Crystallization and Properties of \( p \)-Hydroxybenzoate Hydroxylase from \textit{Pseudomonas putida}*

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SUMMARY

1. The inducible \( p \)-hydroxybenzoate hydroxylase of \textit{Pseudomonas putida} which catalyzes the hydroxylation of \( p \)-hydroxybenzoate to protocatechuate has been obtained in crystalline form as a protein homogeneous upon ultracentrifugation and electrophoresis. The molecular weight is estimated to be 83,600.

2. The enzyme contains approximately 1 mole of flavin adenine dinucleotide per mole of protein. Reduced nicotinamide adenine dinucleotide phosphate, but not reduced nicotinamide adenine dinucleotide, serves as the hydrogen donor.

3. The enzyme is highly specific for \( p \)-hydroxybenzoate. Only four other aromatic compounds could be attacked at rates of less than 5\% of the rate with \( p \)-hydroxybenzoate in all cases.

4. Under anaerobic conditions, the enzyme can catalyze the reduction of FAD by NADPH. This reaction requires the presence of the aromatic substrate to proceed at a significant rate.

5. The absorption spectrum of the enzyme in the visible region is slightly modified by the presence of the aromatic substrate.

Although specific hydroxylations play an important role in the oxidative metabolism of aromatic compounds by bacteria (1), little is known about the properties of the enzymes which mediate these steps. Only one such enzyme, salicylate hydroxylase, has been highly purified and studied in detail (2, 3). This monooxygenase, which converts salicylate to catechol, requires reduced nicotinamide adenine dinucleotide and flavin adenine dinucleotide as cofactors (2).

Many bacteria can use \( p \) hydroxybenzoate (4) as a source of carbon and energy, and the metabolism of this aromatic acid has been reasonably well clarified (4, 5). It is initially attacked by hydroxylation in position 3 to yield protocatechuate (3,4-dihydroxybenzoate). The subsequent oxidation of protocatechuate can take place through two divergent pathways determined by the mode of oxygenative ring cleavage. Some bacteria attack protocatechuate by \textit{ortho} cleavage, yielding \( \beta \)-carboxy-cis-cis-muconate (6); others by \textit{meta} cleavage, yielding \( \alpha \)-hydroxy-\( \gamma \)-carboxy-cis-cis-muconic semialdehyde (7). The \( p \)-hydroxybenzoate hydroxylase of \textit{Pseudomonas desmolytica}, which uses the \textit{meta} cleavage pathway for the metabolism of protocatechuate, has been partially purified and shown to be a monooxygenase which requires FAD and NADPH (8). This report deals with the crystallization and properties of \( p \)-hydroxybenzoate hydroxylase from another bacterium, \textit{Pseudomonas putida}, which uses the \textit{ortho} cleavage pathway for the metabolism of protocatechuate.

EXPERIMENTAL PROCEDURE

Materials—\( \text{cis-cis} \)-muconic acid was prepared by the oxidation of phenol with perpropionic acid (9), and \( \beta \)-carboxy-cis-cis-muconic acid was prepared by the enzymatic oxidation of protocatechuate (4). NADPH (grade II), NADH (Sigma grade), NADP\(^+\) (grade III), NAD\(^+\) (grade III), FMN and FAD (grade III) were purchased from Sigma. Dithioerythritol was obtained from Cyclochemicals Corporation. Ammonium sulfate used for enzyme purification was the special enzyme grade reagent of Mann Research Laboratories. All of the other chemicals employed were of the purest commercial grade available. Aromatic compounds tested as substrates or inhibitors of the enzyme were twice crystallized from water before use.

Glucose-6-P dehydrogenase and glucose-6-P were purchased from Boehringer.

Calcium phosphate gel was prepared according to Keilin and Hartree (10) and aged for more than 3 months before use.

Whatman column Chromedia CF II cellulose powder was soaked in 10\(^{-3}\) M EDTA overnight and the supernatant solution was discarded by decantation. The cellulose powder was then washed 10 times with deionized water and stored in the cold.

DEAE-cellulose, obtained from Mannex Company, was washed, equilibrated with 0.005 M phosphate buffer, pH 6.8, and stored in the cold until used.

