Mechanistic Insights into *p*-Hydroxybenzoate Hydroxylase from Studies of the Mutant Ser212Ala†

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ABSTRACT: In the crystal structure of native *p*-hydroxybenzoate hydroxylase, Ser212 is within hydrogen bonding distance (2.7 Å) of one of the carboxylic oxygens of *p*-hydroxybenzoate. In this study, we have mutated residue 212 to alanine to study the importance of the serine hydrogen bond to enzyme function. Comparisons between mutant and wild type (WT) enzymes with the natural substrate *p*-hydroxybenzoate showed that this residue contributes to substrate binding. The dissociation constant for this substrate is 1 order of magnitude higher than that of WT, but the catalytic process is otherwise unchanged. When the alternate substrate, 2,4-dihydroxybenzoate, is used, two products are formed (2,3,4-trihydroxybenzoate and 2,4,5-trihydroxybenzoate), which demonstrates that this substrate can be bound in two orientations. Kinetic studies provide evidence that the intermediate with a high extinction coefficient previously observed in the oxidative half-reaction of the WT enzyme with this substrate is composed of contributions from both the dienone form of the product and the C4a-hydroxyflavin. During the reduction of the enzyme−2,4-dihydroxybenzoate complex by NADPH with 2,4-dihydroxybenzoate, a rapid transient increase in flavin absorbance is observed prior to hydride transfer from NADPH to FAD. This is direct evidence for movement of the flavin before reduction occurs.

p-Hydroxybenzoate hydroxylase (EC 1.14.13.2, PHBH[1]), which is the most studied aromatic flavoprotein hydroxylase (*1*−*4*), catalyzes the hydroxylation of *p*-hydroxybenzoate (pOHB) to form the catechol, 3,4-dihydroxybenzoate (3,4DOHB). The function of this process in bacterial metabolism is to activate the aromatic ring for subsequent catechol ring cleavage reactions. The product of PHBH, 3,4DOHB, is one of the primary entry points to the β-ketoadipate pathway of aromatic degradation (*5*). The overall catalytic cycle of PHBH depicted in Figure 1 shows that PHBH, like other flavoprotein aromatic hydroxylases, activates molecular oxygen by forming a C4a-hydroperoxyflavin. The distal peroxy oxygen is then incorporated into the aromatic substrate. Although pOHB and NADPH bind in random order to the enzyme, there is an absolute requirement that the aromatic substrate bind before rapid reduction of the flavin by NADPH can occur; binding of pOHB stimulates reduction by 10^5-fold (*6*, *7*). How the enzyme controls this effector role for the substrate is currently under investigation.

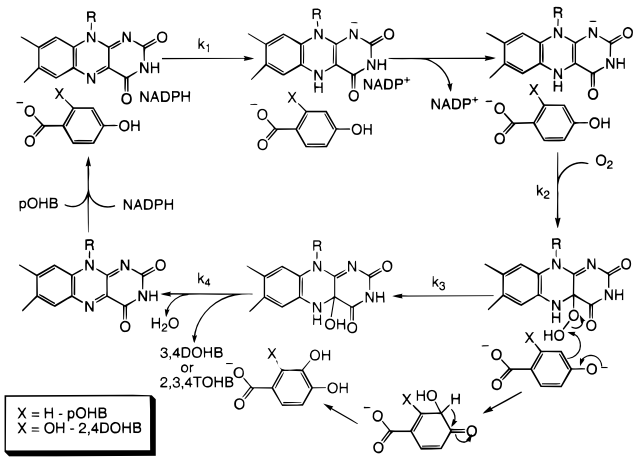

FIGURE 1: Catalytic cycle of *p*-hydroxybenzoate hydroxylase.

PHBH only recognizes activated benzoates as substrates (*1*, *8*). The crystal structure of the wild type enzyme (WT) in complex with pOHB indicates that the carboxyl moiety of the substrate interacts with three active site residues, Arg214, Tyr222, and Ser212 (*2*). These residues form hydrogen bonds with the substrate carboxylate, one each from both Tyr222 and Ser212, and two from the ion-paired Arg214. Several studies with PHBH mutated at these residues have shown their importance in interactions with substrates. Van Berkel et al. (*9*) showed that replacement of arginine

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[1] Abbreviations: PHBH, *p*-hydroxybenzoate hydroxylase; *pob*A, gene encoding *p*-hydroxybenzoate hydroxylase; pOHB, *p*-hydroxybenzoate; 2,4DOHB, 2,4-dihydroxybenzoate; 2,3,4TOHB, 2,3,4-trihydroxybenzoate; 2,4,5TOHB, 2,4,5-trihydroxybenzoate; 3,4DOHB, 3,4-dihydroxybenzoate; WT, wild type.

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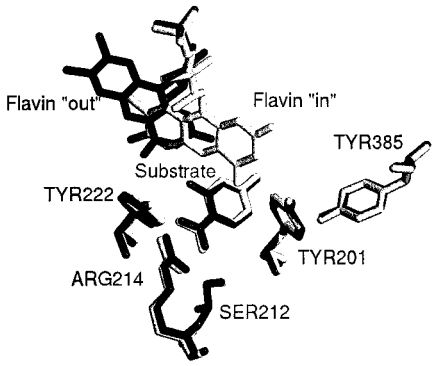


FIGURE 2: Active site of wild type PHBH in complex with pOHB (light shading) compared to the structure of the 2,4DOHB complex (black). The diagram was prepared from PDB file coordinates (1DOD for the 2,4DOHB complex and 1IUW for the pOHB complex).

