Fluoride Elimination from Substrates in Hydroxylation Reactions Catalyzed by p-Hydroxybenzoate Hydroxylase*

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Several fluorinated derivatives of p-hydroxybenzoate were synthesized and examined as substrates in the reaction catalyzed by p-hydroxybenzoate hydroxylase. All the derivatives tested served as substrates, undergoing tightly coupled hydroxylation by molecular oxygen.

Hydroxylation of the difluoro and tetrafluoro derivatives liberated stoichiometric amounts of fluoride. Little or no fluoride was released with monofluoro substrates. The defluorination caused higher consumption of NADPH with an overall NADPH to oxygen ratio of 2, in contrast to the ratio of 1 with the physiological substrate and with the monofluoro derivatives. Evidence was obtained strongly suggestive of a quinonoid species as the primary product formed upon oxygenative defluorination. The additional equivalent of NADPH consumed upon fluoride elimination is presumably used in a nonenzymatic reaction with the quinonoid intermediate, resulting in the observed dihydroxy product.

Stopped flow studies of the reductive and oxidative half-reactions with tetrafluoro-p-hydroxybenzoate substrate were examined. The oxygen half-reaction was analogous to that with p-hydroxybenzoate involving two transient oxygenated flavin intermediates. The decay of the first intermediate, a (4a)-peroxyflavin, results in rupture of the oxygen-oxygen bond and is rate-determining in overall catalysis. This is in contrast to the reaction with the normal substrate, presumably due to a deactivating effect of the fluorine substituents. The above results are consistent with an oxenoid mechanism of oxygen attack.

Fluorine is a unique substituent because of its electronegativity, which affects the acidity and stability of neighboring groups, and its size, which is not much greater than hydrogen. Substitution of fluorine in place of hydrogen, therefore, offers a great potential in the development of useful probes to study enzyme mechanisms. Examples of such studies include the reactions of 3-fluoro-4-hydroxybenzoate with protocatechuate-3,4-dioxygenase (1, 2) and p-fluoroglutamate with d-glutamate cycase (3).

The stabilization energy of the carbon-fluorine bond is among the largest in organic molecules and yet it is cleaved in a number of different enzymatic reactions (4), including those of monoxygenases catalyzing incorporation of 1 atom of molecular oxygen into the substrate. Examples of monoxygenase reactions which involve rupture of a carbon-fluorine bond include conversion of 4-fluorophenylalanine to tyrosine by purified rat and sheep liver phenylalanine hydroxylases (5), 4-fluoroproline to 4-hydroxyproline by proline hydroxylase (6), and p-fluoroalanine to p-aminophenol by the aryI hydroxylase of mammalian liver microsomes (7). The defluorination reaction catalyzed by phenylalanine hydroxylase has been studied in some detail (5, 8). The products of the reaction were identified as L-tyrosine and fluoride, formed in approximately equal amounts. The reaction showed the same requirements as the physiological conversion of phenylalanine to tyrosine, namely the enzyme, molecular oxygen, tetrahydropteridine, and NADPH. However, an unusually high ratio of NADPH oxidized to tyrosine produced was obtained (3 to 4:1). This is in contrast to the physiological reaction which gives a ratio of 1:1.

More recently, salicylate hydroxylase, an NADPH-dependent flavoprotein monoxygenase, has been shown to catalyze a dehalogenation reaction with o-iodophenol as the substrate (9). Catechol and iodide were identified as the products and the ratio of NADPH:catechol or NADPH:I−O2 was 2.0 in contrast to the ratio of 1.0 for the reaction with salicylate as substrate. No satisfactory explanation of the mechanism of elimination of halogens or of how this results in an unusual stoichiometry of the enzyme reaction has been provided. We propose a working hypothesis. The substrate must undergo enzyme-mediated attack of oxygen followed by removal of halide, leaving electron-deficient oxygenated product, which is then reduced by NADPH in a nonenzymatic reaction to give the observed product. The possibility, therefore, existed that this postulated product-level intermediate may be transiently observable.

The reaction catalyzed by p-hydroxybenzoate hydroxylase (EC 1.14.13.2) from Pseudomonas fluorescens, a flavoprotein monoxygenase, has been studied extensively in our laboratory. As a result, a great deal of information on the reaction mechanism of this enzyme is available. The rate of formation and decay of

\[ \text{NADPH} + \text{O}_2 \rightarrow \text{OH} + \text{COO}^- + \text{H}_2\text{O} + \text{NADP}^+ \]

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several charge transfer and oxygenated flavin intermediates, as well as the chemical nature of some of these transient species involved in catalysis, has been established (10–15).