Determination of \( p \)-Hydroxybenzoate and Protocatechuate—Both of these compounds were assayed spectrophotometrically. \( p \)-Hydroxybenzoate was determined from the decrement in absorbance at 340 \text{m}\( \mu \) after addition of crystalline \( p \)-hydroxybenzoate hydroxylase, in the presence of oxygen, to a solution containing \( p \)-hydroxybenzoate and an excess of TPNH. Protocatechuate was determined from the decrement in absorbance at 290 \text{m}\( \mu \) after the addition of protocatechuate oxygenase (3).
as follows. After all of the reagents had been added to the main compartment and to the side arm of the reaction vessel, both the in the center cavity and the cup was filled with the buffer system against which the enzyme solution had been dialyzed. Modified Thunberg tubes. The lower end, was employed. Experiments were conducted in the cell compartment which was designed to accommodate the modified Thunberg tubes. The synthetic boundary centrifugation was performed in a 12-mm cell with a 4° sector by using a Spinco model E analytical ultracentrifuge. The cell was filled by layering 0.6 ml of enzyme solution on 0.1 ml of carbon tetrachloride. The synthetic boundary centrifugation was performed in a 12-mm 4° standard analytical cell (15). The enzyme solution was placed in the center cavity and the cup was filled with the buffer system against which the enzyme solution had been dialyzed.

Anaerobic Experiments—A special type of reaction vessel, consisting of a Thunberg tube with a Pyrex cuvette sealed onto the lower end, was employed. Experiments were conducted in a Cary recording spectrophotometer with a special cover over the cell compartment which was designed to accommodate the modified Thunberg tubes. Anaerobic conditions were established in the reaction system as follows. After all of the reagents had been added to the main compartment and to the side arm of the reaction vessel, both the tube and the cap were flushed for 5 min with \( N_2 \) (99.9% purity) introduced through capillary pipettes. The cap was then positioned for insertion, and the capillary used to gas the tube was withdrawn just before the two elements were connected. The system was then evacuated with a vacuum pump.

Two reactions were followed spectrophotometrically: the reduction of FAD, which was measured directly by the decrement of \( A_{450} \), and the oxidation of NADPH, which was measured by the decrement of \( A_{340} \) and corrected for the change in absorbance at this wave length caused by the reduction of FAD as calculated from the observed change in \( A_{450} \). In computing changes in the reactants from absorbance changes, the following ratios (determined experimentally) were used: \( A_{440} (\text{FAD}) : A_{440} (\text{FADH}_2) : A_{340} (\text{FAD}) : A_{340} (\text{FADH}_2) = 1.000 : 0.070 : 0.440 : 0.330 \).

A value of 1.13 \( \times 10^4 \) was used for the \( e_{1cm} \) of FAD.

**Table I**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Stabilizing mixtures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Glutathione</td>
<td>( 10^{-2} )</td>
</tr>
<tr>
<td>Mercaptoethanol</td>
<td>( 10^{-3} )</td>
</tr>
<tr>
<td>Dithioerythritol</td>
<td>( 10^{-1} )</td>
</tr>
<tr>
<td>EDTA</td>
<td>( 10^{-1} )</td>
</tr>
<tr>
<td>FAD</td>
<td>( 10^{-4} )</td>
</tr>
<tr>
<td>p-Hydroxybenzoate</td>
<td>( 10^{-4} )</td>
</tr>
</tbody>
</table>

**Protein Determinations**—At early stages in the preparation of p-hydroxybenzoate hydroxylase, protein was determined by the biuret method (11). On highly purified preparations determinations were made by the Folin-Ciocalteau method as described by Hawk, Oser, and Summerson (12). Bovine serum albumen (Fraction V, Armour) was used as a standard.

**Starch Gel Electrophoresis**—Starch gel electrophoresis was carried out in the cold according to a modification of Smithies' method (13). Hydrolyzed starch, 54 g (Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada), was suspended in 500 ml of 0.076 m Tris-0.006 m citric acid buffer, pH 8.7, containing \( 10^{-4} \) m EDTA and \( 10^{-3} \) m p-hydroxybenzoate and then dissolved by heating in a water bath. When the mixture had cooled to 50°, FAD and mercaptoethanol were added (\( 10^{-4} \) and \( 10^{-3} \) m final concentrations, respectively) and thoroughly mixed with the starch before it was poured onto the plastic tray. The electrode buffer system consisted of the same concentrations of EDTA, p-hydroxybenzoate, FAD, and mercaptoethanol in 0.3 m boric acid-0.06 m NADH, pH 8.5.

**Stabilizing Mixtures**—Unless protected by the presence of low molecular weight, purified p-hydroxybenzoate hydroxylase is rapidly and irreversibly inactivated. Buffer solutions used in the various steps of purification and in the preparation of the enzyme were consequently always supplemented with one of the three stabilizing mixtures described in Table I. In certain enzyme preparations (specifically indicated in the text), FAD was systematically omitted from all of the stabilizing mixtures.

