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Metabolic Function and Properties of 4-Hydroxyphenylacetic Acid 1-Hydroxylase from *Pseudomonas acidovorans*

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The enzyme 4-hydroxyphenylacetate, NAD(P)H:oxygen oxidoreductase (1-hydroxylating) (EC 1.14.13 . . .; 4-hydroxyphenylacetate 1-monooxygenase; referred to here as 4-HPA 1-hydroxylase) was induced in *Pseudomonas acidovorans* when 4-hydroxyphenylacetate (4-HPA) was utilized as carbon source for growth; homogentisate and maleylacetoacetate were intermediates in the degradation of 4-HPA. A preparation of the hydroxylase that was free from homogentisate dioxygenase and could be stored at 4 C in the presence of dithioerythritol with little loss of activity was obtained by ultracentrifuging cell extracts; but when purified 18-fold by affinity chromatography the enzyme became unstable. Flavin adenine dinucleotide and Mg^{2+} ions were required for full activity. 4-HPA 1-hydroxylase was inhibited by KCl, which was uncompetitive with 4-HPA. Values of K_i determined for inhibitors competitive with 4-HPA were 17 μM *dl*-4-hydroxymandelic acid, 43 μM 3,4-dihydroxyphenylacetic acid, 87 μM 4-hydroxy-3-methylphenylacetic acid, and 440 μM 4-hydroxyphenylpropionic acid. Apparent K_m values for substrates of 4-HPA 1-hydroxylase were 31 μM 4-HPA, 67 μM oxygen, 95 μM reduced nicotinamide adenine dinucleotide (NADH); and 250 μM reduced nicotinamide adenine dinucleotide phosphate (NADPH). The same maximum velocity was given by NADH and NADPH. A chemical synthesis is described for 2-deutero-4-hydroxyphenylacetic acid. This compound was enzymatically hydroxylated with retention of half the deuterium in the homogentisic acid formed. Activity as substrate or inhibitor of 4-HPA 1-hydroxylase was shown only by those analogues of 4-HPA that possessed a hydroxyl group substituent at C-4 of the benzene nucleus. A mechanism is suggested that accounts for this structural requirement and also for the observation that when 4-hydroxyphenoxyacetic acid was attacked by the enzyme, hydroquinone was formed by release of the side chain, probably as glycolic acid. Only one enantiometer of racemic 4-hydroxyhydratropic acid was attacked by 4-HPA 1-hydroxylase; the product, α-methylhomogentisic acid (2-[2,5-dihydroxyphenyl]-propionic acid), exhibited optical activity. This observation suggests that, during its shift from C-1 to C-2 of the nucleus, the side chain of the substrate remains bound to a site on the enzyme while a conformational change of the protein permits the necessary movement of the benzene ring.

The aerobic catabolism of monohydric phenols by microorganisms is usually initiated by introducing a second hydroxyl group into the benzene nucleus at a position either *ortho* or *para* to the first (8, 12). When 4-hydroxyphenylacetic acid (4-HPA) serves as growth substrate, two metabolic routes are available. Hydroxylation at C-3 of the nucleus yields the ring-fission substrate 3,4-dihydroxyphenylacetic acid (1, 5, 32), which has recently been shown to be metabolized to carbon dioxide, pyruvate, and succinate by *Pseudomonas putida* and by a species of *Acinetobacter* (43). Alternatively, Blakley (4) has presented evidence that an unidentified soil bacterium hydroxylates 4-HPA at C-1 to give homogentisic acid (2,5-dihydroxyphenylacetic acid) with simultaneous migration of the side-chain substituent according to the equation:

$$\text{4-hydroxyphenylacetate} + O_2 + \text{NADH} + H^+ \rightarrow \text{homogentisate} + H_2O + NAD^+$$

In these studies (4) the establishment of stoichiometry was hindered by the presence in cell extracts of a very active oxidase for reduced nicotinamide adenine dinucleotide (NADH). We selected the strain of *Pseudomonas*

acidovorans for present work on the basis that cell extracts used in a previous investigation (14) had contained relatively low levels of this enzyme. Further, we now find that although the 4-hydroxyphenylacetic acid 1-hydroxylase (4-HPA 1-hydroxylase) of this organism readily loses activity during conventional purification procedures, it is possible to obtain a stable preparation of the enzyme, effectively free from homogentisate oxygenase (EC 1.13.1.5), by ultracentrifuging cell extracts prepared in phosphate buffer containing dithioerythritol (DTE). Blakley (4) also observed the stabilizing effects on 4-HPA 1-hydroxylase of compounds that possess sulfhydryl groups.

MATERIALS AND METHODS

Organism and cell extracts. The organism, a species of *P. acidovorans*, was originally isolated by Bachrach (2) from poultry house deep-litter and designated *Pseudomonas* A. It is listed in type culture collections as ATCC 17455 and British NCIB 10013 and has been used in several metabolic investigations (14–16, 27). Cells were grown with forced aeration at 30 C in a medium that contained (per liter): $K_2HPO_4 \cdot 3H_2O$, 4.25 g; $NaH_2PO_4 \cdot H_2O$, 1.00 g; NH_4Cl, 2.00 g; $MgSO_4 \cdot 7H_2O$, 0.20 g; $FeSO_4 \cdot 7H_2O$, 0.012 g; $MnSO_4 \cdot H_2O$, 0.003 g; $ZnSO_4 \cdot 7H_2O$, 0.003 g; $CoSO_4 \cdot 7H_2O$, 0.001 g; nitrilotriacetic acid, 0.10 g; and 4-HPA, brought to pH 7 with NaOH, 1.5 g. Cells were collected by centrifugation at the end of logarithmic growth (about 20 h after inoculation) and were washed by resuspension in 0.1 M potassium-sodium phosphate buffer, pH 7.2. This buffer was also used in all reaction mixtures unless otherwise stated.

Extracts were prepared by suspending cell pastes in two volumes of 0.1 M phosphate buffer, pH 7.2, containing 1 mM DTE and 5 mM $MgSO_4$; cells were then broken by sonic treatment (15). The clear supernatant solution obtained by centrifugation at 30,000 × *g* for 30 min is designated "crude extract" and contained 15 to 20 mg of protein per ml. When crude extracts were centrifuged at 100,000 × *g* for 60 min in a Beckman model L ultracentrifuge with a fixed-angle rotor, the extract showed 4-HPA 1-hydroxylase activity but failed to oxidize homogentisate. This preparation, which is designated "ultracentrifuged extract," could be stored without significant loss of 4-HPA 1-hydroxylase activity for 1 week at 4 C. In certain experiments we investigated reactions of the degradative pathway of 4-HPA beyond the formation of homogentisate. On these occasions extracts were prepared by sonic treatment in 0.02 M morpholinopropane sulfonic acid (MOPS) buffer, pH 7.2, containing 1 mM $MgSO_4$ but without addition of DTE. Homogentisate oxygenase was more active in this buffer than in phosphate-DTE; but in the absence of DTE, 4-HPA 1-hydroxylase was unstable and it was necessary to use the extract immediately after preparation.

Enzyme assays and kinetic studies. Two assays for 4-HPA 1-hydroxylase were employed. In the spectrophotometric assay the initial rate of oxidation of NADH was determined from the decrease in absorbance at 340 nm using a value of ϵ = 6,220 for NADH. The standard assay mixture contained 5 μM flavin adenine dinucleotide (FAD), 1 mM DTE, 0.15 mM NADH, and 0.5 mM 4-HPA in 3 ml of 0.1 M phosphate buffer, pH 7.2. One unit of activity is the amount of enzyme that catalyzes the oxidation of 1 μmol of NADH per min. Polarographic assays measured consumption of O_2 and were performed with a Yellow Springs model 55 oxygen monitor equipped with a Clark polarographic oxygen sensor. The reaction mixture (5 ml) was the same as that used in the spectrophotometric assay and was contained in cells designed to prevent diffusion of O_2 from the atmosphere. The instrument was calibrated with air-saturated buffer at 25 C (0.24 mM O_2). In certain studies of the kinetics of 4-HPA 1-hydroxylase, the concentrations of NADH or 4-HPA in the standard assay mixture were raised to values that are stated later. When effects of inhibitors were measured (Table 2), the concentration of 4-HPA was reduced to 60 μM, namely about twice the apparent K_m of 4-HPA (31 μM). Inhibitor constants (K_i) were determined by method 2 of Thorn (47) in which a fixed concentration (x) of substrate is used with various concentrations (i) of inhibitor: the ratio of the maximum velocity to the observed velocity is then plotted against i/x to give a straight line of slope K_m/K_i.

