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Pseudomonas cepacia 3-Hydroxybenzoate 6-Hydroxylase: Induction, Purification, and Characterization†

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ABSTRACT: A single strain of *Pseudomonas cepacia* cells was differentially induced to synthesize salicylate hydroxylase, 3-hydroxybenzoate 6-hydroxylase, or 4-hydroxybenzoate 3-hydroxylase. A procedure was developed for the purification of 3-hydroxybenzoate 6-hydroxylase to apparent homogeneity. The purified hydroxylase appears to be a monomer with a molecular weight of about 44 000 and exhibits optimal activity near pH 8. The hydroxylase contains one FAD per enzyme molecule and utilizes NADH and NADPH with similar efficiencies. The reaction stoichiometry for this enzyme has been determined. In comparison with other aromatic flavohydroxylases, this enzyme is unique in inserting a new hydroxyl group to the substrate at a position para to an existing one.

Microbial flavoprotein hydroxylases are a class of external monooxygenases. Most of such flavohydroxylases are induced in soil pseudomonads to catalyze the hydroxylation of various benzenoids including pollutants and toxicants. The hydroxylated products are readily subject to further catabolism to produce metabolites that can be used by microbes for growth. Through the initial action of hydroxylases, many benzenoid compounds can be degraded and detoxified by microbial actions (Stanier & Ornston, 1973).

Although enzyme induction is an important property shared by microbial flavohydroxylases, the nature of the induction mechanism(s) is not well understood. With respect to the inducer–induced enzyme relationship, our understanding is mostly limited to the observation that a single strain of microorganism can be induced by using a benzenoid compound as the sole carbon source for growth to synthesize a specific flavohydroxylase. This induced hydroxylase is capable of catalyzing the hydroxylation of the very same benzenoid used as the carbon source for growth [Massey and Hemmerich (1975) and references cited therein]. In one case, the differential induction of orcinol and resorcinol hydroxylases (using orcinol and resorcinol, respectively, for induction) in cells of *Pseudomonas putida* ORC has been established (Ohta & Ribbons, 1976). The question as to whether a single strain of microbe can be differentially induced to synthesie multiple species of flavohydroxylases deserves further investigations.

Using a strain of *Pseudomonas cepacia* cells identified in our laboratory, we have previously reported the induction and isolation of salicylate hydroxylase (Tu et al., 1981; Wang & Tu, 1984). In this work, we demonstrate that *m*-hydroxybenzoate hydroxylase and *p*-hydroxybenzoate hydroxylase can also be differentially induced in the same strain of cells. The newly induced *m*-hydroxybenzoate hydroxylase has been purified to apparent homogeneity and its general structural and catalytic properties have been characterized. On the basis of the position of substrate hydroxylation, this hydroxylase is designated 3-hydroxybenzoate 6-hydroxylase. The feature that the new hydroxyl group is inserted at a position para to the existing hydroxyl function is unique among known flavohydroxylases that utilize benzenoid substrates.

Experimental Procedures

Materials. Salicylic acid, *m*-hydroxybenzoic acid, and *p*-hydroxybenzoic acid, obtained from Aldrich, were recrystallized twice from hot water. For large scales of cell growth for enzyme purifications, these benzenoid acids were used directly as supplied. 2,5-Dihydroxybenzoic acid was a product of Eastman, and all other derivatized benzoic acids used were from Aldrich. NADH, dithiothreitol, FAD, venom phosphodiesterase I (type VII), deoxyribonuclease I (type III), and horse liver alcohol dehydrogenase were all purchased from Sigma. FAD was further purified by DEAE-cellulose chromatography (Massey & Swoboda, 1963). Hen egg white lysozyme was from Boehringer-Mannheim GmbH. DEAE-Sephadex A-50, Sephadex G-100, PBE 94 chromatofocusing exchanger, and polybuffer 74 were products of Pharmacia. Hydroxyapatite (Bio-Gel HTP) and horseradish peroxidase

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labeled goat anti-rabbit IgG[1] were both from Bio-Rad. Nitrocellulose Ba85 was purchased from Shleicher & Schuell. Concentrations of the following compounds were determined spectrophotometricaly with extinction coefficients, in M^{-1} cm^{-1}, of 6220 at 340 nm for NADH, 2290 at 288 nm for *m*-hydroxybenzoate (Lang, 1968), 4060 at 320 nm for gentisate (determined in this work), and 11 300 at 450 nm for FAD.

