^{19}F NMR Study on the Regiospecificity of Hydroxylation of Tetrafluoro-4-hydroxybenzoate by Wild-Type and Y385F *p*-Hydroxybenzoate Hydroxylase: Evidence for a Consecutive Oxygenolytic Dehalogenation Mechanism†

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ABSTRACT: The regiospecificity of hydroxylation of tetrafluoro-4-hydroxybenzoate (F_4-POHB) by *p*-hydroxybenzoate hydroxylase (PHBH) and its active site mutant Y385F was investigated by ^{19}F NMR. Evidence is provided that the hydroxylation of F_4-POHB is not restricted to the C3 center of the aromatic ring but rather involves sequential oxygenation and dehalogenation steps. The catalytic efficiency of PHBH and Y385F with F_4-POHB was optimal near pH 6.5. Below pH 7.0, substantial substrate inhibition occurred. Dianionic F_4-POHB was a competent effector, highly stimulating upon binding the rate of flavin reduction by NADPH. Hydroxylation of F_4-POHB involved the formation of quinone intermediates as primary products of oxygenolytic defluorination. Ascorbate competed favorably with NADPH for the nonenzymatic reduction of these reactive intermediates and prevented the accumulation of nonspecific oxidation products. ^{19}F NMR showed that the initial aromatic product 2,5,6-trifluoro-3,4-dihydroxybenzoate (F_3-DOHB) was further converted to 5,6-difluoro-2,3,4-trihydroxybenzoate (5,6-F_2-TOHB). This reaction was most efficient with Y385F. F_3-DOHB was not bound in a unique regiospecific orientation as also 2,6-difluoro-3,4,5-trihydroxybenzoate (2,6-F_2-TOHB) was formed. The oxygenolytic dehalogenation of F_3-DOHB by PHBH and Y385F is consistent with the electrophilic aromatic substitution mechanism proposed for this class of flavoenzymes. Nucleophilic attack of the carbon centers of F_3-DOHB onto the distal oxygen of the electrophilic flavin C(4a)-hydroperoxide occurs when the carbon center has a relatively high HOMO density and is relatively close to the distal oxygen of the flavin C(4a)-hydroperoxide.

Flavoprotein aromatic hydroxylases are inducible enzymes involved in the biodegradation of aromatic compounds by soil microorganisms (Stanier & Ornston, 1973; van Berkel & Müller, 1991). Through the initial action of these NAD(P)H-dependent monooxygenases, many breakdown products of lignin can be metabolized. The enzymatic hydroxylation of an organic compound under physiological conditions requires the activation of oxygen. With flavoprotein monooxygenases, this is achieved through the formation of a transiently stable flavin C(4a)-hydroperoxide species (Entsch et al., 1976a; Vervoort et al., 1986; Massey, 1994).

In addition to the hydroxylation of their natural substrates, flavoprotein hydroxylases can mediate fortuitous dehalogenation reactions (Fetzner & Lingens, 1994). Examples of such reactions include the 3-hydroxylation of tetrafluoro-4-hydroxybenzoate (F_4-POHB)[1] by *p*-hydroxybenzoate hydroxylase (PHBH)[1] (Husain et al., 1980), the dechlorination of 2-chlorophenol by salicylate hydroxylase (Suzuki et al., 1991), and the defluorination of 2-fluorophenol by phenol hydroxylase (Peelen et al. 1995). The oxygenolytic cleavage of the carbon−halogen bond has been proposed to proceed through the formation of a quinonoid product intermediate (Husain et al., 1980). This intermediate presumably is reduced nonenzymatically by an additional equivalent of NAD(P)H, resulting in the dihydroxy product (Husain et al., 1980). A similar unusual reaction stoichiometry was reported for the *para*-hydroxylation of the wood preservative pentachlorophenol by pentachlorophenol hydroxylase (Xun et al., 1992). This enzyme belongs to an emerging group of flavoprotein hydroxylases which use a halogenated organic contaminant as the natural substrate (van Berkel et al., 1997; Wieser et al., 1997).

Relatively little is known about the structural features which determine the substrate specificity of flavoprotein aromatic hydroxylases. Only for PHBH, a three-dimensional model of the enzyme−substrate complex is known in atomic detail in the oxidized and two-electron reduced state (Schreuder et al., 1989, 1992; Gatti et al., 1996), as is the mode of binding of the aromatic product and several substrate analogs (Schreuder et al., 1991, 1994; Gatti et al., 1994). With the

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[1] Abbreviations: E(HOMO), energy of the highest occupied molecular orbital; HOMO, highest occupied molecular orbital; PHBH, *p*-hydroxybenzoate hydroxylase; Y201F, *p*-hydroxybenzoate hydroxylase with Tyr201 replaced by Phe; Y385F, *p*-hydroxybenzoate hydroxylase with Tyr385 replaced by Phe; POHB, 4-hydroxybenzoate; DOHB, 3,4-dihydroxybenzoate; TOHB, 3,4,5-trihydroxybenzoate; F_4-POHB, 2,3,5,6-tetrafluoro-4-hydroxybenzoate; F_3-DOHB, 2,5,6-trifluoro-3,4-dihydroxybenzoate; 2,6-F_2-TOHB, 2,6-difluoro-3,4,5-trihydroxybenzoate; 5,6-F_2-TOHB, 5,6-difluoro-2,3,4-trihydroxybenzoate; 5-F-TeOHB, 5-fluoro-2,3,4,6-tetrahydroxybenzoate; 6-F-TeOHB, 6-fluoro-2,3,4,5-tetrahydroxybenzoate.

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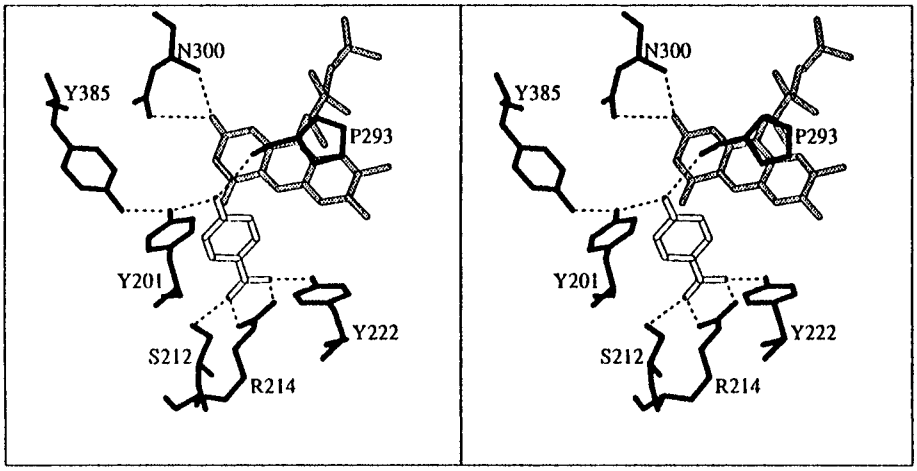

FIGURE 1: Schematic representation of the active site of PHBH complexed with POHB. Data according to the crystal structure at 1.9 Å resolution (Schreuder et al., 1989). The FAD is grey, the aromatic substrate is white, and the protein residues are black.

exception of *p*-mercaptobenzoate, which is hydroxylated at the nucleophilic highly reactive sulfur atom (Entsch et al., 1976b), all PHBH substrates have been proposed to become exclusively hydroxylated at the C3 position of the aromatic ring (Husain et al., 1980). The specificity for C3 hydroxylation is in keeping with the mutual orientation of 3,4-dihydroxybenzoate (DOHB)[1] and the flavin prosthetic group observed in the crystal structure (Schreuder et al., 1988). Recent studies have shown that the active site mutant Y385F has lost the specificity of C3 hydroxylation (Entsch & van Berkel, 1995), leading to toxic 3,4,5-trihydroxybenzoate (TOHB).[1] Tyr385 is part of a hydrogen bond network, including Tyr201 and the 4OH of the substrate (Schreuder et al., 1989; Figure 1). Mutational analysis established that both tyrosines are involved in substrate activation by stimulating the deprotonation of the 4OH group (Entsch et al., 1991; Eschrich et al., 1993; Lah et al., 1994).

