p-Hydroxyphenylacetate-3-hydroxylase
A TWO-PROTEIN COMPONENT ENZYME*

Usha Arunachalam‡, Vincent Massey‡, and C. S. Vaidyanathan‡

From the §Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan 48109-0606 and the ¶Department of Biochemistry, Indian Institute of Science, Bangalore-560012, India

p-Hydroxyphenylacetate-3-hydroxylase, an inducible enzyme isolated from the soil bacterium Pseudomonas putida, catalyzes the conversion of p-hydroxyphenylacetate to 3,4-dihydroxyphenylacetate. The enzyme requires two protein components: a flavoprotein and a colorless protein referred to as the coupling protein. The flavoprotein alone in the presence of p-hydroxyphenylacetate and substrate analogs catalyzes the wasteful oxidation of NADH with the stoichiometric generation of H₂O₂. A 1:1 complex of the flavoprotein and coupling protein is required for stoichiometric product formation. Such complex formation also eliminates the nonproductive NADH oxidase activity of the flavoprotein. A new assay measuring the product formation activity of the enzyme was developed using homoprotocatechuate-2,3-dioxygenase, as monitoring the oxidation of NADH was not sufficient to demonstrate enzyme activity. The coupling protein does not seem to have any redox center in it. Thus, this 2-component flavin hydroxylase resembles the other aromatic hydroxylases in that the only redox chromophore present is FAD.

p-Hydroxyphenylacetate is formed during the degradation of aromatic biogenic amines such as tyramine (1), syringamine (3), and octammine (2). It can be hydroxylated in the 3-position to yield 3,4-dihydroxyphenylacetate or in the 1-position to yield homogentisic acid.

Pseudomonas putida (4–6), Pseudomonas ovalis (7), Acinetobacter sp. (6), Escherichia coli (8), Pseudomonas aerogenosa (2), and Trichosporum cutaneum (9) have all been shown to catabolize p-hydroxyphenylacetate through the meta-cleavage route, with 3,4-dihydroxyphenylacetate being the hydroxylation product. The complete metabolic sequence serving to convert p-hydroxyphenylacetate into CO₂, succinate, and pyruvate was shown by Sparnins et al. (6). p-Hydroxyphenylacetate-β-hydroxylase catalyzes the introduction of the second hydroxyl group into the benzene nucleus at a position ortho to the existing hydroxyl group as shown (Structure I). It is a member of a class of catabolic flavin monooxygenases, requiring pyridine nucleotides as electron donors. Due to its labile nature, studies on this enzyme have been limited to whole cell, crude extracts, or partially purified preparations. Adachi et al. (7) have partially purified this enzyme from P. ovalis and have shown that the enzyme requires NADH and O₂ for activity. Raju et al. (4) purified it from P. putida by affinity chromatography and have shown it to be a single component flavoprotein. The enzyme partially purified from Brevibacterium fuscum was very labile and required thios and FAD for activation (10).

Other examples of enzymes which fall into the class of flavoprotein external monooxygenases are p-hydroxybenzoate hydroxylase (11), salicylate hydroxylase (12), phenol hydroxylase (13, 14), melilotate hydroxylase (15), anthranilate hydroxylase (16), and p-hydroxyphenylacetate-1-hydroxylase (17). Most of these enzymes are of bacterial or fungal origin and are induced by growing the microorganism in cultures containing the substrate as a sole source of carbon. All these enzymes catalyze monooxygenation reactions, hydroxylating the primary substrate either ortho or para to the existing hydroxyl group. In most cases the hydroxylated product is more soluble or more readily metabolized than the substrate. Hence, these enzymes aid considerably in the detoxification of aromatic compounds and constitute an important part of pollution control.

The soil bacterium that we had isolated from garden soil (India) was identified to be P. putida. The enzyme, p-hydroxyphenylacetate-3-hydroxylase isolated from this soil bacterium requires two protein components for complete activity and was obtainable in large quantities in a pure and stable form. This is the first report on the purification and characterization of a multi-component flavin aromatic monooxygenase.