Cell Growth. In all cases, growth of *P. cepacia* cells (Tu et al., 1981; Wang & Tu, 1984) was maintained at 23 °C. For induction experiments, cells from a culture stock were first revived in 1% yeast extract and subcultured in a minimal medium supplemented with 0.2% salicylic acid as the sole carbon source. The minimal medium contained, per liter, 1 mL of a heavy metal solution (White-Stevens & Kamin, 1972), 0.042 g of $CaCl_2 \cdot 2H_2O$, 1.26 g of KNO_3, 0.2 g of $MgSO_4 \cdot 7H_2O$, 2.62 g of K_2HPO_4, 1.36 g of KH_2PO_4, and 0.3 mg of riboflavin. When the cells reached late logarithmic phase, 0.5-mL aliquots were withdrawn, and each was inoculated into 40 mL of the same minimal medium supplemented with 0.2% of a desired benzenoid carbon source. For enzyme isolations, cells were grown with vigorous shaking in multiple flasks (2-L Erlenmeyer) each containing 1 L of the minimal medium supplemented with 0.2% of a desired benzenoid carbon source. The concentration of the benzenoid compound was maintained between 0.05 and 0.25% throughout the cell growth by occasional additions of the benzenoid acid. The pH of the culture medium was usually between 6.5 and 7.5 and should be adjusted to this range during growth if necessary. Bacterial growth was monitored by optical density readings at 600 nm. When OD_{600} reached 4–5, cells were harvested by centrifugation at 4 °C and 10000*g* for 15 min. The yields were about 4 g of wet cells/L of medium.

Enzyme Assays. *m*-Hydroxybenzoate hydroxylase activities were determined at 23 °C by measuring the rate of decrease in absorbance at 340 nm on a Perkin-Elmer 552 absorption spectrophotometer. The reaction was initiated by the addition of 5–10 μL of an enzyme stock solution to 1 mL of 20 mM KP_i, pH 7.6, containing 0.5 mM *m*-hydroxybenzoate, 0.25 mM NADH, and 0.5 mM dithiothreitol. One unit of activity is defined as the conversion of 1 μmol of *m*-hydroxybenzoate to gentisate (or NADH to NAD^+) per minute. This is equivalent to 4.44 ΔA_{340} min^{-1} under our assay conditions on the basis of a decrease in extinction coefficient of 6.22 mM^{-1} cm^{-1} for the oxidation of NADH and a simultaneous increase of 1.78 mM^{-1} cm^{-1} for the conversion of *m*-hydroxybenzoate to gentisate, both at 340 nm. Alternatively, *m*-hydroxybenzoate hydroxylase activities were determined at 23 °C by measuring the oxygen uptake in 1.6 mL of the same assay solution with a Gilson Oxygraph Model 5/6. One unit of enzyme activity is defined as the consumption of 1 μmol of O_2 min^{-1}. Excellent agreement was obtained between these two methods.

Activities of salicylate hydroxylase and *p*-hydroxybenzoate hydroxylase were also determined by these two methods except that salicylate was used instead of *m*-hydroxybenzoate for the former enzyme assay and *p*-hydroxybenzoate and NADPH were utilized to replace *m*-hydroxybenzoate and NADH, respectively, for the latter. For these two enzymes, one activity unit corresponds to 6.22 ΔA_{340} min^{-1}; neither the two benzenoid substrates nor their products absorb at 340 nm. Protein concentrations were determined by the method of Lowry et al. (1951) with lysozyme as a standard.

[1] Abbreviations: PBS, 0.075 M KH_2PO_4–0.075 M Na_2HPO_4 buffer, pH 7.2, containing 0.42% NaCl; M_r, molecular weight; IgG, immunoglobulin G; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

Electrophoreses. Sodium dodecyl sulfate–polyacrylamide gel (12.5%) electrophoresis was conducted following the procedure of Laemmli (1970). Nondenaturing polyacrylamide gel (7.5%) electrophoresis was also carried out at 4 °C following the procedures of Gabriel (1971) for system II except that ammonium persulfate was used for gel polymerization. In the latter case, gels for salicylate hydroxylase and *m*-hydroxybenzoate hydroxylase samples were stained for either protein or activity. For activity stains, gels were each soaked in 5 mL of 0.05 M sodium phosphate/potassium phosphate, pH 7.6, containing 1.5 mg of triphenyltetrazolium chloride, 1.5 mg of phenazine methosulfate, and 5 mM salicylate or *m*-hydroxybenzoate for 5–10 min at 23 °C. Subsequently, 0.5 mL of 50 mM NADH was added and the incubation continued until a red activity band appeared. The gels were then destained and kept in water.