214 with lysine lowers the affinity of the enzyme for benzoate substrates ~1000-fold, reduces the turnover rate, and uncouples hydroxylation of the substrate by inducing futile release of H_2O_2. The mutation of serine 212 to alanine in the *Pseudomonas fluorescens* enzyme weakens the binding of the substrate by 10-fold (*10*), and the replacement of tyrosine 222 with phenylalanine lowers the affinity of the enzyme for the substrate by 6-fold. However, crystallographic studies have shown that on binding of the substrate to Tyr222Phe,the flavin isoalloxazine ring moves ~7 Å toward the solvent (the "out" position). Movement of the flavin in PHBH (Figure 2) is believed to be important for both association and dissociation of substrates and products (*11*−*14*). Thus, in normal catalysis, the flavin moves to the out position so that the substrate can be bound, and then the flavin moves back in to exclude solvent and prevent it from interfering with the hydroxylation reaction.

We report here a more complete description of the catalytic properties of the S212A mutant of PHBH from *Pseudomonas aeruginosa* with the natural substrate, pOHB, and the alternate substrate, 2,4-dihydroxybenzoate (2,4DOHB). The *P. aeruginosa* enzyme is almost identical to the *P. fluorescens* enzyme in every aspect that was investigated. There are only two conservative amino acid residue differences, and these are both on the surface of the enzyme (*15*). Of particular importance are observations made with 2,4DOHB, a substrate used extensively in the study of PHBH. Like pOHB, this molecule is an effector, stimulating the reduction of the flavin by NADPH, and is hydroxylated with high efficiency. Two important findings have emerged from the study of WT PHBH with 2,4DOHB as a substrate; upon binding of 2,4DOHB (Figure 2), the flavin moves to the out position (see above), and an additional process can be detected in the oxidative half-reaction (Figure 1). The spectrum of the observed intermediate corresponding to the additional process (often referred to as intermediate II) is believed to have contributions from both the C4a-hydroxyflavin and a nonaromatic dihydroxycyclohexadienone (*16*); the latter subsequently rearomatizes through tautomerization to form 2,3,4-trihydroxybenzoate (2,3,4TOHB) (Figure 1). Although it has not been directly demonstrated that this intermediate is the immediate product of hydroxylation of 2,4DOHB, rapid quenching experiments show that 2,3,4TOHB is formed simultaneously with decay of the flavin hydroperoxide (*17*). Presumably, the acid quenching conditions brought about tautomerization before analysis of the product was performed. Nevertheless, some controversy persists with regard to the actual chemical nature of intermediate II (*17*−*20*). Studies reported here of the oxidative half-reaction of the mutant S212A with 2,4DOHB provide new evidence that the spectrum of intermediate II includes contributions from the nonaromatic tautomer of the product (Figure 1). In addition, this work suggests that flavin movement is also involved in reduction of the flavin by NADPH.

MATERIALS AND METHODS

The construction of plasmids and methods for expression of the mutant enzyme have been described previously (*21*, *22*). Mutant and WT were purified by the procedure described in ref *23*, but the DEAE-cellulose chromatographic step was eliminated. Directed mutagenesis was carried out according to the Bio-Rad adaptation of the methods of Kunkel et al. (*24*), which are described in ref *21*. The S212A mutation was generated using a 21-mer oligonucleotide with the underlined double base substitution: 5′-CGAACGCTGG-GCACAGGGC-3′. Each side of the mutation was complementary to the pobA gene (*15*).

The standard conditions used to study S212A were 50 mM phosphate buffer (potassium salt) and 10 mM EDTA (sodium salt) at pH 6.5 and 4 °C. These conditions have been demonstrated to be favorable for detecting catalytic intermediates in PHBH (*1*). The procedures used in the measurement of ligand dissociation constants and extinction coefficients for the oxidized enzyme are described in ref *21*. Methods used for the determination of ligand dissociation constants for the reduced enzyme were based on those described in ref *1*. Enzyme-monitored turnover was analyzed according to the methods of Gibson et al. (*25*). Product analyses were a variation on the methods described in ref *21*. Hydroxylated products were analyzed after the complete consumption of a limiting concentration (200 μM) of either NADPH or the aromatic substrate in reaction mixtures containing atmospheric levels of oxygen (about 260 μM), and either a saturating concentration of aromatic substrate or a saturating concentration of NADPH, and 60 nM enzyme. These mixtures were acidified with the addition of H_2SO_4 to a final concentration of 10 mM and centrifuged at 12000*g* for 10 min to remove precipitated protein. The hydroxylated products were then separated on a 3.9 mm × 150 mm Waters Nova-Pak C18-HPLC column, using an isocratic mobile phase of 20% CH_3OH and 5 mM H_2SO_4.

