Mechanism of Action of $p$-Hydroxybenzoate Hydroxylase from Pseudomonas putida

III. THE ENZYME-SUBSTRATE COMPLEX*

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SUMMARY

The mechanism of action of $p$-hydroxybenzoate hydroxylase from Pseudomonas putida, strain M-6, has been investigated. The aromatic substrate analogues, benzoate, $p$-fluorobenzoate, $p$-chlorobenzoate, $p$-nitrobenzoate, $p$-aminobenzoate, and 6-hydroxynicotinate, are found to be competitive inhibitors. This finding differs from the previously reported noncompetitive behavior in a different buffer system.

The optical activity of the enzyme-inhibitor complex has been studied. From the kinetic and circular dichroism (CD) measurements, we have found that the carboxyl moiety is necessary and sufficient for the enzyme-substrate binding, whereas the hydroxyl group alone will not lead to binding. There are two classes of inhibitory analogues: one causes changes in CD spectra of the enzyme similar to those evoked by the substrate, and the other does not cause significant changes. The results indicate that more than one mode of enzyme-inhibitor interaction is involved. The CD of the enzyme-NADPH complex under anaerobic conditions suggests that the oxidized enzyme and reduced pyridine nucleotide form a complex, both in the absence and presence of the substrate, $p$-hydroxybenzoate. Furthermore, evidence for a ternary complex is given.

Hydroxylation plays an important role in the oxidative metabolism of aromatic compounds by bacteria. As a consequence, the mechanisms of action of those enzymes that mediate these hydroxylation reactions have stimulated profound interest. Two mono-oxygenases, $p$-hydroxybenzoate hydroxylase and salicylate hydroxylase, have been purified to homogeneity and investigated actively (1-4). Both mono-oxygenases are inducible. They are flavoproteins requiring FAD and reduced pyridine nucleotides as cofactors; however, they differ in specificity. Salicylate hydroxylase is specific for NADH, whereas $p$-hydroxybenzoate hydroxylase is specific for NADPH (2). The precise role of the flavin prosthetic group and the mechanism of action of the reduced pyridine nucleotide are not clear. Recently, investigation into the nature of the enzyme-substrate ternary complexes of salicylate hydroxylase by fluorometric method has been reported by Takemori et al. (5) and Suzuki, Takemori, and Katalogi (6). Higashi et al. (7) and Nakamura et al. (8) have reported kinetic studies on the reaction mechanism of $p$-hydroxybenzoate hydroxylase from Pseudomonas desmolytica.

To elucidate the mechanism of enzymatic catalysis it is pertinent to study the intermediate enzyme-substrate complexes and also the interactions of various analogues. Hesp, Calvin, and Hosokawa (2) reported that the circular dichroism spectrum of $p$-hydroxybenzoate hydroxylase was perturbed significantly upon addition of the substrate, $p$-hydroxybenzoate. CD measurements thus provide a sensitive method of studying the enzyme-substrate complexes. This report presents the results of a kinetic study which indicates the competitive inhibitory behavior of some of the substituted benzoates, and the CD which suggests the interaction of the substrate and the bound FAD.

$p$-Hydroxybenzoate hydroxylase was induced in Pseudomonas putida. The two subspecies, $P.$ putida and $P.$ desmolytica, have functionally the same $p$-hydroxybenzoate hydroxylases; however, they differ in the subsequent oxidation of protocatechuate. The former uses the ortho cleavage pathway, yielding $\beta$-carboxy-cis-cis-muconate (9); the latter uses meta cleavage, yielding $\alpha$-hydroxy-$\gamma$-carboxy-cis-cis-muconic semialdehyde (10).

EXPERIMENTAL PROCEDURE

Materials—Reagents used routinely for enzyme preparation and assays were the purest grade obtainable and purchased from the University of Texas, Dallas, and the Sigma Chemical Company, St. Louis, Missouri. The abbreviations used are CD, circular dichroism.
sources described previously (1). NADPH (grade II), NADH (Sigma grade), NADP+ (grade III), NAD+ (grade IV), FAD (grade III), p-fluorobenzoate, and p-aminobenzoate were purchased from Sigma. 6-Hydroxynicotinic acid was obtained from Aldrich. Phenol was from Mallinckrodt Chemical, and p-chlorobenzoate and p-nitrobenzoate were from Eastman Kodak. All the benzoate derivatives and phenol were twice crystallized from water before use. All other reagents were used directly without further purification.