The enzyme requires two protein components: a flavoprotein, which has a deceptive substrate-dependent NADH oxidase activity, and a colorless protein, which is absolutely essential for productive hydroxylation. Due to the deceptive substrate-dependent NADH oxidase activity of the flavoprotein, the enzyme could not be assayed either by monitoring the substrate-dependent oxidation of NADH or by monitoring O₂ consumption (which are regularly used to assay other flavin monooxygenases). Hence, the standardization of a simple, quick and continuous coupled enzyme assay using purified homoprotocatechuate-2,3-dioxygenase played an important role in the discovery of this novel two-component flavoprotein hydroxylase.
**Experimental Procedures**

**Materials**

- p-Hydroxyphenylacetic acid, Trizma base, NADH, FAD, and DEAE-Sepharose A-50 were obtained from Sigma. Glycerol, potassium phosphate (monobasic), and potassium phosphate (dibasic) were obtained from Mallinckrodt. Ammonium sulfate was obtained from ICN Biochemicals. DEAE-Sepharose, phenyl-Sepharose, and Sephacryl S-200 were obtained from Pharmacia LKB Biotechnology Inc.

- Homoprotocatechuate-2,3-dioxygenase was purified from Pseudomonas sp. (18).

The other materials used were of analytical grade.

**Methods**

**Isolation, Growth, and Maintenance of the Organism**

A rod shaped Gram-negative microorganism identified as *P. putida* was isolated by the soil enrichment culture technique using p-hydroxyphenylacetate (p-OHPA)\(^1\) as a sole source of carbon and energy. The bacterium was purified by repeatedly isolating a single colony on agar plates. It was maintained on 2% (w/v) agar plates whose chemical composition was as follows: KH\(_2\)PO\(_4\) (anhydrous), 5.8 g; MgCl\(_2\) 6H\(_2\)O, 0.16 g; CaCl\(_2\) 2H\(_2\)O, 20 mg; Na\(_2\)MoO\(_4\) 2H\(_2\)O, 2 mg; FeSO\(_4\) 7H\(_2\)O, 1 mg; MnCl\(_2\) 4H\(_2\)O, 1 mg; p-hydroxyphenylacetate, 1.5 g, and distilled water, 1000 ml. A stock of p-OHPA was prepared and autoclaved separately after adjusting the pH to 7.0 with 10 N NaOH. The medium for the liquid cultures was of identical composition minus the agar. Liquid cultures were grown in a New Brunswick 200-liter fermentor using a 5% inoculum. The temperature was maintained at 28°C and the pH at 7.0 during growth. The medium was aerated (210 liters of O\(_2\)/min) and agitated continuously. The cells were harvested after 7 h of growth using a Sharples super centrifuge just before the cells went into the stationary phase. The yield was between 2 and 2.5 g/liter of culture.

- Isolation of the organism was conducted with ice. The slurry was sonicated with a Branson sonic oscillator at full power for 4-5 min and the temperature was maintained between 4 and 10°C with ice. The extract was centrifuged at 20,000 \(\times\) g for 10 min and the supernatant was labeled as the crude extract.

**Ultracentrifugation**—The crude extract was centrifuged at 100,000 \(\times\) g for 1 h. The supernatant was the particulate-free fraction.

**DEAE-Sephalac Anion Exchange Chromatography**—A 20-ml column of DEAE-Sephalac was equilibrated with 0.05 M KPF, buffer pH 7.2. The particulate-free fraction was loaded on the column and the column was washed extensively with the equilibration buffer. The enzyme was eluted with a gradient of phosphate buffer (0-250 mM), and the active fractions were pooled and brought to 20% ammonium sulfate saturation.

**Phospho-Sepharose Chromatography**—An 8-ml column of phospho-Sepharose was packed and equilibrated with 20% saturated ammonium sulfate, 0.05 M KPF, buffer at pH 7.2. The partially purified dioxygenase was loaded on the column and washed with the equilibration buffer. The enzyme was then eluted with a combined gradient of ammonium sulfate (20-0%) and glycerol (0-20%). The active fractions were pooled, concentrated, and stored on ice.

**Enzyme Assays for p-Hydroxyphenylacetate-3-hydroxylase**

**NADH Oxidation**—p-OHPA dependent oxidation of NADH at 340 nm (\(\Delta\lambda = 6220 \text{ M}^{-1} \text{ cm}^{-1}\)) was used to monitor enzyme activity. Assays were performed in 0.025 M Tris-HCl buffer at pH 8.0 and 25°C (reaction volume = 1 ml), containing 100 μM NADH and 1 mM p-OHPA. A unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 μmol of NADH/min under standard conditions.

**Coupled Enzyme Assay Using Purified Homoprotocatechuate-2,3-dioxygenase**—The product of the dioxygenase reaction (3,4-dihydroxyphenylacetate) is the substrate for homoprotocatechuate-2,3-dioxygenase. The dioxygenase converts 3,4-dihydroxyphenylacetate to 5-carboxymethyl-2-hydroxymuconic semialdehyde, which is colored and has an absorption maximum at 380 nm (\(\Delta\lambda = 38,000 \text{ M}^{-1} \text{ cm}^{-1}\)) at pH 8.0. When saturating amounts of the dioxygenase are present in the reaction mixture (i.e. the same result is obtained with 15 μg/ml dioxygenase as with 30 μg/ml dioxygenase) the product of the dioxygenase is instantaneously converted to the product of the dioxygenase. Thus the rate of formation of the muconic semialdehyde accurately measures the product formation activity of the dioxygenase.