All kinetic data were obtained according to the methods described by Moran et al. (*26*). Composite data sets compiled from single-wavelength absorbance traces recorded at 5 nm increments were fit using global analysis software (SpecFit, Spectrum Software Associates) to calculate the spectral intermediates from the data set. Anaerobic methods were based on the procedures described by Foust et al. (*27*) and Williams et al. (*28*). The inert atmosphere used to exchange oxygen was purified argon.

RESULTS

General Properties. The S212A mutant of PHBH was expressed in *Escherichia coli* to form the active enzyme in amounts comparable to those obtained with WT PHBH (*22*).

Table 1: Selected Properties of S212A Compared to Those of the WT

enzyme	WT	S212A
TN (s^{-1}) at pH 6.5 and 4 °C[a]	6.2	4.2 ± 0.1
% coupled at pH 6.5 and 4 °C[b]	100	100
K_d (pOHB)[c] (μM)	9.5	130 ± 9
K_d (2,4DOHB) (μM)	20	37 ± 2.1
K_d (pOHB−E_{red})[d] (μM)	21	584 ± 21
K_d (2,4DOHB−E_{red}) (μM)	180	1096 ± 112
K_d (NADPH−E−pOHB)[e] (μM)	210	558 ± 28
K_d (NADPH−E−2,4DOHB) (mM)	1.5	1.0 ± 0.2
E_{ox} extinction coefficient at 450 nm (mM^{-1} cm^{-1})	10.3	10.3

[a] Turnover number (TN) refers to the maximum rate of turnover measured in enzyme-monitored turnover experiments with a limiting O_2 concentration and the concentrations of pOHB and NADPH extrapolated to infinity. [b] Hydroxylation stoichiometry for pOHB determined from turnover experiments and subsequent quantitative product analysis. [c] Dissociation constants for pOHB and 2,4DOHB were determined from static titrations at pH 6.5 and 4 °C. Plots of the absorbance changes at wavelengths that demonstrate maximal spectral perturbation against free substrate concentration gave a hyperbolic relationship that could be fit for the determination of dissociation constants (WT dissociation constants taken from ref *21*). [d] The dissociation constants for ligands to the reduced enzyme were determined in the same manner as those for the oxidized enzyme, but anaerobic conditions were maintained in a sealed cuvette. The enzyme was reduced by anaerobically adding a slight excess of dithionite. [e] The dissociation constant for NADPH was determined by fitting the dependence of the rate of reduction by NADPH to a hyperbola (the WT K_d for NADPH from ref *6*).

The absorptivity at 450 nm (10.3 mM^{-1} cm^{-1}) of the purified ligand-free mutant is the same as that of the WT, but the mutant is 1.3-fold more fluorescent. When the mutant is reacting with pOHB, the steps of the catalytic cycle are very similar to those of the WT (Table 1). In enzyme-monitored turnover reactions with pOHB and limiting oxygen, the mutant turns over at a rate of 4.2 s^{-1}, similar to the WT rate of 6.2 s^{-1}. Like the WT, reduction is 100% coupled to hydroxylation, producing one molecule of 3,4DOHB for every molecule of O_2 and NADPH consumed. During steady-state turnover, the flavin is 94% reduced, indicating that processes of the oxidative half-reaction are rate-determining under the conditions that were used. As may be predicted from the crystal structure of the WT−pOHB complex, the absence of the hydroxyl in Ser212Ala causes a 10-fold lower affinity for pOHB, consistent with the loss of one hydrogen bond from the binding (Table 1). The flavin spectral changes seen upon binding of the native substrate are very similar to those observed with the WT enzyme (Figure 3).

In contrast to the above data, considerable differences exist between the properties of S212A and the WT enzyme with the substrate 2,4DOHB (Table 1). The mutant turns over with this substrate at a rate of 0.3 s^{-1}, which is 1.5-fold faster than the rate observed with the WT enzyme. The S212A mutant binds 2,4DOHB with an affinity similar to that displayed by the WT (Table 1), apparently unaffected by the loss of the hydrogen bond to the substrate carboxylate group. However, the flavin difference spectrum produced when 2,4DOHB is complexed to S212A is markedly different from that observed in the WT complex. Large extinction coefficient increases indicative of the flavin moving to the out position are not observed (Figures 2 and 3) (*14*, *29*). Instead, we see a nearly unperturbed 450 nm peak and a decrease in the extinction coefficient of the 380 nm peak

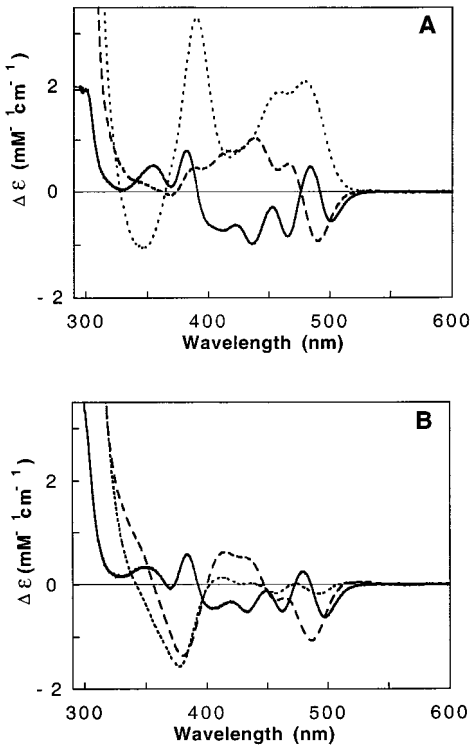


FIGURE 3: Difference spectra (complexed − free) obtained from titrations of oxidized enzyme with ligands: (A) WT PHBH and (B) the mutant S212A with ligands (—) *p*-hydroxybenzoate, (···) 2,4-dihydroxybenzoate, and (- - -)3,4-trihydroxybenzoate.