**Molecular Weight Measurements**—The molecular weight of p-hydroxybenzoate hydroxylase was calculated from the equations of Archibald (14). The purified enzyme was centrifuged in a conventional 12-mm cell with a 4° sector by using a Spinco model E analytical ultracentrifuge. The cell was filled by layering 0.6 ml of enzyme solution on 0.1 ml of carbon tetrachloride. The synthetic boundary centrifugation was performed in a 12-mm 4° standard analytical cell (15). The enzyme solution was placed in the center cavity and the cup was filled with the buffer system against which the enzyme solution had been dialyzed.

Anaerobic experiments were established in the reaction system as follows. After all of the reagents had been added to the main compartment and to the side arm of the reaction vessel, both the tube and the cap were flushed for 5 min with \( N_2 \) (99.9% purity) introduced through capillary pipettes. The cap was then positioned for insertion, and the capillary used to gas the tube was withdrawn just before the two elements were connected. The system was then evacuated with a vacuum pump.

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A value of 1.13 \( \times 10^4 \) was used for the \( e_{1cm} \) of FAD.

**Bacteriological Method**—The strain employed was *Pseudomonas putida* A.3.12 (ATCC 12633). The bacterium was cultivated at 30° in a mineral medium (16) which contained 20 mm p-hydroxybenzoate. Cultures were prepared in batches of 10 liters in a New Brunswick fermenter and subjected to vigorous aeration during growth. Each culture was harvested with the use of an air-driven Sharples centrifuge before the population entered the stationary phase. The wet cell paste was stored at -15° until extraction.

**Enzyme Assay**—The p-hydroxybenzoate hydroxylase of *P. putida* is a soluble enzyme. It can be assayed (even on crude cell-free extracts from which the particulate fraction has been removed by high speed centrifugation) by spectrophotometric measurement of the substrate-dependent oxidation of NADPH. Provided that the particulate fraction has been completely eliminated, the rate of NADPH oxidation in the absence of p-hydroxybenzoate is negligible. The standard assay system contained, in 3.0 ml, 100 \( \mu \)mol of Tris-HCl, pH 8.0, 0.01 \( \mu \)mol of FAD, 0.45 \( \mu \)mol of NADPH, 1.0 \( \mu \)mol of p-hydroxybenzoate, enzyme, and water. As will be described in later sections, FAD (the coenzyme of p-hydroxybenzoate hydroxylase) is extremely tightly bound and drastic treatments are required for even partial resolution of the enzyme. Hence in practice, FAD can be safely omitted from the standard assay system. The control cuvette contained all of the reagents except p-hydroxybenzoate. Assays were conducted at 23° in a Cary or Gilford recording spectrophotometer. The reaction was initiated by addition of p-hydroxybenzoate. The rate declines fairly rapidly, and accurate results can be obtained only by conducting measurements during the first 10 to 20 sec of the reaction. The unit of enzyme activity is defined as that amount of enzyme which oxidizes 1.0 \( \mu \)mol of NADPH per min under the conditions of the spectrophotometric assay. This is equivalent to the hydroxylation of 1 \( \mu \)mol of p-hydroxybenzoate per min (see Table III).

The activity of the enzyme can also be measured by the manometric determination of the rate of oxygen consumption; in this case, an enzymatic system (glucose-6-P dehydrogenase and glucose-6-P) must be added to permit continuous regeneration of NADPH. For manometric measurements, the main compartment contained, in 3.0 ml: Tris-HCl, pH 8.0, 100 \( \mu \)mol; FAD, 0.01 \( \mu \)mol; glucose-6-P, 10 \( \mu \)mol; glucose-6-P dehydrogenase, 1.4 units; MgCl\(_2\), 10 \( \mu \)mol; NADP\(^+\), 0.15 \( \mu \)mol; glutathione, 1 \( \mu \)mol; enzyme; and water. The center well contained KOH. The reaction was initiated by addition from the side arm of 5 \( \mu \)mol of p-hydroxybenzoate. The reaction was conducted at 30°.
RESULTS

Stabilization of p-Hydroxybenzoate Hydroxylase—In crude extracts, the enzyme is stable. Its specific activity remains unchanged for 3 months at -15°; however, stability decreases rapidly as it is purified, and the crystalline material loses about 75% of its activity if it is recrystallized in the unprotected state. Accordingly, an extensive study of various protective agents was undertaken. Thiol compounds are essential; glutathione, dithioerythritol (17), and mercaptoethanol all give good protection at 10^{-4} M. As shown in Figs. 1 and 2, EDTA, FAD, and p-hydroxybenzoate confer additional protection. Of these, p-hydroxybenzoate has the most substantial protective effect. The optimal concentrations of EDTA and p-hydroxybenzoate are above 10^{-4} and 10^{-5} M, respectively. The pure enzyme could be stored satisfactorily at -15° under N_2 in the presence of these protective agents; the loss of activity was less than 10% in 2 months.