Purification of 4-HPA 1-hydroxylase by affinity chromatography. When various purification procedures were applied, the enzyme exhibited marked instability even in the present of DTE which was effective in preserving the activity of crude or ultracentrifuged extracts. We therefore adopted a method which seemed likely to separate the hydroxylase from other proteins under the mildest conditions, namely affinity chromatography. In principle, the procedure consisted in coupling *p*-phenylenediamine to Sepharose essentially as described by Cuatrecasas (11); The diazotized *p*-aminophenyl-Sepharose was then coupled to 4-HPA, presumably at C-3 of the nucleus. Details of the preparation of the column are as follows. Finely divided cyanogen bromide (3 g) was added to stirred suspension of 25 ml of packed Sepharose 4B in 25 ml of water; the pH was immediately brought to 11 with NaOH and maintained at that value by dropwise addition. When NaOH was no longer taken up, a large amount of ice was added to the stirred suspension and the activated Sepharose 4B was transferred to a Buchner funnel and washed under suction with 1 liter of cold 0.1 M $NaHCO_3$ buffer, pH 8.6. A suspension of the activated agarose in this buffer was then stirred for 18 h at 4 C with 0.5 g of *p*-phenylenediamine in 25 ml of the same bicarbonate buffer. The *p*-aminophenyl-agarose was filtered under suction, washed extensively with water to remove unreacted *p*-phenylenediamine, and suspended in 100 ml of 0.5 M HCl at 0 C. Diazotization of the stirred solution was performed by dropwise addition of 3 ml of 1 N $NaNO_2$, followed by 100 ml of 2.5 mM 4-HPA in 0.1 M borate buffer, pH 9.0. The pH was immediately brought back to 9.0 with NaOH, the mixture was gently stirred for 18 h at 4 C, and the

agarose-bound 4-HPA was washed repeatedly with 0.1 M phosphate buffer, pH 7.2, to remove low-molecular-weight components. The agarose derivative was then suspended in phosphate buffer containing 1 mM DTE and 5 mM $MgSO_4$ and used to prepare a column (1 by 25 cm).

A crude extract (15 mg of protein per ml) was prepared from 22 g of cell paste, treated with ribonuclease and deoxyribonuclease to reduce viscosity, and dialyzed for 18 h against 1 liter of 0.02 M phosphate buffer, pH 7.2, containing 0.2 mM DTE and 1 mM $MgSO_4$. The extract was applied to a diethylaminoethyl (DEAE)-cellulose column (3 by 30 cm) previously equilibrated with the buffer specified for dialysis, and the column was washed with 200 ml of the same buffer. Protein was then eluted with a linear gradient of 0 to 1.0 M KCl in 0.02 M phosphate buffer, and fractions of 10 ml were collected at a flow rate of 75 ml/h. 4-HPA 1-hydroxylase was eluted by 0.28 M KCl in fractions 27 to 29. The combined fractions (58 mg of protein), containing 5 μM of added FAD, were applied to the column of agarose-bound 4-HPA, previously equilibrated with 0.1 M phosphate buffer containing 1 mM DTE, 5 mM $MgSO_4$ and 5 μM FAD, and the column was washed with 200 ml of this buffer mixture. Most of the protein applied to the column was removed in the first 50 ml collected at this stage, and these fractions were completely devoid of 4-HPA 1-hydroxylase activity. After the remaining 150 ml of buffer had emerged, essentially free from protein, the enzyme was eluted with 25 ml of the phosphate buffer mixture containing 0.5 M 4-HPA, and 2-ml fractions were collected at a flow rate of 30 ml/h. Activity was confined to four fractions, which were yellow; these were pooled. The protein content could not be determined directly since 4-HPA interferes with both the Folin-Ciocalteau and spectrophotometric determinations. For the purpose of documenting the purification procedure (Table 1), the active fractions were dialyzed free from 4-HPA and analyzed for protein; however, dialysis resulted in a loss of more than half the activity. Attempts at further purification by gel permeation chromatography were attended by similar losses. Further, the purified enzyme lost activity completely in 2 days at 4 C even when DTE, FAD, and Mg^{2+} were added; this contrasts with the stability of ultracentrifuged extracts containing 1 mM DTE, and suggests that purification removed a stabilizing component present in crude extracts. The procedure of Table 1 was repeated only when the use of purified 4-HPA 1-hydroxylase appeared to be particularly desirable, as when kinetic parameters for NADH and NADPH were compared, and when the stoichiometry of the oxidation of 2-deutero-4-hydroxyphenylacetic acid was investigated.

TABLE 1. *Purification of 4-HPA 1-hydroxylase*

Step	Protein (mg)	Total activity (units)	Sp act (units/mg)	Yield (%)
Crude extract	1,500	180	0.12	100
Diethylaminoethyl-cellulose column	58	54	0.93	30
Affinity column	21	47	2.2	26

Synthesis of 2-deutero-4-hydroxyphenylacetic acid. 4-HPA labeled with deuterium at C-2 of the benzene nucleus was synthesized by the reaction sequence of Fig. 1. First, 3-deuteroanisole (II) was prepared from 3-bromoanisole (I) by the action of D_2O on the Grignard reagent. A dry 250-ml, three-necked flask was equipped with sealed stirrer, separating funnel, and reflux condenser fitted with a $CaCl_2$ guard. To the cooled flask, containing 1.2 g of magnesium turnings and 10 ml of sodium-dried ether, a dry ethereal solution of 9.5 g of 3-bromoanisole was added slowly with stirring. The solution was refluxed for 15 min on a steam bath and cooled to 0 C, when 5 ml of D_2O (99.7%; Aldrich Chemical Co.) was added to the stirred reaction mixture. This was then acidified with 10% sulfuric acid and extracted with three portions (50 ml) of ether, and the combined ethereal extracts were washed with water and dried over anhydrous $MgSO_4$. The ether was removed under reduced pressure, and 3-deuteroanisole was collected by distillation (151 to 153 C, yield 3.6 g). Mass spectral analysis showed that the compound was enriched 80% in deuterium. 2-Deutero-4-methoxyacetophenone (III) was obtained from 3-deuteroanisole by a procedure similar to that used by Snyder and Elston (42) to prepare 4-hydroxyacetophenone from phenol and acetic acid. 2-Deutero-4-methoxyphenylacetic acid (V) was then synthesized from 2-deutero-4-methoxyacetophenone (III) by a modified Willgerodt reaction (37). A misture of crude 2-deutero-4-methoxyacetophenone, 0.06 mol of morpholine, and 0.075 mol of sulfur was refluxed for 5 h with stirring. The reaction mixture solidified upon the addition of ice water, and the crude thioacetomorpholide derivative (IV) was separated by filtration and dissolved in 50 ml of 95% ethanol. NaOH (5 g) was added to the ethanol solution in a 250-ml flask and the mixture was refluxed for 12 h. Ethanol was removed by distillation under reduced pressure, the residue was dissolved in water and acidified with sulfuric acid, and the aqueous solution was extracted with three 100-ml portions of ether. The combined ether extracts were reextracted with three 50-ml portions of 5% sodium carbonate, and the aqueous solution was acidified with sulfuric acid and extracted with three 50-ml portions of ether. The combined ethereal extracts when evaporated to near dryness yielded a reddish-brown oil. 2-Deutero-4-methoxyphenylacetic acid (V) was extracted from the oil with hot water and crystallized upon cooling to give white crystals, mp 84 to 85.5 C.