- The assays were conducted in 0.025 M Tris-HCl, pH 8.0, buffer (reaction volume = 1 ml) containing 100 μM NADH, 1 mM p-OHPA, and 27-30 μg of purified homoprotocatechuate-2,3-dioxygenase. When assayed for the coupling protein, at least 3 different concentra-

**Ammonium Sulfate Fractionation**—Solid ammonium sulfate was added with constant stirring to 30% saturation. The mixture was stirred for 30 min and the brown precipitate which pelleted down after centrifugation at 16,300 \(\times\) g for 30 min. The precipitate was resuspended in a small volume of buffer and used as before. After centrifugation, the supernatants were combined and labeled as the crude extract.

**Protonate Sulfate Precipitation**—To clarify the crude extract from ribosomal material, it was precipitated with a polyclation (proteinate sulfate). A 2% (w/v) stock of proteinate sulfate was prepared in water and added dropwise to give a final concentration of 1 mg/ml. The precipitate was stirred for 30 min after addition and centrifuged at 16,300 \(\times\) g for 30 min. The pellet was discarded.

**Ultracentrifugation**—The supernatant obtained after proteinate sulfate precipitation was centrifuged at 100,000 \(\times\) g for 2 h. The pellet was discarded and the supernatant was labeled as the particulate-free fraction.

**Further Purification of the Coupling Protein**

**DEAE-Sepharose Column Chromatography**—A 50 × 4-cm diameter column was packed with DEAE-Sepharose (fast flow) and equili-

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\(^1\) The abbreviations used are: p-OHPA, p-hydroxyphenylacetate; KPF, potassium phosphate; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.
Further Purification of the Flavoprotein

**DEAE-Sepharose Column Chromatography**—The extensively dialyzed 45-70% ammonium sulfate fraction was applied on a 1200-ml DEAE-Sepharose column equilibrated with 0.025 M KP, buffer, pH 7.0. The flavoprotein binds to form a distinct yellow band at the top of the column. The column was washed with 4 liters of the equilibrium buffer. The flavoprotein was eluted with a 1.5-liter gradient of 0-10% ammonium sulfate in 0.025 M KP buffer at pH 7.0. Fractions were assayed by the coupled enzyme assay (using 640 nm fluorescent flavoprotein) and those which had a specific activity of over 20 units/mg protein were pooled and labeled as the main fraction. The coupling protein was concentrated with ammonium sulfate (0-50%) and stored either as an ammonium sulfate pellet or was desalted on a Sephadex G-25 column equilibrated with 0.025 M KP buffer containing 10% glycerol and stored on ice. As the coupling protein was inactivated upon freezing and thawing it could not be stored frozen.

**Phenyl-Sepharose Column Chromatography**—The extensively dialyzed 45-70% ammonium sulfate fraction was applied on a 1200-ml phenyl-Sepharose column previously equilibrated with 0.025 M KP, buffer containing 10% ammonium sulfate at pH 7.0. After extensively washing the column with the equilibrium buffer the enzyme was eluted with a combined gradient of glycerol (0-10%) and ammonium sulfate (10-0%) in 0.025 M KP buffer at pH 7.0. Fractions were assayed by the coupled enzyme assay (using 640 nm fluorescent flavoprotein) and those which had a specific activity of over 20 units/mg protein were pooled and labeled as the main fraction. The coupling protein was concentrated with ammonium sulfate (0-50%) and stored either as an ammonium sulfate pellet or was desalted on a Sephadex G-25 column equilibrated with 0.025 M KP buffer containing 10% glycerol and stored on ice. As the coupling protein was inactivated upon freezing and thawing it could not be stored frozen.

**Protein Estimation**

Protein was estimated by using bicinchoninic acid, with bovine serum albumin (Fraction V, Armour) as a standard (19).

**Molecular Weight Determinations**

For the determination of native molecular weight, an HPLC gel filtration column (Sephacryl S-200) was calibrated with standards ranging in molecular weight from 40,000 to 200,000. The standards used were ovalbumin (43,000), bovine serum albumin (66,000), p-hydroxybenzoate-3-hydroxylase (83,000), and proteocatechuate dioxygenase (200,000). The native molecular weight of the flavoprotein was determined using the calibration curve obtained with the above proteins.