Purification of p-Hydroxybenzoate Hydroxylase

Unless otherwise noted, all of the steps were performed below 4°.

Step 1—Frozen bacterial paste, 190 g, was suspended in 186 ml of 0.05 M phosphate buffer (stabilizing mixture A), pH 7.2. Ratches of 40 ml were treated for 3 hr in a 10-kc Raytheon sonic oscillator cooled with circulating ice water, and then centrifuged for 30 min at 13,200 × g. After centrifugation, the supernatant liquid was carefully decanted from the lower creamy layer. The lower layers were combined, resuspended in 93 ml of the same buffer mixture, and reextracted. After centrifugation, the supernatant was combined with that obtained in the first extraction. The balance sheet for subsequent steps in purification is shown in Table II.

Step 2—The crude extract was adjusted to pH 6.8 by dropwise addition of 1 M acetic acid and dialyzed overnight against 6 liters of 0.05 M phosphate buffer (stabilizing mixture A), pH 6.8. After dialysis, protamine sulfate (2% solution in the same buffer) was added at a ratio of 0.1 mg per mg of protein. The mixture was stirred for 30 min and then centrifuged at 13,200 × g for 30 min.

Step 3—The supernatant from Step 2 was fractionated with solid ammonium sulfate containing 0.2% sodium pyrophosphate; during this procedure, the pH of the solution was maintained close to pH 6.8 by periodic addition of 10% ammonium hydroxide. The fractions precipitating at 0.3 to 0.4, 0.4 to 0.5, and 0.5 to 0.6 saturation were collected and redissolved in the minimal volume of 0.1 M phosphate buffer (stabilizing mixture A), pH 7.6. They were then dialyzed overnight against 0.02 M phosphate buffer (stabilizing mixture A), pH 6.8.

Step 4—As shown in Table II, most of the p-hydroxybenzoate hydroxylase is contained in the fractions precipitated between 0.3 and 0.5 saturation with ammonium sulfate. These two fractions were pooled after dialysis and chromatographed in two lots (each containing 4.5 g of protein) on a column of Sephadex G-100 (5 × 100 cm, 100 g, dry weight). The column was eluted with 0.02 M phosphate buffer (stabilizing mixture A), pH 6.8, at an average flow rate of 350 drops per hour. Fractions of 11.5 ml were collected. Elution resulted in the separation of three colored bands: a leading brown band, followed by two yellow bands. Protocatechuate oxygenase is entirely contained in the leading brown band. p-Hydroxybenzoate hydroxylase is associated exclusively with the first yellow band. Sephadex treatment accordingly separates completely the enzymes that catalyze the first and second steps in the p-hydroxybenzoate pathway (Fig. 3). The successive fractions of the first yellow band from the Sephadex column were pooled and condensed by treatment with ammonium sulfate. The material precipitating between 0.3 and 0.6 saturation was collected and resuspended in 15 ml of 0.1 M phosphate buffer (stabilizing mixture A), pH 7.6.
**TABLE II**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Total protein (^a)</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cell-free extract</td>
<td>414.5</td>
<td>37,181</td>
<td>6,204</td>
<td>0.17</td>
<td>100.0</td>
</tr>
<tr>
<td>2. Protamine sulfate treatment</td>
<td>505.3</td>
<td>24,709</td>
<td>4,901</td>
<td>0.20</td>
<td>79.0</td>
</tr>
<tr>
<td>3. First ammonium sulfate fractionation</td>
<td>32.0</td>
<td>2,560</td>
<td>924</td>
<td>0.36</td>
<td>48.7</td>
</tr>
<tr>
<td>4. Sephadex G-100 treatment and concentration by ammonium sulfate precipitation</td>
<td>55.5</td>
<td>6,161</td>
<td>3,793</td>
<td>0.62</td>
<td>61.2</td>
</tr>
<tr>
<td>5. Calcium phosphate gel column</td>
<td>68.6</td>
<td>3,670</td>
<td>199</td>
<td>0.05</td>
<td>3.2</td>
</tr>
<tr>
<td>6. DEAE-cellulose column</td>
<td>20.0</td>
<td>972</td>
<td>3,083</td>
<td>1.67</td>
<td>49.7</td>
</tr>
<tr>
<td>7. Crystallization</td>
<td>90.0</td>
<td>143</td>
<td>2,280</td>
<td>18.9</td>
<td>26.6</td>
</tr>
<tr>
<td>8. DEAE-cellulose column</td>
<td>75.0</td>
<td>84</td>
<td>1,188</td>
<td>15.0</td>
<td>19.2</td>
</tr>
</tbody>
</table>

\(^a\) Protein determinations were made by the biuret method in Steps 1 through 3, and by the Folin-Ciocalteu method in Steps 4 through 7.