To gain more insight in the mechanism of flavin-mediated oxygenolytic dehalogenation of haloaromatic compounds and the role of substrate and enzyme dynamics (Peelen et al., 1993), we have addressed in the present study the regiospecificity of hydroxylation of F_4-POHB by PHBH. ^{19}F NMR was used for the unambiguous assignment of aromatic products, and mutant Y385F was selected as a tool to modulate the enzyme specificity. Because quinones are highly electrophilic compounds involved in several toxicological processes (Rietjens et al., 1997), the effect of ascorbate as chemical reducing agent was studied as well. Evidence is presented that the PHBH-mediated conversion of F_4-POHB involves several consecutive oxygenation and dehalogenation steps including hydroxylation at the substrate C2 position. The results are discussed in relation to the frontier orbital characteristics of the aromatic substrate and products and their orientation in the active site. A preliminary account of this work has been presented elsewhere (van Berkel et al., 1997).

MATERIALS AND METHODS

Materials. Mes, Hepes, Hepps, and Tris were from Sigma. NADH, NADPH, catalase, and superoxide dismutase were purchased from Boehringer Mannheim, Inc. F_4-POHB was prepared from pentafluorobenzoic acid (Aldrich) as described (Husain et al., 1980).

Enzyme Purification. PHBH and Y385F were purified as described before (Eschrich et al.,1993). *p*-Hydroxybenzoate 1-hydroxylase (decarboxylating) from the yeast *Candida parapsilosis* was isolated according to the procedure reported by van Berkel et al. (1994).

Enzyme Kinetics. The activity of PHBH was determined spectrophotometrically by measuring the F_4-POHB-stimulated oxidation of NADPH at 340 nm (25 °C). pH-dependent activity measurements were performed in 80 mM Mes (pH 5.6−6.6) and 80 mM Hepes (pH 7.0−8.0), in either the absence or the presence of 2 mM ascorbate. Buffers were brought to constant ionic strength ($I = 0.1$ M) with sodium sulfate as described (van Berkel & Müller, 1989). For estimation of steady state kinetic parameters, NADPH and F_4-POHB were varied using 0.15 mM (F_4-POHB) and 0.18 mM (NADPH) of the fixed substrate. Kinetic data were analyzed by nonlinear least-squares fitting routines (van Berkel et al., 1991; Eschrich et al., 1993). Rapid-reaction kinetics were carried out at 25 °C using a temperature-controlled single-wavelength stopped-flow spectrophotometer, type SF-51, from High-Tech Scientific Inc. with 1.3 ms deadtime. Rate constants for the anaerobic reduction of F_4-POHB complexed enzymes were estimated from kinetic traces recorded at 450 nm at variable concentrations of NADPH (Eppink et al., 1995).

Oxygen Uptake Experiments. Oxygen consumption was measured polarigraphically at 25 °C by using a Clark electrode. Assay mixtures contained 100 mM Mes, pH 6.0, or 50 mM potassium phosphate, pH 7.0, and varying amounts of aromatic substrate, NADPH, and enzyme. The degree of uncoupling of hydroxylation was determined with catalase (Eschrich et al., 1993). For the establishment of the reaction stoichiometry, the oxygen consumption experiments were performed in either the absence or the presence of 2 mM ascorbate. F_4-POHB (150 μM) was incubated with 50, 100, 150, 200, 250, 300, and 350 μM NADPH, respectively, and the reaction was started by the addition of 1.5 μM enzyme. At the end of the initial rapid phase of oxygen consumption (5−10 min), the residual amount of NADPH was determined by the addition of 1 mM POHB.

Substrate Binding Studies. Dissociation constants of binary complexes between oxidized enzyme and F_4-POHB were determined fluorimetrically using an Aminco SPF-500 spectrofluorimeter (van Berkel et al., 1992).

NMR Measurements. ^{19}F NMR measurements were performed on a Bruker AMX 300 or Bruker DPX-400 NMR spectrometer, essentially as described elsewhere (Peelen et al., 1993, 1995). Proton-decoupled spectra were obtained at 7 °C. Between 5000 and 15 000 scans were recorded, depending on the concentrations of the fluorinated products and the signal to noise ratio required. The sample volume was 2.0 mL. A coaxialary capillary contained 4-fluorobenzoate as a standard and ^2H_2O for locking the magnetic field. Concentrations of fluorinated products were calculated by comparison of the integral of the ^{19}F NMR resonances of the products to the integral of the ^{19}F NMR resonance of 4-fluorobenzoate. Chemical shifts are reported relative to $CFCl_3$. ^1H NMR analysis was carried out on a Bruker AMX 500 MHz spectrometer. Samples were prepared as described for ^{19}F NMR. At the end of the reaction the incubation mixtures were freeze-dried and dissolved in 0.5 mL ^2H_2O.

Product Identification. ^{19}F NMR product analysis revealed that the addition of ascorbate to the incubation mixtures was essential to prevent the accumulation of nonspecific oxidation products. Incubation mixtures (25 °C) contained 100 mM Mes, pH 6.0, or 50 mM potassium phosphate buffer, pH 7.0, 0.17 mM F_4-POHB, 0.05−0.3 mM NADPH, 1 mM ascorbate, 150 units of catalase, and 1.5 μM enzyme. The reaction was monitored spectrophotometrically by following the absorbance decrease at 340 nm until all NADPH was consumed. In time-dependent experiments, reactions with the fluorinated substrate were stopped by the addition of 1 mM POHB. Before recording the NMR spectra, samples were made anaerobic by five cycles of flushing with argon and degassing. Oxidative decarboxylation of fluorinated products was achieved by incubation with *p*-hydroxybenzoate 1-hydroxylase from *C. parapsilosis* (van Berkel et al., 1994). Initial incubations (total volume 2.0 mL, 25 °C) contained 50 mM potassium phosphate buffer, pH 7.0, 0.17 mM F_4-POHB, 0.3 mM NADPH, 1 mM ascorbate, 150 units of catalase, 30 μM FAD, and 1.5 μM Y385F. After analyzing the sample by ^{19}F NMR, 0.6 mM NADH, 50 units of superoxide dismutase, and 1 μM *p*-hydroxybenzoate 1-hydroxylase from *C. parapsilosis* were added, and the reaction was followed spectrophotometrically until no absorption decrease at 340 nm was observed. The sample was then again analyzed by ^{19}F NMR for the formation of fluorinated phenolic products.

Identification of fluorinated aromatic products was achieved by (a) the ^{19}F-^{19}F coupling patterns and (b) on the basis of the chemical shift values of the ^{19}F NMR resonances of a set of fluorinated benzene derivatives (Rietjens et al., 1993; Peelen et al., 1995). No proton coupling to the fluorine resonances was observed on comparison of ^1H decoupled ^{19}F NMR spectra with ^1H coupled ^{19}F NMR spectra. The quality of the spectra was good enough to observe small coupling constants (0.3−0.5 Hz). From the *ortho*-hydroxylation of fluorinated phenols by phenol hydroxylase from *Trichosporon cutaneum* (Peelen et al., 1995), it is known that substitution of a fluorine atom by a hydroxyl group results in a chemical shift of a fluorine substituent located at an *ortho* position of +2.2 (± 0.9) ppm, at a *meta* position of +1.0 (± 0.9) ppm, and at a *para* position of −5.4 (± 0.9) ppm.