Purification of m-Hydroxybenzoate Hydroxylase. Unless otherwise indicated, all operations were carried out at 4 ± 2 °C, and centrifugations were performed at 14000*g* and 4 ± 1 °C for 20 min. Buffer A contained 0.5 mM dithiothreitol in 5 mM KP_i, pH 7.6.

(*1*) *Cell Lysis.* About 130 g of wet paste of the *m*-hydroxybenzoate-induced cells was suspended in 250 mL of 20 mM KP_i, pH 7.6, containing 0.5 mM dithiothreitol. The suspension was sonicated for 3 min at full power by using a Branson cell disruptor W185 with a large probe. The suspension was centrifuged, and the supernatant was collected. The pellet was then subject to two cycles of resuspension, sonication, and centrifugation as before. All the supernatants were combined and brought to 20 mM $MgSO_4$. About 5 mg of deoxyribonuclease was added, and the sample solution was stirred for 2 h. At this stage, the crude extract contained a dioxygenase, which converted gentisate (i.e., 2,5-dihydroxybenzoate) to maleyl pyruvate accompained by a marked increase in absorbance at 340 nm (Hamzah, 1984). The existence of this dioxygenase thus interferes with the spectrophotometric assay of *m*-hydroxybenzoate hydroxylase. To obviate this problem, aliquotes of crude lysate were first incubated in 30 mM KP_i, pH 7.6, containing 0.5 mM dithiothreitol and 50 mM EDTA for 2 min and then used for activity assays. EDTA was used to inactivate this iron-containing dioxygenase (Hamzah, 1984).

(*2*) *Ammonium Sulfate Fractionation.* The supernatant was fractionated between 30 and 50% ammonium sulfate saturation. At each stage, centrifugation was carried out after 1 h of gentle stirring of the sample solution. The precipitate from 50% ammonium sulfate saturation was dissolved in about 120 mL of buffer A and dialyzed against three changes of 2 L of this buffer overnight. The dialyzate was centrifuged to remove any insoluble material. From this stage on, enzyme assays were carried out without preincubation with EDTA.

(*3*) *Heat Treatment.* The supernatant was brought to 45 °C and incubated for 20 min with occassional stirring. The sample was then cooled in an ice–water bath to 4 °C. Denatured proteins were removed by centrifugation for 40 min at 12000*g*.

(*4*) *DEAE-Sephadex Chromatography.* The supernatant was applied to a DEAE-Sephadex column (3 × 13 cm) preequilibrated with buffer A. The column was then eluted with buffer A, and 5-mL fractions were collected. The *m*-hydroxybenzoate hydroxylase activity was very weakly retarded by this column. Fractions with activities of more than 3 units mL^{-1} were pooled and brought to 60% ammonium sulfate saturation. After 1 h of gently stirring, protein precipitate was collected by centrifugation, taken up in ~15 mL

of buffer A, and centrifuged to remove any insoluble material.

(5) *Molecular Sieve Chromatography.* The sample was applied to a 2.5 × 70 cm Sephadex G-100 column previously equilibrated with buffer A. The column was eluted with the same buffer, and 5-mL fractions were collected. Fractions with activities of >5 units mL^{-1} were pooled.

(6) *Hydroxyapatite Chromatography.* The pooled fractions were applied to a hydroxyapatite column (2.5 × 21 cm) preequilibrated with buffer A. The column was eluted with 60 mL of the same buffer and then with a 600-mL linear gradient from 5 to 50 mM KP_i, pH 7.6, containing 0.5 mM dithiothreitol. Fractions containing most of the activity were pooled, subjected to 60% ammonium sulfate fractionation, and centrifuged. The pellet was resuspended in 5–10 mL of 25 mM imidazole hydrochloride buffer, pH 7.4, containing 0.5 mM dithiothreitol and dialyzed against the same buffer overnight.