It was dialyzed successively against 0.005 M and 0.001 M phosphate buffer (stabilizing mixture A), pH 6.8.

**Step 5**—The dialyzed solution was placed on a column (4.5 × 30 cm) filled with a mixture of Whatman Chromedia CF II cellulose powder (100 g, dry weight) and calcium phosphate gel (6.7 dry weight). Before use, the column was washed with 0.001 M phosphate buffer (stabilizing mixture A), pH 6.8, until the pH of the effluent was 6.8. Elution was conducted with the same buffer at a flow rate of 50 ml per hour. The enzyme emerged in a front running yellow band. This step gives a purification of 6- to 7-fold with recovery of 70%.

**Step 6**—The eluted enzyme from the preceding step was chromatographed on a DEAE-cellulose column (110 ml in a column 2.2 × 30 cm) previously equilibrated with 0.005 M phosphate buffer (stabilizing mixture A), pH 6.8, made 0.1 M with respect to NaCl. It was subjected to linear gradient elution with the same buffer containing NaCl of between 0.1 and 0.35 M at a flow rate of 50 ml per hour; successive fractions of 8.8 ml were collected. The enzyme was eluted in a single peak at approximately 0.15 M NaCl (Fig. 4).

The eluate was concentrated by precipitation with ammonium sulfate and the fraction precipitating between 0.35 and 0.55 saturation was collected and redissolved in 4.5 ml of 0.1 M Tris-HCl buffer. The material obtained at first crystallization appeared pure as judged by its electrophoretic behavior and sedimentation pattern in the ultracentrifuge. Such material was used in all of the subsequent experiments with the crystalline enzyme.

**Criteria of Purity of Crystalline Enzyme**—The crystalline enzyme sedimented with a single symmetric moving boundary when examined with the schlieren optical system in a Spinco model E ultracentrifuge (Fig. 6). The sedimentation coefficient, calculated from another experiment, corresponded to 

\[ s_{20,w} = 5.13 \]

for a 1.4% solution.

Starch gel electrophoresis was carried out by using the two

![Fig. 3. Purification of hydroxylase on a Sephadex G-100 column. See text for details.](image-url)

![Fig. 4. Purification of p-hydroxybenzoate hydroxylase on a DEAE-cellulose column. See text for details.](image-url)
buffer systems described under "Experimental Procedure." After completion of the run, the gel was bisected in the long axis, and one-half was stained with amido black to locate protein (13). The other half (1 cm in width) was cut into squares of 1 x 1 cm, and each piece was eluted with 0.02 M Tris-HCl buffer (stabilizing mixture A), pH 7.5, by homogenization and centrifugation. Enzyme activity was then determined on the eluates. The stained section revealed a single spot moving to the anode, which coincided with the location of enzyme hydroxylase activity, as shown semidiagrammatically in Fig. 7.

**Stoichiometry**

The stoichiometry of the reaction catalyzed by the enzyme is shown in Table III. The over-all reaction catalyzed by the enzyme is evidently

\[ \text{p-Hydroxybenzoate} + \text{NADPH} + \text{H}^+ + \text{O}_2 \rightarrow \text{protocatechuate} + \text{NADP}^+ + \text{H}_2\text{O} \]

**Properties of Purified Enzyme**

**Identification of Flavin Component**—The crystalline enzyme in the oxidized state has an absorption spectrum with maxima at 275, 370, and 448 m\(\mu\), similar to that of many other flavoproteins (18–20). The enzyme can be reversibly reduced to the colorless state with dithionite (Fig. 8). The spectrum in the visible region is characteristic of flavin nucleotide (21), the ratios \(A_{275} : A_{370}, A_{370} : A_{448}\), and \(A_{275} : A_{448}\) being 11.51, 9.73, and 0.85, respectively. Solutions of the enzyme are strongly fluorescent. The binding of flavin is so strong that it is impossible to resolve the enzyme to any appreciable extent by dialysis for 3 days against water or by prolonged electrophoresis on a starch column as judged by the constancy of the \(A_{440}\) to protein ratio.