Molecular Orbital Calculations. Molecular orbital calculations were performed on a Silicon Graphics Indigo 2 workstation with Insight (Biosym Technologies, CA). The semiempirical molecular orbital method was used, applying the AM1 Hamiltonian from the MOPAC program. All calculations were carried out, essentially as described by Peelen et al. (1995).

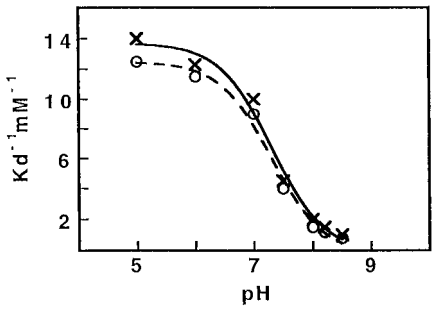

FIGURE 2: pH-dependent binding properties of PHBH and Y385F with F_4-POHB. The dissociation constants of the complexes were determined from flavin fluorimetric titration experiments: PHBH (×), Y385F (○).

RESULTS

Fluorescence Binding Studies. Previous studies have shown that PHBH preferentially binds F_4-POHB between pH 6 and 7 with an apparent pK_a of about 7.6 in the oxidized state (van Berkel & Müller, 1989). Because the pK_a of the fluorinated substrate free in solution is 5.3 (Husain et al., 1980), weak binding of F_4-POHB at pH 8 was tentatively ascribed to the ionization of one of the tyrosines in the active site (van Berkel and Müller, 1989). Titration of Y385F with F_4-POHB was accompanied by quenching of the fluorescence of protein-bound FAD and followed simple 1:1 binding. Figure 2 shows that the binding properties of F_4-POHB to Y385F were nearly indistinguishable from PHBH, excluding the possibility that the apparent pK_a observed originates from the deprotonation of Tyr385.

Catalytic Properties of PHBH with F_4-POHB. To identify the optimal conditions for the enzymatic conversion of F_4-POHB, steady state kinetic parameters were estimated as a function of pH. As can be seen from Table 1, Y385F catalyzed the F_4-POHB-stimulated oxidation of NADPH at approximately the same rate as PHBH. With both enzymes, substantial substrate inhibition occurred below pH 7.0 (Table 1). A similar type of inhibition was reported for other fluorinated substrate analogs (Husain et al., 1980). Table 1 also shows that the apparent K_m values for F_4-POHB and NADPH decreased with decreasing pH, resulting in an optimal catalytic efficiency near pH 6.5. This is clearly different from the reaction with the physiological substrate where optimal catalysis takes place around pH 8.0 (van Berkel & Müller, 1989).

The turnover rate of Y385F with F_4-POHB was about 1 order of magnitude higher than with POHB (Eschrich et al., 1993) where flavin reduction is rate limiting in catalysis (Entsch et al., 1991; Eschrich et al., 1993). This suggests that F_4-POHB is a better effector for Y385F than POHB. The effector role of F_4-POHB was addressed in more detail by studying the reductive half-reaction by anaerobic stopped flow experiments. At pH 7.0, 25 °C, flavin reduction of the F_4-POHB complexed enzymes was a simple monoexponential process. The extrapolated rate constants for reduction at infinite NADPH concentrations were 12.5 s^{-1} for PHBH (K_d of NADPH = 0.81 mM) and 19.3 s^{-1} for Y385F (K_d of NADPH = 0.79 mM), respectively. This

Table 1: Steady State Kinetic Parameters of PHBH and Y385F with F_4-POHB

enzyme	pH	apparent k_{cat}[a] (s^{-1})	apparent K_m F_4-POHB (μM)	apparent K_m NADPH (μM)	excess substrate inhibition (mM)
PHBH	6.0	3.7	36	51	>0.08
	6.5	3.8	43	73	>0.1
	7.0	4.1	67	145	>0.3
	8.0	4.0	156	280	>1.0
+ascorbate	6.0	3.0	36	30	>0.1
	6.5	3.2	68	66	>0.15
	7.0	3.5	102	112	>0.5
	8.0	3.4	120	225	>1.0
Y385F	6.0	3.9	43	47	>0.08
	6.5	4.1	36	67	>0.15
	7.0	5.1	66	127	>0.3
	8.0	4.1	154	154	>1.0
+ascorbate	6.0	2.7	41	36	>0.1
	6.5	3.1	51	49	>0.15
	7.0	3.5	68	105	>0.4
	8.0	3.0	112	152	>1.0

[a] Turnover numbers and apparent K_m values for F4-POHB and NADPH were determined from the initial velocity of NADPH consumption at 25 °C in air-saturated buffer, variable [F4-POHB] or [NADPH] and 0.2 mM of the fixed substrate. Experiments ($n = 2$) were performed in the absence or presence of 2 mM ascorbate. Turnover numbers are apparent maximum values extrapolated to infinite concentrations of F4-POHB and NADPH and taking into account substrate inhibition. The mean standard error of values of kinetic parameters was about 10%.

shows that the F_4-POHB stimulated rate of reduction of both enzymes is not rate limiting in catalysis (cf. Table 1) and confirms that F_4-POHB is a better effector for Y385F than POHB.

Stoichiometry of the Reaction. The stoichiometry of the reaction of PHBH and Y385F with F_4-POHB was studied by oxygen consumption experiments. In analogy with published results (Husain et al., 1980), PHBH fully coupled oxygen consumption to F_4-POHB hydroxylation. With Y385F, some uncoupling of hydroxylation was observed as evidenced by the formation of hydrogen peroxide. The efficiency of hydroxylation by Y385F was somewhat dependent on the reaction conditions yielding about 0.15 mol of hydrogen peroxide/mol of oxygen consumed at pH 7.0. This degree of uncoupling is in the same range as observed with the Y385F catalyzed conversion of POHB (Entsch et al., 1991; Eschrich et al., 1993).

When the reaction of PHBH (or Y385F) with F_4-POHB was performed with varying concentrations of NADPH (pH 7.0), about 2 equiv of NADPH was consumed/equiv of oxygen (Figure 3). Comparable results were obtained at pH 6.0 (data not shown). These results are consistent with an earlier proposal (Husain et al., 1980) that the utilization of 2 mol of NADPH/mol of oxygen can be ascribed to the initial formation of a quinonoid product intermediate. When the same set of experiments was repeated in the presence of ascorbate, nearly equal amounts of oxygen and NADPH were consumed (Figure 3). The reaction stoichiometry did not change when the concentration of ascorbate was varied beween 1 and 10 mM, indicating that 1 mM ascorbate is sufficient to compete favorable with NADPH for quinone reduction. In line with this, the presence of ascorbate significantly decreased the rate of NADPH consumption (Table 1). Interestingly, in the presence of ascorbate and at high NADPH/F_4-POHB ratios, more than 1 equiv of NADPH

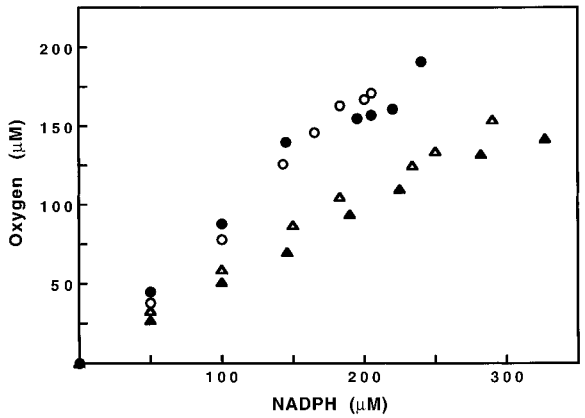

FIGURE 3: Reaction stoichiometry of the reaction of PHBH and Y385F with F_4-POHB as determined by oxygen consumption experiments. F_4-POHB (150 μM) was incubated with varying concentrations of NADPH in air-saturated buffer, pH 7.0, and the reaction was initiated by the addition of 1.5 μM enzyme: PHBH in the absence (△) or presence (○) of ascorbate, Y385F in the absence (▲) or presence (●) of ascorbate. The plotted *x*-axis values are the actual amounts of NADPH consumed. For other experimental details, see Materials and Methods.

and oxygen was consumed per substrate molecule (Figure 3). This prompted us to study the reaction stoichiometry in more detail by ^{19}F NMR.