2-Deutero-4-hydroxyphenylacetic acid was obtained from the methyl ether by heating with pyridine hydrochloride. A mixture of 100 mg of V and 5 g of pyridine hydrochloride was maintained at 200 to 220 C for 1.5 h in a round-bottom flask fitted with an air condenser. The reaction mixture was then cooled, acidified with hydrochloric acid, and extracted with ether. The ethereal solution was dried with anhydrous $MgSO_4$, the ether was removed by evaporation, and the solid residue was recrystallized from an ether-hexane mixture to yield 2-deutero-4-hydroxyphenylacetic

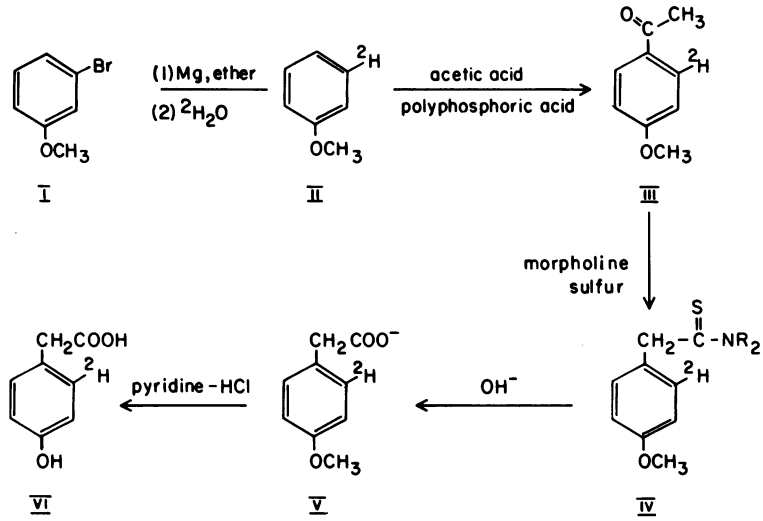

FIG. 1. *Chemical synthesis of 2-deutero-4-hydroxyphenylacetic acid.*

acid (VI), mp 150 to 151.5 C. A portion of the yield was treated with diazomethane to give methyl 2-deutero-4-methoxyphenylacetate, which was examined by mass spectrometry and shown to be enriched 80% in deuterium.

Chemical analyses. 4-HPA was determined colorimetrically by coupling with diazotized 4-nitroaniline (6). Protein was determined either spectrophotometrically or by a modification of the Folin-Ciocalteau procedure (45). Analysis using an LKB 9000A gas-chromatograph mass spectrometer, and also separation of materials by means of silica gel thin-layer chromatography, were performed as previously described (35, 43). Optical rotation was measured at the wavelength of the sodium D-line in a Bendix automatic polarimeter (27).

Chemicals. The following compounds that served as substrates or inhibitors in enzymatic investigations were obtained from Aldrich Chemical Co., Milwaukee, Wis.: 4-hydroxyphenylpropionic acid, 4-hydroxybenzyl alcohol, 4-hydroxybenzoic acid, 3-hydroxyphenylacetic acid, 2-hydroxyphenylacetic acid, phenylacetic acid, 4-chlorophenylacetic acid, and 4-fluorophenylacetic acid. The following were from Sigma Chemical Co., St. Louis, Mo.: 4-hydroxyphenylacetic acid, *dl*-4-hydroxymandelic acid, 3,4-dihydroxyphenylacetic acid, 2,5-dihydroxyphenylacetic acid, and 4-hydroxy-β-phenyllactic acid. 4-Hydroxyphenoxyacetic acid was from Eastman Organic Chemicals, Rochester, N.Y. The following reagents used in chemical syntheses were from Aldrich Chemical Co.: morpholine, cyanogen bromide, 4-hydroxy-2-methylacetophenone, 4-hydroxy-3-methylacetophenone, and 3-bromoanisole. Hydratropic acid (2-phenylpropionic acid) was from Eastman Organic Chemicals.

4-Hydroxy-2-methylphenylacetic acid (mp 152 to 153 C) and 4-methoxyphenylacetic acid (mp 85.5 to 86.5 C) were synthesized by the reactions already described for the 2-deuterated compounds. 4-Hydroxyhydratropic acid (mp 128 C) was synthesized by nitrating hydratropic acid with a mixture of concentrated nitric and sulfuric acids. The crude product was reduced directly with Sn and HCl yielding 4-aminohydratropic acid (mp 128 C) which was converted to 4-hydroxyhydratropic acid by hot aqueous decomposition of the diazonium salt. 4-Hydroxy-3-methylphenylacetic acid (mp 103 C) was prepared from 4-hydroxy-3-methylacetophenone by the Willgerodt reaction (37) of its benzyloxy derivative, using procedures described for a similar synthesis of 5-fluoro-2-hydroxyphenylacetic acid (46).

RESULTS

Metabolism of homogentisate by cells grown with 4-HPA. A cell suspension (4 mg dry weight of cells) oxidized various aromatic acids (5 μmol of each) at the following rates (microliters of O_2 per min): 4-HPA, 10,0; homogentisic, 7.5; homoprotocatechuic, 1.2; 3-hydroxyphenylacetic, 1.0; 2-hydroxyphenylacetic, 1.0. The endogenous respiration rate was 0.5 μliters of O_2 per min. The total oxygen consumption for 4-HPA exceeded that for homogentisate by 1 μmol of O_2 per mol of substrate. These results suggest that 4-HPA is oxidized through the homogentisate pathway of metabolism, and the low rates observed with other acids may be ascribed to nonspecific induction. This was supported by the observation that, in the presence of 20 μM orthophenanthroline, the rates of oxidation of 4-HPA and homogentisate were reduced, respectively, to 41 and 23% of the rates measured in the absence of inhibitor; moreover, after partial oxidation of 4-HPA, a compound that gave the spectrum of the derivative of homogentisic acid was formed when the

contents of the manometric flasks were taken through the procedure of Stoner and Blivaiss (44).

Evidence was also obtained that a cell extract metabolized 4-HPA to give, successively, homogentisate, maleylacetoacetate, and fumarylacetoacetate. Each of three cuvettes contained, in 3 ml of 0.02 M MOPS buffer (pH 7.2), 1 mM $MgSO_4$ and 0.1 ml of a crude extract (15 mg of protein per ml) freshly prepared in MOPS buffer. Two cuvettes contained 0.22 μmol of NADH, and to the first of these was added 0.1 mM orthophenanthroline to inhibit oxidation of homogentisate; no inhibitor was added to the second. After incubating for 1 min, the addition of 0.20 μmol of 4-HPA resulted in a rapid decrease in the absorption at 340 nm of both reaction mixtures, and this decrease continued in the first cuvette until all of the NADH was oxidized. However, in the second cuvette containing no orthophenanthroline, the absorbance began to increase at about 2 min after the start of the experiment and reached a value of 0.82 after 5 min (Fig. 2a). The increase in absorbance was ascribed to the conversion of homogentisate into maleylacetoacetate which absorbs strongly at 340 nm (9, 31). This interpretation was confirmed by adding 0.1 ml of 0.3 mM reduced glutathione, the coenzyme for the enzymatic isomerization of maleylacetoacetate to fumarylacetoacetate (9); the addition caused a rapid decrease in absorbance (Fig. 2a). The reaction in the third cuvette was started by adding 0.20 μmol of homogentisate, an amount equivalent to that of 4-HPA which was added to the first two reaction mixtures. There was an immediate rapid rise in absorbance to 0.82, followed by a decrease on addition of reduced glutathione (Fig. 2b). To obtain the spectrum of the maleylacetoacetate produced, experiments were repeated for homogentisate (2 μmol), and for 4-HPA (2 μmol) with NADH (3 μmol), using 1 ml of cell extract in a reaction volume of 20 ml and with an incubation time of 10 min at 30 C. Solutions were then cooled to 0 C, deproteinized with 0.3 volume of 30% metaphosphoric acid, and centrifuged at 30,000 × *g* for 10 min. The absorption spectra of the products from homogentisate and 4-HPA were those reported for maleylacetoacetate (9).

