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Phthalate and 4-Hydroxyphthalate Metabolism in *Pseudomonas testosteroni*: Purification and Properties of 4,5-Dihydroxyphthalate Decarboxylase

TERUKO NAKAZAWA†* AND EMIKO HAYASHI

Department of Bacteriology, School of Medicine, Juntendo University, 2-1-1 Hongo, Bunkyoku, Tokyo, Japan

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Phthalate is degraded through 4,5-dihydroxyphthalate and protocatechuate in *Pseudomonas testosteroni* NH1000. The enzyme 4,5-dihydroxyphthalate decarboxylase, catalyzing the conversion of 4,5-dihydroxyphthalate to protocatechuate and carbon dioxide, was purified approximately 130-fold from phthalate-induced cells of a protocatechuate 4,5-dioxygenase-deficient mutant of *P. testosteroni*. The most purified preparation showed a single protein band on sodium dodecyl sulfate-acrylamide disc gel electrophoresis with a molecular weight of 38,000. The apparent molecular weight of the native enzyme determined by Sephadex G-200 column chromatography was 150,000. Among the substrate analogs tested, only 4-hydroxyphthalate served as a substrate, which was decarboxylated to form *m*-hydroxybenzoate. The apparent K_m values for 4,5-dihydroxyphthalate and 4-hydroxyphthalate were estimated to be 10.5 μM and 1.25 mM, respectively, and the V_{max} for the former was 10 times larger than that for the latter. Whereas the wild-type strain could utilize 4-hydroxyphthalate as a sole source of carbon, none of the following could grow with the compound: 4,5-dihydroxyphthalate decarboxylase-deficient, *m*-hydroxybenzoate-nondegradable, and protocatechuate 4,5-dioxygenase-deficient mutants. Since one-step revertants of these mutants could utilize 4-hydroxyphthalate, the compound appears to be metabolized through *m*-hydroxybenzoate and protocatechuate in *P. testosteroni* NH1000.

Phthalate esters have been synthesized on a massive scale for the last two to three decades, mainly for the formation of plastics, and considerable attention has been directed to the possible toxicity of these compounds (2). In recent years, several reports on the biodegradation of phthalate esters have been written. These reports have established that phthalate esters are easily biodegradable in the labroatory with pure cultures of bacteria (6, 9), as well as in activated-sludge digestion (18) and hydrosoils (8). Keyser et al. (9) demonstrated the presence of catabolic pathways of phthalate esters in pseudomonads and micrococci that are initiated by successvie hydrolysis of the diesters to give phthalate, which is then metabolized through 4,5-dihydroxyphthalate and protocatechuate. An enzyme, 4,5-dihydroxyphthalate decarboxylase (DHP decarboxylase), catalyzing the conversion of 4,5-dihydroxyphthalate to protocatechuate and carbon dioxide has been purified from a strain of *Pseudomonas fluorescens* (B. G. Pujar and D. W. Ribbons, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, K51, p. 145). Whereas *P. fluorescens* uses the β-ketoadipate pathway for the degradation of protocatechuate, *Pseudomonas testosteroni* generally uses a *meta*-cleavage pathway in which protocatechuate 4,5-dioxygenase catalyzes the opening of the benzene ring of protocatechuate (5).

We have been studying the phthalate metabolism in a strain of *P. testosteroni* and reported the isolation of a mutant that accumulates 4,5-dihydroxyphthalate after incubation with phthalate (14). This paper reports the purification and some properties of DHP decarboxylase of *P. testosteroni*. Evidence is presented that the enzyme is also involved in the biodegradation of 4-hydroxyphthalate in *P. testosteroni*.

MATERIALS AND METHODS

Bacterial strains. A strain of *P. testosteroni*, NH1000, and its derivative mutants (14) were used. For the source of DHP decarboxylase, a protocatechuate 4,5-dioxygenase-deficient mutant, NH1027, was used. This and other mutants were obtained as described previously (14).