**Enzyme Preparation**—p-Hydroxybenzoate hydroxylase from *P. putida*, strain M-6 (ATCC 17428), was prepared and purified by a modification of the method described in Paper I of this series. The method is similar to that reported in Paper II. The purified enzyme was stored in a stabilizing mixture at −70°C until use, as described previously (2). The enzyme was found to denature slowly over a period of 1 year. The preservation of the enzyme was improved by storing it under an oxygen-free helium atmosphere.

**Enzyme Assays**—p-Hydroxybenzoate hydroxylase was assayed by spectrophotometric measurement of the substrate-dependent oxidation of NADPH. The procedure has been described earlier (1). The standard assay system is slightly modified from the previous method. The standard system contained 3.0 ml, 67 mmoles of K2HPO4-KH2PO4 (pH 7.0), 3.3 mmoles of FAD, 0.13 mmole of NADPH, 0.67 mmole of p-hydroxybenzoate, enzyme, and deionized water. The unit of enzyme activity is defined as that amount of enzyme which oxidizes 1.0 μmole of NADPH per min under the conditions of the spectrophotometric assay.

**Kinetic Measurements**—The kinetics were studied spectrophotometrically by measuring the substrate-dependent oxidation of NADPH at 340 nm. Various substrate analogues were incubated for 2 min (or longer) before the measurement. All substrate analogues were adjusted to pH 7.5. All experiments were carried out at 20°C ± 1°C. Each experiment was repeated at least twice until consistent results were obtained.

**Spectroscopic Methods**—The absorption spectra were measured in a Cary model 15 spectrometer. The circular dichroism measurements were obtained with a Cary model 60 spectropolarimeter with a standard model 6001 CD accessory attachment. A Cary thermostatable cell compartment was used. CD spectra of the enzyme and the p-fluorobenzoate and NADPH complexes with the enzyme were studied at both 20°C and 2°C. Since spectra at the two temperatures were identical, subsequent inhibitor studies were carried out at 2°C ± 0.1°C where the enzyme is more stable. The slit width program was set for 15 A resolution and the time constant and scan speed were adjusted for best signal to noise ratio for each individual experiment. In all cases, an optical path length of 1.0 cm was used.

**Anaerobic Experiment**—We found it difficult to achieve anaerobic conditions efficiently without denaturing the enzyme in a cuvette suitable for optical activity measurement, while still permitting the addition of various reagents. We have used the method described previously (2). The space above the enzyme solution in the cuvette is first flushed with oxygen-free helium for 30 min. The enzyme solution is then bubbled with O2-free helium at a rate of 1.5 cc per min for 15 min. If it is noticed that the enzyme is undergoing denaturation during the assay procedure, the result is discarded. We have been able to achieve strict anaerobic conditions with a negligible amount of denaturation.

**RESULTS**

**Chemical Structure of Substrate Analogues**—Several aromatic compounds which are structurally related to *p*-hydroxybenzoate but cannot serve as a substrate, inhibit the enzyme. The benzoic acid derivatives vary in strength according to the nature of the substituent and its ring position. The relative strength of a substituted benzoic acid is characterized by a substituent constant σ. The more electron-attracting a substituent is, the more positive its σ value (relative to benzoate as zero). Conversely, the more strongly a substituent donates electrons, the more negative is its σ value. These values for the inhibitory benzoates are given in Table I.

| Substrate analogues   | σ values were based on ionization of substituted benzoic acids (II). |
|-----------------------|---------------------------------------------------------------|
| p-Nitrobenzoate       | 0.778 ± 0.02                                                   |
| 6-Hydroxynicotinate   | 0.227 ± 0.02                                                   |
| p-Chlorobenzoate      | 0.062 ± 0.02                                                   |
| Benzoate              | 0.37 ± 0.04                                                   |
| p-Aminobenzoate       | 0.66 ± 0.1                                                     |
| p-Hydroxybenzoate     | 0.012 ± 0.02                                                   |