Stoichiometry of oxidation of 4-HPA and homogentisate by cell extracts. The buffer and crude cell extract were those used in obtaining the data of Fig. 2. In one series of experiments the amount of 0.15 mM NADH oxidized was determined spectrophotometrically for 3-ml mixtures containing, respectively, 0.1, 0.2, and 0.3 μmol of 4-HPA in the presence and absence of 0.1 mM orthophenanthroline. In a parallel series, the amount of oxygen consumed was determined polarographically. In separate determinations with homogentisate as substrate, crude extract oxidized 1 μmol of homogentisate with the consumption of 1 μmol of O_2. Table 2 shows that when the oxidation of 4-HPA does not proceed beyond homogentisate, 1 μmol each of O_2 and NADH are consumed per 1 μmol of 4-HPA.

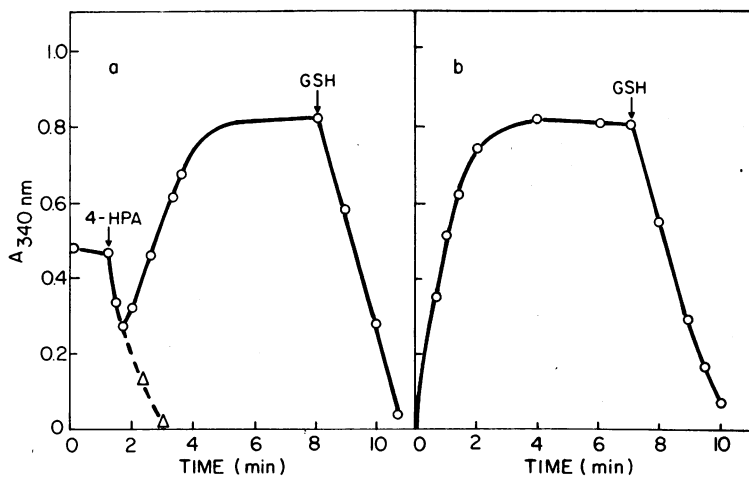

FIG. 2. *Metabolism of 4-HPA and homogentistic acid by a crude cell extract. Absorbance at 340 nm was measured for cuvettes that contained, initially, crude cell extract and (a) NADH, with additions of 4-HPA and reduced glutathione at the times indicated; (b) homogentisic acid, with subsequent addition of reduced glutathione. The dotted line was obtained for a reaction mixture that contained orthophenanthroline to inhibit homogentisate dioxygenase.*

TABLE 2. *Stoichiometry of oxidation of 4-HPA by cell extracts in the presence and absence of orthophenanthroline*

4-HPA added (μmol)	NADH oxidized (μmol)	Oxygen consumed (μmol)	Ratios	
			NADH/ 4-HPA	O_2/ 4-HPA
0.1	0.108	0.196	1.08	1.96
0.1[a]	0.104	0.100	1.04	1.00
0.2	0.214	0.400	1.07	2.00
0.2[a]	0.210	0.204	1.05	1.02
0.3	0.313	0.610	1.04	2.03
0.3[a]	0.315	0.303	1.05	1.01

[a] Orthophenanthroline (0.1 mM) present in reaction mixture.

Induction of 4-HPA 1-hydroxylase. *P. acidovorans* was grown to the stationary phase in 500 ml of growth medium in which 1% Difco yeast extract replaced 4-HPA as carbon source. The cells were centrifuged, washed twice with phosphate buffer, and suspended in 1 liter of growth medium containing 0.1% 4-HPA. The culture was shaken in a 3-liter Erlenmeyer flask at 30 C, growth was followed by measuring the change in absorbance at 540 nm, and 40-ml volumes of the culture were withdrawn at intervals. These samples were centrifuged, the clear superantant solutions were analyzed for 4-HPA, and the cells were suspended in 10 ml of phosphate buffer containing 1 mM DTE and 5 mM $MgSO_4$. After sonic treatment and centrifugation at 30,000 × *g* for 30 min, 0.5 ml of each crude extract (0.8 to 1.6 mg of protein) was taken for assay of 4-HPA 1-hydroxylase. The specific activity of this enzyme began to increase about 1 h before growth at the expense of 4-HPA (Fig. 3).

Cofactor requirements of 4-HPA 1-hydroxylase. The activity of a crude extract, prepared in phosphate buffer and stabilized by the presence of 1 mM DTE, was unaffected by additions of FAD. However, a fraction collected between 33 and 50% saturation with ammonium sulfate (43) showed only 20% of the specific activity of the crude extract when the precipitated protein was taken up in 0.1 M phosphate buffer (pH 7.2), containing 1 mM DTE and 5 mM $MgSO_4$, and was assayed as in Materials and Methods using a reaction mixture from which FAD was omitted. Determinations made with the standard assay mixtures in which the concentration of FAD was progressively increased showed that activity reached a maximum at about 10 μM FAD (Fig. 4). The same ammonium sulfate fraction was used in the standard assay, and the concentration of $MgSO_4$ in the reaction mixture was varied. The maximum activity, reached at 3.3 mM $MgSO_4$, was 2.5 times the value obtained when $MgSO_4$ was omitted. The enzyme was not activated by any of the other salts of divalent cations added to the growth medium, namely $FeSO_4$, $MnSO_4$, $ZnSO_4$, and $CoSO_4$.

Kinetic properties of 4-HPA 1-hydroxylase. The pH optimum of the enzyme was 7.3 when either MOPS buffer or phophate buffer was used in the assay mixture; however, at pH 7.3 activity in phosphate buffer was 80% that in MOPS buffer.

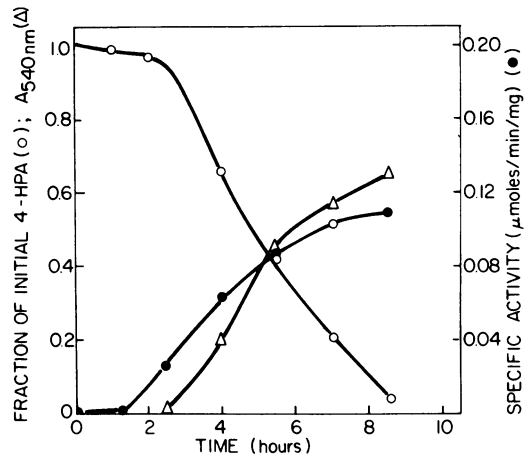

FIG. 3. *Induction of 4-HPA 1-hydroxylase. Cells were grown in the absence of 4-HPA and then incubated in a growth medium with 4-HPA as carbon source. At intervals of times the following were measured: cell density (Δ); fraction of initial 4-HPA remaining (○); specific activity of 4-HPA 1-hydroxylase in cell extracts (●).*

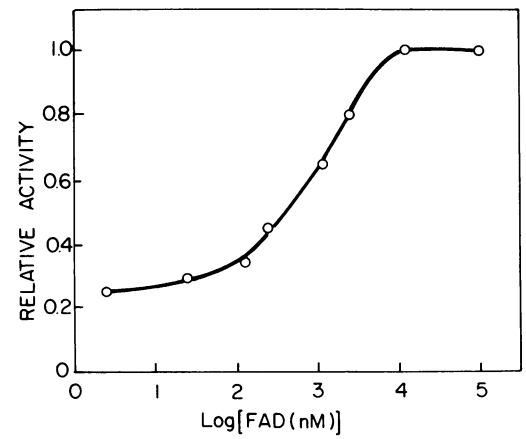

FIG. 4. *Stimulation of 4-HPA 1-hydroxylase activity by additions of FAD to an ammonium sulfate-fractionated cell extract.*

Initial rates of oxidation of NADH were determined spectrophotometrically using reaction mixtures with all components at the concentrations of the standard assay (0.15 mM NADH) except 4-HPA, which was varied within the range of 10 to 500 μM 4-HPA. A Lineweaver and Burk plot was linear throughout this range and gave apparent K_m = 31 μM 4-HPA. An ultracentrifuged extract (20 mg of protein per ml) was used in these determinations. Oxidation of NADH in the absence of substrate was too low to be measured during an assay period of 1 min.