Chemicals. 4,5-Dihydroxyphthalate was isolated from a reaction mixture containing phthalate-induced

† Present address: Department of Biochemistry, Yamaguchi University School of Medicine, Ube, Japan 755.

cells of a DHP decarboxylase-deficient mutant, using procedures described previously (14). 4-Hydroxyphthalate was obtained from Pfaltz and Bauer Inc., Stamford, Conn., ovalbumin was from Nutritional Biochemicals Corp., Cleveland, Ohio, and other standard proteins for gel filtration chromatography and electrophoresis were from Sigma Chemical Co., St. Louis, Mo. Diethylaminoethyl-cellulose, diethylaminoethyl-Sephadex A-50, and Sephadex G-200 were from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. All other chemicals were of reagent grade and commercially available.

Media and culture conditions. Medium M9 (4) without carbon sources was used as a basal medium. The basal medium was solidified with 1.5% agar (Eiken Chemicals Co. Ltd., Tokyo, Japan) and supplemented with aromatic substrates for studying the growth response. Incubations were carried out at 27°C. For the purification of DHP decarboxylase, cells were grown in basal medium supplemented with 20 mM L-glutamate, 5 mM phthalate, and 0.05% yeast extract (Difco Laboratories, Detroit, Mich.). Growth was allowed to proceed for approximately 20 h at 27°C with vigorous agitation. Cells were harvested by centrifugation and washed with saline. Cells were kept frozen at −20°C until needed.

Enzyme assay. The activity of DHP decarboxylase was assayed at 27°C by following the decrease in absorbance at 230 nm due to the conversion of 4,5-dihydroxyphthalate to protocatechuate (14). Measurements were made with a Union SM-401 high-sensitivity recording spectrophotometer. The reaction mixture contained 125 μmol of tris(hydroxymethyl)-aminomethane(Tris)-acetate (pH 7.5), 0.25 μmol of 4,5-dihydroxyphthalate, and a suitable addition of enzyme in a total volume of 2.5 ml. Under the standard assay conditions, the conversion of 1 μmol of the substrate to the product corresponded to a decrease of 2.2 optical density units at 230 nm. One unit of enzyme activity was defined as the amount of enzyme catalyzing the conversion of 1 μmol of substrate into product per min at 27°C. Protein was determined spectrophotometrically from the absorbance at 280 and 260 nm (10). The specific activity was defined as units of enzyme activity per milligram of protein.

Enzyme purification. All purification steps were carried out at 0 to 4°C, and all centrifugations were carried out at 10,000 × *g* for 60 min.

Acrylamide disc gel electrophoresis. For disc gel electrophoresis, the method described by Gabriel (7) was used, with 7.5% polyacrylamide gels at pH 8.3 and room temperature. Gels were stained for protein by immersion in 0.25% Coomassie brilliant blue R250 (Nakarai Chemicals, Ltd., Kyoto, Japan) for 2.5 h and destained in 7.5% acetic acid. Electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed as described by Weber et al. (19). The sample was reduced with 4% 2-mercaptoethanol in the presence of 2% SDS at room temperature overnight, and about 5 μg of DHP decarboxylase was applied to the gel.

Molecular weight determination by gel filtration. The method of Andrews (1) was slightly modified. The data were treated as described by Mann and Fish (12). The sample containing DHP decarboxylase (0.2 mg), protein markers (0.5 to 1.5 mg of each), blue dextran (0.5 mg), dinitrophenyl-alanine (0.3 mg), and sucrose (100 mg) in 0.5 ml of 0.05 M Tris-acetate (pH 7.5) supplemented with 0.2 mM dithiothreitol (DTT) was applied on a Sephadex G-200 column (1.5 by 90 cm) previously equilibrated with the same buffer. One milliliter of effluent with a flow rate of 3 ml/h was collected, and elution positions were determined by measuring for proteins at 230 nm, blue dextran at 630 nm, cytochrome *c* at 420 nm, and dinitrophenyl-alanine at 360 nm. The elution positions of alkaline phosphatase and glucose oxidase were determined by measuring the hydrolysis of *p*-nitrophenylphosphate (11) and glucose-dependent oxygen uptake (16), respectively.

