be occupying the "in" conformation. However, the K_d of pOHB for this complex is approximately 12-fold larger than that of WT (see Table 1). The weaker binding of pOHB to reduced R220K compared to WT was also illustrated by the significant drop of the FAD redox potential (34 mV—see Table 1) when pOHB was bound to the enzyme compared to the enzyme without ligand (Figure 6). This potential shift is consistent with the binding data, indicating a 14-fold drop in association upon reduction of the enzyme. It was also found that pOHB binds significantly faster to reduced R220K than it does to reduced WT (a factor of 6.5). A lower barrier to the formation of the "out" conformation in the reduced R220K may explain the higher rate of substrate binding and dissociation, even though the equilibrium state of the flavin still favors the "in" position for the reduced complex. Since the mutation does not involve a residue interacting directly with the substrate or the flavin, these results suggest that there is a region near the active site that is linked to flavin movement and that this is in some way important in substrate binding. Whether the observed changes are a consequence of effects to either/both the rate of flavin movement or the fraction "in" versus "out" is not known.

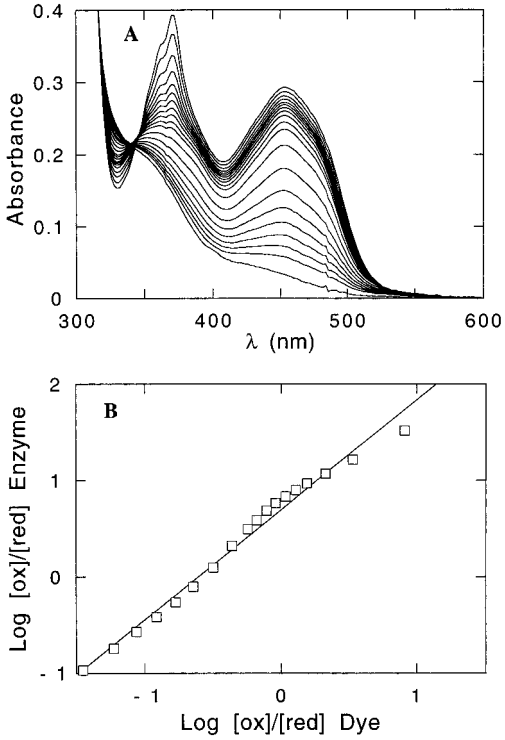


FIGURE 6: Determination of the redox potential of R220K·pOHB complex. (A) Successive spectra recorded in the simultaneous anaerobic reduction of R220K·pOHB and 1-hydroxyphenazine by xanthine oxidase (Massey, 1990). (B) Plot of log([ox]/[red] R220K) vs log([ox]/[red] 1-hydroxyphenazine). The calculated midpoint potential of R220K·pOHB complex is −212 mV.

Reductive Half-Reaction. The catalytic chemistry of the enzyme can be conveniently studied in two parts: reduction of enzyme bound FAD by NADPH in the presence of pOHB and, separately, the reaction of oxygen with the reduced enzyme in complex with pOHB to yield oxidized FAD bound to enzyme, 3,4-DOHB, and water (see Figure 1).

The reduction of R220K differed from that of WT and other mutants of this enzyme that have been studied. The oxidized enzyme in complex with pOHB was reacted under anaerobic conditions with a range of NADPH concentrations in the stopped-flow apparatus. The results are shown in Figure 7. NADPH formed a much tighter complex with the oxidized mutant enzyme (K_d of 5 μM) than with WT enzyme (K_d of 210 μM) (Howell *et al.*, 1972; Husain & Massey, 1979). However, the rate of reduction was approximately 17-fold lower than for WT (see the legend of Figure 7). The ternary complex of enzyme, pOHB, and pyridine nucleotide did form charge-transfer absorption bands (see Figure 7), as observed during the reduction of WT enzyme, but the intensities were 10-fold lower. This agrees with previous observations that charge-transfer bands correlate with a reductively competent ternary complex (Entsch & van Berkel, 1995).