(Figure 3B). HPLC product analysis of the steady-state turnover reaction of S212A with 2,4DOHB reveals two hydroxylated products (Figure 4). One of these products was identified by HPLC as 2,3,4TOHB by co-injection of the authentic compound. The other product eluted just before 2,3,4TOHB from the column and has been assigned to be the alternate *ortho* hydroxylation product, 2,4,5-trihydroxybenzoate (2,4,5TOHB). Although a standard for this compound is not available, several considerations support this assignment. The fact that the retention time is similar to that of 2,3,4TOHB is consistent with it being a trihydroxybenzoate. The only activated positions in addition to 3 for hydroxylation are 1 and 5, and position 1 would not yield a benzoate. A similar loss of regioselectivity has been observed with the Y385F mutant, which hydroxylates 3,4DOHB to give 3,4,5-trihydroxybenzoate (*21*). An estimate of the proportions of each of the two products was made by monitoring the oxidation of NADPH in steady-state reactions with limiting concentrations of 2,4DOHB. At low 2,4DOHB concentrations, the S212A mutant is close to fully coupled (ca. 92% of the NADPH consumed results in product molecules). Thus, monitoring the amount of NADPH consumed provides an estimate of the total amount of substrate hydroxylated in the reaction. From HPLC analysis of 2,3,4TOHB in the acidified reaction mixtures, the 2,3,4TOHB and 2,4,5TOHB products were estimated to be approximately equimolar (Figure 4).

Reductive Half-Reaction. The reduction of PHBH by NADPH was studied under anaerobic conditions and could be monitored at all wavelengths above 420 nm. Like the WT, NADPH reduced S212A at a negligible rate in the absence of benzoate ligands. The reduction of the flavin of S212A in complex with pOHB was a single process observed as an

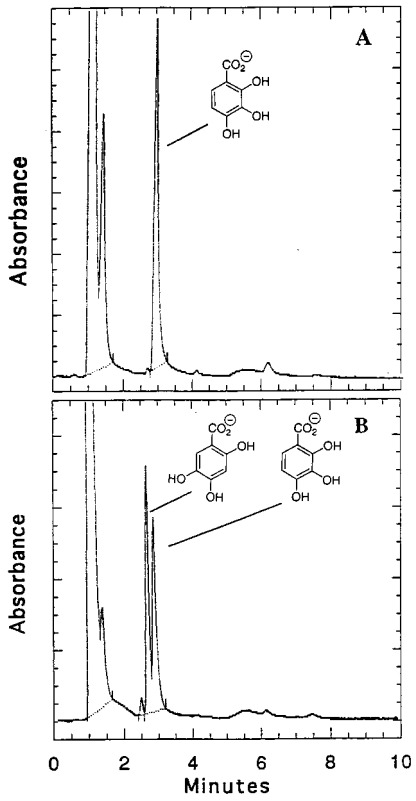


FIGURE 4: HPLC product analysis of S212A−2,4DOHB turnover mixtures after complete conversion of limiting 2,4DOHB to product: (A) the chromatogram (254 nm) from the WT reaction and (B) the chromatogram (254 nm) from the S212A reaction.

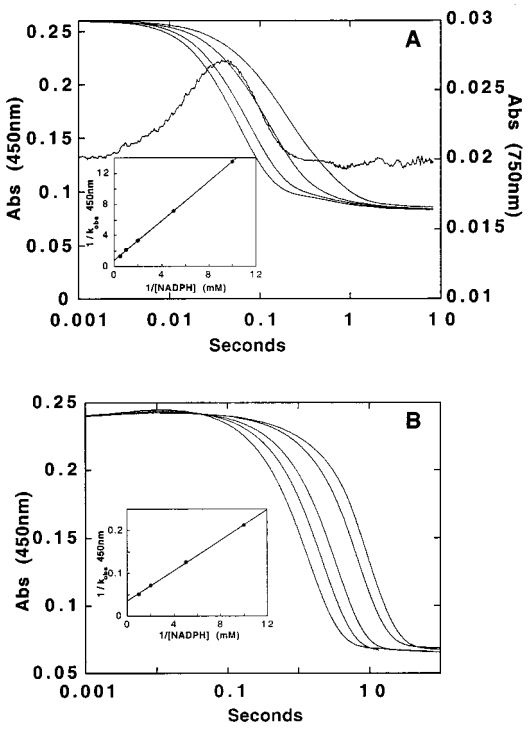


FIGURE 5: Reductive half-reaction kinetics of the PHBH mutant S212A. (A) Reduction of the S212A−pOHB complex with NADPH. The charge-transfer interaction was assessed at 750 nm using 1000 μM NADPH (noisier trace). The 450 nm traces are shown for concentrations of NADPH of 100, 200, 500, and 1000 μM (right to left). The concentration of the enzyme used in the reaction was 19 μM. The limiting reduction rate determined from the experiment was 28.1 s^{-1}, and the K_d for NADPH was 500 μM (see the inset). (B) Reduction of the S212A−2,4DOHB complex with NADPH concentrations of 100, 200, 500, 1000, and 2000 μM (right to left). The concentration of the enzyme used in the reaction was 19 μM. The limiting reduction rate determined from the experiment was 1.20 s^{-1}, and the K_d for NADPH was 1.0 mM (see the inset).