*Inhibition* | *K*<sub>i</sub>*
---|---
| 16 | (4.2 ± 1.3) × 10<sup>-4</sup> |
| 17 | (3.1 ± 0.4) × 10<sup>-4</sup> |
| 12 | (1.8 ± 0.3) × 10<sup>-4</sup> |
| 35 | (7.6 ± 1.9) × 10<sup>-5</sup> |
| 20 | (9.9 ± 0.6) × 10<sup>-3</sup> |
| 81 | (4.2 ± 0.9) × 10<sup>-6</sup> |

**K*<sub>i</sub>* values were calculated, assuming a completely competitive inhibition, by a linear regression analysis of the equation

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[S] - [S] = \frac{K_m}{V_{max}} + \frac{1}{K_i}\]

where \( S \) and \( I \) denote the substrate, *p*-hydroxybenzoate, and the inhibitory substrate analogue, respectively, \( v \) and \( V_{max} \) are the reaction velocity and maximal reaction velocity, \( K_m \) is the Michaelis-Menten constant, and \( K_i \) is the inhibitor dissociation constant. In our kinetic measurement, the velocity determinations probably are reasonably homogeneous in variance. If the variance of \( v \) is \( σ_v^2 \), the variance of \( [S]/v \) can be shown to be \( σ_v^2/[S]/v^2 \) (12). Therefore, in fitting the linear form to the above equation, the proper weight \( σ_v^2/[S]/v^2 \) is used. The standard error of \( K_i \) is estimated by standard procedure of propagation of error. All computations were done on a CDC 6600 computer.
Kinetic Study—It was reported previously (1) that benzoate and a series of its analogues were inhibitory to the activity of p-hydroxybenzoate hydroxylase. With Tris-HCl buffer system for assay, the inhibition was found to be noncompetitive. However, enzyme activity drops very quickly during the assay in Tris buffer system (pH 8.0). Accordingly, there was inaccuracy in determining the initial velocity of the reaction. We could overcome this problem by replacing Tris-HCl buffer with a phosphate buffer system (pH 7.5) in which oxidation of NADPH proceeded almost linearly even at very low concentrations of NADPH and enzyme.

By employing the improved assay system, we have found that benzoate, p-fluorobenzoate, p-chlorobenzoate, p-nitrobenzoate, p-aminobenzoate, and 6-hydroxynicotinate are all competitive inhibitors. Fig. 1 shows the Lineweaver-Burk plots of the enzyme activity in the presence of these inhibitors. All of them are typical competitive inhibitions. The $K_i$ values were determined by the method described by Wilkinson (12) and Cleland (14). The results are given Table I.

A Hammett plot of the action of the substituted benzoate derivatives is given in Fig. 2, in which the logarithms of the inhibition constants ($K_i$) are plotted against their $\sigma$ values. They fit the Hammett equation very well. The "reaction constant," $\rho$, which is the slope of the Hammett plot, is estimated to be 1.78. The significance of $\rho$ is that it measures the sensitivity of the reaction to the electrical effects of substituents in the meta and para positions. A positive $\rho$ value suggests that the inhibition is favored by increasing the nucleophilic reactivity of the carboxyl group.

Phenol shows no inhibition at concentrations below 0.01 m.

![Fig. 1. Effect of benzoate analogues on the activity of p-hydroxybenzoate hydroxylase: double reciprocal plot. The data are plotted by the method of Lineweaver and Burk (13). Assay conditions were described under "Experimental Procedure." The reciprocal velocity, $1/v$, is in arbitrary units. The molar concentrations of benzoate are indicated on the plot.](http://www.jbc.org/Downloaded.html)
Inhibitory effects occur at concentrations greater than 0.01 M, due presumably to nonspecific binding to the enzyme. The results are given in Table II.