This paper presents investigations with various fluorine-substituted analogues of p-hydroxybenzoate primarily to determine whether or not p-hydroxybenzoate hydroxylase is capable of catalyzing the cleavage of the C-F bond, what effect such a cleavage would have on the mechanism of catalysis, and whether it can be used to test the hypothesis for halide elimination. Results presented show that all the fluoro analogues tested serve as substrates, undergoing hydroxylation during catalysis, and that defluorination indeed occurs with suitably substituted analogues. A mechanism involving a quinonemid intermediate as the primary oxygenated product is presented.

MATERIALS AND METHODS

The following reagents were used as purchased: F2O, 2-fluorophenol, and 2-fluorophenol-4-carboxylic acid from Sigma Chemical Co., 3,5-difluorophenol and 3,5-difluorophenol-4-carboxylic acid from Aldrich Chemical Co., and 4-fluorophenol and 4-fluorophenol-4-carboxylic acid from Fisher Scientific Co. Materials were purified prior to use as described by Fisher (71) and Klotz (125). F2O was stored in a vacuum desiccator and was distilled twice before use with sodium metal. 2-Fluorophenol was stored in a cold, dark, dry place and was distilled twice before use with sodium metal. 4-Fluorophenol was stored at -70°C and was distilled twice before use with sodium metal.

p-Hydroxybenzoates. The following p-hydroxybenzoates were synthesized: 2-F-pOHB, 3-F-pOHB, 3,5-F2-pOHB, F2-pOHB, and F-pOHB. The procedure was that described by Nakazato et al. (13) modified by the addition of 2-fluorophenol or 4-fluorophenol. The reactions of the enzyme with oxygen were catalyzed by 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (13), and the reaction was started by adding a volume of ABTS to the enzyme solution. The reaction was stopped by adding a volume of 1 M acetic acid to the reaction mixture, and the absorbance was measured at 414 nm.

The dianion of each compound was measured spectrophotometrically by measuring the ultraviolet absorption spectra of the compounds at 25°C with HCl or NaOH. The pK values were determined from the changes in the ultraviolet absorption spectra of the compounds at 25°C. The pH values of the solutions were measured using a pH meter.

RESULTS

Properties of Fluorine-substituted Substrates—The ionization of substituents on the benzene ring of potential substrates seems to play an important role in the reaction catalyzed by p-hydroxybenzoate hydroxylase (12, 22). It was necessary to determine the altered pK values of carboxyl and hydroxyl groups in the various fluorinated p-hydroxybenzoates used in this paper.

The dianion was measured by adding an appropriate amount of HCl or NaOH to the sample in 0.1 M HCl or NaOH. The sample was stirred for 10 minutes, and the pH was measured using a pH meter. The pK values were determined from the changes in the ultraviolet absorption spectra of the compounds at 25°C.

The data presented show that all the fluoro analogues tested serve as substrates, undergoing hydroxylation during catalysis, and that defluorination indeed occurs with suitably substituted analogues. A mechanism involving a quinonemid intermediate as the primary oxygenated product is presented.

Vegetal Growth Measurements—Some observations of substantial interest were made spectrophotometrically by measuring the ultraviolet absorption spectra of the compounds at 25°C with HCl or NaOH. The pH values of the solutions were measured using a pH meter.

An aqueous solution containing 50 to 60 μM p-hydroxybenzoate was titrated at 25°C with HCl or NaOH. The pH values were determined from the changes in the ultraviolet absorption spectra of the compounds at 25°C. The pH values of the solutions were measured using a pH meter.

The data presented show that all the fluoro analogues tested serve as substrates, undergoing hydroxylation during catalysis, and that defluorination indeed occurs with suitably substituted analogues. A mechanism involving a quinonemid intermediate as the primary oxygenated product is presented.

Table 1: Ionization of various fluorinated analogues of p-hydroxybenzoate and absorption properties of different toxic species produced

Aqueous solutions containing 50 to 60 μM compounds were titrated at 25°C with HCl or NaOH. The pH values were determined from the changes in the ultraviolet absorption spectra of the compounds at 25°C. The pH values of the solutions were measured using a pH meter.

The data presented show that all the fluoro analogues tested serve as substrates, undergoing hydroxylation during catalysis, and that defluorination indeed occurs with suitably substituted analogues. A mechanism involving a quinonemid intermediate as the primary oxygenated product is presented.

1 Portions of this paper (including "Materials and Methods") and all the figures and their legends are presented in miniprint exactly as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Office of Biological Chemical, 9650 Rockville Pike, Bethesda, Md. 20814. Request Document No. 79M232, cite authors, and include a check or money order for $1.00 per set of photocopies.