The subunit molecular weight was determined by 11% SDS-polyacrylamide gel electrophoresis. The molecular weight markers used were proteocatechuate dioxygenase (31,000 and 26,500) subunits, phthalate dioxygenase reductase (34,000), phthalate dioxygenase (48,000), p-hydroxybenzoate hydroxylase (44,000), and bovine serum albumin (66,000).

**Anaerobic Technique**

The enzyme solutions were placed in a stopped cell fitted with two side arms and made anaerobic by repetitive cycles of evacuation and equilibration with oxygen-free argon. This procedure was repeated 8 times to ensure complete anaerobiosis.

**Photoreduction**

Anaerobic light irradiation was performed at 4°C. The enzyme was reduced by deazaflavin (10-4 M)-catalyzed photochemical reduction with EDTA (9 x 10-4 M) as photodonor (20).

**Equilibrium Ligand Binding Measurements**

Titrations were performed in a Cary 219 double beam recording spectrophotometer. This was temperature controlled and flushed with dry air to prevent condensation at low temperatures.

**RESULTS**

**Enzyme Purification**—A typical purification sequence for the flavoprotein and the coupling protein is presented in Tables I and II, respectively. The coupling protein was homogeneous on SDS-polyacrylamide gel electrophoresis (Fig. 1). The flavoprotein had minor contaminants corresponding to molecular weights of 28,000 and 26,000 as shown in Fig. 1. These contaminants could be separated by fast protein liquid chromatography on an analytical Mono-Q column using a gradient of ammonium sulfate (20-300 mM) in 0.025 Tris-HCl buffer at pH 8. Each of the fractions eluted from the fast protein liquid chromatography column were electrophoresed on SDS-PAGE and the visible absorption spectrum of each of the fractions was also recorded. The flavoprotein, which migrated on SDS-PAGE corresponding to a molecular weight of 30,750 was the only component with a visible absorption spectrum and the minor contaminants clearly did not contribute to the spectral characteristics of the flavoprotein. The purified coupling protein, unlike the flavoprotein, was unsta-

### Table I

<table>
<thead>
<tr>
<th>Purification of flavoprotein</th>
<th>Volume Protein</th>
<th>Activity</th>
<th>Specific activity</th>
<th>Purification Yield</th>
<th>Fold</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulfate (45-70%)</td>
<td>128</td>
<td>11.0</td>
<td>4.17</td>
<td>0.38</td>
<td>3.12</td>
<td>91</td>
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<tr>
<td>S-200</td>
<td>124</td>
<td>8.7</td>
<td>2.48</td>
<td>0.67</td>
<td>5.48</td>
<td>52</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>64</td>
<td>1.5</td>
<td>3.08</td>
<td>2.05</td>
<td>16.80</td>
<td>33</td>
</tr>
</tbody>
</table>

* 1 U (unit), 1 µmol of product formed per min at 25°C using 600 nm coupling protein.

### Table II

<table>
<thead>
<tr>
<th>Purification chart for coupling protein</th>
<th>Volume Protein</th>
<th>Activity</th>
<th>Specific activity</th>
<th>Purification Yield</th>
<th>Fold</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulfate (30-45%)</td>
<td>165</td>
<td>27.0</td>
<td>2.37</td>
<td>0.099</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>DAE-Sepharose</td>
<td>234</td>
<td>2.8</td>
<td>0.95</td>
<td>0.28</td>
<td>9.3</td>
<td>57</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>134</td>
<td>0.58</td>
<td>1.22</td>
<td>2.120</td>
<td>24.1</td>
<td>42</td>
</tr>
</tbody>
</table>

* 1 U (unit), 1 µmol of product formed per min at 25°C using 600 nm flavoprotein.
0.025 M KPi buffer containing 10% glycerol, 36 pM flavoprotein at purified coupling protein, 5 pg. Lane pH 7.0.

Fig. 1. 11% SDS-polyacrylamide gel electrophoresis. Lanes a, b, e, and f, markers (see "Experimental Procedures"). Lane c, purified coupling protein, 5 µg. Lane d, purified flavoprotein, 5 µg.

Fig. 2. Absorption spectrum of flavoprotein. Conditions: 0.025 M KPi buffer containing 10% glycerol, 36 µM flavoprotein at pH 7.0.