85% decrease in the 450 nm absorbance (Figure 5A). Reaction traces were fit to a single primary phase with a correction for a small contribution by contaminating O_2 (<5%). The dependence of the observed rate upon NADPH could be described by a hyperbolic function, yielding a calculated dissociation constant of 500 μM; this is compared with 210 μM for the WT enzyme. The limiting rate of reduction from the ternary complex was 28 s^{-1}, approximately 2-fold slower than that of the WT (50 s^{-1}). Charge-transfer interactions between the pyridine nucleotide and FAD were observed at 750 nm during the reduction of S212A in the presence of pOHB (Figure 5A). When traces at 750 nm were fit to two sequential exponential functions, the increase in absorbance at 750 nm was found to have a constant rate of 40 s^{-1}, and its amplitude increased with NADPH concentration. The rates of absorbance decrease at this wavelength were dependent upon NADPH concentration and were equal to the observed rates at 450 nm. These observations are consistent with the rate of dissociation of NADPH from the enzyme always being faster than the rate of hydride transfer; thus, the dissociation rate controls the amplitude of the increase in absorbance at 750 nm (*30*). Taken together, our results indicate that NADPH associates rapidly with the oxidized enzyme (K_d of 500 μM), and this is followed by transfer of a hydride at 28 s^{-1} and dissociation of NADP from the reduced enzyme at a rate of 40 s^{-1}, 1.5-fold faster than dissociation from the WT (27 s^{-1}, unpublished results from B. Entsch).

The substrate 2,4DOHB induces a maximum rate of flavin reduction by NADPH of 1.22 s^{-1} (Figure 5B) with the S212A mutant compared with 0.81 s^{-1} for the WT enzyme (*26*). The binding of NADPH to the S212A−2,4DOHB complex is characterized by a K_d of 1.5 mM, which is similar to that observed for the WT (Table 1). Like the WT with 2,4DOHB, no charge-transfer absorbance bands were detected under these conditions. An unusual aspect of the reductive half-reaction of S212A with 2,4DOHB, but not with the WT, is a fast increase in visible flavin absorbance just prior to reduction (Figure 6A). The amplitude of this extinction increase at 10 ms was plotted for selected wavelengths between 420 and 500 nm to provide an approximation of the difference spectrum of the transient intermediate state that is involved (Figure 6B). The results were qualitatively consistent with the known flavin out difference spectrum (*13*, *29*).

Oxidative Half-Reaction. The oxygen reaction of S212A was studied by stopped-flow spectrophotometry by mixing anaerobic dithionite-reduced enzyme−substrate complex with various concentrations of oxygen. The reaction was monitored by absorbance at wavelengths of >340 nm, where free substrate and PHBH protein do not contribute to the absorbance, except as noted. The half-reaction was studied using pOHB or 2,4DOHB at concentrations that were sufficient to saturate the reduced enzyme.

The native substrate, pOHB, was hydroxylated efficiently (Table 1), and the rates of all oxidative processes proved to

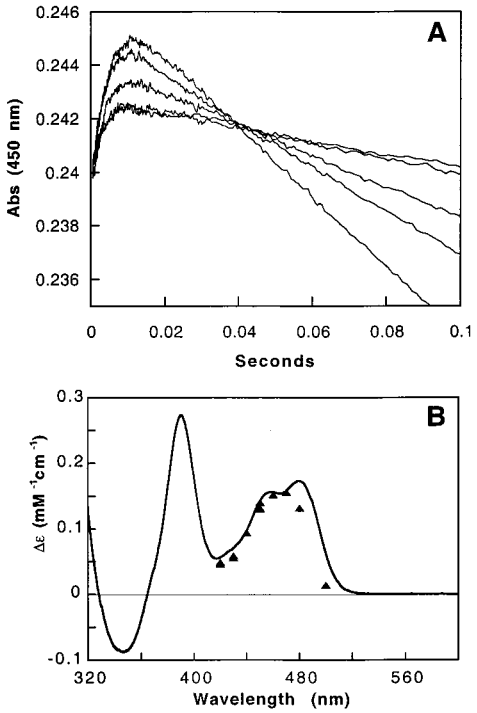


FIGURE 6: Formation of an intermediate observed upon addition of NADPH to the S212A−2,4DOHB complex. (A) The initial increases in extinction observed prior to flavin reduction are shown as a function of the concentration of NADPH. These traces are expansions of Figure 5B. (B) The difference spectrum (the spectrum 10 ms after mixing 2000 μM NADPH with the S212A−2,4DOHB complex minus the spectrum of the S212A−2,4DOHB complex) (▲) is compared to the known flavin out difference spectrum caused by the binding of 2,4DOHB to WT. The spectrum for the WT has been scaled to match that of the mutant for comparison. The transient increases are 15% of the total difference upon binding 2,4DOHB.