RESULTS

Purification of DHP decarboxylase. A typical purification scheme is given in Table 1. In step 1, frozen cells (140 g, wet weight) of *P. testosteroni* NH1027 were thawed and suspended in 1,400 ml of 0.05 M Tris-acetate (pH 7.5) containing 0.2 mM each DTT and ethylenediaminetetraacetate. Cells were disrupted by sonic oscillation with a 20-kilocycle Tomy sonic oscillator, model UR 105P, for 15 min. After centrifugation, 1,470 ml of crude extract was obtained. In step 2, 78 ml of a 10% solution of streptomycin sulfate was added to the crude extract with stirring. The mixture was stirred for 60 min, and the precipitate was removed by centrifugation. In step 3, 376 g of ammonium sulfate (40% saturation) was added to the supernatant solution of step 2 (1,550 ml) with stirring. After the mixture was stirred for 120 min, the

TABLE 1. *Purification of DHP decarboxylase from P. testosteroni NH1027*

Step	Procedure	Vol (ml)	Protein (mg)	Total activity (units)	Sp act (units/mg)	Yield (%)	Purification (fold)
1	Crude extract	1,470	7,330	3,370	0.46	(100)	(1)
2	Streptomycin sulfate	1,550	7,170	3,370	0.47	100	1
3	Ammonium sulfate (40 to 60%)	167	3,120	1,840	0.59	55	1.3
4	DEAE-cellulose[a]	66	229	1,520	6.64	45	14.4
5	DEAE-Sephadex A-50	13.4	43.5	596	13.7	17.7	30.0
6	Sephadex G-200	34.1	9.2	542	58.9	16.1	128

[a] DEAE, Diethylaminoethyl.

precipitate was removed by centrifugation. Upon further addition of 222 g of ammonium sulfate to 1,690 ml of supernatant solution (60% saturation), the mixture was stirred for 120 min, and the precipitate was collected by centrifugation. The precipitate was dissolved in 160 ml of 0.05 M Tris-acetate (pH 7.5), containing 0.2 mM DTT, and dialyzed against 3,000 ml of the same buffer for 2 h and then overnight with a change of buffer. In step 4, the dialyzed preparation (167 ml) was passed through a diethylaminoethyl-cellulose column (4.4 by 50 cm) previously equilibrated with 0.07 M Tris-acetate (pH 7.5) containing 0.2 mM DTT. The column was washed with the same buffer, and nonadsorbed protein fractions were collected. The combined solution (1,075 ml) was concentrated with a Pellicon ultrafiltration system (Pellicon membrane PTGC; Millipore Corp., Bedford, Mass.) to a volume of 60 ml and dialyzed against 1,000 ml of 5 mM Tris-acetate (pH 7.5) containing 0.2 mM DTT. In step 5, the enzyme (66 ml) was applied to a column of diethylaminoethyl-Sephadex A-50 (3 by 28 cm) equilibrated with 5 mM Tris-acetate (pH 7.5) containing 0.2 mM DTT. After washing with 300 ml of the same buffer, the column was eluted with 0.05 M Tris-acetate (pH 7.0) containing 0.2 mM DTT and then with a linear gradient of 0 to 0.1 M ammonium sulfate in 400 ml of the same buffer. About 60% of the activity was lost by this step with a twofold purification. Fractions having a specific activity higher than 10 were combined (300 ml) and concentrated by ultrafiltration to a volume of 13.4 ml. In step 6, the enzyme solution was applied to a Sephadex G-200 column (2.7 by 70 cm) equilibrated with the same buffer. Fractions (5 ml) were collected, and the samples with a specific activity higher than 58.5 units/mg were combined.

The above steps gave a 128-fold purification from the crude extract, with a yield of 16.1%.

Molecular properties of DHP decarboxylase. The purified enzyme preparation gave one protein band when subjected to SDS-disc gel electrophoresis after reduction in the presence of SDS. When the same sample was subjected to polyacrylamide disc gel electrophoresis, however, several protein bands appeared, including a concentrated band at the interface between the stacking and separating gels. This latter band might be due to aggregation of the enzyme protein during electrophoresis. By chromatography on Sephadex G-200 gels, the activity eluted in a single protein peak (Fig. 1).