The above findings suggest strongly that the carboxyl group is necessary for the substrate analogues to inhibit the enzyme and possibly is involved in the binding at the active site.

**Circular Dichroism Spectra of Enzyme-Inhibitor Complexes**

The CD spectrum of the holoenzyme of $p$-hydroxybenzoate hydroxylase has been reported previously (2). The effect of the substrate, $p$-hydroxybenzoate, on the holoenzyme manifests itself in the visible region of the FAD absorption. The perturbation of the holoenzyme CD spectrum upon addition of $p$-hydroxybenzoate was given in Paper II. The CD spectra of the enzyme-bound FAD holoenzyme in the absence and presence of the inhibitors and substrate, $p$-hydroxybenzoate, are shown in Figs. 3 and 4. The effect of benzamide on the CD spectrum of $p$-hydroxybenzoate hydroxylase was described earlier (2). A large change was observed in the CD spectrum of $p$-hydroxybenzoate hydroxylase upon addition of benzamide.

**$p$-Fluorobenzoate**—The CD spectrum of the enzyme was changed by the addition of $10^{-3}$ M $p$-fluorobenzoate. The 367 nm CD was slightly shifted and decreased. The 455 nm band also decreased. Addition of $10^{-3}$ M $p$-hydroxybenzoate to the enzyme $p$-fluorobenzoate system produced the familiar change of the CD spectrum characteristic of the enzyme $p$-hydroxybenzoate complex.

**6-Hydroxynicotinate**—The effect on the CD spectrum of the enzyme caused by addition of $10^{-5}$ M 6-hydroxynicotinate was significant. Both the 367 nm and 455 nm CD bands decreased markedly. When $10^{-2}$ M $p$-hydroxybenzoate was added to the system, enhancement of the 455 nm band was observed. The general appearance of the CD spectrum resembled that of the enzyme $p$-hydroxybenzoate complex.

**$p$-Aminobenzoate**—In contrast to the effect produced by $p$-fluorobenzoate, benzamide, and 6-hydroxynicotinate, the addition of $10^{-3}$ M $p$-aminobenzoate caused almost no modification of the CD spectrum of the enzyme except a slight decrease of the shoulder at 340 nm. Even after the addition of $10^{-3}$ M $p$-hydroxybenzoate, no further change was observed. This experiment was repeated with the order of addition of $p$-aminobenzoate and $p$-hydroxybenzoate reversed. The same final CD spectrum was obtained. Since $p$-aminobenzoate did inhibit the enzyme, this may suggest that the interaction of $p$-aminobenzoate with the enzyme is such that it does not manifest itself in a CD modification. This result indicates the possibility of a different mode of binding between the enzyme and $p$-aminobenzoate from that between the enzyme and $p$-fluorobenzoate.

**$p$-Chlorobenzoate and $p$-Nitrobenzoate**—These substrate analogues have been shown to be the weakest inhibitors (Table I). The CD spectra of the substrate analogue-enzyme mixtures are not significantly modified.

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**Table II**

| Phenol concentration | Inhibition |
|----------------------|------------|
| $10^{-1}$            | 78         |
| $5 \times 10^{-2}$   | 51.5       |
| $10^{-3}$            | 0          |
| $8.3 \times 10^{-4}$ | 0          |
| $0$                  | 0          |

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**Fig. 2.** Action of the substituted benzoate derivatives. $K_i$ represents the inhibition constants evaluated in Table II. The standard errors in log $K_i$ are indicated by the vertical error bars. The Hammett substituent constants ($c$) are summarized in Table I. The slope of the line is 1.78.

**Fig. 3.** CD spectrum of $p$-hydroxybenzoate hydroxylase in the presence of 6-hydroxynicotinate and $p$-hydroxybenzoate. The enzyme ($6.7 \times 10^{-5}$ M) is in 0.05 M potassium phosphate buffer, pH 7.5; temperature 20°; path length, 1.0 cm.