Enzyme purified by affinity chromatography was used to determine apparent Michaelis constants for NADH and reduced nicotinamide adenine dinucleotide phosphate (NADPH), with 1.7 mM 4-HPA present in each assay mixture. The same value for V_{max} (6.2 μmol per min per ml of enzyme containing 7.1 mg of protein) was measured for each pyridine dinucleotide, but different apparent Michaelis constants were obtained: for NADH, the K_m was 95 μM; and for NADPH, the K_m was 250 μM (Fig. 5).

For oxygen, the concentration was monitored polarographically as this substrate was consumed in a reaction mixture contained in a cell designed to prevent replenishment of O_2 by diffusion from the atmosphere. Initially, the solution contained 0.21 mM O_2 and 0.3 mM NADH, decreasing to 0.03 mM O_2 and 0.12 mM NADH for the period of time when measurements were made. By drawing tangents to the curve relating O_2 concentration to absorbance at 340 nm, rates of reaction were obtained which gave a linear Lineweaver and Burk plot from which apparent K_m = 67 μM O_2. These determinations were made with an ultracentrifuged extract; the initial concentration of 4-HPA was 1.0 mM.

Substrate analogues and inhibitors of 4-HPA 1-hydroxylase. The behavior of these compounds was investigated using 0.1 ml of ultracentrifuged extract added to 3 ml of reaction mixtures in phosphate buffer. Potassium chloride inhibited the enzyme at concentrations greater than 10 mM. In a series of standard assay mixtures containing a fixed concentration of KCl, rates of oxidation of NADH were measured at various concentrations of 4-HPA. Lineweaver and Burk plots (Fig. 6) indicated that inhibition by KCl was uncompetitive.

The ability of various aromatic acids to serve as substrates was investigated by replacing 4-HPA with each compound at 10 mM in the standard assay. In a parallel series, 60 μM 4-HPA was also added to each reaction mixture to ascertain whether any substrate analogue acted as inhibitor. In addition to 4-HPA, six compounds (Table 3) were capable of promoting measurable rates of oxidation of NADH; of these the most active, 4-hydroxyphenoxyacetic acid and 4-hydroxyhydratropic acid, were se-

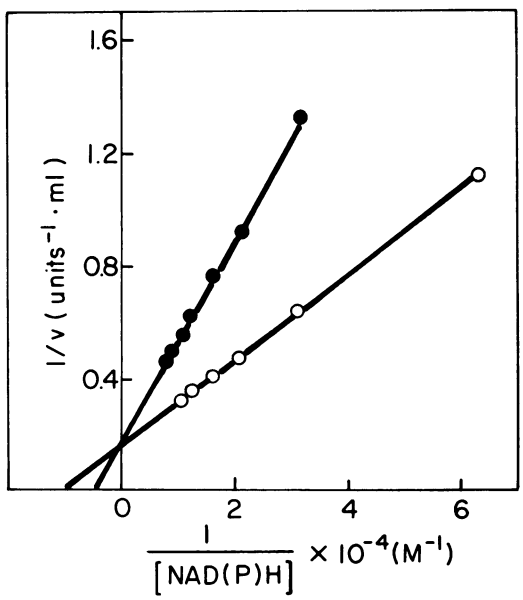

FIG. 5. *Lineweaver and Burk plots of rates of reaction catalyzed by 4-HPA 1-hydroxylase at various concentrations of NADH (○) and NADPH (●).*

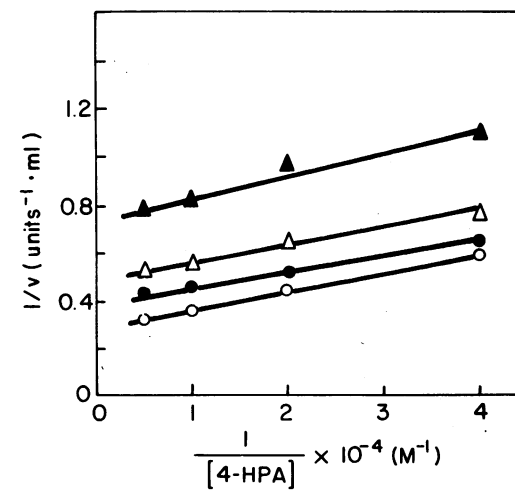

FIG. 6. *Uncompetitive inhibition of 4-HPA 1-hydroxylase by potassium chloride. Reaction rates were determined at the following concentrations of potassium chloride: 10 mM (○), 25 mM (●), 50 mM (△), and 100 mM (▲).*

TABLE 3. *Activities of analogues of 4-HPA as substrates or inhibitors*

Compound	Relative rates of oxidation[a]	
	Compound alone[b]	Compound with 4-HPA[c]
4-Hydroxyphenylacetate (4-HPA)	1.00	1.00
4-Hydroxyphenoxyacetate	0.53	—[d]
4-Hydroxyhydratropate	0.48	—
4-Hydroxy-2-methylphenylacetate	0.24	0.10
4-Hydroxyphenylpropionate	0.12	0.12
4-Hydroxy-3-methylphenylacetate	0.12	ND[e]
3,4-Dihydroxyphenylacetate	0.06	ND
4-Hydroxymandelate	ND	ND
Methyl 4-hydroxyphenylacetate	ND	0.65
4-Hydroxyphenyllactate	ND	0.60
4-Hydroxybenzoate	ND	0.41
4-Hydroxybenzyl alcohol	ND	0.76
3-Hydroxyphenylacetate	ND	0.41
2-Hydroxyphenylacetate	ND	0.41
Phenylacetate	ND	0.65
2,5-Dihydroxyphenylacetate	ND	0.60
4-Fluorophenylacetate	ND	0.41
4-Methylphenylacetate	ND	0.71
4-Chlorophenylacetate	ND	0.35
4-Methoxyphenylacetate	ND	—

[a] Oxidation of NADH assayed spectrophotometrically.
[b] Concentration 10 mM.
[c] A 60-μmol amount of 4-HPA used in 3-ml assay.
[d] —, Not determined.
[e] ND, No activity detected.

lected for further investigations described later. Of the five most powerful inhibitors tested, three reduced the activity of 4-HPA below detectable levels. These three compounds, and also 4-hydroxyphenylpropionic acid, were shown to be competitive inhibitors from double reciprocal plots of velocities and corresponding 4-HPA concentrations using four different concentrations of each inhibitor. Graphs for 4-hydroxy-3-methylphenylacetic acid are shown in Fig. 7. Similar graphs were obtained for the three other competitive inhibitors studied in detail, and the method of Thorn (47) was applied to the data to provide the following inhibitor constants (K_i, μM): *dl*-4-hydroxymandelic acid, 17; 3,4-dihydroxyphenylacetic acid, 43; 4-hydroxy-3-methylphenylacetic acid, 87; 4-hydroxyphenylpropionic acid, 440.

Oxidation of 2-deutero-4-hydroxyphenylacetic acid. To 60 ml of the standard assay mixture were added 30 μmol of 2-deutero-4-hydroxyphenylacetic acid and 1.2 ml of 4-HPA hydroxylase (8.5 mg of protein) that had been purified by affinity column chromatography. The solution was shaken for 30 min at 30 C and then deproteinized by addition of 2 ml of metaphosphoric acid followed by centrifugation to remove precipitated protein. The solution was extracted three times with 10 ml of ethyl acetate, fractions were pooled, and the solution was taken down to dryness. The residue was dissolved in ether and treated with diazomethane to form a methylated derivative (35) which was then analyzed by mass spectrometry. Parent ions were observed having *m/e* of 210 (trimethyl derivative of homogentisate) and of 211 (monodeutero trimethyl derivative). When corrected for the contribution of C^{13}, the ratio of peak heights showed an enrichment of 40% in deuterium for the homogentisate formed in the reaction. Since the substrate for 4-HPA was 80% enriched in deuterium, the course of the reaction can be represented by Fig. 8a which shows an equal probability for migration of the side chain either to C-2 or C-6.