Based upon filtration by Sephadex G-200, an apparent molecular weight of 150,000 was estimated (Fig. 2). From SDS-disc gel electrophoresis, the molecular weight of 38,000 was estimated (Fig. 3). Thus, it can be tentatively assumed that DHP decarboxylase of *P. testosteroni* consists of four identical subunits.

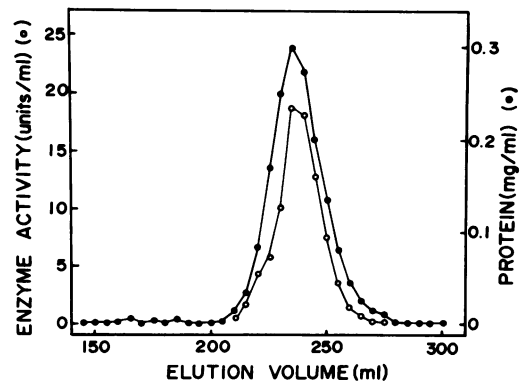

FIG. 1. *Sephadex G-200 column chromatography of purified DHP decarboxylase. Symbols:* (○) *activity of DHP decarboxylase;* (●) *protein.*

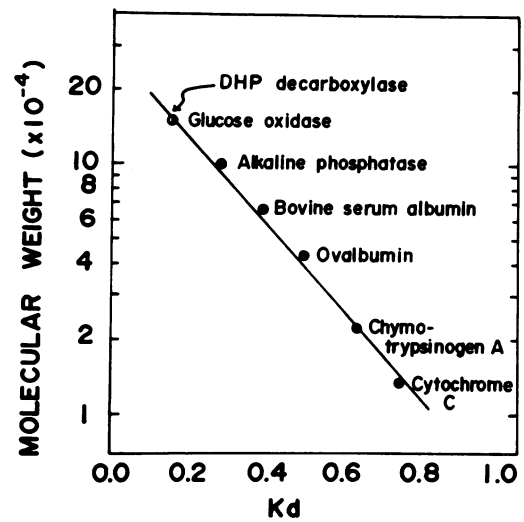

FIG. 2. *Determination of molecular weight of DHP decarboxylase by Sephadex G-200 column chromatography. Kd, Distribution coefficient.*

The purified enzyme was colorless and showed an ultraviolet spectrum with λ_{max} at 280 nm in 0.05 M Tris-acetate (pH 7.5).

Enzymatic properties. The purified enzyme was relatively unstable and lost half of its activity in 4 days in 0.05 M Tris-acetate (pH 7.5) containing 0.2 mM DTT at 4°C. It lost about 20% of its activity during incubation at 27°C for 3.5 h in 0.05 M Tris-acetate (pH 7.5), and more rapid inactivation occurred at a pH higher than 8.5 or lower than 6.5. The loss of activity was partially prevented by the addition of 0.2 mM DTT.

The pH optimal for DHP decarboxylase reaction was determined in 0.05 M Tris-acetate or

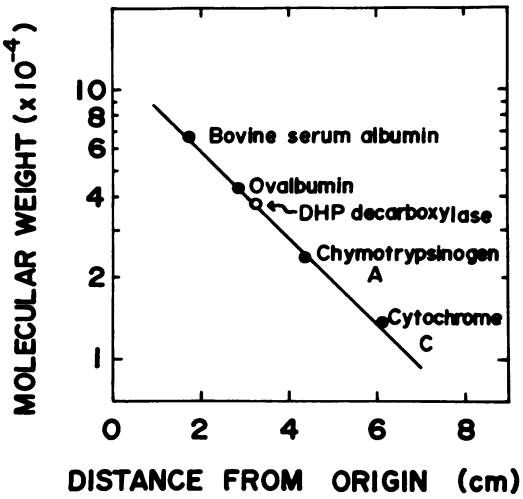

FIG. 3. *Determination of molecular weight of DHP decarboxylase by SDS-disc gel electrophoresis.*

potassium phosphate buffer in the range of pH 7.0 to 9.0 at 27°C. The optimum pH was approximately 7.5 with either buffer, and Tris-acetate permitted slightly higher activity than potassium phosphate buffer.