Oxidative Half-Reaction. To study reactions with oxygen, the anaerobic enzyme complexed with pOHB was reduced with dithionite and then mixed in the stopped-flow apparatus with oxygen-equilibrated buffer containing pOHB. The reaction was monitored at selected wavelengths over a range of oxygen concentrations to measure oxygen-dependent processes, and also at high oxygen concentration over a range of wavelengths between 300 and 550 nm to permit determination of spectra of transient chemical species in the reaction.

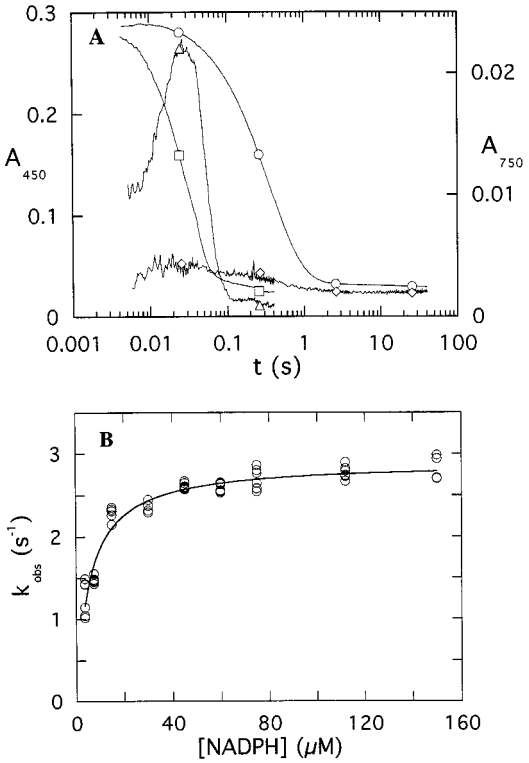


FIGURE 7: Reduction reaction of R220K. (A) Comparison of traces of reduction (450 nm) and charge-transfer bands (750 nm) for WT and R220K enzymes. The symbols □ and △ denote traces of WT reduction and charge transfer, respectively, while ○ and ◊ indicate respective reduction and charge-transfer absorbance traces for R220K. (B) Determination of the limiting reduction rate (k_1, 2.9 s^{-1}) and the dissociation constant of NADPH (5 μM) for R220K.

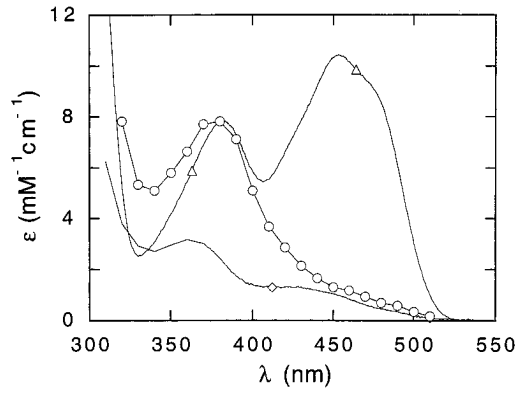


FIGURE 8: Spectra of reduced, oxidized, and flavin C4a-hydroperoxide of the R220K mutant. Reduced enzyme−pOHB complex (◊) was reacted with O_2 equilibrated buffer in the stopped-flow spectrophotometer. A flavin hydroperoxide was formed by 20 ms (○) that decayed in a single phase to the oxidized form (◊).