2 The abbreviations used are: pOHB, 4-hydroxybenzoate; 2-F-pOHB and 3-F-pOHB, 2-fluorophenol and 3-fluorophenol-4-carboxylic acid; 3,5-F2-pOHB, 3,5-difluorophenol-4-carboxylic acid; F2-pOHB, 4-fluorophenol-4-carboxylic acid; PCA, protocatechuate; F2-PCA, 2-fluorophenol; F-PCA, 4-fluorophenol; and F2, 3,5-difluorophenol-4-carboxylic acid.

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* The diaminof 3-F-pOHB exhibits a broad absorption peak.

ND, not determined.
mation of H$_2$O$_2$. It is of importance, therefore, to determine the fate of a potential substrate.

Fig. 1 shows the spectral changes that occur when 3-F-pOHB (A), 3,5-F$_2$-pOHB (B), and F$_4$-pOHB (C) derivatives are hydroxylated by the enzyme in the presence of an NADPH-generating system. The spectral changes with 2-F-pOHB (not shown) were similar in form to those of 3-F-pOHB. It should be noted that the absorption spectra of the final products from 3-F-pOHB and 3,4-F$_2$-pOHB derivatives are identical. Identical products would be expected if both of the compounds were hydroxylated, but only the difluoro analogue undergoes fluoride elimination as illustrated below:

Using gas chromatography-mass spectrometry, the products from 3-fluoro, 2-fluoro, and the tetrafluoro derivatives were identified as 5-fluoro-, 2-fluoro-, and 2,5,6-trifluoroprotocatechuates. The formation of the trifluoroprotocatechu e from tetrafluoro-pOHB demands that fluorine be eliminated during the reaction. In addition to 5-fluoroprotocatechu e, a small amount of protocatechu e was also identified as a hydroxylation product of 3-fluoro-pOHB, suggesting some elimination of fluoride during the reaction.

Fluoride was identified as a product during oxidation of the difluoro and the tetrafluoro derivatives. The amount of fluoride released in these reactions as measured by the fluoride-specific electrode (see "Materials and Methods") was equal to the amount of the fluoro derivative consumed (Table II). A low but finite amount of fluoride was also released with 3-F-pOHB as the substrate, in agreement with the small amount of protocatechu e detected by gas chromatography-mass spectrometry analysis. Fluoride was not released in the reaction mixture with 2-F-pOHB.

The stoichiometry of NADPH consumption with fluoro derivatives as the limiting substrate was established spectrophotometrically by monitoring the decrease in absorbance at 340 nm. The stoichiometry was normal when monofluoro derivatives were used as substrates; equimolar consumption of NADPH occurred. In contrast, however, 2 mol of NADPH were utilized/mol of fluoro analogue with the difluoro and tetrafluoro compounds as the limiting substrates. Since the reactions with the latter two substrates involve removal of fluoride in the reduced state as fluoride, consumption of an extra equivalent of NADPH would be expected (cf. "Discussion").

Studies using the oxygen electrode with NADPH as the limiting substrate showed that in the presence of either 2-F-pOHB or 3-F-pOHB, 1 mol of oxygen was consumed/mole of NADPH supplied. However, with the difluoro and the tetrafluoro compounds, the utilization of NADPH and oxygen was

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<table>
<thead>
<tr>
<th>Substrate added</th>
<th>NADPH/substrate $^a$</th>
<th>NADPH$^+/O_2$</th>
<th>Fluoride/ NADPH$^+$</th>
<th>Fluoride/substrate $^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pOHB</td>
<td>1.0</td>
<td>1.0</td>
<td>0</td>
<td>ND$^-$</td>
</tr>
<tr>
<td>2-F-pOHB</td>
<td>1.0</td>
<td>1.0</td>
<td>0</td>
<td>ND$^-$</td>
</tr>
<tr>
<td>3-F-pOHB</td>
<td>1.0</td>
<td>1.0</td>
<td>0</td>
<td>ND$^-$</td>
</tr>
<tr>
<td>3,5-F$_2$-pOHB</td>
<td>2.0</td>
<td>2.0</td>
<td>0.92$^+$</td>
<td>ND$^+$</td>
</tr>
<tr>
<td>F$_4$-pOHB</td>
<td>2.0</td>
<td>2.0</td>
<td>ND$^+$</td>
<td>0.95$^+$</td>
</tr>
</tbody>
</table>

$^a$ Limiting substrate.

$^b$ Based on NADPH to substrate ratio of 2.0. NADPH was not limiting in this experiment.

$^c$ ND, not determined.

$^d$ An approximate number, because the purity of the substrate was unknown.

in a ratio of 2:1. Oxygen was fully coupled to hydroxylation for all substrates as indicated by the failure to detect H$_2$O$_2$. Some uncoupling (20 to 40%) occurred, however, when the reaction was carried out at 4°C in the presence of high concentrations of F$_4$-pOHB (50 to 100 mM).