When the initial velocity of the reaction was determined with varying substrate concentrations, a linear Lineweaver-Burk plot was obtained. The apparent K_m value for 4,5-dihydroxyphthalate was estimated to be 10.5 μM, and the V_{max} was 100 units/mg of purified enzyme.

In addition to 4,5-dihydroxyphthalate, 4-hydroxyphthalate was also attacked by the enzyme. The absorption spectrum of 0.2 mM 4-hydroxyphthalate in 0.05 M Tris-acetate (pH 7.5) was changed by incubation with the enzyme, and the final spectrum obtained was identical to that of 0.2 mM *m*-hydroxybenzoate in the same buffer (Fig. 4). Since the difference between the absorbance of 4-hydroxyphthalate and that of *m*-hydroxybenzoate was maximum at 250 nm, the reaction velocity was determined by following the decrease in the absorbance at this wavelength. The conversion of 1 μmol of 4-hydroxyphthalate to *m*-hydroxybenzoate corresponded to a decrease of 2.1 optical density units at 250 nm in a reaction mixture of 2.5 ml. Thus, the apparent K_m for 4-hydroxyphthalate was estimated to be 1.25 mM from a Lineweaver-Burk plot, and the V_{max} value was 10 μmol/min per mg of purified enzyme, which was 10 times smaller than that when 4,5-dihydroxyphthalate was used as a substrate. The ultraviolet spectra of the following compounds at a concentration of 0.2 mM in 0.05 M Tris-acetate (pH 7.5) did not change after incubation with 11 μg of DHP decarboxylase at 27°C for 16 h: phthalate, terephthalate, isophthalate, anthranilate, benzoate, salicylate, *p*-hydroxybenzoate, *m*-hydroxybenzoate, and gentisate.

Substrate analogs were also tested as inhibitors (Table 2). Several di- and monocarboxylic compounds inhibited the reaction at a concentration 10 times greater than that of the substrate, whereas hydroxylic compounds were less inhibitory. Inhibition of the enzyme by terephthalate or anthranilate was found to be competitive with respect to the substrate. These findings suggest that the carboxyl group of 4,5-dihydroxyphthalate is more important than the hydroxyl group for the formation of an enzyme-substrate complex.

The enzyme was not inactivated by dialysis

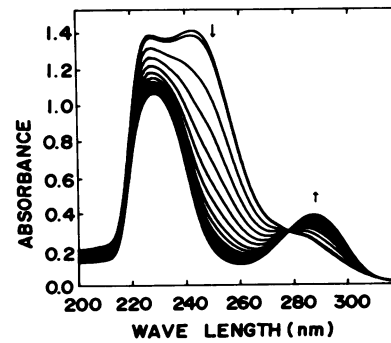

FIG. 4. *Absorption spectra of reaction mixture of 4-hydroxyphthalate and DHP decarboxylase. The reaction mixture contained 0.2 mM 4-hydroxyphthalate, 50 mM Tris-acetate (pH 7.5), and purified enzyme (8.8 μg) in a total volume of 2.5 ml. Reference cuvette contained the reaction mixture without 4-hydroxyphthalate. The initial spectrum was recorded before the addition of enzyme. Spectra were recorded automatically at 5-min intervals at 27°C, using a repeat scan programmer. Arrows show a decrease (↓) or increase (↑) in absorption at the indicated wavelength.*

TABLE 2. *Inhibition of DHP decarboxylase by substrate analogs*[a]

Inhibitor	Inhibition (%)
None	0
Terephthalate	50
Anthranilate	39
Salicylate	33
Phthalate	32
Benzoate	31
p-Hydroxybenzoate	28
Protocatechuate	24
m-Hydroxybenzoate	23
Gentisate	15
3-Methylcatechol	10
4-Methylcatechol	3

[a] The initial rate of reaction was determined with a reaction mixture containing 0.05 μmol of 4,5-dihydroxyphthalate, 125 μmol of Tris-acetate (pH 7.5), and DHP decarboxylase (44 μg) in the absence or presence of 0.5 μmol of inhibitor.