The reduced enzyme−pOHB complex reacted with oxygen to quantitatively form a flavin C4a-hydroperoxide (see k_2 in Figure 1 and spectra in Figure 8) in a second order reaction, with a rate constant of 2.0×10^5 M^{-1} s^{-1}, similar to the reaction of oxygen with the reduced WT enzyme (2.9×10^5 M^{-1} s^{-1}). The spectrum of the intermediate formed was characteristic of the C4a-hydroperoxy flavin observed in the WT oxidative half-reaction (Figure 8). This intermediate then decayed in a single phase to the complex between oxidized enzyme and pOHB (with a measured rate of 10.6 s^{-1}). However, the second reaction cannot be a single process, since separate measurements of product formation in the oxidative half-reactions showed that 16.5 ± 3.0% of the enzyme formed 3,4-dihydroxybenzoate (with the remainder eliminating H_2O_2 during formation of oxidized FAD (k_5 in Figure 1)). Thus, the observed reaction must involve two paths of decay of the hydroperoxide that cannot be distinguished by absorbance measurements. Based upon the amount of product formed and taking into account the fact that the observed rate of reaction is the sum of the rates of reactions involved in the decay of a single species (the flavin hydroperoxide), the rate of hydroxylation was calculated to be 1.6 s^{-1}, and the rate of H_2O_2 (and oxidized FAD) formation was 9.0 s^{-1}. Since no slow phase due to decay of the flavin hydroxide that formed upon hydroxylation could be observed (rate k_4 in Figure 1), elimination of water from the putative flavin hydroxide via k_4 must have been faster than the measured rate for the hydroxylation step (k_3). The product release reaction has a rate of 14 s^{-1} in the WT reaction. If the mutant displayed a similar rate, this step would not be detected in the experiment.

Enzyme Monitored Turnover. The reaction of the mutant was studied in steady state by enzyme monitored turnover (Gibson *et al.*, 1964), which has been used with this enzyme to confirm the relevance to catalysis of reactions measured by transient state kinetic techniques (Husain & Massey, 1979; Entsch *et al.*, 1991). The steady state composition of the enzyme species observed by monitoring the FAD during enzyme monitored turnover was independent of oxygen and pOHB concentrations when they were high—the normal situation in steady-state kinetics. In contrast to observations with the WT enzyme, high levels of NADPH were found to inhibit turnover. Figure 9 A shows a series of 450 nm turnover traces measured at various concentrations of NADPH. With increasing NADPH concentration the turnover number decreases, as does the proportion of oxidized enzyme in the steady state. Since the reductive process is essentially saturated with NADPH above 0.05 mM and the flavin hydroperoxide and hydroxide have little absorbance at 450 nm, it appears that the inhibition by NADPH is exerted upon one of the steps involved in the formation or decay of the flavin C4a adducts. When we monitored the enzyme at 410 nm (a wavelength that has minimal difference between the absorbance of the oxidized enzyme and hydroperoxyflavin and does not have a significant absorbance contribution from NADPH), we observed a similar trend; the absorbance during the steady state is dependent on NADPH concentration. These data are consistent with the accumulation of the C4a-hydroxyflavin (we have assumed that the spectrum of this intermediate is blue-shifted relative to the hydroperoxyflavin (Entsch *et al.*, 1976)). In a second experiment we determined where the inhibitory effect of NADPH is exerted by approaching steady state turnover from the pre-reduced enzyme and varying the concentration of NADPH in the reaction (Figure 9B). The rate of the initial reaction of the reduced flavin with molecular oxygen, and the subsequent rate of hydroxylation and hydrogen peroxide elimination, could be observed prior to steady state. Both of these rates were unaffected by NADPH concentration, implying that NADPH was exclusively inhibiting the decay of the flavin hydroxide in the R220K mutant. A scheme representing the proposed mechanism of inhibition is shown in Scheme 1. The turnover number calculated from transient state kinetic constants is 2.4 s^{-1}, and this theoretical value is only approached when NADPH is below 0.05 mM.