(7) *Chromatofocusing Chromatography.* A chromatofocusing exchanger PBE 94 column (0.8 × 12 cm) was prepared and preequilibrated with 25 mM imidazole hydrochloride, pH 7.4, containing 1 mM dithiothreitol. The eluent, polybuffer 74, containing 1 mM dithiothreitol was prepared according to the manual provided by Pharmacia and the pH adjusted to 5.5 with 4 N HCl. One milliliter of the eluent was first applied to the column, followed by the enzyme sample and then again the eluent. Fractions (1.2 mL each) were collected in test tubes containing 60 μL of 0.8 M KP_i, pH 7.6. Fractions were pooled on the basis of measurements of specific enzyme activity. Samples were dialyzed exhaustively against 20 mM KP_i, pH 7.6, containing 1 mM dithiothreitol and stored at −80 °C.

Salicylate Hydroxylase and p-Hydroxybenzoate Hydroxylase. Salicylate hydroxylase was purified from the salicylate-induced cells to homogeneity as previously described (Tu et al., 1981). For *p*-hydroxybenzoate hydroxylase, about 4 g of the *p*-hydroxybenzoate-induced cells were sonicated in 20 mL of 50 mM KP_i, pH 7.6, containing 0.1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.1 mM *p*-hydroxybenzoate (buffer B). The supernatant was collected after centrifugation and to which protamine sulfate was added to 0.3%. After about 30 min of gentle stirring, insoluble materials were removed by centrifugation. The supernatant was then made to 65% saturation with ammonium sulfate, stirred for 30 min, and centrifuged. The protein precipitate was collected and dissolved in and extensively dialyzed against buffer B. One milliliter of this sample was applied to a DEAE-cellulose column preequilibrated with buffer B. The column was then eluted with a linear gradient of 0.05–0.35 M KP_i, pH 7.6, containing all other components specified for buffer B. Fractions contained the highest specific activity were combined and concentrated by vacuum dialysis. Following such a procedure, *p*-hydroxybenzoate hydroxylase was purified ~55-fold to a specific activity of 5 units mg^{-1} with NADPH and *p*-hydroxybenzoate as substrates.

Apoprotein of m-Hydroxybenzoate Hydroxylase. Apoprotein of *m*-hydroxybenzoate hydroxylase was prepared by the acidic $(NH_4)_2SO_4$–KBr method as described previously (Strittmatter, 1961) except that the pH of acidic $(NH_4)_2SO_4$ was adjusted to 3.1 and the buffer used was 20 mM KP_i, pH 7.6, containing 0.5 mM dithiothreitol.

Antibody against Salicylate Hydroxylase. Procedures used for antibody prepration were similar to that described previously (Klass & Hirsh, 1981). For each of several New Zealand rabbits, about 50–75 μg of salicylate hydroxylase in 0.125 mL of complete Freund's adjuvant was injected into a toe pad.

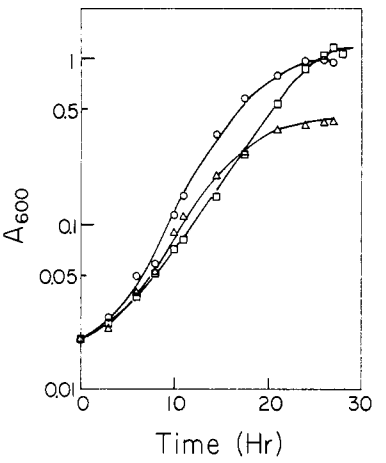


FIGURE 1: Growth rates of *P. cepacia* cells with salicylate, *m*-hydroxybenzoate, or *p*-hydroxybenzoate as the sole carbon source. Cells were first cultured to late logarithmic phase in a minimal medium containing 0.2% salicylate as the carbon source and then subcultured in the same minimal medium containing 0.2% salicylate (□), *m*-hydroxybenzoate (△), or *p*-hydroxybenzoate (○).

After 2 weeks, each rabbit was injected again with 0.1–0.15 mg of the enzyme in 0.125 mL of incomplete Freund's adjuvant. Blood was collected from ear veins about 6 weeks from the time of the initial injection. Samples were allowed to clot, and the whole serum was collected. The immunoglobulin proteins were collected by $(NH_4)_2SO_4$ precipitation and stored in PBS at −80 °C.