Fig. 3. Spectrophotometric titration of the oxidized flavoprotein with p-hydroxyphenylacetate. The figure shows the difference spectra of the flavoprotein titrated with different concentrations of p-hydroxyphenylacetate taken in the sample cuvette versus the flavoprotein titrated with an equal volume of buffer in the reference cuvette. Concentrations of p-hydroxyphenylacetate (from the base line outwards) were 4, 9, 14, 38, and 87 µM. Conditions: 0.025 M KPi buffer, pH 7.0, 4 °C.

Table III: Substrate specificity

<table>
<thead>
<tr>
<th>Substrate/substrate analog</th>
<th>Turnover number (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavoprotein alone</td>
<td>148</td>
</tr>
<tr>
<td>Flavoprotein + coupling protein</td>
<td>142</td>
</tr>
<tr>
<td>Flavoprotein + coupling protein + pOHPa</td>
<td>151</td>
</tr>
</tbody>
</table>

No substrate 7 2 148
p-Hydroxyphenylacetate 87 208
p-Fluorophenylacetate 49 1 142
p-Chlorophenylacetate 55 0 145
p-Nitrophenylacetate 18 0 151
p-Amino-4-hydroxyphenylacetate 11 1 115
p-Phenylpropionate 39 21 136
p-Hydroxybenzoate 14 13 81
p-Hydroxybenzoate 10 3 151
Salicylate 7 4 130

Concentration of substrate/substrate analogs used was 250 µM.
1 mM p-hydroxyphenylacetate was added after measuring the activity in the presence of the coupling protein to show that the loss of activity was not due to enzyme inactivation. This concentration exhibits some excess substrate inhibition, but was used to compete with the binding of the alternate substrates, present at a concentration of 250 µM.

Fig. 4. Demonstration of coupling using spectrophotometric assays. Conditions: 0.025 M Tris-HCl buffer, pH 8.0, 25 °C contained 100 µM NADH, 1 mM p-OHPA, and in addition, for the coupled enzyme assay 27 µM of purified homoprotocatechuate-2,3-dioxygenase was added. The figure shows the effect of various concentrations of the coupling protein (0-900 nM) on the substrate-dependent NADH activity (A) and the product formation activity as assayed by the coupled enzyme assay (B) at a fixed concentration of the flavoprotein (109 nM). The coupling ratio (moles of product formed to moles of substrate oxidized) is represented by the dashed line. A coupling ratio of 1 is obtained when all the NADH oxidized is coupled to substrate hydroxylation without the generation of H2O2.

The native molecular weight of the coupling protein could not be determined using the above method as the protein aggregates and elutes from the column as a broad band.

Absorption Spectrum and Extinction Coefficients—The absorption spectrum for the flavoprotein is presented in Fig. 2 and has maxima at 458, 376, and 278 nm. The spectrum is resolved with shoulders around 360 and 490 nm. The purified flavoprotein has an A360/A458 ratio of 7.5. The extinction coefficient of the flavoprotein at pH 7.0 in 0.025 M sodium phosphate buffer at 4 °C was determined by recording the spectrum of the flavoprotein before and after the addition of 0.1% SDS. The denaturation of the flavoprotein was complete in less than 30 s without the formation of a precipitate, permitting the determination of the extinction coefficient of the enzyme-bound flavin relative to that of free FAD (11,300 M⁻¹ cm⁻¹). The spectrum of FAD is unaffected by SDS. An
extinction coefficient of $12,400 \text{ M}^{-1} \text{ cm}^{-1}$ was calculated for the enzyme-bound flavin. When the flavin was treated with Naja naja snake venom, there was the marked enhancement of the flavin fluorescence typical of the conversion of FAD to FMN, confirming that the prosthetic group is FAD. Concentrations of the flavoprotein are given throughout in terms of flavoprotein; the flavoprotein at pH 7.0, containing 10% glycerol, results in a marked spectral change.

**Table IV**

<table>
<thead>
<tr>
<th>Chelator</th>
<th>Activity remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tiron (4,5-dihydroxy-1,3-benzene disulfonic acid)</td>
<td>%</td>
</tr>
<tr>
<td>1 mM</td>
<td>71</td>
</tr>
<tr>
<td>5 mM</td>
<td>39</td>
</tr>
<tr>
<td>10 mM</td>
<td>14</td>
</tr>
<tr>
<td>1.10-Phenanthroline, 277 μM</td>
<td>50</td>
</tr>
</tbody>
</table>

**FIG. 5.** Anaerobic photoreduction of flavoprotein. The flavoprotein (14.5 μM) in 0.025 M KPi buffer containing 10% glycerol, 9 mM EDTA, and 1 μM 5-deazaflavin was made anaerobic and mixed with p-hydroxyphenylacetate (1 mM) at pH 7.0 and 4 °C, the spectrum of the flavoprotein after mixing with p-hydroxyphenylacetate; - - -, spectrum of the flavoprotein after irradiation for 100 s; - - -, after irradiation for 160 s; - - - - - - , after irradiation for 220 s; - - - - - - - - - , reduced enzyme after irradiation for 1000 s; ø, spectrum of the flavoprotein in complex with the substrate after reoxidation. Inset, the isocratic separation of reactants by HPLC on an RP-18 column. p-Hydroxyphenylacetate elutes at a retention time of 11.5 min.