The cofactor was liberated from the enzyme by heat inactivation followed by trypsin digestion to destroy the coagulated protein. As also shown in Fig. 8, the positions of the peaks of the free coenzyme occur at slightly longer wave lengths than in the enzyme itself and coincide exactly with the peak positions of authentic FAD. Despite this spectral shift, the absorbance of the enzyme and of the free coenzyme at 450 m\(\mu\) are virtually identical, so that the absorbance of the coenzyme content of an enzyme preparation may be determined from its absorbance at 450 m\(\mu\) with the use of the extinction coefficient of the free coenzyme.

The following experiment was undertaken to establish the nature of the bound flavin. A solution of crystalline enzyme

![Fig. 5. Phase contrast photomicrograph of crystals of hydroxylase. 400 X.](image)

![Fig. 6. Sedimentation analysis of crystalline p-hydroxybenzoate hydroxylase. The pictures were taken at 10, 32, 48, and 64 min after the maximum speed was attained. The protein concentration was 1.4% in 0.02 M Tris-HCl buffer (pH 7.5) containing 5 x 10^{-5} M dithioerythritol, 10^{-4} M EDTA, and 10^{-6} M FAD. Temperature, 22°; bar angle, 60°; speed, 59,780 rpm.](image)
Table III
Stoichiometry of reaction catalyzed by p-hydroxybenzoate hydroxylase

The reactions were conducted in Warburg vessels containing KOH in the center well at 30°C in air. The main compartment contained: Tris-HCl, pH 8.0, 100 μmoles; NADPH, 3 μmoles; crystalline enzyme, 1.1 units; and water to 2.8 ml. The side arm contained: p-hydroxybenzoate, 5 μmoles; NADPH, 4.39 and 5 μmoles in Experiments 1 and 2, respectively; and water to 0.2 ml. After the reaction had reached completion as judged from oxygen consumption, an aliquot (0.3 ml) was immediately withdrawn for the measurement of residual NADPH from A340, which was performed after appropriate dilution and then corrected for NADPH oxidation in a control vessel containing all of the ingredients except enzyme. The rest of the reaction mixture was placed in a boiling water bath for 5 min, and then centrifuged to remove the precipitate; p-hydroxybenzoate and protocatechuate were determined on aliquots of the supernatant liquid. The Kₚ value was 0.362 in tertiary amyl alcohol-formic acid-water (3:1:1) (22). Authentic FAD gave a value of 0.350 in this solvent system; the value for FMN was 0.525, and 0.310 for riboflavin. Cochromatography of the extracted pigment with authentic FAD gave a single spot. The identity of the coenzyme with FAD was further confirmed by comparative paper electrophoresis.

The enzyme was largely resolved for FAD by the following procedure. A solution of crystalline material was supplemented with 10⁻³ m dithioerythritol, 5 × 10⁻⁴ m p-hydroxybenzoate, and 10⁻³ m EDTA, and brought to 0.55 saturation with ammonium sulfate. It was then adjusted to pH 3.0 with 2 N HCl. The precipitate was removed by centrifugation, washed once with 0.55 saturated ammonium sulfate, pH 2.5, containing the same components, resuspended in a small amount of 0.33 mM Tris-HCl, pH 8.0, containing the same components, and dialyzed overnight against the same buffer mixture. As judged by its absorption spectrum, this preparation was not completely resolved for flavin; however, it was essentially inactive in the absence of added flavin. Activity could be restored either by the extracted coenzyme or by authentic FAD, but not by FMN or riboflavin (Table IV). Equal quantities of the extracted coenzyme and of authentic FAD restored nearly equal levels of activity.

No evidence for other cofactor requirements has been obtained. Ferrous ion (10⁻⁴ M) is slightly inhibitory; ascorbic acid had no effect.

Substrate Specificity for Reduced Pyridine Nucleotide—The enzyme reacts specifically with NADPH and is wholly inactive with NADH.

Substrate Specificity for Aromatic Compounds—The enzyme is almost completely specific for p-hydroxybenzoate. Very slight activities (measured in terms of NADPH oxidation) were observed with four other aromatic compounds. Expressed as percentages of the activity with p-hydroxybenzoate, the activities were: 0.29 with p-toluolate; 3.2 with 3-bromo-4-hydroxy-

Table IV
Effect of flavin derivatives on activity of p-hydroxybenzoate hydroxylase partly resolved for coenzyme by treatment with acid ammonium sulfate

Preparation of the free coenzyme and of the partly resolved enzyme are described in the text. Flavin derivatives were added separately to aliquots of the partly resolved enzyme, and the mixtures were incubated for 15 hours at 4°C. A sample (0.1 ml) of each mixture was tested for activity by the standard assay method, except that FAD was omitted, and 0.01 μmole of the flavin derivative with which the mixture had been incubated was added in its place. The untreated control was assayed without addition of any flavin derivative.