Product Identification by ^{19}F NMR. Conversion of F_4-POHB by PHBH or Y385F did not result in the exclusive formation of 2,5,6-trifluoro-3,4-dihydroxybenzoate (F_3-DOHB). Especially with Y385F, several other fluorinated aromatic products were formed. Figure 4A shows the ^{19}F NMR spectrum of an incubation of Y385F in the presence of equimolar amounts of F_4-POHB and NADPH. The ^{19}F NMR resonances at −150.3, −157.1, and −171.1 ppm are assigned to F_3-DOHB, whereas the resonances at −153.1 ppm and −170.7 ppm are ascribed to residual F_4-POHB (Table 2). The resonance at −123.1 ppm originates from fluoride anion, released upon dehalogenation. The two additional main resonances at −154.7 and −177.2 ppm in Figure 4A showed identical integrals, and both resonances have the same *J* coupling, suggesting that these resonances are derived from an aromatic product with two fluorine substituents *ortho* positioned to each other. This indicates that the initial product F_3-DOHB was subject to further hydroxylation at the C2 position of the aromatic ring. The formation of 5,6-difluoro-2,3,4-trihydroxybenzoate (5,6-F_2-TOHB) was in good agreement with chemical shift predictions of −176.5 ppm for the C5 fluorine substituent and −156.1 ppm for the C6 fluorine substituent, respectively (Table 2). All together, the results presented in Figure 4A suggest that conversion of F_4-POHB by Y385F involves the consecutive formation of F_3-DOHB and 5,6-F_2-TOHB.

Further evidence for hydroxylation at the C2 center of the aromatic ring was obtained when the NADPH/F_4-POHB ratio in the incubation mixture of Y385F was increased. Addition of an extra equivalent of NADPH resulted in the complete depletion of F_4-POHB and in a nearly 2-fold increase in the intensities of the resonances assigned to 5,6-F_2-TOHB (Figure 4B). Moreover, some extra ^{19}F NMR resonances of relatively low intensity were observed at −153.6 ppm and −171.4 ppm, indicative for the formation of additional fluorinated products. The intensities of these two resonances were different, suggesting that they belong to different hydroxylation products. Furthermore, both these resonances

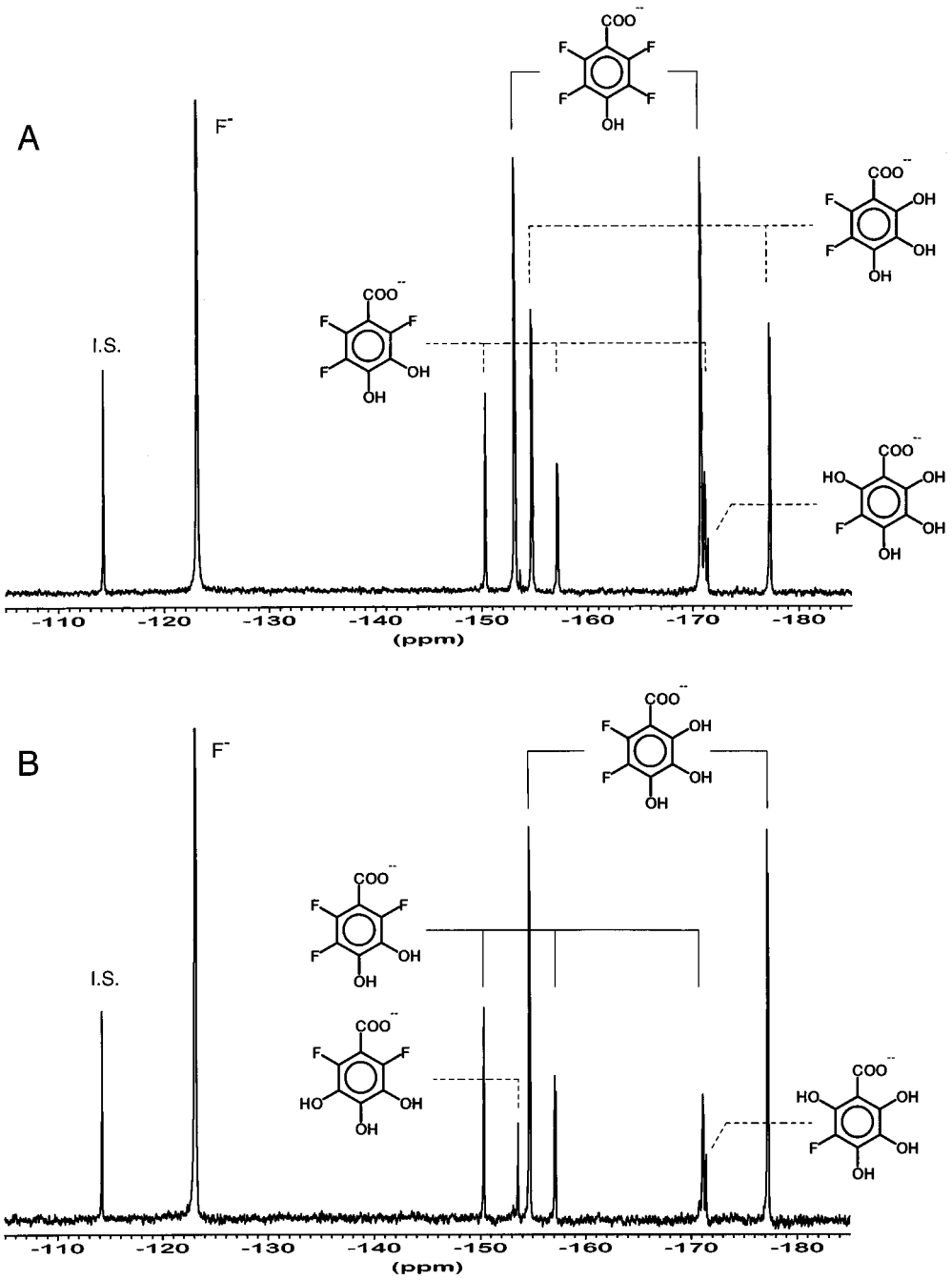

FIGURE 4: ^{19}F NMR product analysis of the reaction of F_4-POHB with Y385F: (A) NADPH/F_4-POHB ratio 0.9; (B) NADPH/F_4-POHB ratio 1.8. 170 μM F_4-POHB was incubated with NADPH in air-saturated buffer pH 7.0, containing 1 mM ascorbate. Resonances were identified on the basis of the results summarized in Table 2. The resonance at −114.2 ppm is from the internal standard, 4-fluorobenzoate.

showed no J_{F-F} coupling. Earlier studies have shown that Y385F hydroxylates the physiological product DOHB to TOHB (Entsch et al., 1991; Eschrich et al., 1993). On the basis of these data, the resonance at −153.6 ppm is assigned to 2,6-difluoro-3,4,5-trihydroxybenzoate (2,6-F_2-TOHB) (Table 2). The ^{19}F NMR resonance at −171.4 ppm (Figure 4B) could originate from either 5-fluoro-2,3,4,6-tetrahydroxybenzoate (5-F-TeOHB) or 6-fluoro-2,3,5,6-tetrahydroxybenzoate (6-F-TeOHB). On the basis of chemical shift values of the difluorinated products and theoretical considerations (Peelen et al., 1995), this resonance is assigned to 5-F-TeOHB (Table 2).