Immunoblotting. Proteins were separated by sodium dodecyl sulfate–polyacrylamide slab gel electrophonesis and electrophoretically transferred at 35 V overnight to nitrocellulose paper in a transfer buffer of 25 mM Tris and 192 mM glycine, pH 8.3, containing 20% (V/V) methanol (Towbin et al., 1979). Excess protein binding sites on paper were masked by incubation in a blocking buffer (i.e., PBS containing 5% Carnation nonfat milk) for 1 h at 4 °C. The nitrocellulose paper was then incubated with a solution consisted of 36 mL of the blocking buffer, 4 mL of goat serum (from Gibco), and 50 μL of anti-salicylate hydroxylase antibodies (containing 1.65 mg of protein) at 23 °C for 2.5 h. The paper was washed extensively with 0.05% Tween (Sigma) in PBS, incubed overnight with peroxidase-conjugated goat anti-rabbit antibodies diluted 2000-fold in PBS–10% goat serum, and washed as before. Antigen–antibody reactions were visualized by staining with a development solution prepared by mixing 12 g of 4-chloro-1-naphthol in 4 mL of methanol with 20 mL of 50 mM Tris, pH 7.4, containing 0.9% NaCl and 6.2 μL of 30% H_2O_2. Color development was terminated by rinsing the paper with water.

Results and Discussion

When 3-methylsalicylate, 4-aminosalicylate, 2,3-dihydroxybenzoate, 2,4-dihydroxybenzoate, 2,5-dihydroxybenzoate, or 2,6-dihydroxybenzoate was each used as the sole carbon source in the minimal medium, no cell growth was detected. However, *P. cepacia* cells can utilize salicylate, *m*-hydroxybenzoate, or *p*-hydroxybenzoate for growth with comparable efficiencies (Figure 1). As will be shown below, 2,5-dihydroxybenzoate is a reaction product for 3-hydroxybenzoate-6-hydroxylase induced in the *m*-hydroxybenzoate-grown cells. The fact that 2,5-dihydroxybenzoate itself fails to support the cell growth as a carbon source suggests that *m*-hydroxybenzoate trigers the induction of more than one enzyme involved in the *m*-hydroxybenzoate degradation pathway. The culture stock used in this work had gone

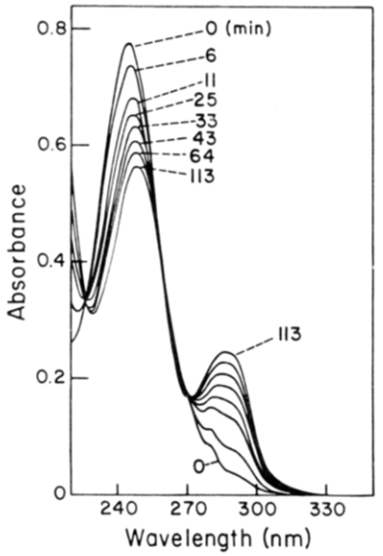


FIGURE 2: Conversion of *p*-hydroxybenzoate to protocatechuate by *p*-hydroxybenzoate hydroxylase. One milliliter of 25 mM KP_i, pH 7.5, containing 15 μM NADPH, 0.33 mM glucose 6-phosphate, 24 units of glucose-6-phosphadte dehydrogenase, and 0.027 unit of *p*-hydroxybenzoate hydroxylase, was added to each of a reference and a sample cuvette. The reaction was initiated at 23 °C by the addition of 10 μL of a 7 mM *p*-hydroxybenzoate stock solution to the sample cuvette. Absorption spectral changes during the reaction were recorded at different times, indicated in min, after the initiation of the reaction. The time zero spectrum is that of the *p*-hydroxybenzoate whereas the final spectrum taken at the completion of reaction is identical with that of an equal molar quantity of a protocatechuate standard.

through single-colony selection on salicylate-containing minimal medium plates. Furthermore, the inoculants used in the growth experiments described above had been first grown in the salicylate-containing medium. Therefore, the similar lag phases and growth rates shown in Figure 1 for subsequent cell growth with salicylate and the two new carbon sources argue strongly against the possibility that the observations are due to a secondary selection of minor cell populations by *m*-hydroxybenzoate or *p*-hydroxybenzoate.