**Fig. 4.** CD spectrum of $p$-hydroxybenzoate hydroxylase in the presence of $p$-fluorobenzoate and $p$-hydroxybenzoate. The enzyme ($6.7 \times 10^{-5}$ M) is in 0.05 M potassium phosphate buffer, pH 7.5; temperature 20°; path length, 1.0 cm.
Hydroxybenzoate hydroxylase or, at least, it does not manifest itself in a CD modification. 

There is probably no interaction between NADP and p-hydroxybenzoate under aerobic conditions. These facts indicate that the enzyme-substrate complex, we extended CD measurements to the study of the interaction of NADPH with the enzyme.

Evidence for Direct Binding of NADPH to p-Hydroxybenzoate Hydroxylase—Since CD spectra of p-hydroxybenzoate hydroxylase served as a sensitive means of detecting the formation of the enzyme-substrate complex, we extended CD measurements to the study of the interaction of NADPH with the enzyme.

Under anaerobic conditions, which were produced by flushing the system with helium, the effect of NADPH on the CD spectra of free p-hydroxybenzoate hydroxylase was examined. Upon addition of NADPH, there were marked changes (as shown in Fig. 5) in the CD spectra of the enzyme. In the presence of 10^{-4} M NADPH, both the negative and positive CD bands of the free enzyme (maxima at 455 nm and 367 nm, respectively) are blue-shifted. In addition, a negative contribution to the optical rotation is observed. When p-hydroxybenzoate is added in addition to NADPH under anaerobic conditions, the broad negative band of the CD spectrum of the enzyme-NADPH mixture becomes even more negative. The results suggest that p-hydroxybenzoate hydroxylase forms a complex with NADPH both in the absence and presence of p-hydroxybenzoate.

In order to find whether NADPH or its oxidation product, NADP, is responsible for this binding, the effect of NADP on the CD spectra of the enzyme was examined. Under anaerobic conditions, there are no significant changes in the CD spectra of the enzyme upon addition of NADP. NADP does not affect the familiar substrate-induced changes in CD spectra of the enzyme under aerobic conditions. These facts indicate that there is probably no interaction between NADP and p-hydroxybenzoate hydroxylase or, at least, it does not manifest itself in a CD modification.

NADH does not induce significant changes in CD spectra of free enzyme solution under anaerobic conditions. Therefore, the enzyme-NADPH complex is highly specific, which is consistent with previous reports (1, 2).

From the results described above, we cannot determine the order of the binding of the substrates, NADPH and p-hydroxybenzoate, to the holoenzyme in the ternary enzyme-p-hydroxybenzoate-NADPH complex. We leave it for future study.

**DISCUSSION**

As has been reported, discrepancies were found among the modes of inhibition of p-hydroxybenzoate hydroxylase by a number of substrate analogues. The analogues, such as p-fluorobenzoate, p-aminobenzoate, benzoate, and others, inhibited p-hydroxybenzoate hydroxylase from *P. putida* A3.12 in a noncompetitive manner (1), whereas these analogues were competitive inhibitors to the p-hydroxybenzoate hydroxylase from *P. putida* M-6 (this report) and *P. desmolytica* (7). These discrepancies may be ascribed to the different buffer systems used. In the Tris-HCl buffer, pH optimum is 8.0 as compared to 7.5 (1) and 7.0 (2) found in the potassium phosphate buffer system, and furthermore, the enzyme activity is found to be inhibited, increasingly and markedly, during the assay due to the presence of chloride ion. This inhibitory action of chloride ion made the calculation of enzyme activity inaccurate and resulted earlier in an erroneous interpretation of the mode of inhibition.

The competitive behaviors of the benzoate derivatives indicate strongly that the carboxyl group is essential in the binding. Phenol does not inhibit the enzyme up to a very high concentration at which nonspecific binding may take place. The above findings, in conjunction with the fact that the enzyme will only catalyze p-hydroxybenzoate and will not catalyze the other analogues to any significant extent (1), lead to the following hypotheses: the carboxyl moiety participates in binding with the primary site, providing an “anchoring” and, subsequently, the hydroxyl group is bound to a secondary site to facilitate the catalytic activity. This is in agreement with the two binding site model proposed previously (2, 7, 8). The positive “reaction constant” (\(\rho = 1.78\)) obtained from the Hammett plot (Fig. 2) suggests that the primary binding site is favored by a nucleophilic attack.