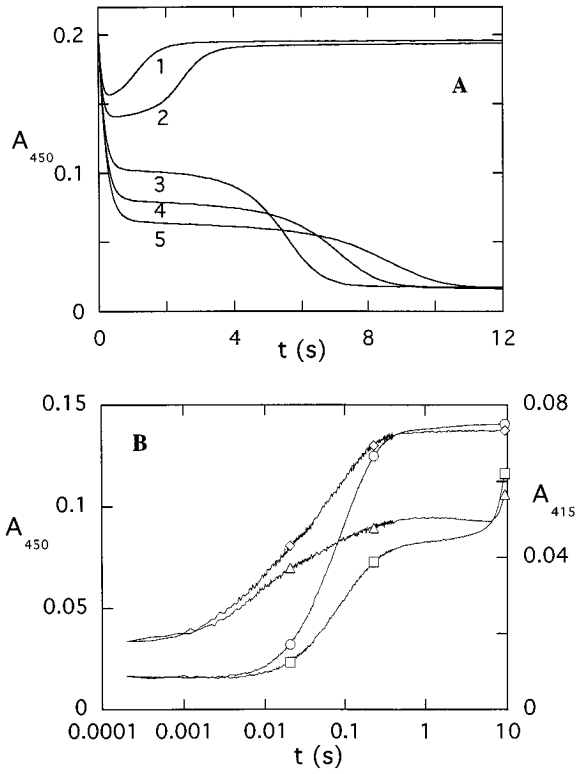
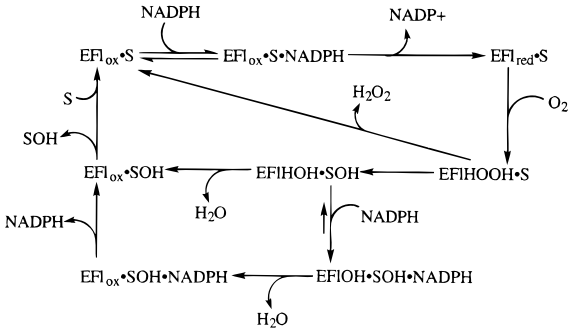


FIGURE 9: Enzyme monitored turnover experiment demonstrating the inhibitory effect of NADPH. In (A) both oxygen and pOHB concentrations were fixed for each trace at 130 μM and 2.5 mM, respectively. (1) Absorbance trace in turnover with NADPH (40 μM) as the limiting substrate. (2) Absorbance trace with NADPH (80 μM) as the limiting substrate. (3) Absorbance trace with oxygen as the limiting substrate and an NADPH concentration of 320 μM. (4) Absorbance trace with oxygen as the limiting substrate and an NADPH concentration of 640 μM. (5) Absorbance trace with oxygen as the limiting substrate and an NADPH concentration of 1280 μM. Note that the amount of oxidized enzyme at steady state decreases with increased NADPH concentration. In (B) the approach to steady state is from the pre-reduced mutant enzyme. In this experiment the processes of the oxidative half-reaction can be observed prior to steady state in the presence of various NADPH concentrations. In each case, oxygen (620 μM) was reacted with enzyme (15 μM) in the presence of saturating pOHB. (○) 450 nm trace, 0 μM NADPH; (□) 450 nm trace, 200 μM NADPH; (◇) 415 nm trace, 0 μM NADPH; (△) 415 nm trace, 200 μM NADPH.

DISCUSSION

In the last three years, different conformational states of the flavin of PHBH have been proposed to be important to the function of the enzyme (Lah *et al.*, 1994b; Gatti *et al.*, 1994; Entsch *et al.*, 1994; van Berkel *et al.*, 1994a,b; Schreuder *et al.*, 1994; Entsch & van Berkel, 1995). Although R220 is outside the apparent active site of the enzyme (where hydroxylation occurs), it is one of the few residues to show a change of position in crystal structures in which the flavin ring system moves to the "out" conformation (Figure 2). The rationale for this work was that mutations at position 220 may cause changes in enzyme catalysis by changing the relative stability of flavin conformational states. Support for the importance of this residue comes indirectly from comparisons between the published amino acid sequences of the protein from several species. At present, there are sequences from *Pseudomonas aeruginosa* (Entsch *et al.*, 1988), *Pseudomonas fluorescens* (Weijer *et al.*, 1983; Shuman & Dix, 1993), *Acinetobacter calcoaceticus* (DiMarco *et al.*, 1993), and *Rhizobium leguminosarium*

Scheme 1



(Wong *et al.*, 1994). Arginine 220 and many residues near this position in the structure are conserved in all these sequences. In fact, an extended area around the active site is highly conserved, including amino acid residues in the region of the flavin in its two observed conformations. The mutation R220K is a fairly conservative alteration. It retains the general charge distribution in the protein, but shortens the side chain and changes the charge density from a dispersed positive charge to a point charge, and thus is likely to modify the local electrical field in the active site.