**FIG. 6.** Anaerobic photoreduction of flavoprotein in complex with coupling protein. The flavoprotein (13 μM) and coupling protein (1.2 eq) in 0.025 M KPi buffer containing 10% glycerol, 9 mM EDTA, and 1 μM 5-deazaflavin was made anaerobic and mixed with p-hydroxyphenylacetate (1 mM) at pH 7.0 and 4 °C. ——, the spectrum of the flavoprotein in complex with the coupling protein after mixing with p-hydroxyphenylacetate; — — — — — — — , spectrum taken after irradiation with light for 100 s; ø, spectrum taken after irradiation for 160 s; — — — , spectrum after 220 s; — — — — — , spectrum after 460 s of irradiation; - - - - - - - , spectrum of reduced enzyme; - - - - - - - - - , spectrum of the enzyme after reoxidation. Inset, isocratic separation of reactants by HPLC on an RP-18 column.

the difference spectra nor the dissociation constant. A $K_d$ of $2.3 \times 10^{-6}$ M was calculated for p-hydroxyphenylacetate when the coupling protein was present in saturating amounts (data not shown).

**Activity of the Flavoprotein**—In the absence of the coupling protein, the flavoprotein catalyzes the oxidation of NADH in the presence of the substrate, p-hydroxyphenylacetate, without the hydroxylation of the substrate. The reduction product of oxygen was confirmed to be H$_2$O$_2$ by testing the effect of catalase in the assay. When monitoring the O$_2$ consumption with a Clark O$_2$ electrode, the addition of catalase in the reaction mixture reduced the rate of O$_2$ consumption by half. As shown in Table III, the flavoprotein also catalyzes the oxidation of NADH in the presence of a variety of substrate analogs. However, the oxidation of NADH in the presence of the natural substrate p-hydroxyphenylacetate is the fastest.

**Substrate Hydroxylation Specificity**—Although the flavoprotein quite nonspecifically oxidizes NADH in the presence of a variety of substrate analogs, in the presence of the coupling protein the enzyme becomes highly specific for p-hydroxyphenylacetate (Table 3), hydroxylating it at full efficiency to 3,4-dihydroxyphenylacetate. p-Hydroxyphenylpropionate, the next best substrate for the enzyme, is only hydroxylated with 50% efficiency when the coupling protein is present. None of the other compounds tested was hydroxylated.

**Demonstration of Coupling by Spectrophotometric Enzyme Assays**—Enzyme activity at a fixed flavoprotein concentra-
FIG. 7. Evidence for absence of a redox center in the coupling protein. The flavoprotein (13 μM) in 0.025 M KP, buffer containing 10% glycerol, 9 mM EDTA, and 1 μM 5-deazaflavin was made anaerobic and mixed with 1 mM p-OHPA. - - - - spectrum of the flavoprotein after mixing with p-OHPA; -- -- -- spectrum of the reduced flavoprotein after irradiation for 1110 s; -- -- -- spectrum of the reduced flavoprotein after mixing with 1.2 eq of coupling protein; --- spectrum after reoxidation. Inset, isocratic separation of reactants by HPLC on an RP-18 column.

TABLE V

Redox potential of p-hydroxyphenylacetate-3-hydroxylase

<table>
<thead>
<tr>
<th>Redox potential</th>
<th>mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavoprotein</td>
<td>-218</td>
</tr>
<tr>
<td>Flavoprotein + pOHPA</td>
<td>-211</td>
</tr>
<tr>
<td>Flavoprotein + coupling protein</td>
<td>-211</td>
</tr>
<tr>
<td>Flavoprotein + coupling protein + pOHPA</td>
<td>-208</td>
</tr>
</tbody>
</table>

The redox potential of the flavoprotein was measured in the presence of the coupling protein and the substrate, p-hydroxyphenylacetate in 0.025 M phosphate buffer containing 10% glycerol, pH 7.0, at 25 °C, using anthraquinone-2-sulfonate (-225 mV) as a redox standard, and xanthine oxidase as a source of reducing equivalents (22).