The identity of the reaction products was confirmed by ^{1}H-NMR. For this purpose, F_4-POHB was incubated with a 2-fold excess of NADPH either in the absence or presence of Y385F. In the presence of Y385F, no extra resonances were observed in the 6−8 ppm region of the ^{1}H NMR spectrum. This shows that no products with aromatic protons were formed. Moreover, in all ^{19}F NMR spectra, no ^{1}H proton coupling could be observed.

Further identification of fluorinated aromatic products was achieved by incubation of the reaction samples with *p*-hydroxybenzoate 1-hydroxylase from the yeast *C. parapsilosis*. Recent studies have shown that this NAD(P)H-dependent flavoenzyme catalyzes the oxidative decarboxylation of POHB, yielding 1,4-dihydroxybenzene (van Berkel et al., 1994). Figure 5A illustrates that *p*-hydroxybenzoate 1-hydroxylase from *C. parapsilosis* forms a single aromatic product from F_4-POHB which is ascribed to the formation of tetrafluoro-1,4-dihydroxybenzene. Figure 5B shows the ^{19}F NMR spectrum of F_4-POHB, successively incubated with Y385F and *p*-hydroxybenzoate 1-hydroxylase from *C. parap-*

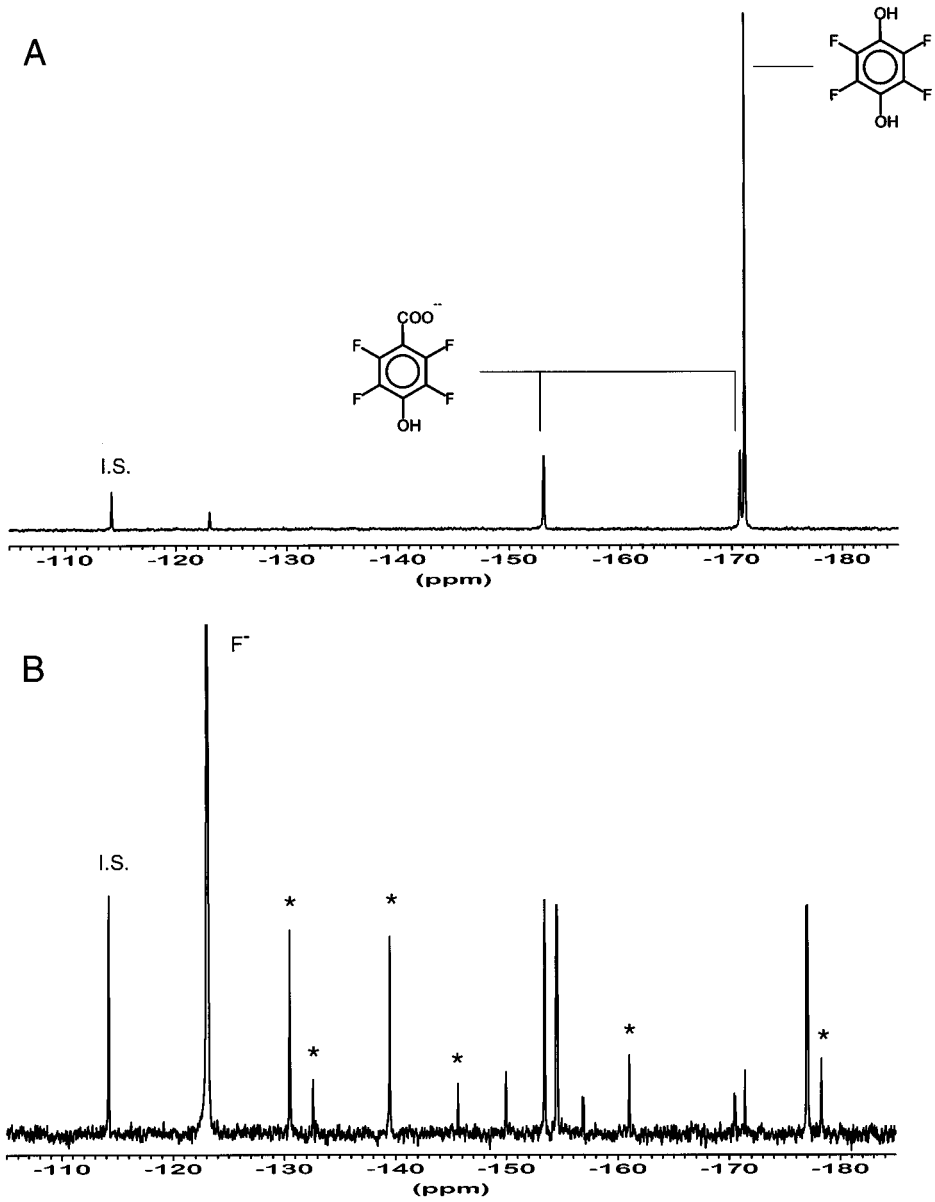

FIGURE 5: ^{19}F NMR spectra of the conversion of fluorinated POHB derivatives by *p*-hydroxybenzoate 1-hydroxylase from *C. parapsilosis*: (A) conversion of F_4-POHB; (B) conversion of hydroxylation products obtained from the incubation of F_4-POHB with Y385F at a NADPH/F_4-POHB ratio of 1.8 (cf. Figure 4B). New resonances are indicated by an asterisk. The resonance at −114.2 ppm is from the internal standard, 4-fluorobenzoate.

silosis. This spectrum revealed six new resonances in relation to the same sample incubated in the absence of the yeast enzyme (cf. Figure 4B). From the difference spectrum, it could be established that three of the new resonances were derived from 2,5,6-trifluoro-1,3,4-trihydroxybenzene, one resonance from 5,6-difluoro-1,2,3,4-tetrahydroxybenzene, one resonance from 2,6-difluoro-1,3,4,5-tetrahydroxybenzene and one resonance from 5-fluoro-1,2,3,4,6-pentahydroxybenzene. This confirmed that all hydroxylation products formed by Y385F contained a carboxylic group. The polyhydroxylated fluorobenzene products formed by *p*-hydroxybenzoate 1-hydroxylase from *C. parapsilosis* were rather stable in the presence of ascorbate. This is concluded from the nearly identical ^{19}F NMR spectrum recorded after several hours.

Reaction Stoichiometry as Measured by ^{19}F NMR. The hydroxylation pattern of the enzymatic conversion of F_4-POHB was strongly dependent on the reaction conditions. With PHBH (pH 7.0), depletion of F_4-POHB resulted in nearly equal amounts of F_3-DOHB and 5,6-F_2-TOHB (Figure 6A, Table 3). With Y385F, the initial product F_3-DOHB was rather efficiently converted to 5,6-F_2-TOHB (Figure 6B) and, to a minor extent, to 2,6-F_2-TOHB (Table 3). Unlike Y385F, PHBH produced minor amounts of 5,6-F_2-TOHB at pH 6.0 (Table 3). During the conversion of F_4-POHB and in contrast to published data (Husain et al., 1980), considerable higher amounts of fluoride anion were produced as expected from the amount of hydroxylated fluorinated aromatic products formed. From the concentrations of fluorinated aromatic substrate and products observed at the end of the reactions (Table 3), it could be calculated that, during the conversion of F_4-POHB, 10−20% of the fluorinated aromatic reaction products were lost and that the excess fluoride released increased with increasing concentrations of NADPH. Increasing the concentration of ascorbate in the incubation mixtures to 10 mM or addition of superoxide dismutase did not significantly change the amount of fluoride anion formed. On the other hand, conversion of F_4-POHB