<table>
<thead>
<tr>
<th>Flavin derivative</th>
<th>Concentration (X 10⁻⁴ M)</th>
<th>Activity (μmoles substrate hydroxylated/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>3.3</td>
<td>0</td>
</tr>
<tr>
<td>FMN</td>
<td>3.3</td>
<td>0</td>
</tr>
<tr>
<td>FAD</td>
<td>3.3</td>
<td>96</td>
</tr>
<tr>
<td>Extraceted coenzyme</td>
<td>3.3</td>
<td>106</td>
</tr>
</tbody>
</table>

* Material eluted from a paper chromatogram. The concentration was determined by measurement of A₄₄₀, with the use of the extinction coefficient of free FAD.
benzoate; 1.5 with 2,4-dihydroxybenzoate; and 0.34 with benzene sulfonate. No activity was observed with benzoate, salicylate, m-hydroxybenzoate, p-fluorobenzoate, p-iodobenzoate, p-chlorobenzoate, p-aminobenzoate, p-nitrobenzoate, 2,3-dihydroxybenzoate, 2,5-dihydroxybenzoate, protocatechuate (3,4-dihydroxybenzoate), 3,5-dihydroxybenzoate, 2,4,6-trihydroxybenzoate, p-methoxybenzoate, p-methiminothiazobenzoate, p-hydroxyphenylacetate, phenoxacetate, p-cresol, p-phenolsulfate, p-toluenesulfonate, and catechol.

**Substrate Affinities**—Rates of reaction of the crystalline enzyme as a function of the concentration of NADPH and of p-hydroxybenzoate gave typical Lineweaver-Burk plots. The \( K_m \) values are 2.18 \( \times 10^{-4} \) for p-hydroxybenzoate and 2.27 \( \times 10^{-4} \) for NADPH.

**Inhibition by Aromatic Compounds**—The enzyme is subject to quite severe inhibition by its aromatic substrate. Maximal activity is observed at p-hydroxybenzoate concentrations between \( 10^{-4} \) and \( 10^{-3} \) M; concentrations above \( 10^{-3} \) M abruptly inhibit activity (Fig. 9). Several structurally related compounds which cannot serve as substrates (benzoate, salicylate, m-hydroxybenzoate, p-fluorobenzoate, p-iodobenzoate, p-chlorobenzoate) are likewise inhibitory at concentrations greater than \( 10^{-3} \) M, the pattern of inhibition being in every case similar to that produced by p-hydroxybenzoate. Kinetic studies have shown that these inhibitions are noncompetitive.

\( \beta \)-Carboxy-cis-cis-muconate (5.6 \( \times 10^{-4} \) M), cis-cis-muconate (10\( ^{2} \) M), and catechol (10\( ^{-2} \) M) are not inhibitory.

**Effects of Heavy Metals and Chelating Agents**—As might be expected from the protective effect of thiol compounds, the enzyme is very sensitive to heavy metals and other inhibitors of thiol groups. At a concentration of \( 10^{-4} \) M, p-hydroxymercurobenzoate completely inhibited activity; inhibitions of 60 and 70\% were observed with iodoacetate and iodoacetamide, respectively, at a concentration of \( 10^{-3} \) M.

EDTA (10\( ^{-3} \) M), 8-hydroxyquinoline (10\( ^{-4} \) M), \( \alpha,\alpha' \)-dipyridyl (10\( ^{-3} \) M), and cyanide (10\( ^{-3} \) M) were without inhibitory effect.

Total inactivation of the enzyme by p-hydroxymercurobenzoate (5 \( \times 10^{-4} \) M) or Zn\( ^{+2} \) (2 \( \times 10^{-4} \) M) could be largely reversed by \( 10^{-3} \) M glutathione. Recovery of activity was 65 and 94\%, respectively.

**pH Optimum**—The enzyme has a fairly narrow pH range. In Tris buffer, it is active between pH 7.0 and 9.0, with a sharp optimum at pH 8.0. In phosphate buffer, the optimal pH is about 7.5.

**Molecular Weight**—Determination of the molecular weight was performed by the Archibald method (14). The molecular weight was calculated from pictures taken at 16, 32, 48, and 64 min, which yielded values of 8.38, 8.40, 8.29, and 8.38 \( \times 10^{4} \), respectively. The average molecular weight is accordingly 8.36 \( \times 10^{4} \). Since the maximal specific activity is 26, the turnover number is approximately 2200 moles of p-hydroxybenzoate decomposed per min per mole of enzyme.

An independent estimation of molecular weight by measurement of the relative rate of migration on a Sephadex G-200 column gave a value of 7.8 \( \times 10^{4} \) (23).