The difference spectrum upon binding pOHB to the oxidized mutant enzyme (Figure 4) was recognized as characteristic of the formation of the "out" conformation of the flavin (Gatti *et al.*, 1994; Schreuder *et al.*, 1994). Thus, the equilibrium position of the flavin had changed compared to WT with no significant change to the K_d for pOHB. As with WT, kinetic experiments showed binding of the substrate to be complete within the dead time of the stopped-flow instrument (2 ms), reflecting the ability of the oxidized enzyme to rapidly exchange pOHB. The observation that the isoalloxazine is "out" in the oxidized mutant enzyme−pOHB complex suggested that there may be substantial changes to the stability of this complex in reduced enzyme (where the "in" conformation has a central role in the subsequent hydroxylation reaction) (Gatti *et al.*, 1994; Entsch *et al.*, 1994). The K_d for the complex between reduced enzyme and pOHB was 12-fold larger in R220K than in WT, yet the rate of substrate binding to reduced enzyme was 6.5-fold higher. Thus, the substrate complex with reduced mutant enzyme was less stable and the substrate exchanged faster, consistent with the isoalloxazine of the flavin being more mobile than in WT, permitting substrate exchange. The difference absorption spectra for the complex between reduced enzyme and pOHB were essentially the same for mutant and for WT enzymes, and the mutant quantitatively forms the flavin hydroperoxide upon reaction with oxygen. Both of these observations suggest that the equilibrium conformation of the flavin is mainly "in" as in WT.

The most significant difference in the catalytic mechanism of mutant and WT enzymes was the partial hydroxylation of pOHB by the mutant. This was due to the preferential loss of H_2O_2 from the flavin hydroperoxide instead of oxygen transfer to the substrate (see Figure 1). The substrate analogue, 2,4DOHB, is a somewhat more efficient substrate with this mutant. There are two ionizable groups that are known to affect the reactivity of the flavin hydroperoxide, the substrate 4-hydroxyl (Entsch *et al.*, 1991) and the isoalloxazine N5 (Anderson, 1982). Deprotonation of the former activates the substrate to electrophilic attack by the hydroperoxide (facilitating hydroxylation), and deprotonation

of the latter destabilizes the hydroperoxide, favoring the elimination of H_2O_2. The pH dependence of hydroxylation efficiency observed in R220K (see Figure 3) may be a function of the protonation state of these two groups. We observed a one unit shift of the pK_a of the basic branch of the hydroxylation profile opon substituting 2,4DOHB for pOHB. This shift is consistent with stabilizing the flavin hydroperoxide with a single hydrogen bond (2 kcal mol^{-1}). A hydrogen bond between the 2-hydroxyl of 2,4DOHB and N5 of the flavin would reduce the acidity of the N5 group and favor hydroxylation over hydrogen peroxide elimination.

At the optimum pH for NADPH oxidation (7.5−8.0) the mutant produced almost no product from pOHB while the WT enzyme is completely coupled. This property of the mutant is similar to that of the mutant Y222F (Entsch *et al.*, 1994), which is known from crystallographic studies to also favor the "out" conformation of the flavin (Lah *et al.*, 1994b; Gatti *et al.*, 1994). The simplest explanation for the lack of product formation is that the "in" conformation is destabilized in both of these mutants and after formation of the flavin hydroperoxide there is a catalytically significant rate of movement of the flavin hydroperoxide to the "out" conformation. This would lead to solvent exposure and a greater rate of elimination of H_2O_2 (9.0 s^{-1} for the mutant versus immeasureable in WT). The rate of hydroxylation in the mutant is 1.6 s^{-1} versus 50 s^{-1} in the WT. This suggests that the optimal position of the flavin hydroperoxide relative to the substrate occurs rarely with R220K (ca. 3% of WT).