Table 2: Chemical shifts of ^{19}F NMR Resonances of Identified Fluorinated Products Formed from the Conversion of F_4-POHB by PHBH and Y385F[a]

compound	chemical shift (ppm)	basis of identification
F_4-POHB	−153.1 (F2/F6)	2,3,5,6-tetrafluorophenol (F3/F5) (−149.3 ppm, $^3J_{FF}$ = 18.0 Hz)
	−170.7 (F3/F5)	2,3,5,6-tetrafluorophenol (F2/F6) (-170.5 ppm, $^3J_{FF}$ = 18.0 Hz)
F_3-DOHB	−150.3 (F2)	*o*-OH shifts F2 by +2.8 ppm ($^4J_{FF}$ = 5.1 Hz, $^5J_{FF}$ = 7.6 Hz)
	−171.1 (F5)	*m*-OH shifts F5 by −0.4 ppm ($^3J_{FF}$ = 24.2 Hz, $^5J_{FF}$ = 7.6 Hz)
	−157.1 (F6)	*p*-OH shifts F6 by −4.0 ppm ($^3J_{FF}$ = 24.2 Hz, $^4J_{FF}$ = 5.1 Hz)
2,6-F_2-TOHB	−153.6 (F2/F6)	*o*-OH shifts F6 by +3.5 ppm *p*-OH shifts F2 by −3.3 ppm
5,6-F_2-TOHB	−154.7 (F6)	*m*-OH shifts F6 by +2.4 ppm ($^3J_{FF}$ = 22.9 Hz)
	−177.2 (F5)	*p*-OH shifts F5 by −6.1 ppm ($^3J_{FF}$ = 22.9 Hz)
5-F-TeOHB	−171.4 (F5)	*o*-OH shifts F5 by +5.8 ppm

[a] The chemical shifts (as determined in 50 mM potassium phosphate, pH 7.0) are relative to $CFCl_3$. Chemical shifts were ascribed to specific fluorine substituents on the basis of relative peak intensities, proton-decoupled splitting patterns, and the knowledge that *meta* substituents influence chemical shifts far less than *para* or *ortho* substituents.

in the absence of ascorbate led to F_3-DOHB, several unidentified fluorinated reaction products, and only minor amounts of 5,6-F_2-TOHB (data not shown). This raised the possibility that the fluorinated quinone formed upon enzymatic attack of F_4-POHB undergoes a Michael addition by water at the 2-position, leading to fluoride elimination and, after reduction, the trihydroxy product. Because fluorinated benzoquinones are highly reactive species which become dehalogenated upon reaction with nucleophiles (den Besten et al., 1993), such a nonenzymatic mechanism could also explain the high amount of side-products formed in the absence of ascorbate.

In order to establish whether the conversion of F_3-DOHB to 5,6-F_2-TOHB was enzyme mediated, the following experiments were performed. F_4-POHB was completely converted by PHBH (cf. Figure 6A, Table 3) and the resulting mixture of F_3-DOHB and 5,6-F_2-TOHB was incubated in aerated buffer in the absence or presence of excess NADPH. ^{19}F NMR analysis revealed that in the absence of NADPH no reaction occurred. In the presence of NADPH, F_3-DOHB was further converted to 5,6-F_2-TOHB, providing direct evidence for an enzyme-mediated hydroxylation step. As already noted from the ^{19}F NMR experiments reported above, the extent of conversion of F_3-DOHB was strongly dependent on the reaction conditions. With PHBH, formation of 5,6-F_2-TOHB was most efficient at pH 7.0, but in contrast to pH 6.0, also minor amounts of 2,6-F_2-TOHB and 5-F-TeOHB were formed. Furthermore, the reaction of F_3-DOHB with PHBH was not complete, most probably as a result of product (5,6-F_2-TOHB) inhibition. This is in keeping with the results presented in Figures 3 and 6 and the fact that the NADPH left was readily consumed after the addition of 1 mM POHB. The reaction of F_3-DOHB with Y385F was rather efficient. At both pH 6.0 and 7.0, more than 80% of F_3-DOHB was converted to 5,6-F_2-TOHB. Again, additional formation of 2,6-F_2-TOHB and 5-F-TeOHB was only observed at pH 7.0.

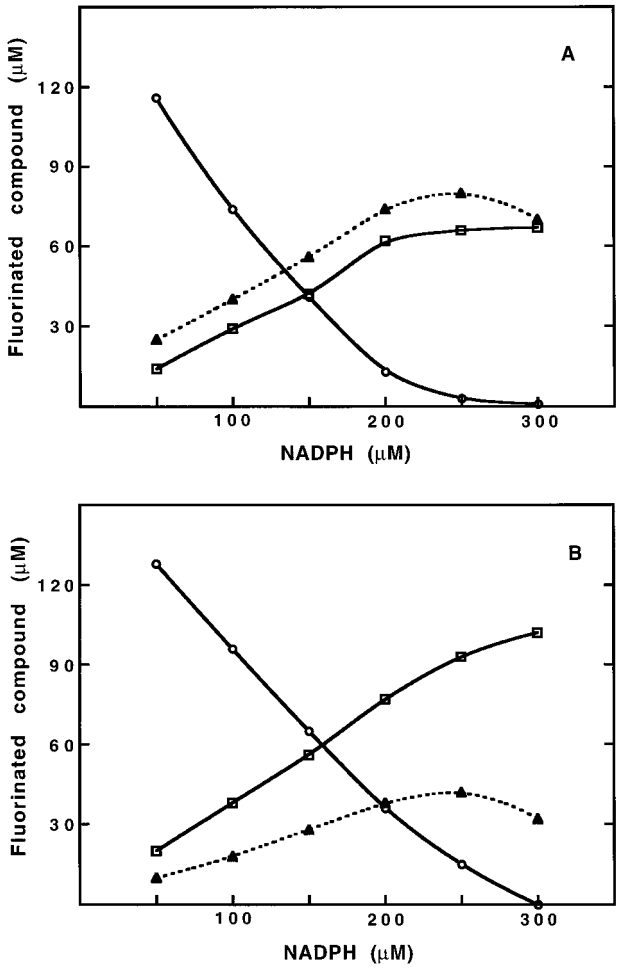

FIGURE 6: NADPH dependence of the conversion of F_4-POHB by PHBH and Y385F as determined by ^{19}F NMR. F_4-POHB (170 μM) was incubated with varying concentrations of NADPH in air-saturated buffer pH 7.0, containing 1 mM ascorbate and the reaction was initiated by the addition of 1.5 μM enzyme: (a) PHBH, (b) Y385F; F_4-POHB (○), F_3-DOHB (▲), 5,6-F_2-TOHB (□). The standard error in the concentrations of fluorinated compounds was about 5%. For other experimental details, see Materials and Methods.

Table 3: Conversion of F_4-POHB by PHBH and Y385F as determined by ^{19}F NMR[a]

fluorinated compound	pH	concentration of fluorinated product (μM) at NADPH/F_4-POHB ratio of 0.9 (1) or 1.8 (2)			
		PHBH		Y385F	
		(1)	(2)	(1)	(2)
F_4-POHB	6.0	32		56	
	7.0	41	1	65	
F_3-DOHB	6.0	79	101	32	49
	7.0	56	68	28	32
2,6-F_2-TOHB	6.0				
	7.0		1	1	6
5,6-F_2-TOHB	6.0	30	41	52	89
	7.0	42	66	56	102
5-F-TeOHB	6.0				
	7.0		1	1	3
fluoride anion	6.0	191	245	203	327
	7.0	203	302	217	392

[a] The initial F_4-POHB concentration was 170 ± 8 μM and the enzyme concentration was 1.5 μM. All experiments (n = 2) were performed in the presence of 1 mM ascorbate. Concentrations of fluorinated products have a standard error <5%.