The results summarized in Table II show the following stoichiometries for the enzyme-catalyzed hydroxylations of the fluoro compounds:

1. $2$ F$_2$-pOHB (3-F-pOHB) + NADPH + H$^+$ + O$_2$ $\rightarrow$ 2 F$_2$-PCA (5-F-PCA) + NADP$^+$ + H$_2$O

2. 3,5-F$_2$-pOHB (F$_4$-pOHB) + 2 NADPH + H$^+$ + O$_2$ $\rightarrow$ 6 F$_2$-PCA (6-F-PCA) + 2 NADP$^+$ + H$_2$O + F

Steady State Kinetics with Fluorine-substituted Substrates—All the fluoro compounds tested were found to be substrates, being tightly coupled to hydroxylation. In common with most other substrates, they also have an effector role (23) greatly enhancing the rate of enzyme flavin reduction by NADPH.

Initial rate measurements for varied concentrations of pOHB (for comparison) and its fluoro derivatives at a fixed concentration of NADPH gave linear Lineweaver-Burk plots at low concentrations of substrate (Fig. 2). Linear Lineweaver-Burk plots were also obtained when the concentration of NADPH was varied at a fixed concentration of the fluoro compounds (data not shown).

The apparent $K_m$ and $V_{max}$ values for the various substrates are presented in Table III. The monofluorinated substrates have apparent $V_{max}$ and $K_m$ values comparable to those for pOHB, whereas the di- and tetrafluorinated substrates have higher apparent $K_m$ values and considerably lower $V_{max}$ values. Attention is drawn to the remarkably strong excess substrate inhibition exhibited by 2-F$_2$-pOHB (Fig. 2). This inhibition is found at much lower concentrations than with other fluorinated substrates or with substrates studied previously (11, 12, 14, 16).

Since F$_4$-pOHB was chosen for detailed rapid reaction study
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for reasons apparent in later sections, a more detailed steady state analysis with this substrate was performed under conditions of previous stopped flow experiments. Fig. 3 shows the results for varying concentrations of F₄-pOHB at three different fixed concentrations of NADPH. When analyzed by the method of Dalziel (24), the data yield an apparent reaction rate that is about 20-fold higher than that for F₄-pOHB; the Kₘ for pOHB is 5-fold lower.

These constants are the same within experimental error as those determined from data in Fig. 3 by the method described previously (14) were both found to have the same value (0.16 mM). The values given were derived from Lineweaver-Burk plots of substrate-stimulated NADPH consumption versus substrate concentrations (cf. Fig. 2). The initial velocity measurements were made in air-saturated 33 mM Tris/SO₄, 0.5 mM EDTA buffer, pH 8, at 25°C.

Comparison of steady state data for p-hydroxybenzoate hydroxylase with its various fluorinated derivatives as substrates

The values given were derived from Lineweaver-Burk plots of substrate-stimulated NADPH consumption versus substrate concentration (cf. Fig. 2). The initial velocity measurements were made in air-saturated 33 mM Tris/SO₄, 0.5 mM EDTA buffer, pH 8, at 25°C.

Table III

<table>
<thead>
<tr>
<th>Aromatic substrate</th>
<th>Apparent Kₘ</th>
<th>Apparent Vₘₙₐₓ</th>
<th>Excess substrate inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>min⁻¹</td>
<td>mM</td>
</tr>
<tr>
<td>pOHB</td>
<td>11.0</td>
<td>26.0</td>
<td>3000</td>
</tr>
<tr>
<td>3-F-pOHB</td>
<td>43.0</td>
<td>24.0</td>
<td>2100</td>
</tr>
<tr>
<td>2-F-pOHB</td>
<td>3.4</td>
<td>4.5</td>
<td>1050</td>
</tr>
<tr>
<td>F₄-pOHB</td>
<td>200</td>
<td>145.0</td>
<td>84</td>
</tr>
<tr>
<td>3,5-F₂-pOHB</td>
<td>400</td>
<td>100</td>
<td>550</td>
</tr>
</tbody>
</table>

*Apparent Kₘ values for the aromatic substrates were determined in the presence of 0.28 mM NADPH. Apparent Kₘ values for NADPH were determined in the presence of the aromatic substrate at concentrations approximately 10 times that of its apparent Kₘ value, except for 3,5-F₂-pOHB (60 µM).