Oxidation of 4-hydroxyhydratropic acid. This compound [2-(4-hydroxyphenyl)-propionic acid] appeared to be a substrate for 4-HPA 1-hydroxylase (Table 3). Measurements to determine stoichiometry were made with an ultracentrifuged extract and indicated that only half of the substrate was oxidized; and since the molecule possesses an asymmetric carbon atom in the side chain, it appeared possible that only

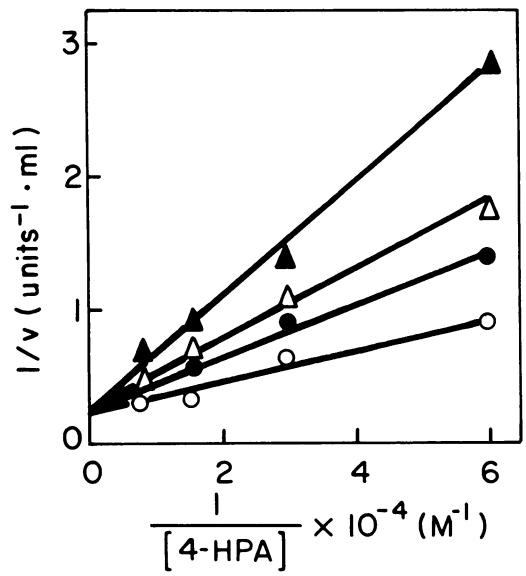

FIG. 7. *Competitive inhibition of 4-HPA 1-hydroxylase by 4-hydroxy-3-methylphenylactic acid. Concentrations of inhibitor: 165 μM (●), 330 μM (△), 660 μM (▲); and no inhibitor (○).*

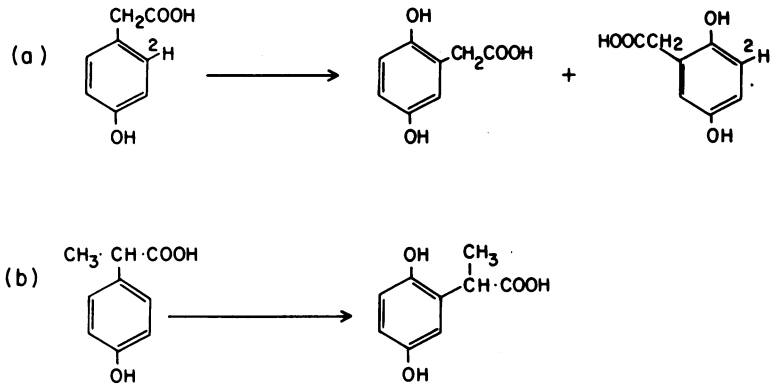

FIG. 8. *Reaction schemes to show retention of 50% of the deuterium of 2-deutero-4-hydroxyphenylacetic acid during hydroxylation (a); and formation of an analogue of homogentisic acid by hydroxylation of 4-hydroxyhydratropic acid (b).*

one enantiomer of synthetic 4-hydroxyhydratropic acid might be hydroxylated by the enzyme. To investigate this possibility, the product of the reaction was first isolated and shown to be α-methylhomogentisic acid (Fig. 8b).

To 4.5 ml of a solution containing 0.1 M phosphate buffer (pH 7.2), 3 mM orthophenanthroline, 1 mM DTE, 5 mM $MgSO_4$, and 10 μM FAD were added 60 μmol of *dl*-4-hydroxyhydratropic acid, 30 μmol of NADH, and 0.5 ml (10 mg of protein) of a fresh crude extract. The solution was stirred slowly at 25 C for 60 min and then deproteinized with metaphosphoric acid and centrifuged. The clear supernatant solution was extracted with a mixture of chloroform-acetone (1:1, vol/vol) and the combined solvent phases dried over anhydrous Na_2SO_4. The solvent was evaporated to leave a residue that was then converted to trimethylsilyl (TMS) derivatives as previously described (43) and analyzed by gas chromatography-mass spectrometry. Only two components were observed by gas chromatography; one gave the mass spectrum of the TMS derivative of 4-hydroxyhydratropic acid and the other gave a parent ion of m/e = 398 which is expected for the derivative of α-methylhomogentisic acid (3 TMS). Hydroquinone was not present in the residue from the enzymatic reaction.

Properties of enzymatically formed α-methylhomogentisic acid. This compound was isolated as follows from a reaction mixture of 20 ml of phosphate buffer containing orthophenanthroline, DTE, and cofactors used in the previous experiment. After the addition of 120 μmol of *dl*-4-hydroxyhydratropic acid, 200 μmol of NADH, and 2 ml of crude extract (40 mg of protein), the solution was stirred slowly at 25 C for 3 h, deproteinized with metaphosphoric acid, clarified by centrifugation, and saturated with NaCl. This solution was extracted five times with 100-ml portions of ethyl acetate and then with the same quantities of diethylether. Combined solvent phases were dried over Na_2SO_4 and concentrated to about 0.1 ml by evaporation under a stream of N_2 at room temperature. The concentrated extract was applied as a band to a preparative thin-layer chromatography plate which was developed in benzene-ethyl acetate-formic acid (10:2:1, vol/vol). *dl*-4-Hydroxyhydratropic acid and homogentisic acid were spotted at the edge of the plate as standards to assist in locating the position of α-methyl-homogentisic acid. Bands were located visually under short-wave ultraviolet light. Two bands were observed: one due to residual 4-hydroxyhydratropic acid and the other formed by a compound with approximately the same R_f as homogentisic acid (homogentisate, 0.25; unknown, 0.30). Both bands were scraped from the plate and compounds were eluted from the silica gel with absolute ethanol. Ethanol was removed by evaporation in the dark at room temperature under a stream of N_2. The yield of presumed α-methylhomogentisic acid was 8.0 mg. Analysis by mass spectrometry (direct probe) showed a parent ion having m/e = 182 as expected for α-methylhomogentisic acid and also a prominent peak at m/e = 137 corresponding to the loss of one carboxyl group from the molecular ion. The ultraviolet spectrum of α-methylhomogentisic acid was very similar to that of homogentisic acid (39), showing strong light absorption between 250 nm and 350 nm (λ_{max} at 290 nm; log ε = 3.58; pH 4.8). When each compound was allowed to stand in air in phosphate buffer, pH 7.2, spectral changes took place similar to those

investigated for homogentisate by Milch et al. (39). Enzymatically formed α-methylhomogentisic acid was optically active. The observed optical rotation for a solution of 8.0 mg in 7 ml of deionized water was −20 millidegrees for a cell of 0.2-decimeter light path. The yield of 8.0 mg of α-methyl-homogentisic acid obtained is 73% of that expected from 60 μmol of one enantiomer of 4-hydroxyhydratropic initially present in the enzymatic reaction mixture.

The 4-hydroxyhydratropic acid remaining after completion of the enzymatic reaction was recovered as the second band observed in thin-layer chromatography; the yield was 55 μmol, or 45.8% of the synthetic 4-hydroxyhydratropate used. The isolated compound had mp 155 to 158 C compared with mp 129 C for *dl*-4-hydratropic acid, suggesting an efficient resolution of the racemic compound. When a solution of 2.5 mg of recovered 4-hydroxyhdyratropic acid in 7.0 ml of deionized water was examined in the polarimeter, an optical rotation of −4 millidegrees was observed. Recovered 4-hydroxyhydratropate was not a substrate for 4-HPA 1-hydroxylase. The mass spectra of *dl*-4-hydroxyhydratropic acid and the recovered compound were identical, each showing a parent ion at m/e = 166.