The induction of salicylate hydroxylase in *P. cepacia* cells has already been documented (Tu et al., 1981; Wang & Tu, 1984). In cells grown on *p*-hydroxybenzoate, the induction of a *p*-hydroxybenzoate hydroxylase species has now been identified on the basis of absorption spectral analysis of the reaction product (Figure 2). The enzyme is specific for NADPH and stoichiometrically converts *p*-hydroxybenzoate to protocatechuate (i.e., 3,4-dihydroxybenzoate). Furthermore, *p*-hydroxybenzoate hydroxylase activity assays were carried out in a Gilson Oxygraph reaction chamber in the absence and presence of catalase. Upon completion of the reaction, the same level of oxygen consumption was observed under both conditions, indicating that no H_2O_2 was formed during the reaction. On the other hand, the synthesis of an enzyme in *m*-hydroxybenzoate-induced cells, which catalyzes a stoichiometric conversion of *m*-hydroxybenzoate to gentisate (i.e., 2,5-dihydroxybenzoate), has also been demonstrated (Figure 3). The identity of gentisate as a product was further confirmed by the comparison of the fluorescence emission spectrum with that of a standard (result not shown). Specifically, the two hydroxylases induced by *p*-hydroxybenzoate and *m*-hydroxybenzoate are designated as 4-hydroxybenzoate 3-hydroxylase and 3-hydroxybenzoate 6-hydroxylase, respectively.

The three species of hydroxylases mentioned above are apparently differentially induced. For each of the crude lysadtes prepared from salicylate-, *m*-hydroxybenzoate-, and

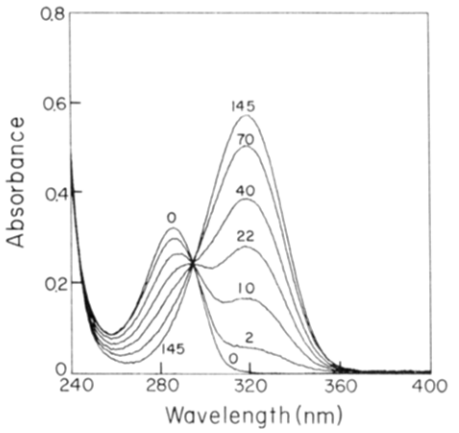


FIGURE 3: Enzymatic conversion of *m*-hydroxybenzoate to gentisate as determined by absorption spectral changes. Both sample and blank cuvettes contained 0.4 M ethanol, 0.5 mM dithiothreitol, 0.4 unit of alcohol dehydrogenase, and 0.24 unit of *m*-hydroxybenzoate hydroxylase in 1 mL of 20 mM KP_i, pH 7.6. The sample cuvette also contained 0.14 mM *m*-hydroxybenzoate. Two microliters of 2.5 mM NADH was added to both the sample and reference cuvettes, and absorption spectra were recorded at different times (inducated in min). The time zero spectrum is that of the *m*-hydroxybenzoate whereas the final spectrum taken at the completion of reaction is identical with that of an equal molar quantity of a gentisate standard.

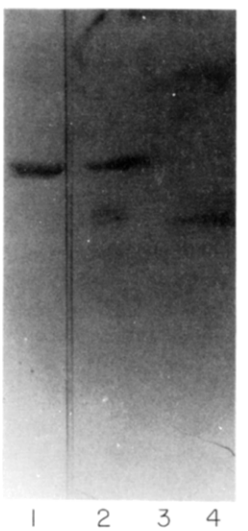


FIGURE 4: Immunoblotting of crude lysates from salicylate-, *m*-hydroxybenzoate-, and *p*-hydroxybenzoate-induced cells with anti-salicylate hydroxylase serum as a probe. Samples were purified salicylate hydroxylase standard for lane 1 and crude lysates from salicylate-, *m*-hydroxybenzoate-, and *p*-hydroxybenzoate-induced cells for lanes 2–4, respectively.

p-hydroxybenzoate-grown cells, no hydroxylase activities were detected when the two benzenoid compounds not used for carbon source were tested as a substrate. Furthermore, the crude lysate from the salicylate-induced cells showed a single positive band (with the mobility identical with that of a salicylate hydroxylase standard) by the immunoblotting test with rabbit anti-salicylate hydroxylase serum as a probe. However, no positive band was detected for the crude lysates prepared from *m*-hydroxybenzoate- or *p*-hydroxybenzoate-grown cells (Figure 4).