...tion and varied coupling protein concentrations was determined by both substrate-induced NADH oxidation (monitored at 340 nm) and the product formation activity (monitored at 400 nm by the coupled enzyme assay using the purified dioxygenase in saturating amounts). The ratio of NADH oxidation to productive hydroxylation for each of the coupling protein concentrations was calculated. As shown in Fig. 4 a coupling ratio of 1 was obtained upon increasing the concentration of the coupling protein in the reaction mixture.

Demonstration of Coupling by Single Turnover Photoreduction Experiments—The flavoprotein in the main compartment of an anaerobic cuvette equipped with 2 side arms was made anaerobic by repeated cycles of evacuation and equilibration with O₂-free argon, and the spectrum of the enzyme was recorded. The substrate, p-hydroxyphenylacetate was added from the side arm to give a final concentration of 10⁻³ M. The enzyme-substrate complex was irradiated in the presence of EDTA and catalytic amounts of 5-deazaflavin with light for short intervals after which the spectra were recorded (Fig. 5). After complete reduction the cuvette was opened to air in the dark. The reaction mixture was subjected to ultrafiltration using a Centricon 30 membrane and the flow through was acidified with 6 N HCl and analyzed for products by HPLC on a reverse phase RP-18 column. An isocratic solvent system of 25 parts methanol and 75 parts of 1% acetic acid was used for elution. The product, 3,4-dihydroxyphenylacetate, eluted earlier at a retention time of 8.5 min, and the substrate, p-hydroxyphenylacetate, eluted at a retention time of 11.5 min. As shown in the inset to Fig. 5, the flavoprotein alone does not form the product, 3,4-dihydroxyphenylacetate. However, when the coupling protein (1.2 eq) was present with the flavoprotein and the p-hydroxyphenylacetate (Fig. 6), photoreduction and reoxidation yielded stoichiometric (0.85 product/FAD) formation of product, 3,4-dihydroxyphenylacetate (inset to Fig. 6). A comparison of Figs. 5 and 6 confirms that the coupling protein is absolutely necessary for hydroxylation.

Absence of a Prosthetic Group on the Coupling Protein—The activity of the enzyme as determined by the coupled enzyme assay was markedly inhibited by iron chelators such as Tiron (4,5-dihydroxy-1,3-benzene disulfonic acid) and 1,10-phenanthroline (Table IV). The dioxygenase was insensitive to both Tiron and 1,10-phenanthroline at the same concentrations. However, when 20 mg of the coupling protein was precipitated with 20% trichloroacetic acid and estimated for acid-extractable iron using 1,10-phenanthroline (21), no iron was detected. To test whether the coupling protein might have an unidentified redox active center, the flavoprotein in the main compartment, with the coupling protein and p-hydroxyphenylacetate in 2 separate side arms of the anaerobic cuvette was made anaerobic. p-Hydroxyphenylacetate was mixed with the flavoprotein and the enzyme photoreduced, with the coupling protein protected from light. After complete reduction of the flavoprotein the coupling protein was tipped from the side arm and mixed with the reduced flavoprotein. A very small absorbance change was observed with the addition of the coupling protein to the reduced flavoprotein (Fig. 7), indicating that the reducing equivalents from the reduced flavoprotein were not transferred to the coupling protein. When the cuvette was reopened to air as previously and the products analyzed on HPLC (inset to Fig. 7), stoichiometric amounts of product were formed (0.92 product/FAD). These observations demonstrated that the role of the coupling protein is probably not as the hydroxylating agent itself, but as a "helper" protein in complex with the flavoprotein component. This conclusion is supported by the results from rapid reaction studies, which will be detailed in a subsequent paper.

Calculation of the Dissociation Constant for the Flavoprotein and the Coupling Protein—Enzyme activity at 3 flavoprotein concentrations and varied concentrations of coupling protein were determined by both substrate-induced NADH oxidation and the coupled enzyme assay using the purified dioxygenase in saturating amounts, as shown in Fig. 4. The ratio of the product formed to the NADH that disappeared gave the coupling ratio. The coupling ratio for each of the flavoprotein and coupling protein concentrations was calculated. The coupling ratio was then taken as an estimate of complex formation between the flavoprotein and the coupling protein. From the initial slope of the coupling ratio versus concentration of...
coupling protein (Fig. 4) it can be concluded that a 1:1 complex of the flavoprotein and coupling protein is required for productive hydroxylation. A dissociation constant of $2.6 \times 10^{-8}$ M was estimated.