Oxidation of 4-hydroxyphenoxyacetic acid. Ultracentrifuged extracts catalyzed oxidation of NADH on addition of 4-hydroxyphenoxyacetic acid (Table 3), but the stoichiometry of the reaction differed from that observed for 4-HPA insofar as 2 mol of NADH were oxidized for each mol of O_2 consumed. Moreover, an investigation of the products formed showed that the enzymatic reaction differed chemically: release of the side chain, rather than migration, appeared to be involved. In this experiment, the reaction mixture in 4.5 ml of 0.1 M phosphate buffer, pH 7.2, was the same as that used to isolate α-methylhomogentisic acid, except that 4-hydroxyphenoxyacetic acid (10 mg) was added as substrate in place of 4-hydroxyhydratropic acid. After stirring for 90 min at 25 C, the mixture was deproteinized with metaphosphoric acid and the supernatant solution was clarified by centrifugation. The pH was adjusted to 2 by addition of sulfuric acid, and the solution was then extracted three times with 100-ml portions of chloroform-acetone (1:1, vol/vol). The solvent phases were combined, dried over Na_2SO_4, and taken to dryness. Components of the mixture were then separated as TMS derivatives by gas chromatography, and peaks were scanned in the mass spectrometer. The only TMS derivative observed, in addition to that of unchanged 4-hydroxyphenoxyacetic acid, had the same retention time as the TMS derivative of authentic hydroquinone; moreover, the parent ions of authentic and isolated material both had m/e = 254.

It is evident that the side chain of 4-hydroxyphenoxyacetate was removed by cell extracts when hydroquinone was formed, but it proved difficult to identify the compound released. Our chromatographic procedures were similar to those adopted by Gamar and Gaunt (20) when they demonstrated that glyoxylate was released during the conversion of 4-chloro-2-methylphenoxyacetate into 5-chloro-*o*-cresol by extracts of a species of *Pseudomonas*. We found that the residue from which the TMS derivative of hydroquinone was isolated contained material that appeared to be polymeric in nature since it was immobile in the solvent systems used; however, no trace of glyoxylic, glycolic, or oxalic acids could be detected. Similar polymeric material was obtained when glycolic acid was taken through the procedure used to isolate hydroquinone; however, direct determinations of glycolate formed in reaction mixtures were not attempted since products from the acid decomposition of NADH interfered with the method of estimation used (13). Glycolic acid was eventually identified as a product by applying the solution from the enzymatic reaction directly to a column (33 by 1 cm) of Dowex 1-X8 resin (formate form), followed by elution with 0.5% formic acid. The elution profile for the reaction product, determined by the procedure of Dagley and Rogers (13), was the same as that for authentic glycolic acid. Compounds that interfered with these estimations were removed by the Dowex column. Yields of glycolate were not determined, since recovery from the column was not quantitative.

Hydroquinone was also formed by washed suspensions of cells grown with 4-HPA. A solution containing 50 mg of 4-hydroxyphenoxyacetate, 20 mg of chloramphenicol, and 1 g (wet weight) of cells was shaken at 30 C for 4 h, the cells were removed by centrifugation, and the reddish colored supernatant was brought to pH 2 with 2 N sulfuric acid. After extracting three times with 100-ml portions of chloroform-acetone (1:1, vol/vol) and once with 100 ml of chloroform, organic solvent phases were combined, dried over Na_2SO_4, and evaporated to dryness. Analysis by thin-layer chromatography showed spots due to unchanged 4-hydroxyphenoxyacetic acid, chloramphenicol, hydroquinone, and various unidentified colored products, probably arising from oxidation of

hydroquine. Preparative thin-layer chromatography in benzene-ethyl acetate-formic acid (10:2:1, vol/vol) yielded 3 mg of crystalline material showing a mass spectrum identical to that of authentic hydroquinone.

DISCUSSION

We have shown that when 2-deutero-4-hydroxyphenylacetic acid is oxidized by 4-HPA 1-hydroxylase, migration of the intact side-chain substituent to give homogentisic acid results in retention of half the deuterium originally present (Fig. 8a). Some features of this reaction resemble those of the enzymatic decarboxylation of 4-hydroxyphenylpyruvic acid, which also gives rise to homogentisic acid. This system has been studied extensively in mammalian liver (23, 33, 34) and is generally assumed to operate when tyrosine is degraded by bacteria. However, the mammalian enzyme system differs from bacterial 4-HPA 1-hydroxylase in several respects. Thus, the side chain of 4-hydroxyphenylpyruvate does not migrate intact (33). Hydroxylation of the nucleus, and the shift and simultaneous decarboxylation of the side chain, appear to proceed by a concerted reaction during which two atoms of oxygen are incorporated into homogentisate (36). Accordingly, the recommended name of this enzyme is now 4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27, formerly EC 1.14.2.2). Further, whereas 4-HPA 1-hydroxylase like other bacterial hydroxylases (19, 21, 26, 38, 40, 48) requires FAD as coenzyme and a reduced pyridine nucleotide as substrate, the electrons necessary for hydroxylation by 4-hydroxyphenylpyruvate dioxygenase are furnished through decarboxylation of the 2-ketoacid side chain, and not by a second donor. Blakley (4) reported that 4-hydroxyphenylpyruvate was a substrate, though a poor one, for 4-HPA 1-hydroxylase. We found that 4-hydroxyphenylpyruvate was not a substrate for our preparations of the enzyme when assayed from rates of oxidation of NADH; however, cell extracts readily attacked 4-hydroxyphenylpyruvate, without addition of NADH, to give compounds having the same spectral characteristics as intermediates of the homogentisate catabolic pathway. This enzymatic activity was retained by extracts when stored at 4 C for 2 days in the absence of DTE, whereas 4-HPA 1-hydroxylase activity was lost completely under these conditions. It is evident that 4-HPA 1-hydroxylase was not responsible for the ability of our strain of *P. acidovorans* to degrade 4-hydroxyphenylpyruvate. Extracts apparently contained a bacterial 4-hydroxyphenylpyruvate dioxygenase, and the factors influencing the derepression of this enzyme are currently under investigation. It may be noted that another hydroxylase for 4-HPA has been reported, namely 4-HPA 3-hydroxylase (EC 1.14.13.3). This enzyme was present in cell extracts of *Pseudomonas ovalis* grown with 4-HPA as carbon source (1), and it functions to initiate a degradative pathway by which pyruvate and succinic semialdehyde (43) are formed from homoprotocatechuate (3,4-dihydroxyphenylacetate). Our strain of *P. acidovorans* does not elaborate this enzyme; moreover, homoprotocatechuate is a strong competitive inhibitor of 4-HPA 1-hydroxylase.

In agreement with Blakley's observations (4), we found that 4-HPA 1-hydroxylase from *P. acidovorans* differed from other bacterial hydroxylases (19, 21, 26, 38, 40, 48) in showing a specific requirement for Mg^{2+} ions. Also, NADPH could replace NADH; however, the apparent K_m for the former was 2.6 times greater than for NADH, although the observed maximum velocities were the same. In this respect, and also with regard to the inhibition by chloride ions, the enzyme resembles salicylate hydroxylase (48); but, in contrast, inhibition of the latter enzyme was competitive with salicylate, whereas for 4-HPA 1-hydroxylase inhibition by chloride was uncompetitive with 4-HPA. Cleland (10) has observed that uncompetitive inhibition appears to be encountered exclusively in multireactant systems.