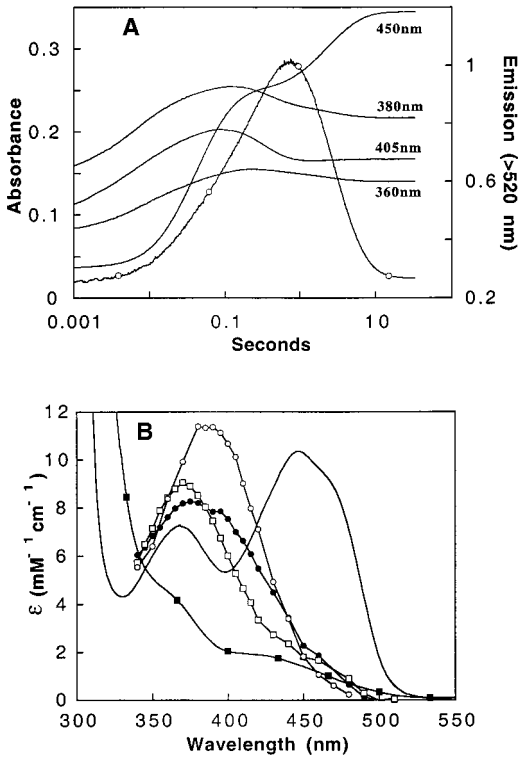


FIGURE 7: Oxidative half-reaction of the PHBH mutant S212A complexed with substrate analogue 2,4DOHB (20 mM). (A) The graph shows selected absorbance traces observed in the half-reaction (28 μM enzyme) and (○) fluorescence emission beyond 520 nm when excited at 400 nm (10 μM enzyme). (B) Spectra of species calculated (using SpecFit) from the observed reaction traces in the half-reaction: (■) the reduced S212A−2,4DOHB complex, (●) C4a-hydroperoxyflavin, (○) 1-carboxy-2,3-dihydroxycyclohexa-1,5-dien-4-one and C4a-hydroxyflavin, (□) the C4a-hydroxyflavin complex with product, and (—) the oxidized S212A−2,4DOHB complex.

be very similar to those observed for the WT under the same conditions (*31*). Consistent with all previously studied PHBH mutants and analogues, the reaction of the reduced flavin of S212A with oxygen is second-order, and forms a flavin hydroperoxide. Unlike the case for the WT enzyme, a fourth (slow) phase was discernible in the oxygen half-reaction. This phase (1.0 s^{-1}) was not part of normal catalysis, since with pOHB as a substrate, S212A has a turnover rate of 4.2 s^{-1}. This slow phase is probably a result of the high concentration of pOHB required to saturate the reduced enzyme in the half-reaction. At high concentrations, substrate can exchange with product to trap a substantial fraction of the hydroxyflavin in a quite stable dead-end complex (*32*). The concentration of the substrate that was used with S212A (6 mM) was approximately 10-fold greater than the K_d for the reduced enzyme (Table 1) and 15-fold greater than required for the WT enzyme in the same type of experiment.

Product analysis of the oxidative half-reaction with 2,4DOHB showed that two products are formed, just as in steady-state turnover with limited substrate (Figure 4), suggesting that 2,4DOHB can be hydroxylated when bound in either of two possible orientations in the active site of S212A. The observation of the individual hydroxylation steps for each orientation of 2,4DOHB in the active site was obscured by a simultaneous uncoupling reaction that forms H_2O_2 from the C4a-hydroperoxyflavin and thereby prevents hydroxylation. Interestingly, the rates observed in the oxidative half-reaction of 2,4DOHB with S212A proved to be similar to those observed in the WT oxidative half-reaction with this substrate. The reaction was monitored using both absorbance and fluorescence techniques (Figure 7A). When mixed with oxygen, the reduced enzyme reacted in an oxygen-dependent manner to form a nonfluorescent flavin hydroperoxide at an estimated rate of 2.8×10^5 M^{-1} s^{-1} (comparable to that of the WT). The spectrum of the hydroperoxyflavin intermediate (Figure 7B) is identical to that observed for the WT (*31*). It is difficult to measure this rate accurately with S212A because the hydroperoxide that is formed has negligible fluorescence, and at concentrations of oxygen at or below 0.3 mM, the absorbance changes in this reaction cannot be easily resolved from the subsequent steps.