Among all known flavohydroxylases that utilize benzenoid substrates (referred to as aromatic hydroxylases), 3-hydroxybenzoate 6-hydroxylase is the only one that inserts the new hydroxyl function at a position para to the existing hydroxyl group. With all other aromatic flavohydroxylases (including another species of *m*-hydroxybenzoate hydroxylase, namely, 3-hydroxybenzoate 4-hydroxylase), the new hydroxyl

Table I: Summary of Results of *m*-Hydroxybenzoate Hydroxylase Purification

step	vol (mL)	protein concn (mg mL^{-1})	total activity (units)	sp act. (units mg^{-1})	yield (%)
crude extract	725	21.6	7185	0.5	100
ammonium sulfate fractionation	210	30.8	4397	0.7	61
heat treatment	150	21.2	3406	1.1	47
DEAE-Sephadex	174	6.1	2821	2.7	39
Sephadex G-100	126	4.1	2267	4.4	32
hydroxy-apatite	220	0.4	1289	14.6	18
chromato-focusing	8	5.8	1099	23.7	15

group is added to a position ortho to the existing one (Massey & Hemmerich, 1975; Ballou, 1984). Prior to this work, our knowledge of 3-hydroxybenzoate 6-hydroxylase is limited to a single literature report on the detection and isolation of this enzyme from *Pseudomonas aeruginosa* cells (Groseclose & Ribbons, 1973). Much remains to be learned about this unusual flavohydroxylase. We have therefore chosen to study the 3-hydroxybenzoate 6-hydroxylase newly detected in *P. cepacia* in detail.

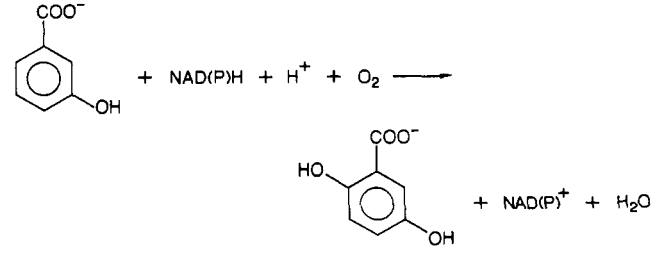
As described under Experimental Procedures, a scheme has been developed for the isolation of *P. cepacia m*-hydroxybenzoate hydroxylase. Following such a procedure, this hydroxylase has been purified ~50-fold to a specific activity of 23.7 units mg^{-1} with about 15% yield. The results of a typical purification run are summarized in Table I. The *m*-hydroxybenzoate hydroxylase so obtained was apparently homogeneous; only a single band was observed after sodium dodecyl sulfate and nondenaturing polyacrylamide gel electrophoresis with gels stained for protein and, in the latter case, activity. Several schemes analogous to the purification of other flavohydroxylases have been tried for the isolation of this enzyme without success for obtaining a high-purity preparation. A crucial new step for our purification procedure is the chromatofocusing chromatography.

The molecular weight of *m*-hydroxybenzoate hydroxylase was determined to be 45 000 by sodium dodecyl sulfate (12.5%) gel electrophoresis with bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme as standards. In a separate experiment, a M_r of 40 000 was obtained by Sephadex G-100 column chromatography in 0.15 M KP_i, pH 7, with horse liver alcohol dehydrogenase, bovine serum albumin, ovalbumin, and β-lactoglobulin as standards. In this case, *m*-hydroxybenzoate hydroxylase was identified by both A_{280} and activity assay. The native enzyme apparently existed in a monomeric form.

The visible absorption spectrum of the enzyme (Yu et al., 1987) is characteristic of a flavoprotein. The flavin cofactor was released from the hydroxylase by heat denaturation and identified to be FAD on the basis of fluorescence characterizations: (a) the ratio of fluorescence emission (measured at 535 nm) at pH 7.7 to that at pH 2.6 was 0.22, in excellent agreement with that reported for FAD (Faeder & Siegel, 1973:; (b) a 10-fold fluorescence enhancement was observed upon treating the free flavin with phosphodiesterase, indicating a conversion of FAD to FMN. FAD (based on ϵ_{450} = 11 300 M^{-1} cm^{-1}) and protein contents of a purified enzyme sample were determined, and a M_r of 44 000 per FAD site was obtained. The purified enzyme was also tested for sugar content (Dubois et al., 1956), but none was detected other than that in the bound FAD.