Redox Potential—The redox potential for the flavoprotein in the presence and absence of the coupling protein was determined at 25 °C, pH 7.0, 0.025 M phosphate buffer by a spectrophotometric method employing a reducing system of xanthine and xanthine oxidase (22). As presented in Table V the redox potential of the flavoprotein is changed only marginally by complex formation with coupling protein, by substrate, or by a mixture of both.

**DISCUSSION**

We have described here the purification of $p$-hydroxyphenylacetate-3-hydroxylase from a soil bacterium, *P. putida*. The enzyme is the first example of an aromatic hydroxylase that requires two protein components to catalyze the hydroxylation of its substrate, $p$-hydroxyphenylacetate to 3,4-dihydroxyphenylacetate. The flavoprotein alone can only catalyze the substrate-stimulated oxidation of NADH without substrate hydroxylation. A second colorless protein, the coupling protein, is crucial for coupling the oxidation of NADH to substrate hydroxylation.

Hydroxylases, including $p$-hydroxyphenylacetate-3-hydroxylase (4, 5, 7, 8, 10) are commonly assayed by the substrate-dependent oxidation of reduced pyridine nucleotide followed at 340 nm or by substrate-dependent O$_2$ consumption. These assays, however, were insufficient to demonstrate enzyme activity for the two-component $p$-hydroxyphenylacetate-3-hydroxylase. Hence, the development and standardization of a sensitive product formation assay was crucial for the purification of the enzyme components.

Although the product of the reaction could be analyzed by HPLC, this is very time consuming and tedious, hence, a simple, quick and sensitive product formation assay (coupled enzyme assay) was developed using purified homoprotocatechuate-2,3-dioxygenase. The product of the dioxygenase (5-carboxymethyl-2-hydroxymuconic semialdehyde) which is yellow in color was followed by the increase in absorbance at 400 nm ($\Delta \epsilon = 21,300$ M$^{-1}$ cm$^{-1}$).

Uncoupling of flavin reduction from substrate hydroxylation has been shown with practically every flavoprotein hydroxylase. This uncoupling is usually brought about by substrate analogs which are commonly referred to as “non-substrate effectors.” Salicylate hydroxylase, a flavoprotein from *P. putida* catalyzes the hydroxylation of salicylate to yield catechol. This enzyme effectively hydroxylates the substrate salicylate, whereas benzoate, a nonsubstrate analog, stimulates NADH oxidation, but uncouples oxygen activation from hydroxylation, resulting in stoichiometric generation of H$_2$O$_2$ (12). Similarly, $p$-hydroxybenzoate hydroxylase efficiently hydroxylates $p$-hydroxybenzoate but 6-hydroxynicotinate, which binds to the active site of the enzyme, enhances the rate of flavin reduction by NADPH without itself being hydroxylated (23).

$p$-Hydroxyphenylacetate behaves as a nonsubstrate effector of the flavoprotein, but in the presence of the coupling protein it is a perfect substrate with virtually no leakage of oxygen to H$_2$O$_2$. Furthermore, the flavoprotein component by itself clearly has a binding site for both NADH and the primary substrate, $p$-hydroxyphenylacetate, and also readily reacts with oxygen. However, despite the existence of the usual catalytic features, it still requires the coupling protein, which has no redox center, to help in the hydroxylation reaction.

The presence or absence of the coupling protein clearly decides whether the flavoprotein behaves as an oxidase or as a hydroxylase (Scheme I). Furthermore, it appears that it is the coupling protein which is chiefly responsible for the hydroxylation specificity of the enzyme being restricted to $p$-hydroxyphenylacetate and partially to $p$-hydroxyphenylpropionate. While the flavoprotein component by itself will catalyze the oxidation of NADH dependent on the presence of a variety of aromatic compounds, it is remarkable that in most cases, NADH oxidation is practically eliminated when the coupling protein is present (Table III). Thus it is possible that the two-protein component nature of this enzyme has evolved as a secondary defense against the wasteful oxidation of NADH in the absence of the substrate to be hydroxylated. All flavoprotein hydroxylases so far studied have a common defense mechanism against wasteful NAD(P)H oxidation in that binding of the substrate is required for the rapid reduction of the enzyme flavin by NAD(P)H. However, this has resulted in the phenomenon that many compounds sufficiently similar to the real substrate will also bind and stimulate flavin reduction with most flavoprotein hydroxylases. In the present case, complex formation of the flavoprotein and the coupling protein is required for the hydroxylation of $p$-hydroxyphenylacetate.
tate. Such complex formation also eliminates the nonproductive oxidation of NADH found in the presence of a variety of nonsubstrate effectors. The way in which this exquisite control mechanism is exerted is presently not understood.

REFERENCES