**ND, not determined.

Table IV

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Conditions</th>
<th>Dissociation constant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>T °C</td>
</tr>
<tr>
<td>pOHB</td>
<td>6.5-7.6</td>
<td>2 and 25</td>
</tr>
<tr>
<td>F₄-pOHB</td>
<td>7.0</td>
<td>25</td>
</tr>
<tr>
<td>3-F-pOHB</td>
<td>8.0</td>
<td>4</td>
</tr>
<tr>
<td>F₄-pOHB</td>
<td>7.0</td>
<td>25</td>
</tr>
</tbody>
</table>

*The values for the dissociation constant for p-hydroxybenzoate have been taken from earlier work (14, 25).
Thus, when enzyme in the presence of thermodynamic stabilization of the radical were observed. When photoreacted, a considerable amount of radical was observed in the spectrum shown by Curve 4, after which no further changes occurred over a very long time period. That Curve 4 represents the level of thermodynamic stabilization of the radical was shown in a separate experiment. Enzyme, in the absence of substrate, was photoreduced to half-reduction (Curve 2) and F4-pOHB (38 mM) was added from a side arm; Curves 3 and 4 show the proportionality of oxidized and reduced enzyme to the radical state, with Curve 4 representing the final equilibrium position.

Kinetic Analysis of the Reduction Half-Reaction — The substrate-stimulated reduction of the enzyme by NADPH has been studied using F4-pOHB as a function of NADPH concentration (Fig. 7). At pH 6.5, 4°C, the extrapolated rate constant for reduction at infinite NADPH concentration is 0.43 s-1, about one-tenth of that obtained previously with pOHB (14). The dissociation constant of NADPH from the enzyme-F4-pOHB complex was calculated from the slope and intercept to be 0.43 mM, compared to a value of 0.18 mM in the presence of pOHB (14). Little or no absorbance change was detected in the long wavelength region (>550 nm) upon reduction of the enzyme in the presence of F4-pOHB. This is in contrast to the reaction in the presence of pOHB and other related compounds which involve formation and decay of long wavelength absorbance due to charge transfer intermediates (12, 14, 16).

Reduction of the enzyme in the presence of 2-F-pOHB is considerably faster. At pH 6.8 and 4°C and in the presence of 50 μM substrate, the extrapolated rate constant at infinite NADPH concentration is approximately 25 s-1. The value for the rate constant is independent of NADPH concentration in the range 48 μM to 1.0 mM, suggesting a tight binding of NADPH in the presence of the 2-fluoro substrate. The reduction was also studied at a higher concentration of 2-F-pOHB (7.5 x 10-4 M), which is highly inhibitory in the catalytic reaction (cf. Fig. 2). No effect on the rate of reduction was observed, suggesting that the enzyme is inhibited due to an effect in the oxygen half-reaction. Long wavelength absorbance was observed upon reduction of the enzyme. The rate of decay of this charge transfer species as measured by subtracting the final spectrum from the first recorded spectrum shows a peak at 420 nm with an observed extinction coefficient of -1500 M-1 cm-1 (Fig. 8).

Further evidence for quinone as the primary product was obtained from turnover experiments in which reduced cytochrome c was used as a trapping agent. These experiments were based on the measured reactivity of o-chloranil, (tetra-chloro-o-quinone), which was shown to react rapidly with NADPH or reduced cytochrome c. The difference spectrum obtained by subtracting the final spectrum from the first recorded spectrum shows a peak at 420 nm with an observed extinction coefficient of ~1500 M-1 cm-1 (Fig. 8).

As much as 50% of the oxidation of reduced cytochrome c that is theoretically possible was observed, strongly suggesting that a quinone is the primary product of F4-pOHB oxidation. This value is lower than theoretical, presumably for two reasons. First, as shown in Scheme 1, the reduced cytochrome c would compete with NADPH for the quinone. Second, it could be low due to reduction of oxidized cytochrome c by the reduced enzyme formed during turnover. In separate static experiments, the reduced enzyme was shown to rapidly reduce cytochrome c.
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No oxidation of reduced cytochrome c was observed in the absence of F₄-pOHB or when p-hydroxybenzoate was substituted for the tetrafluoro derivative.

Reaction of Reduced Enzyme Plus F₄-pOHB with Oxygen.—Reaction of the reduced enzyme with oxygen can be followed independently of the reduction of enzyme by NADPH (28). Unlike most known substrates (11), F₄-pOHB was found to bind poorly to the reduced enzyme, compared to the oxidized form. It bound so poorly that secondary binding processes prevented a direct measurement of the association. An estimate of the $K_d$ of 10 to 20 mM was obtained from spectral perturbation studies. The only other substrate known to exhibit a large differential in binding affinity between oxidized and reduced enzyme is p-mercaptobenzoate (12). These two compounds exist principally as dianions in neutral solution. This suggests that the reduced enzyme binds only the monoanion of substrates, the p-hydroxyl group remaining uncharged, as has been proposed before (11).