**FAD Content**—As already mentioned, the pure enzyme is not detectably resolved for FAD, and the \( A_{450} \) of the enzyme is virtually identical with that of free FAD. Consequently, the FAD content of the crystalline enzyme can be directly deter-
determined by measurement of the $A_{465}$. Such determinations give values of approximately 0.8 mole of FAD per mole of protein.

**Specific Activity**—Maximal specific activities were always obtained at Step 6 of purification, following elution of the enzyme from the DEAE-cellulose column, and they decreased slightly after the subsequent crystallization (see Table II). The highest specific activity observed after chromatography on DEAE-cellulose was 26; values of approximately 20 were more usual. Crude extracts freshly prepared from cultures of *P. putida* growing exponentially at the expense of p-hydroxybenzoate have a specific p-hydroxybenzoate hydroylase activity of approximately 0.4. Hence this enzyme represents about 1.5% of the total soluble protein in fully induced cells. Its activity is undetectable by spectrophotometric assay in uninduced cells grown with either succinate or glucose as carbon source.

**Catalysis of FAD Reduction**—Under anaerobic conditions, the enzyme can catalyze a reduction of FAD by NADPH provided that the aromatic substrate is present in the system; in the absence of p-hydroxybenzoate, the rate of the reaction is negligible (Table V). As shown in other experiments, this reaction has the expected stoichiometry. The ratio of FAD reduced to NADPH oxidized is close to unity.

**Influence of p-Hydroxybenzoate on Spectral Properties of Enzyme**—The catalytic role of p-hydroxybenzoate in mediating anaerobic electron transfer by p-hydroxybenzoate hydroxylase suggests that the combination of the aromatic substrate with the enzyme may induce a conformational change which converts the enzyme to an active state. As shown in Fig. 10, the addition of p-hydroxybenzoate to crystalline enzyme previously dialyzed free of p-hydroxybenzoate causes slight but significant absorptive changes in the visible region of the spectrum where p-hydroxybenzoate itself does not absorb.

**DISCUSSION**

The p-hydroxybenzoate hydroxylase of *Pseudomonas putida* appears to be similar in its properties to the isofunctional enzyme of *Pseudomonas desmolita* (8) which has been less highly purified. Both enzymes contain bound FAD and are specific for NADPH. Furthermore, the extreme specificity for p-hydroxybenzoate, the only aromatic substrate attacked at a significant rate by the enzyme from *P. putida*, is shared by the enzyme from *P. desmolita*. The similarities are of particular interest in view of the fact that this is the only enzyme of p-hydroxybenzoate metabolism shared by the two bacterial species in which the specific metabolic pathways diverge at the next step as a result of different chemical modes of cleavage of protocatechuic acid.

The only other bacterial enzyme of this class which has been highly purified and studied in detail is the salicylate hydroxylase of *P. desmolytica*.2 Highly purified and studied in detail is the salicylate hydroxylase of different chemical modes of cleavage of protocatechuate. The similarities are of particular interest in view of the fact that this is the only enzyme of p-hydroxybenzoate metabolism shared by the two bacterial species in which the specific metabolic pathways diverge at the next step as a result of different chemical modes of cleavage of protocatechuic acid.

Salicylate hydroxylase shows a far lower degree of specificity for aromatic substrates attacking at rates comparable to the rate of salicylate oxidation several other hydroxybenzoic acids and 1-hydroxy-2-naphthionic acid.

We have shown that in the presence of the aromatic substrate, p-hydroxybenzoate hydroxylase can catalyze an anaerobic reduction of FAD by NADPH. A precisely analogous phenomenon has been shown for salicylate hydroxylase by Katagiri et al. (3). The virtual inability of both enzymes to mediate anaerobic electron transfer when the aromatic substrate is absent suggests that the reduction of FAD observable under anaerobic conditions may well be a component step in the over-all aerobic reaction. Specifically, the over-all reaction could be envisaged as the resultant of the following three reactions.

$$
\text{AR} + \text{enzyme-FAD} \rightarrow \text{AR-enzyme-FADH}_2
$$

$$
\text{NAD(P)}^+ + \text{H}^+ + \text{AR-enzyme-FADH}_2
$$

$$
\rightarrow \text{NAD(P)}^+ + \text{AR-enzyme-FADH}_2
$$

AR-enzyme-FADH$_2$ + $O_2$ → AR-OH + enzyme-FAD + $H_2O$

where AR and AR-OH designate the aromatic substrate and its hydroxylated product, respectively.

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Crystallization and Properties of \( p \)-Hydroxybenzoate Hydroxylase from 
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