In the WT enzyme, the pK_a of pOHB shifts from 7.4 in oxidized enzyme (Entsch *et al.*, 1991; Eschrich *et al.*, 1993; Shoun et al., 1983) to ≥8.6 in the reduced enzyme (unpublished observation, B. Entsch). This pK_a shift must be part of the compensatory changes involved in accommodating the now negatively charged reduced FAD in the active site (Palfey *et al.*, 1994). Upon reduction of the mutant, reprotonation of pOHB should be more difficult (because of the lower pK_a of 5.8; Figure 5) and may provide an added explanation for the increased K_d in the reduced state relative to WT. The complex between oxidized WT PHBH and pOHB is partly characterized by the shift of substrate pK_a in the active site. The shift in the phenolic pK_a (7.4) from that in free solution (9.3) has been attributed to a positive electrical field in the active site and facilitated proton abstraction by the protein (Palfey *et al.*, 1994; Gatti *et al.*, 1996; Schreuder *et al.*, 1994). Substrate deprotonation is suppressed by the presence of a new negative charge in the active site (as illustrated by the mutant, N300D (Palfey *et al.*, 1994), and the presence of anions such as chloride (Shoun *et al.*, 1983)). The mutant described here, R220K, may modify the positive field around the active site, as illustrated by the lower pK_a of 5.8.

The reduction of R220K with NADPH requires some comment. PHBH has attracted great interest with respect to its interaction with NADPH. The importance of this interaction is the tight control over the reduction of the flavin obtained from the substrate acting as an effector (Spector & Massey, 1972). In the structures of other enzymes that catalyze reactions with pyridine nucleotides, the cofactor is commonly bound by a $\beta\alpha\beta$ fold in the protein (Rossman *et al.*, 1974). No site for NADPH binding has been identified in PHBH (Schreuder *et al.*, 1989). The measured turnover number for NADPH oxidation by R220K (2.1 s^{-1}) is consistent with reduction of flavin being a rate-limiting process. NADPH binds more tightly to R220K than to WT (40-fold decrease in K_d), but reduction is slowed (17-fold to 2.9 s^{-1}). Additionally, charge-transfer interactions between NADPH and FAD (whose intensity normally correlates with the rate of reduction) are diminished (Entsch & van Berkel, 1995). Thus, this mutant appears to favor a conformation that binds NADPH strongly at the expense of enhanced binding to the transition state in reduction. Studies are in progress to try to obtain appropriate crystal structures of the mutant with various substrates.

The discussion above has shown that the properties of the mutant, R220K, are consistent with it shifting the balance of conformational states of flavin in the enzyme toward the "out" conformation (shown in Figure 2) and that the mechanism of the enzyme is extremely dependent upon the balance between conformational states at all stages in the catalytic cycle. The relationship between flavin movement and protein in the active site of this enzyme remains an intriguing and complex matter for study of protein dynamics.

REFERENCES

Anderson, R. F. (1982) in *Flavins and Flavoproteins 1981* (Massey, V., & Williams, C. H., Eds.) pp 278−283, Elsevier North Holland, New York.

DiMarco, A., Averhoff, B., Kim, E. E., & Ornston, L. N. (1993) *Gene 125*, 25−33.

Entsch, B. (1990) *Methods Enzymol. 188*, 138−147.

Entsch, B., & van Berkel, W. J. H. (1995) *FASEB J. 9*, 476−483.

Entsch, B., Ballou, D. P., & Massey, V. (1976) *J. Biol. Chem. 251*, 2550−2563.

Entsch, B., Nan, Y., Weaich, K., & Scott, K. F. (1988) *Gene 71*, 279−291.

Entsch, B., Palfey, B. A., Ballou, D. P., & Massey, V. (1991) *J. Biol. Chem. 266*, 17341−17349.

Entsch, B., Palfey, B. A., Lumberg, M., Ballou, D. P., & Massey, V. (1994) in *Flavins and Flavoproteins 1993* (Yagi, K., Ed.) pp 211−220, de Gruyter, Berlin.

Eppink, M. H. M., Scheuder, H. A., & van Berkel, W. J. H. (1995) *Eur. J. Biochem. 231*, 157−165.