against a 0.2 mM concentration of either ethylenediaminetetraacetate or *o*-phenanthroline in 0.05 M Tris-acetate (pH 7.5) containing 0.2 mM DTT at 4°C overnight, nor was it activated by 1 mM $MgCl_2$ without or with 1 μM pyridoxal 5'-phosphate. On the other hand, the enzyme was inactivated by sulfhydryl reagents such as *p*-chloromercuribenzoate, $HgCl_2$, $AgCl_2$, and 5,5'-dithiobis(2-nitrobenzoate) at a concentration of 0.01 to 0.1 mM, and the inactivated enzyme was partially reactivated by further incubation with 1 mM DTT. The enzyme was also inactivated by 0.01 mM 2,4,6-trinitrobenzene-sulfonate, and the inactivation was partially prevented in the presence of 4,5-dihydroxyphthalate or 4-hydroxyphthalate at a concentration of 1 mM. Carbonyl group reagents such as hydrazine, phenylhydrazine, and hydroxylamine, at a concentration of 1 mM, did not inactivate DHP decarboxylase. Addition of 10 mM $NaBH_4$ during the reaction did not affect the reaction velocity.

4-Hydroxyphthalate metabolism in *P. testosteroni*. Since 4-hydroxypthalate was found to be a substrate for DHP decarboxylase, the biodegradation of this compound was studied. The wild-type strain, NH1000, could utilize 4-hydroxyphthalate as a sole source of carbon, whereas the DHP decarboxylase-deficient mutant, NH1024, could not grow with this compound (Table 3). A revertant obtained by spreading a thick cell suspension of NH1024 on phthalate agar also gained the ability to grow with 4-hydroxyphthalate. Accordingly, DHP decarboxylase is required for the biodegradation of both phthalate and 4-hydroxyphthalate. Based upon the absorption spectra of the reaction mixture of 4-hydroxyphthalate and purified DHP decarboxylase, the reaction product was assumed to be *m*-hydroxybenzoate. The identity of this reaction product was also confirmed by the finding that an *m*-hydroxybenzoate-nondegradable mutant, NH1028, was unable to grow with 4-hydroxyphthalate, whereas the one-step revertant of NH1028 selected on *m*-hydroxybenzoate could grow with 4-hydroxyphthalate. In *P. testosteroni*, *m*-hydroxybenzoate is commonly metabolized to protocatechuate by the action of 3-hydroxybenzoate 4-hydroxylase (13). Therefore, 4-hydroxyphthalate is metabolized in *P. testosteroni* through *m*-hydroxybenzoate and protocatechuate by the successive action of DHP decarboxylase and 3-hydroxybenzoate 4-hydroxylase (Fig. 5). Protocatechuate is then degraded via the *meta*-cleavage pathway initiated by the action of protocatechuate 4,5-dioxygenase.

TABLE 3. *Growth response of P. testosteroni NH1000 and derived mutants*

Strain	Growth substrate phenotype[a]				
	Pht	4Hp	Mhb	Phb	Pro
NH1000 (wild type)	+	+	+	+	+
NH1024	−	−	+	+	+
NH1024 (revertant)	+	+	+	+	+
NH1028	+	−	−	+	+
NH1028 (revertant)	+	+	+	+	+
NH1027	−	−	−	−	−
NH1027 (revertant)	+	+	+	+	+
NH1032	+	+	+	−	+

[a] +, Growth; −, no growth. Phenotype abbreviations and concentrations of compounds in M9 agar: Pht, phthalate, 10 mM; 4Hp, 4-hydroxyphthalate, 5 mM; Mhb, *m*-hydroxybenzoate, 10 mM; Phb, *p*-hydroxybenzoate, 10 mM; Pro, protocatechuate, 5 mM.