Substrate concentrations high enough to approach saturation of the reduced enzyme resulted in interfering absorbance and greatly reduced reaction rates with oxygen, presumably by formation of dead-end or secondary complexes. A compromise was therefore necessary to follow the reaction with oxygen so a concentration of 40 mM F₄-pOHB was chosen.

Oxygen reactions were investigated at pH 6.6 and 2°C (the same conditions most frequently employed previously to follow transient states in the reaction of this enzyme). The reaction is most clearly illustrated by the absorbance changes at 400 and 470 nm (Fig. 9). In the first 400 ms, two separate reactions were clear. At 400 nm, a rapid increase merged with a slower increase. At 470 nm, only the slower increase was found, preceded by a lag. Both of these reactions were found to be dependent on oxygen concentration. Two slower consecutive reactions followed. One occurred in a 4- to 8-s time frame (Fig. 9), followed by a final, very slow reaction, resulting in an oxidized flavin spectrum some minutes after the start of the reaction. This last reaction involved an increase in absorbance at 470 nm and a decrease at 400 nm (Fig. 9). The last two reactions were completely independent of oxygen concentration, and analysis of rates showed no change with wavelength.

The observed rate constants of the first two reactions at different oxygen concentrations were obtained by a computer-assisted curve-stripping procedure. Only three oxygen concentrations (a 5-fold change) were studied since, when oxygen was too low, the reaction phases could not be resolved. The rate measured for the first absorbance increase at 470 nm varied in direct proportion to oxygen concentration, indicating a second order reaction with a rate constant of $2.1 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ at 2°C. This is in excellent agreement with the rate of oxidation of enzyme without substrate under these conditions (11). Thus, it is clear that the reaction of enzyme in complex with the substrate is a sequence of three reactions with the fourth being due to reaction of free reduced enzyme. The result is analogous to the sequence detected for the natural substrate, p-hydroxybenzoate. The rate constant of the O₂-dependent reaction was the most difficult to measure, but estimates were in the range of $8 \times 10^5$ to $1.4 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$, approximately 3-fold slower than the comparable reactions in the presence of other substrates (11), but still about 4-fold faster than that of the free reduced enzyme. The observed reactions are summarized in the sequence below.

$$
	ext{ES} + \text{O}_2 \rightarrow \text{I} \rightarrow \text{II} \rightarrow \text{II} \rightarrow \text{III} \rightarrow \text{Eox} + \text{H}_2\text{O}_2
$$

The Roman numerals represent transient states detected in the reaction and follow nomenclature used before with this enzyme (11). It should be noted that the conversion of I to III is the same rate as the overall rate of catalysis. The slow observed rate of conversion of III to oxidized enzyme is

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**Scheme 1**

No oxidation of reduced cytochrome c was observed in the absence of F₄-pOHB or when p-hydroxybenzoate was substituted for the tetrafluoro derivative.

**Reaction of Reduced Enzyme Plus F₄-pOHB with Oxygen.**—Reaction of the reduced enzyme with oxygen can be followed independently of the reduction of enzyme by NADPH (28). Unlike most known substrates (11), F₄-pOHB was found to bind poorly to the reduced enzyme, compared to the oxidized form. It bound so poorly that secondary binding processes prevented a direct measurement of the association. An estimate of the $K_d$ of 10 to 20 mM was obtained from spectral perturbation studies. The only other substrate known to exhibit a large differential in binding affinity between oxidized and reduced enzyme is p-mercaptobenzoate (12). These two compounds exist principally as dianions in neutral solution. This suggests that the reduced enzyme binds only the monoanion of substrates, the p-hydroxyl group remaining uncharged, as has been proposed before (11).

Substrate concentrations high enough to approach saturation of the reduced enzyme resulted in interfering absorbance and greatly reduced reaction rates with oxygen, presumably by formation of dead-end or secondary complexes. A compromise was therefore necessary to follow the reaction with oxygen so a concentration of 40 mM F₄-pOHB was chosen.

Oxygen reactions were investigated at pH 6.6 and 2°C (the same conditions most frequently employed previously to follow transient states in the reaction of this enzyme). The reaction is most clearly illustrated by the absorbance changes at 400 and 470 nm (Fig. 9). In the first 400 ms, two separate reactions were clear. At 400 nm, a rapid increase merged with a slower increase. At 470 nm, only the slower increase was found, preceded by a lag. Both of these reactions were found to be dependent on oxygen concentration. Two slower consecutive reactions followed. One occurred in a 4- to 8-s time frame (Fig. 9), followed by a final, very slow reaction, resulting in an oxidized flavin spectrum some minutes after the start of the reaction. This last reaction involved an increase in absorbance at 470 nm and a decrease at 400 nm (Fig. 9). The last two reactions were completely independent of oxygen concentration, and analysis of rates showed no change with wavelength.