Examination of the enzyme-inhibitor complex by CD spectroscopy showed that there are two classes of substrate analogues which competitively inhibit enzyme activity. One class of substrate analogues causes changes in CD spectra, and the

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**Table III**

Effect of reduced and oxidized pyridine nucleotides on CD spectra of p-hydroxybenzoate hydroxylase

| Condition | p-Hydroxybenzoate | NADPH | NADP | NADH |
|-----------|-------------------|-------|------|------|
| Aerobic   | Absent            | No changes, but the conclusion cannot be deduced, because NADPH is oxidized in this system. | Marked changes. | No effect. |
| Anaerobic | Present           | NADPH is oxidized quickly. | Further changes, more than above. | Familiar changes. |
|           | Absent            | No significant changes. | No significant changes. |

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*Fig. 5* The effect of NADPH on the CD spectrum of p-hydroxybenzoate hydroxylase. The enzyme is in 0.05 M potassium phosphate buffer, pH 7.5; temperature 28°C; path length, 1.0 cm; anaerobic conditions. ---, enzyme alone (6.9 X 10^{-4} M); ---, enzyme in the presence of 10^{-4} M NADPH; ---, enzyme in the presence of 10^{-4} M NADPH and 10^{-4} M p-hydroxybenzoate, indicates region of the curve where signal to noise ratio is poor.

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other class shows no significant changes. \( p \)-Fluorobenzoate belongs to the former, and \( p \)-aminobenzoate is an example of the latter.

\( p \)-Fluorobenzoate binds to the enzyme, as evidenced by a change in the CD spectrum upon addition to free enzyme. If \( p \)-hydroxybenzoate is added to the \( p \)-fluorobenzoate-enzyme complex, the CD spectrum is converted to one similar to the spectrum for the \( p \)-hydroxybenzoate-enzyme complex. This indicates that \( p \)-fluorobenzoate binds to the same site as the substrate, and can be displaced by the \( p \)-hydroxybenzoate. On the other hand, \( p \)-aminobenzoate does not produce significant changes in the CD spectrum of the free enzyme, although it inhibits the enzyme activity in a competitive manner. \( p \)-Aminobenzoate may bind to a site other than the one binding \( p \)-fluorobenzoate, and thus may interfere with the over-all reaction of \( p \)-hydroxybenzoate hydroxylase. Furthermore, the CD spectrum of the \( p \)-aminobenzoate-enzyme mixture is not affected significantly upon addition of \( p \)-hydroxybenzoate. Conversely, the CD spectrum characteristic of the enzyme-substrate complex is converted to the one similar to the free enzyme spectrum by the addition of \( p \)-aminobenzoate. The results show that the binding of \( p \)-aminobenzoate to the enzyme causes a secondary effect on the substrate binding site so that \( p \)-hydroxybenzoate can no longer bind to its site, resulting in the inhibition of activity.

The different modes of binding may be understood in terms of a steric hindrance effect at the hydroxyl binding site. Examination of the CPK space filling models and interatomic distances and configurations (15) of the inhibitors indicates that the class of inhibitors which does not modify the CD spectrum of the enzyme has a benzoate substituent (\(-\text{NH}_2, -\text{NO}_2, -\text{Cl}\)) bulkier than that of the other class (\(-\text{H}, -\text{F}, -\text{OH}\)).

By CD studies, we obtained evidence that NADPH binds to free \( p \)-hydroxybenzoate hydroxylase. The binding is so specific that NADH does not become associated with the enzyme. These findings well explain the specific requirement of NADPH for the enzymatic hydroxylation of \( p \)-hydroxybenzoate.

A preliminary proton magnetic resonance study of the relaxation times of the NADPH protons in the presence of \( p \)-hydroxybenzoate hydroxylase revealed NADPH binding with the enzyme. This result supports the above-described CD study that NADPH specifically interacts with \( p \)-hydroxybenzoate hydroxylase.

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