Eschrich, K., van der Bolt, F., de Kok, A., & van Berkel, W. J. H. (1993) *Eur. J. Biochem. 216*, 137−146.

Gatti, D. L., Palfey, B. A., Lah, M. S., Entsch, B., Massey, V., Ballou, D. P., & Ludwig, M. L. (1994) *Science 266*, 110−114.

Gatti, D. L., Entsch, B., Ballou, D. P., & Ludwig, M. L. (1996) *Biochemistry 35*, 567−578.

Gibson, Q. H., Swoboda, B. E. P., & Massey, V. (1964) *J. Biol. Chem. 239*, 3927−3934.

Howell, L. G., Spector, T., & Massey, V. (1972) *J. Biol. Chem. 247*, 4340−4350.

Husain, M., & Massey, V. (1979) *J. Biol. Chem. 254*, 6657−6666.

Kemal, C., & Bruice, T. C. (1977) *J. Am. Chem. Soc. 99*, 7064−7067.

Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−382.

Lah, M. S., Palfey, B. A., Schreuder, H. A., & Ludwig, M. L. (1994a) *Biochemistry 33*, 1555−1564.

Lah, M. S., Gatti, D., Schreuder, H. A., Palfey, B. A., & Ludwig, M. L. (1994b) in *Flavins and Flavoproteins 1993* (Yagi, K., Ed.) pp 221−229, de Gruyter, Berlin.

Massey, V. (1990) in *Flavins and Flavoproteins 1990* (Curti, B., Ronchi, S., & Zanetti, G., Eds.) pp 59−66, de Gruyter, Berlin.

McCammon, J. A., & Harvey, S. C. (1987) *Dynamics of Proteins and Nucleic Acids*, Cambridge University Press, Cambridge.

Moran, G. R., & Entsch, B. (1995) *Protein Expression Purif. 6*, 164−168.

Palfey, B. A., Entsch, B., Ballou, D. P., & Massey, V. (1994) *Biochemistry 33*, 1545−1554.

Rossman, M. G., Moras, D., & Olsen, K. W. (1974) *Nature 250*, 194−199.

Schreuder, H. A., Prick, P. A. J., Wierenga, R. K., Vriend, G., Wilson, K. S., Hol, W. G. J., & Drenth, J. (1989) *J. Mol. Biol. 208*, 679−696.

Schreuder, H. A., van der Laan, J. M., Swarte, M. B. A., Kalk, K. H., Hol, W. G. J., & Drenth, J. (1992) *Proteins: Struct., Funct., Genet. 14*, 178−190.

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.

Shoun, H., Arima, K., & Beppu, T. (1983) *J. Biochem. 93*, 169−176.

Shuman, B., & Dix, T. A. (1993) *J. Biol. Chem. 268*, 17057−17062.

Spector, T., & Massey, V. (1972) *J. Biol. Chem. 247*, 4679−4687.

Van Berkel, W., Westphal, A., Eschrich, K., Eppink, M., & De Kok, A. (1992) *Eur. J. Biochem. 210*, 411−419.

Van Berkel, W. J. H., Eppink, M. H. M., & Schreuder, H. A. (1994a) *Protein Sci. 3*, 2245−2253.

Van Berkel, W. J. H., van der Bolt, F. J. T., Eppink, M., De Kok, A., Rietjens, I. M. C. M., Veeger, C., Vervoort, J., & Schreuder, H. A. (1994b) in *Flavins and Flavoproteins 1993* (Yagi, K., Ed.) pp 231−234, de Gruyter, Berlin.

Weijer, W. J., Hofsteenge, J., Beintema, J. J., Wierenga, R. K., & Drenth, J. (1983) *Eur. J. Biochem. 133*, 109−118.

Wierenga, R. K., de Jong, R. J., Kalk, K. H., Hol, W. G. J., & Drenth, J. (1979) *J. Mol. Biol. 131*, 55−73.

Wong, C. M., Dilworth, M. J., & Glenn, A. R. (1994) *Microbiology 140*, 2775−2786.

BI960360S