We tested 19 analogues of 4-HPA as possible substrates or inhibitors (Table 3). The three most powerful inhibitors, namely 4-hydroxy-3-methylphenylacetic acid, 3,4-dihydroxyphenylacetic acid, and 4-hydroxymandelic acid, were competitive with 4-HPA. Each of these compounds possesses a free carboxyl group joined through one carbon atom to a benzene nucleus that bears a hydroxyl group substituent at C-4. These structural features are also exhibited by all of the six analogues of 4-HPA that behaved as substrates in Table 3, with two exceptions: 4-hydroxyphenoxyacetic acid and 4-hydroxyphenylpropionic acid. An oxygen atom and a methylene group are interposed, respectively, between the benzene nucleus and the two-carbon side chain in these compounds. These structural requirements may reflect the presence of binding sites on the enzyme serving, respectively, to accommodate the hydroxyl group at C-4 of the nucleus and the side chain at C-1. Enzymatic activity was shown towards hydroxyphenylpropionic acid and by one enantiomer of 4-hydroxyhydratropic acid, whereas

dl-4-hydroxymandelic acid was a strong inhibitor. It therefore appears that binding is not abolished when the side chain is lengthened by one carbon atom or when a hydrogen atom on the α-carbon is substituted by a methyl or hydroxyl group placed in the appropriate configuration. However, enzymatic activity was no longer shown when the side chain was shortened or the carboxyl group was esterified, since 4-hydroxybenzoic acid and methyl 4-hydroxyphenylacetate served neither as substrates nor as effective inhibitors. It may be mentioned that ability to stimulate oxidation of NADH (Table 3) does not necessarily indicate that an analogue is hydroxylated. Thus, certain analogues of substrates merely facilitate transfer of electrons from the reduced pyridine nucleotide to oxygen, forming hydrogen peroxide; this "uncoupling" of hydroxylation has been reported for benzoate with salicylate hydroxylase (48), 6-hydroxynicotinate with 4-hydroxybenzoate hydroxylase (28), and *m*-cresol with orcinol hydroxylase (41). Since our preparations of 4-HPA 1-hydroxylase possessed some catalase activity, we did not invesitgate uncoupling, but we confined attention to showing that the two analogues with activities approaching that of 4-HPA, namely 4-hydroxyphenoxyacetic acid and 4-hydroxyhydratropic acid (Table 3), were in fact oxygenated.

Substrate activity, assayed by measuring NADH oxidation, was not detected for compounds in which the hydroxyl group at C-4 was replaced by H, Cl, F, methyl, or methoxyl, nor for 2- and 3-hydroxyphenylacetic acids. The reaction schemes of Fig. 9 provide an explanation for this particular structural requirement, and also for the fact that the reaction takes a totally different course when 4-hydroxyphenoxyacetic acid serves as substrate. The precise nature of active oxygen that attacks a carbon atom during enzymatic hydroxylation is not known; but since substitution invariably occurs at positions in the nucleus where electrons are most readily available, namely *ortho* or *para* to the hydroxyl already present (8, 12), it is assumed that the attacking species is electrophilic and that it may be shown symbolically as OH^+ (Fig. 9). A plausible scheme to explain the role of reduced FAD in generating an electrophilic species of oxygen has been discussed by Hamilton (24). In Fig. 9 it is suggested that the indispensable function of the hydroxyl group at C-4 stems from its ability to ionize and thereby supply electrons required for hydroxylation at C-1. The resulting benzoquinone derivative shows structural similarities to certain intermediates that are thought to be formed during catalysis by 4-hydroxyphenylpyruvate dioxygenase (36). A rearrangement of electrons then leaves C-2 with a positive charge and results in a shift of the carboxymethyl side chain to this position. Molecular rearrangements by 1,2 shifts of alkyl groups or hydrogen atoms are not uncommon when carbonium ions are generated by electrophilic reagents (25), and such a mechanism has been suggested to account for the migration of groups observed during enzymatic conversion of squalene to cholesterol (3).

The enzymatic conversion of 4-HPA into homogentisate bears some resemblance to the "NIH shifts," observed for isotopes of hydrogen,

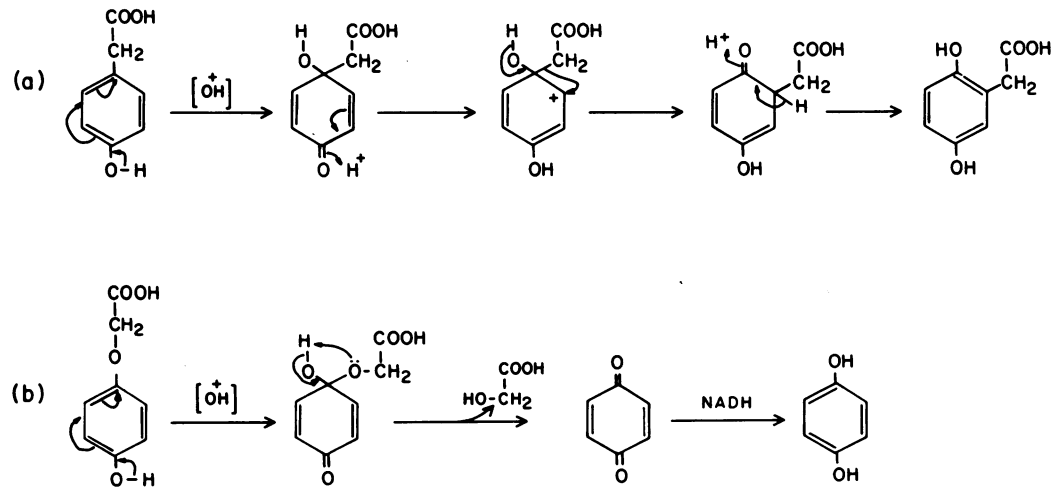

FIG. 9. *Reaction mechanisms suggested for hydroxylation of 4-hydroxyphenylacetic acid (a), and 4-hydroxyphenoxyacetic acid (b).*

halogen atoms, and methyl groups which take place during hydroxylation of various aromatic compounds by liver microsomes (22) and enzymes from other sources (18). There is strong evidence that arene oxides (epoxides) are formed as reaction intermediates during hydroxylation by these enzymes (18, 30). However, it is probable that 4-HPA 1-hydroxylase operates by an entirely different mechanism. Thus, when the microsomal system catalyzes hydroxylation *para* to an ionizable group (such as OH in Fig. 9a), deuterium is eliminated from the nucleus and does not undergo a shift to an adjacent carbon atom (17); whereas, by contrast, all the substrates and effective inhibitors of 4-HPA possessed a hydroxyl group at this position.

When 4-hydroxyphenoxyacetate was attacked by 4-HPA 1-hydroxylase, the side chain was released and hydroquinone was identified unequivocally as a reaction product. The formation of glycolate was difficult to establish; but the side chain was not removed as glyoxylate, neither was this compound reduced by NADH in the presence of cell extracts. It may be concluded, therefore, that the α-carbon of the side chain was not oxidized, as was found for 4-chloro-2-methylphenoxyacetate (20); instead, we propose that 4-hydroxyphenoxyacetate, like other substrates of 4-HPA 1-hydroxylase, is attacked at C-1 of the nucleus (Fig. 9b). The presence of oxygen in the side chain would be expected to facilitate fission and so divert the shift of electrons from the formation of a carbonium ion that favors migration. Benzoquinone would be a direct product of side chain release. However, cell extracts contained an active benzoquinone reductase, so that in the presence of NADH the expected product would be the compound actually isolated, namely hydroquinone, formed from 4-hydroxyphenoxyacetate with concomitant oxidation of 2 mol of NADH per 1 mol of O_2 consumed. A benzoquinone reductase was reported to be present in bacterial extracts capable of degrading thymol (7). Despite the differences mentioned, the side chains of 4-chloro-2-methylphenoxyacetate and 4-hydroxyphenoxyacetate are probably released by similar mechanisms. Hydroxylation of the former compound gives a hemiacetal, and of the latter, a hemiketal (Fig. 9b). Decomposition of these unstable intermediates would give, in each case, two products containing respectively a carbonyl and a hydroxyl group arising from cleavage: 4-chloro-2-methylphenoxyacetate yielding glyoxylate and a phenol, 4-hydroxyphenoxyacetate yielding benzoquinone and hydroxyacetate (glycolate).

Although the side chain of 4-HPA is shown schematically to migrate from C-1 to C-2, structural features common to substrates and effective inhibitors suggest that the enzyme possesses a rather discriminating binding site for the side chain. Further, the fact that only one enantiomer of 4-hydroxyhydratropate was attacked while molecular asymmetry was retained in the product indicates that the side chain does not "migrate" but remains bound to the enzyme surface throughout the reaction. The protein may undergo a conformational change sufficient to bring C-2 of the nucleus into close proximity to the bound side chain: such a movement might occur as a consequence of carbonium ion formation shown in Fig. 9.

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