The observed rate constants of the first two reactions at different oxygen concentrations were obtained by a computer-assisted curve-stripping procedure. Only three oxygen concentrations (a 5-fold change) were studied since, when oxygen was too low, the reaction phases could not be resolved. The rate measured for the first absorbance increase at 470 nm varied in direct proportion to oxygen concentration, indicating a second order reaction with a rate constant of $2.1 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ at 2°C. This is in excellent agreement with the rate of oxidation of enzyme without substrate under these conditions (11). Thus, it is clear that the reaction of enzyme in complex with the substrate is a sequence of three reactions with the fourth being due to reaction of free reduced enzyme. The result is analogous to the sequence detected for the natural substrate, p-hydroxybenzoate. The rate constant of the O₂-dependent reaction was the most difficult to measure, but estimates were in the range of $8 \times 10^5$ to $1.4 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$, approximately 3-fold slower than the comparable reactions in the presence of other substrates (11), but still about 4-fold faster than that of the free reduced enzyme. The observed reactions are summarized in the sequence below.

$$
	ext{ES} + \text{O}_2 \rightarrow \text{I} \rightarrow \text{II} \rightarrow \text{II} \rightarrow \text{III} \rightarrow \text{Eox} + \text{H}_2\text{O}_2
$$

The Roman numerals represent transient states detected in the reaction and follow nomenclature used before with this enzyme (11). It should be noted that the conversion of I to III is the same rate as the overall rate of catalysis. The slow observed rate of conversion of III to oxidized enzyme is

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**Scheme 1**

The observed rate constants of the first two reactions at different oxygen concentrations were obtained by a computer-assisted curve-stripping procedure. Only three oxygen concentrations (a 5-fold change) were studied since, when oxygen was too low, the reaction phases could not be resolved. The rate measured for the first absorbance increase at 470 nm varied in direct proportion to oxygen concentration, indicating a second order reaction with a rate constant of $2.1 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ at 2°C. This is in excellent agreement with the rate of oxidation of enzyme without substrate under these conditions (11). Thus, it is clear that the reaction of enzyme in complex with the substrate is a sequence of three reactions with the fourth being due to reaction of free reduced enzyme. The result is analogous to the sequence detected for the natural substrate, p-hydroxybenzoate. The rate constant of the O₂-dependent reaction was the most difficult to measure, but estimates were in the range of $8 \times 10^5$ to $1.4 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$, approximately 3-fold slower than the comparable reactions in the presence of other substrates (11), but still about 4-fold faster than that of the free reduced enzyme. The observed reactions are summarized in the sequence below.

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The Roman numerals represent transient states detected in the reaction and follow nomenclature used before with this enzyme (11). It should be noted that the conversion of I to III is the same rate as the overall rate of catalysis. The slow observed rate of conversion of III to oxidized enzyme is
presumably due to complex formation of III with excess substrate, a phenomenon observed before with high concentrations of this adduct with the enzyme melilotate hydroxylase (11). However, a similar spectrum was obtained directly at 7.5 min (Fig. 11). This spectrum is completely analogous to the spectrum of only oxidized flavin and the transient form of flavin occurring at this stage in the reaction. When a correction was made for oxidized enzyme, the spectrum of Intermediate III was obtained (Fig. 11). This spectrum is completely analogous to the species named Intermediate III in earlier studies with this enzyme, and thus almost certainly represents the presence of C(4a)-hydroxyflavin.

DISCUSSION

The results presented in this study show that a number of fluorine-substituted p-hydroxybenzoates can serve as substrates in the reaction catalyzed by p-hydroxybenzoate hydroxylase (12, 14, 16) and furnish further examples of mono-oxygenase-catalyzed cleavage of carbon-halogen bonds of aryl fluorides. In analogy to other substrates previously studied, all the fluoro derivatives enhance enormously the enzyme-catalyzed oxidation of NADPH by molecular oxygen, undergoing hydroxylation during the reaction to yield the corresponding dihydroxy products. Oxygen was fully coupled to hydroxylation as indicated by the absence of any peroxide. In substrates that are hydroxylated by p-hydroxybenzoate hydroxylase, the second hydroxyl group originating from molecular oxygen is introduced at positions ortho to carbon 4 (positions 3 and 5). The fluorine-substituted substrates tested reacted in agreement with this rule. The only known exception is p-mercaptopbenzoate, which undergoes oxygen attack at the thiol substituent, subsequently resulting in a disulfide as an unusual product (12).