Further support for the consecutive hydroxylation at the C3 and C2 position of the aromatic ring was obtained by

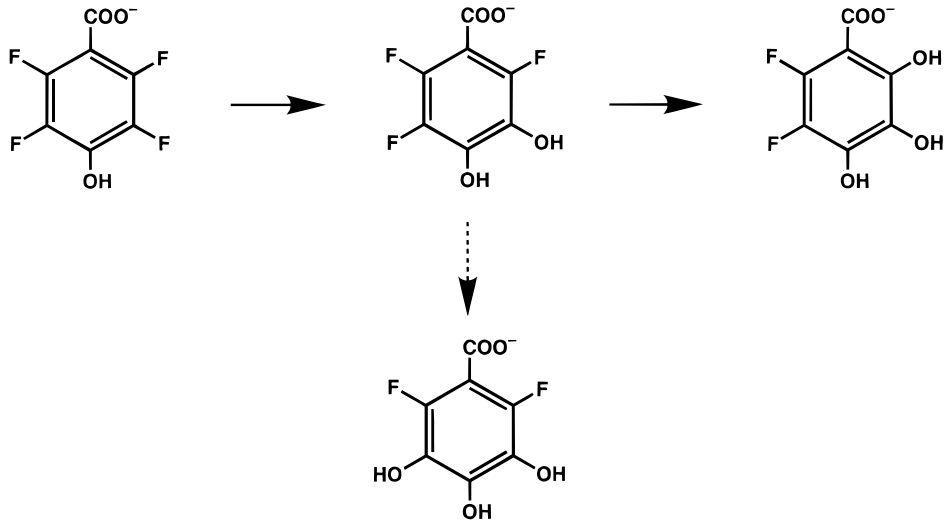

FIGURE 7: Proposed hydroxylation pathway of F_4-POHB by PHBH and Y385F.

Table 4: E(HOMO) and HOMO Density Characteristics of Dianionic POHB Derivatives[a]

compound	dp	*E*(HOMO) eV	HOMO density on carbon center C1	C2	C3	C4	C5	C6
POHB	4	0.44	0.29	0.01	0.21	0.05	0.21	0.01
F_4-POHB	4	−0.49	0.25	0.01	0.20	0.05	0.20	0.01
F_3-DOHB	4	−0.43	0.25	0.00	0.18	0.06	0.18	0.02
	3	−0.23	0.00	0.18	0.07	0.18	0.01	0.25
5,6-F_2-TOHB	4	−0.30	0.25	0.00	0.19	0.06	0.18	0.02
	3	−0.15	0.00	0.16	0.08	0.17	0.02	0.22
2,6-F_2-TOHB	4	−0.32	0.24	0.01	0.17	0.08	0.17	0.01
	3	−0.13	0.00	0.18	0.06	0.17	0.02	0.25

[a] Molecular orbital characteristics of dianionic forms of POHB derivatives of importance for nucleophilic attack on the flavin C(4a)-hydroperoxide, as calculated by the AM1 Hamiltonian of the MOPAC module of the Insight II package (Biosym, San Diego). Calculations were carried out with either a deprotonated 4-hydroxyl or 3-hydroxyl group (dp). eV, electronvolts.

recording ^{19}F NMR spectra at different time intervals. Spectra recorded of samples which were incubated for only 30 s after initiating the reaction of PHBH with equimolar amounts of F_4-POHB and NADPH showed nearly the exclusive formation of F_3-DOHB. With Y385F, the formation of F_3-DOHB as the first product was more difficult to assign because of its rapid conversion to 5,6-F_2-TOHB. The spectra recorded with time clearly showed the accumulation of 5,6-F_2-TOHB and rather slow formation of 2,6-F_2-TOHB and 5-F-TeOHB. All together, the above results from NMR product analysis are consistent with the hydroxylation pathway presented in Figure 7.

Frontier Orbital Characteristics of Fluorinated Hydroxylation Products. In order to explain the observed hydroxylation pattern from a chemical point of view, molecular orbital calculations were performed to investigate the intrinsic electronic characteristics of the fluorinated POHB derivatives. For PHBH it was reported that the turnover rates for the conversion of a series of fluorinated POHB analogs correlate with the *E*(HOMO) of their dianionic forms (Vervoort et al., 1992). From this it was suggested that a high *E*(HOMO) and HOMO density on C3 of the aromatic substrate will favor the electrophilic attack by the flavin C(4a)-hydroperoxide. Table 4 shows that the dianionic form of F_4-POHB has a relatively low *E*(HOMO) explaining the low turnover rate (Vervoort et al., 1992), but that the electron density at the C3 (C5) atom is comparable to that of POHB. This may explain the rather efficient C3 hydroxylation of F_4-POHB by PHBH at pH 6. In analogy to F_4-POHB, the fluorinated aromatic products formed from the enzymatic conversion of F_4-POHB showed considerable HOMO density (reactive electron density) at the fluorinated carbon atom *ortho* or *para* to a deprotonated hydroxyl group (Table 4). This suggests that the formation of 5,6-F_2-TOHB from F_3-DOHB results from the (partial) ionization of the 3OH of F_3-DOHB. In line with this proposal, formation of 2,6-F_2-TOHB must then result from the (partial) ionization of the 4OH of F_3-DOHB.

DISCUSSION

The flavoprotein-mediated oxygenolytic dehalogenation of haloaromatic substrates requires two NAD(P)H molecules per turnover (Husain et al., 1980; Suzuki et al., 1991; Xun et al., 1992; Peelen et al., 1995; Wieser et al., 1997). On the basis of studies of PHBH with fluorinated substrate analogs it was proposed that this unusual stoichiometry is due to the nonenzymatic reduction of a quinonoid species formed as the primary product of oxygenolytic dehalogenation (Husain et al., 1980). In the present study, the stoichiometry of the PHBH-catalyzed conversion of F_4-POHB was studied in the presence of ascorbate. Addition of ascorbate nearly restored the 1:1 reaction stoichiometry, indicative for the rapid chemical reduction of quinone intermediates by ascorbate. In line with earlier results on the oxygenolytic dehalogenation of *ortho*-fluorinated phenols (Peelen et al., 1995), ascorbate prevented the accumulation of nonspecific oxidation products. However, ^{19}F NMR analysis revealed that even in the presence of high concentrations of ascorbate, part of the fluorinated quinones were dehalogenated in a nonenzymatic process. These findings are of physiological relevance for flavoprotein hydroxylases which use a polyhalogenated aromatic compound as their natural substrate, because *in vivo* quinone reduction might compete with covalent binding to cellular macromolecules (den Besten et al., 1993; Rietjens et al., 1997).

The pH optimum of PHBH catalysis with F_4-POHB was around pH 6.5. This acidic shift in pH optimum with respect to the reaction with POHB is ascribed to the weak binding of dianionic F_4-POHB at pH 8.0. From mass spectral analysis, it was previously concluded that the conversion of F_4-POHB by PHBH results in the formation of F_3-DOHB

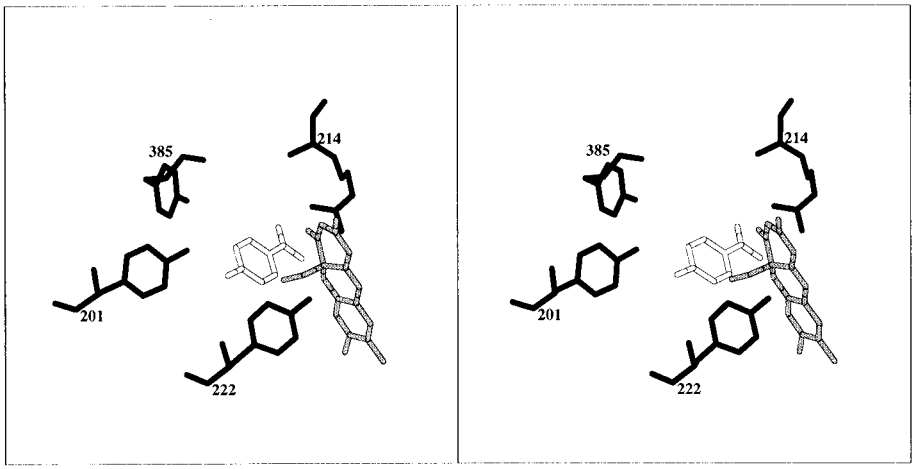

FIGURE 8: Stereo view of the flavin C(4a)-hydroperoxide model in the active site of the enzyme substrate complex of PHBH. Data according to Schreuder et al. (1990). In the depicted orientation, the distal peroxide oxygen is closest (2.4 Å) to the C3 and at 3.2 Å from the C2 of POHB. The FAD is grey, the aromatic substrate is white, and the protein residues are black.