The C4a-flavin intermediates, as well as reduced FAD, have very little absorbance in the 450−520 nm region. Thus, most of the absorbance change in this region is due solely to the formation of oxidized FAD. After formation of the C4a-hydroperoxide with oxygen, two phases are observed in this region; a rate of 30 s^{-1} accounts for 65% of the absorbance increase, and the remaining oxidized flavin appears at a rate of 0.3 s^{-1}. Product analysis from this reaction (using 20 mM 2,4DOHB) shows that 35% of the reduced enzyme forms product (2,3,4TOHB and 2,4,5TOHB). Thus, the phase with the larger amplitude (65%) is due to elimination of hydrogen peroxide, with no oxygenation of the substrate, and the slower phase (35% amplitude) is due

to hydroxylation. The yield of products in this reaction is much lower than reported above for steady-state turnover (92%). This can be attributed to the high substrate concentration that is required to saturate the reduced enzyme for studying the oxygen half-reaction. Uncoupling at high 2,4DOHB concentrations has been documented previously for the WT (*1*). The observed rate of decomposition of the flavin hydroperoxide is the sum of the rates for the two paths, and the fraction of each reaction defines the rate of the individual processes. Thus, the elimination of H_2O_2 occurs at a rate of 20 ± 3 s^{-1}, and the concurrent hydroxylation reaction occurs at a rate of 10 ± 3 s^{-1}; the latter is similar to that observed for the WT with this substrate (*31*).

The high degree of uncoupling of the flavin hydroperoxide made it difficult (from the absorbance data) to accurately define the rates for the 35% of the enzyme that achieved hydroxylation. However, C4a-hydroxyflavin intermediates are the most fluorescent intermediates in the reaction pathway of PHBH. Since the hydroxyflavin is only formed via the hydroxylation step (Figure 1), fluorescence detection provides a selective probe for hydroxylation. When excited in the region in which intermediates absorb maximally (about 390 nm), traces of the fluorescence emission above 520 nm (Figure 7A) displayed a biphasic increase followed by a monophasic decrease. The faster phase in the fluorescence increase is 30 s^{-1}, the same rate as the bifurcating decay of the hydroperoxyflavin observed by absorbance experiments. This rate constant represents the 65% loss of H_2O_2 and the formation of the 35% of the C4a-hydroxyflavin−dihydroxycyclohexadienone product complex (Figure 1). The second phase (3 s^{-1}) parallels the decline in absorbance at 405 nm (Figure 7A), and represents the tautomerization of the dihydroxycyclohexadienone to the trihydroxybenzoate products. The slowest phase that is observed (0.3 s^{-1}) is a fluorescence decrease, and is due to the elimination of water from the hydroxyflavin (35% of the total) to form the oxidized flavin; this correlates with the slow phase in absorbance at 450 nm (Figure 7A). This formation of oxidized FAD from the flavin hydroxide is 4-fold faster than that observed with the WT enzyme.

The rates obtained from the fluorescence data were used for global fitting of the absorbance data and calculation of the intermediate spectra. The calculated spectra of the first and third intermediates (Figure 7B) are essentially the same as those of the WT. However, the extinction coefficient of the second intermediate is 11.5 mM^{-1} cm^{-1}, compared to 14.5 mM^{-1} cm^{-1} in the equivalent WT reaction.

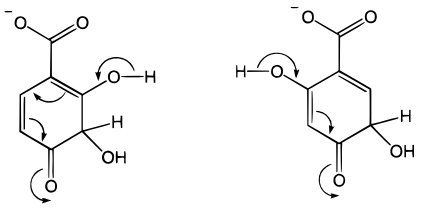
DISCUSSION

The crystal structure of the WT−pOHB complex has the side chain hydroxyl of Ser212 within 2.7 Å of one of the carboxylate oxygens of pOHB, which implies that Ser212 forms a H-bond to the substrate. This may enhance the affinity of the enzyme for the substrate and help orient the substrate correctly in the active site (*2*). Serine 212 is conserved in all known PHBH protein sequences. The mutant S212A is one in a series of mutants that have probed the interaction of pOHB with PHBH (*10*, *14*, *21*, *33*). The data presented here for the catalytic activity of S212A with pOHB are consistent with the assertion that the Ser212 residue is not required for catalysis, since each chemical process of the catalytic cycle of this mutant is highly analogous to that observed in the WT enzyme. The only significant differences from the properties of the WT are the increases in the dissociation constants for pOHB to the oxidized and reduced enzymes (Table 1). In each case, weaker substrate binding can be attributed to the loss of the proposed hydrogen bond to pOHB. This conclusion is supported by the crystal structure of the oxidized S212A mutant in complex with pOHB, where no significant alterations to the geometry of any active site residue are observed (personal communication from D. Gatti, Wayne State University, Detroit, MI).

The oxidized form of the S212A mutant has a higher affinity for 2,4DOHB than it does for pOHB (Table 1), and the difference spectrum observed upon titration of the oxidized mutant with 2,4DOHB (Figure 3B) indicates that, in contrast to the WT enzyme (*29*), this substrate does not induce the out conformation of the flavin. Consistent with this conclusion, the crystal structure of the S212A mutant in complex with 2,4DOHB and bromide shows that the position of the flavin isoalloxazine is in (personal communication from D. Gatti). The reduced form of S212A binds 2,4DOHB with nearly the same affinity as it binds pOHB, which is weaker than that of the WT by approximately the energy of one H-bond. The fact that with S212A there are two hydroxylation products formed in turnover with 2,4DOHB [approximately 50% 2,3,4TOHB and 50% 2,4,5TOHB (Figure 4)] suggests that there is nearly equal probability for binding this substrate in each of two distinct orientations. In this mutant, the benzene ring of the substrate may even be free to rotate in the active site at one or more stages in catalysis. Since the WT only forms 2,3,4TOHB, unfavorable steric hindrance between the 2-hydroxyl of 2,4DOHB and the β-hydroxyl of serine 212 (Figure 2) must prevent 2,4DOHB from binding in the orientation required for forming 2,4,5TOHB.