Fluorine substitution in the p-hydroxybenzoate molecule has a marked influence on the phenolic pK as well as the pK values of the parent compound, as expected for electron-withdrawing substituents. The decreased carboxyl and phenolic pK values and effectors, the fluoro substrates stabilize a blue, neutral semiquinonoid flavin species on photochemical reduction of the enzyme. Whereas substitution of fluorine at carbon 2 and carbon 3 results only in kinetic stabilization, the semiquinone flavin species on photochemical reduction of the enzyme. Whereas substitution of fluorine at carbon 2 and carbon 3 results only in kinetic stabilization, the semiquinone flavin species on photochemical reduction of the enzyme. Whereas substitution of fluorine at carbon 2 and carbon 3 results only in kinetic stabilization, the semiquinone flavin species on photochemical reduction of the enzyme. Whereas substitution of fluorine at carbon 2 and carbon 3 results only in kinetic stabilization, the semiquinone
produced in the presence of \( F_{p}OHB \) is also thermodynamically stabilized.

Hydroxylation of the 3,5-difluoro and 2,3,5,6-tetrafluoro substrates catalyzed by the enzyme resulted in elimination of fluoride. The reaction with the difluoro or the tetrafluoro substrate shows an unusual NADPH stoichiometry. Two moles of NADPH are utilized/mol of the fluoro derivatives hydroxylated, resulting in an NADPH to oxygen ratio of 2:1. This is in contrast to the physiological substrate as well as to other substrates studied, which give an NADPH to oxygen ratio of 1:1.

\( o \)-Benzoquinones are strong oxidizing agents which are unstable in dilute aqueous solution (31). The high reactivity of NADPH with \( o \)-chloranil (a model compound for the \( o \)-quione produced by the enzyme) clearly showed that such a nonenzymatic reaction could account for the overall stoichiometry observed when fluoride was eliminated in the enzyme reaction. Thus, only indirect evidence could be used to illustrate the production of \( o \)-quinone. This paper presents spectral evidence for a unique, unstable product (Figs. 8 and 10B) with a spectrum typical of \( o \)-quinones (absorbance maximum about 400 nm and extinction of 1 to 2000 \( M^{-1} \) \( cm^{-1} \)), which reacts with the trapping agent, reduced cytochrome \( c \), in competition with NADPH. Hence, the data strongly suggest that the enzyme catalyzes the formation of an \( o \)-quinone from substrate, which is then reduced by an additional molecule of NADPH in a nonenzymatic reaction (Scheme 2).

The higher consumption of NADPH during the dehalogenation reactions catalyzed by phenylalanine hydroxylase (5) and salicylate hydroxylase (9) strongly suggests that a similar mechanism operates with these enzymes. The formation of quinone is also likely to be the primary result of hydroxylation of \( o \)-nitrophenol, with the elimination of nitrite, by salicylate hydroxylase (9). The same conclusion is likely when glyoxylate is eliminated upon hydroxylation of 4-hydroxyphenoxyacetate by 4-hydroxyphenylacetic acid 1-hydroxylase (32).

Rapid reaction studies of the reduced enzyme with oxygen in the presence of \( F_{p}OHB \) demonstrate a sequence with two transient species as seen previously with \( p \)-hydroxybenzoate as substrate (11). The initial reaction of the reduced enzyme-\( F_{p}OHB \) complex with oxygen to give Intermediate I proceeds at a rate only slightly lower than that for other substrates (11). However, the decay of the \( C(4a) \)-peroxy intermediate (I) to the \( C(4a) \)-hydroxyflavin (III) involving transfer of oxygen to the substrate is about 100 times slower with \( F_{p}OHB \) than with \( p \)-hydroxybenzoate so that this now becomes the rate-limiting step in catalysis. In contrast to the deactivating effect observed with \( F_{p}OHB \), the process of oxygen transfer is greatly accelerated when \( p \)-mercaptobenzoate is the substrate, as expected with the highly reactive aromatic mercaptan (12).

Elimination of fluorine as fluoride from the 3,5-difluoro and the tetrafluoro substrates upon oxygen transfer is consistent with the oxenoid mechanism (electrophilic attack of oxygen) previously proposed for the enzyme (11), as illustrated below for \( F_{p}OHB \) as substrate (Scheme 3).

The results presented in this study clearly rule out a recent
propose by Kemal and Bruce (33) which involves peroxidation of the substrate from the flavin peroxide as an early step in the mechanism of flavoprotein hydroxylases. Although their proposal involves the formation of a quinonoid intermediate, if applied to the case of F₄-pOHB, it would require the fluorine atom to be eliminated as F⁻, rather than F⁻ as observed. Further evidence in favor of an electrophilic attack of oxygen is provided by the reaction with p-mercaptobenzoate reported earlier. With this substrate, the electron-rich, highly reactive sulfur atom is attacked rather than the benzene ring (12).

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REFERENCES