DISCUSSION

DHP decarboxylase, an enzyme responsible for the biodegradation of phthalate, has been purified about 130-fold from the crude extract of a protocatechuate 4,5-dioxygenase-deficient mutant of *P. testosteroni*. The purified preparation showed a single protein band on SDS-disc gel electrophoresis after reduction in the presence of SDS. The same preparation, however, showed several bands upon acrylamide disc gel electrophoresis. A similar observation was made on purified DHP decarboxylase from *P. fluorescens* (Pujar and Ribbons, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, K51, p. 145). Pujar and Ribbons reported that the purified preparation showed six bands on acrylamide disc gel electrophoresis, but a single band was obtained after reduction and treatment with SDS. Therefore, DHP decarboxylase, in general, appears to have the ability to aggregate, showing many bands on acrylamide disc gel electrophoresis. The homogeneity of DHP decarboxylase from *P. testosteroni*, however, was not extensively studied, and the possibility still remains that the purified preparation contained contaminant proteins with the same mobility on SDS-disc gel electrophoresis. The purified enzyme was rather unstable, and further attempts at purification have been unsuccessful.

The apparent molecular weight of the native enzyme determined by Sephadex G-200 filtration was 150,000, which was about one-third that of the *P. fluorescens* enzyme (Pujar and Ribbons, personal communication). Since the reduced enzyme in the presence of SDS showed a molecular weight of 38,000 by SDS-disc gel electrophoresis, it was tentatively assumed that DHP decarboxylase from *P. testosteroni* consists of four identical subunits.

The purified enzyme appeared to have no prosthetic group with absorption in the visible range and was inactivated by a low concentration of 5,5'-dithiobis(2-nitrobenzoate). This inactivation was prevented in the presence of sub-

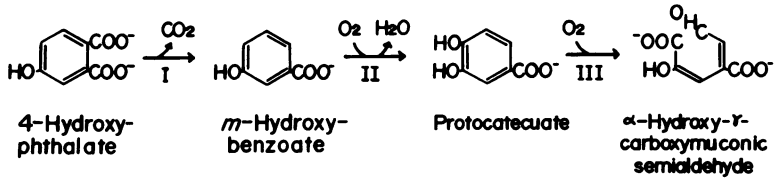

FIG. 5. *Metabolism of 4-hydroxyphthalate in P. testosteroni. I, DHP decarboxylase; II, 3-hydroxybenzoate 4-hydroxylase; III, protocatechuate 4,5-dioxygenase.*

strate. These results suggest a possible involvement of an ϵ-amino group of lysine residue for the enzyme activity. Furthermore, a predominant role of the carboxyl group of 4,5-dihydroxyphthalate has been suggested by a competitive-type inhibition of DHP decarboxylase by terephthalate and anthranilate. Therefore, an ionic interaction between the lysine ϵ-amino group of the enzyme and a carboxyl group of the substrate may be important for the formation of an enzyme-substrate complex. Amino acid decarboxylases usually contain pyridoxal phosphate as a prosthetic group and require Mg^{2+} for the reaction (3). Histidine decarboxylase from *Lactobacillus*, however, has no pyridoxal phosphate but contains covalently bound pyruvate as a prosthetic group (17). On the other hand, acetoacetate decarboxylase has lysine residue at the active center (15). Although these decarboxylases contain different prosthetic groups, they are exclusively inactivated by carbonyl reagents and also by $NaBH_4$. None of these compounds inactivates DHP decarboxylase from *P. testosteroni*. Further studies are necessary to elucidate the reaction mechanism of DHP decarboxylase.

Among the substrate analogs tested, only 4-hydroxyphthalate was found to serve as a substrate for DHP decarboxylase. Although the catalytic activity of the enzyme was unfavorable on 4-hydroxyphthalate, our studies with mutants showed that DHP decarboxylase is responsible for the biodegradation of 4-hydroxyphthalate in *P. testosteroni*. Thus, the wild-type strain, but not a DHP decarboxylase-deficient mutant, could utilize the compound as a sole source of carbon. The involvement of *m*-hydroxybenzoate as an intermediate from 4-hydroxyphthalate to protocatechuate was evidenced by the finding that an *m*-hydroxybenzoate-nondegradable mutant could not grow on 4-hydroxyphthalate. As far as we know, this is the first report on the biodegradation of 4-hydroxyphthalate by bacteria.

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