as sole aromatic product (Husain et al., 1980). However, the ^{19}F NMR results presented in this paper provide clear evidence for a consecutive hydroxylation process leading to successive fluoride elimination at C3 and C2 of the substrate aromatic ring. The extent of C2 hydroxylation was dependent on the reaction conditions and the type of enzyme used. With PHBH, conversion of F_3-DOHB to 5,6-F_2-TOHB was less efficient than with Y385F, confirming that the hydroxylation of F_3-DOHB is enzyme mediated. The observed differences in the extent of C2 hydroxylation might be caused by the relative binding strengths of F_4-POHB and F_3-DOHB and the different degree of substrate and product inhibition. However, other factors like the effector properties of F_3-DOHB might also influence the outcome.

Fluoride elimination from F_4-POHB was not restricted to C2 and C3 of the aromatic ring. In the presence of excess NADPH, Y385F catalyzed the production of significant amounts of 2,6-F_2-TOHB from F_3-DOHB, a reaction nearly abolished in PHBH. These results are consistent with earlier observations (Entsch et al., 1991; Eschrich et al., 1993) that Y385F, unlike PHBH, can catalyze the C5 hydroxylation of DOHB, presumably by binding the physiological product with the 3OH rotated around the C1-C4 axis of the aromatic ring. From the structure of Y385F in complex with POHB it was deduced that the lost hydrogen bond between Tyr201 and Tyr385 in Y385F probably allows some displacement of Tyr201 (Lah et al., 1994). This would abolish the unfavorable contact between the 3OH of DOHB and the 4OH of Tyr201, presumably present in PHBH with DOHB bound in the flipped orientation (Lah et al., 1994). The predominant formation of 5,6-F_2-TOHB observed in the present study indicates that Y385F favors F_3-DOHB binding with the 3OH pointing toward the flavin. However, a similar reorientation of Tyr201 as envisioned from the Y385F structure, might induce the alternative binding mode of F_3-DOHB and lead to the formation of significant amounts of 2,6-F_2-TOHB. Moreover, the lost interaction between the side chains of Tyr201 and Tyr385 in Y385F might also explain the formation of small amounts of 5F-TeOHB from 5,6-F_2-TOHB (cf. Figure 4B).

As already pointed out before (Husain et al., 1980; Vervoort et al., 1992), dianionic F_4-POHB is a rather slow substrate for PHBH because of the deactivating effect of the fluorine substituents. The frontier orbital substrate characteristics presented in this paper suggest that C2 hydroxylation of F_3-DOHB is feasible when this substrate is activated at the C3 position, i.e., by deprotonation of the 3OH group. In keeping with their electron-withdrawing properties, the fluorine substituents of F_3-DOHB obviously stimulate the deprotonation of the 3OH moiety by delocalizing the negative charge. Upon deprotonation of the 3OH moiety, HOMO density and, thus, nucleophilic reactivity is redistributed and becomes located to a significant extent at the C2 center of F_3-DOHB. In line with this, the inability to deprotonate the 3OH group explains why DOHB is not a substrate for PHBH but rather an effector.

The enzymatic conversion of F_3-DOHB to 5,6-F_2-TOHB implies that F_3-DOHB binding induces the stabilization of the flavin C(4a)-hydroperoxide. Furthermore, it indicates that the peroxide bond of the oxygenated flavin intermediate becomes favorably oriented to allow C2 hydroxylation. Using the crystal structure of the reference compound 4a,5-epoxyethano-3-methyl-4a,5-dihydrolumiflavin (Bolognesi et al., 1978), Schreuder et al. (1990) have built a structural model of the flavin C(4a)-hydroperoxide in the active site of PHBH with bound POHB. In this model (Figure 8), the distal oxygen of the oxygenated flavin intermediate is in close vicinity (2.4 Å) of the C3 but also rather close (3.2 Å) to the C2 of the substrate. In the structure of the DOHB-complexed enzyme (Schreuder et al., 1989), the plane of the aromatic ring of DOHB is slightly rotated around the C1-C4 axis with respect to the plane of POHB, and the 3OH of the product is in short hydrogen bond distance of the carbonyl oxygen of Pro293. Because of steric constraints, this geometry will likely prevent the peroxide moiety of the oxygenated flavin to occupy the same orientation as modeled in the enzyme−substrate complex. From the conserved mode of binding of several substrate analogs (Schreuder et al., 1994), it is reasonable to assume that the binding mode of F_3-DOHB will resemble that of DOHB, leaving the salt bridge with Arg214 intact (cf. Figure 1). From this and the above considerations, we conclude that, in the F_3-DOHB complexed enzyme, the distal oxygen of the flavin C(4a)-hydroperoxide will approach the C2 atom. Moreover, enzyme dynamics will allow some rotational mobility of the substrate aromatic ring, facilitating a proper attack (see also below).

Several lines of evidence have indicated that the ionic state of the substrate is linked to the rate of flavin reduction by NADPH (Entsch & van Berkel, 1995). Our results are in accordance with this hypothesis. Unlike POHB binding (Entsch et al., 1991; Eschrich et al., 1993), binding of dianionic F_4-POHB highly stimulates the rate of reduction of Y385F. This supports the view that replacement of Tyr385 by Phe influences the ionization state of bound POHB (Eschrich et al., 1993). Crystallographic studies have indicated that deprotonation of POHB induces a conformational change in the active site loop extending from Pro293 to Ala296 (Gatti et al., 1996). It was inferred from these data that deprotonation of POHB might change the orientation of hydrogen bonds in the hydrogen bond network around the 4OH of the substrate. Binding of dianionic F_4-POHB might induce similar changes in the conformation of the active site loop. However, it seems obvious that in Y385F, the lost interaction between Tyr385 and Tyr201 will disrupt the potential chain of hydrogen bonds, which extends from the 4OH of POHB to His72 at the protein surface (Schreuder et al., 1994; Gatti et al., 1996). The conformation of the Pro293-Ala296 segment is probably also linked to the ionic state of F_3-DOHB. For instance, the deprotonated 3OH species of F_3-DOHB might be stabilized by hydrogen bond formation between the protonated 4OH and the carbonyl oxygen of Pro293. The deprotonation of the 3OH could be a transient one, i.e., under the influence of enzyme dynamics (Gatti et al., 1996). This suggests that the hydroxylation of the C2 center of F_3-DOHB predominantly is dictated by the HOMO density distribution of the π electrons in the substrate aromatic ring which is in accordance with the electrophilic aromatic substitution mechanism proposed for this class of flavoenzymes (Entsch et al., 1976a; Vervoort et al., 1992; Massey, 1994).

In conclusion, this paper demonstrates for the first time that C2 hydroxylation by PHBH is feasible when the chemical properties of the substrate favor (or allow) it. To get more insight in the dynamic factors which determine the regiospecificity of the hydroxylation reaction, we are presently addressing the mode of binding of fluorinated substrate analogs by ^{19}F NMR.

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