Measurements of the reduction of S212A with 2,4DOHB as the substrate showed that the rate of reduction and the dissociation constant for NADPH were similar to those of the WT reaction under the same conditions (see Figure 5B). However, a notable difference from the WT reaction was a fast increase in absorbance of the flavin prior to reduction of the flavin (Figure 6A), which might be attributable to a movement of the flavin to the out position. With the WT enzyme, movement of the flavin from the in to the out conformation is accompanied by a similar spectral change with an extinction coefficient increase of 2000 M^{-1} cm^{-1} at 450 nm (*29*, *34*). Before NADPH is added to S212A, however, the flavin is in (see above); therefore, it is tempting to attribute this increase in absorbance to flavin movement prior to reduction, implying that the flavin then becomes reduced in the out position. When the observed absorbance increase under the conditions studied was extrapolated to saturating NADPH concentrations, 15% of the extinction coefficient change for all in to all out was determined (Figure 6B). The calculated difference spectrum of the absorbance change was similar to a change for the flavin moving to the out position (Figure 6B). The shoulder usually seen at 480 nm in out difference spectra is not observed in this experiment. This may be due to the presence of NADPH. These observations are consistent with there being an equilibrium with approximately 15% of the flavin in the out conformation in the ternary complex of enzyme, 2,4DOHB, and NADPH.

Scheme 1



Similarly, the WT enzyme does not achieve the optimum flavin conformation for maximum rates of reduction at pH 6.55 (these conditions), but only at pH ≥8.0 (*34*).

Manstein et al. (*35*) demonstrated that hydride transfer is to the *re* face of the flavin isoalloxazine. However, analysis by Schreuder et al. (*36*) of the crystal structure of the enzyme with the flavin in shows that the close packing of the loops around the flavin isoalloxazine in the active site does not allow sufficient space for the nicotinamide of the NADPH to bind to the re side. Therefore, the movement of the flavin isoalloxazine to the out position prior to reduction would facilitate interaction of the isoalloxazine and pyridine rings by avoiding these steric constraints. The association of pyridine and isoalloxazine rings can be modeled in PHBH with the flavin out (*37*), and more experimental evidence has now been accumulated to support reduction with the flavin out (*34*). Immediately after hydride transfer, however, the flavin must return to the in position to be sufficiently secluded from solvent for a controlled reaction with molecular oxygen, and to be appropriately oriented for hydroxylating the substrate. This observation with S212A during reduction provides a fortuitous and rare piece of direct evidence for the mechanism of conformational control of flavin reduction.

Observation of the hydroxylation steps for each orientation of 2,4DOHB in the active site was obscured by the simultaneous uncoupling reaction that forms H_2O_2 from the C4a-hydroperoxyflavin without hydroxylation. Moreover, because the 3- and 5-positions of the substrate are similarly activated to electrophilic substitution, the rates of hydroxylation may not be very different. The 3-position is activated *ortho, ortho,* and the 5-position is activated *ortho, para*. Thus, during hydroxylation, a simultaneous single phase of increase in absorbance and fluorescence is observed at all wavelengths that are monitored. In the WT reaction, the second observed intermediate (intermediate II) has a large extinction coefficient at 390 nm, which is thought to be a consequence of two absorbing species, a nonaromatic cyclohexadienone that must rearomatize to form product (Figure 1) and the C4a-hydroxyflavin (*16*). The calculated absorbance spectrum of intermediate II with S212A (Figure 7B) has an extinction coefficient at 390 nm that is approximately 3.0 mM^{-1} cm^{-1} smaller than that of the equivalent intermediate observed in the WT enzyme 2,4DOHB oxidative half-reaction. The diminished extinction for intermediate II from S212A may be due to a smaller extinction contribution from that fraction of nonaromatic product that leads to 2,4,5TOHB, rather than from 2,3,4TOHB. Therefore, the spectrum of the second intermediate would be a sum of the flavin hydroxide, and 50% each of the two nonaromatic product tautomers (Scheme 1). Of the two products, the 2,4,5TOHB precursor is expected to be blue shifted, because the conjugated system is smaller and would have considerably less absorbance at the absorbance maximum for intermediate II. Moreover, the nonaromatic product from hydroxylation at the 5-position may rearomatize quickly so that it does not contribute significantly to the spectrum of the observed second intermediate.

All of the contributions of the flavin species (including the rates) in the hydroxylation of 2,4DOHB are very similar to those of the WT enzyme; therefore, the difference in the observed intermediate II for S212A from that for the WT enzyme must be due to the difference in the nonaromatic products that are formed. This provides clear evidence for explaining the puzzling unusual spectrum of intermediate II; it is due to the spectral contributions from the transient nonaromatic product to the spectrum of the C4a-hydroxyflavin.

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