The identification of FAD as a cofactor was further confirmed by a reconstitution experiment. To 0.98 mL of 20 mM KP_i, pH 7.6, containing 0.7 μg of apoprotein, 5- or 10-μL aliquots of stock solutions of FAD, NADH, and *m*-hydroxybenzoate at appropriate concentrations were added and mixed in different sequence. The final concentrations were 0.25 mM for NADH, 0.5 mM for *m*-hydroxybenzoate, and either 0 or 1 μM for FAD. After the addition of the last component(s) at time zero, the reaction was followed at 23 °C by monitoring the absorbance change at 340 nm. No enzyme activity was observed with the apoenzyme in the absence of FAD. Fully active holoenzyme was obtained by mixing apoprotein with FAD but not with 10 μM FMN or riboflavin. Interestingly, the kinetics for activity recovery depended on the sequence of FAD and NADH additions. When apoenzyme was mixed with FAD prior to NADH, catalytic activity was regained immediately. On the other hand, when apoenzyme was mixed with NADH prior to FAD, the rate of activity recovery was markedly retarded. The sequence of *m*-hydroxybenzoate addition in relation to that of FAD exhibited no effect on the rate of activity recovery. Among all flavohydroxylases, only salicylate hydroxylase has been examined with respect to effects of the addition sequence of FAD and NAD(P)H on the kinetics of holoenzyme formation (Wang et al., 1984). In that case, the observations are qualitatively the same as those described for *m*-hydroxybenzoate hydroxylase.

The reaction stoichiometry for *m*-hydroxybenzoate hydroxylase has been determined. Reactions were carried out in 1 mL 0.02 M KP_i, pH 7.6, containing 10 μg of *m*-hydroxybenzoate hydroxylase, limiting (0.1 mM) NADH, and 0.2, 0.4, or 0.5 mM *m*-hydroxybenzoate. In each case, absorption changes were followed at both 320 and 340 nm until the completion of the reaction. Neither NAD^+ nor *m*-hydroxybenzoate absorbs at 320 or 340 nm. On the basis of the known extinction coefficients of NADH and gentisate at these two wavelengths, the molar ratio of gentisate formed over NADH oxidized was calculated to be 0.98 ± 0.02. In another experiment, changes in oxygen concentration in 1.6 mL of 0.02 M KP_i, pH 7.6, containing 12 μg of *m*-hydroxybenzoate hydroxylase, 0.25 mM O_2, 0.5 mM *m*-hydroxybenzoate, and 0.05, 0.10, and 0.15 mM NADH were followed until reactions reached completion. The addition of catalase to final reaction solutions did not change oxygen concentrations, indicating that no H_2O_2 was formed. The molar ratio of oxygen consumed over NADH oxidized was found to be 0.94 ± 0.01. Therefore, the overall reaction catalyzed by *m*-hydroxybenzoate hydroxylase can be summarized as



Activities of *m*-hydroxybenzoate hydroxylase were determined at 23 °C in 20 mM KP_i at pH ranging from 6.0 to 9.2. Each assay was carried out in 1 mL of buffer containing 0.26 mM NADH, 0.5 mM *m*-hydroxybenzoate, and 0.5 mM dithiothreitol. The reaction was initiated by the addition of 4 μL of buffer containing 2 μg of *m*-hydroxybenzoate hydroxylase. Under such conditions, an activity maximum was

Table II: Kinetic Constants for *m*-Hydroxybenzoate Hydroxylase[a]

parameter	varying NADH, fixed MHB	varying NADPH, fixed MHB	varying MHB, fixed NADH	varying MHB, fixed NADPH
K_m (μM)	40 ± 3	64 ± 6	162 ± 10	168 ± 9
V_{max} (min^{-1})	1330 ± 30	1100 ± 40	1520 ± 30	1340 ± 30

[a] Reactions were carried out at 23 °C in 1 mL of air-saturated 20 mM KP_i, pH 7.6, containing 1.5 μg of *m*-hydroxybenzoate hydroxylase, 0.5 mM dithiothreitol, and a constant *m*-hydroxybenzoate concentration at 0.5 mM and various levels of NAD(P)H or a constant level of NAD(P)H at 0.25 mM and various amounts of *m*-hydroxybenzoate. MHB is *m*-hydroxybenzoate.

observed near pH 8. To test the preference of this hydroxylase for reduced pyridine nucleotide, enzyme activities were measured at a constant level of *m*-hydroxybenzoadte and variable amounts of NADH or NADPH and at a fixed level of NADH or NADPH and variable concentrations of *m*-hydroxybenzoate. As shown in Table II, this hydroxylase exhibited a slightly smaller apparent K_m for NADH than that for NADPH whereas quite comparable apparent K_m values were obtained for *m*-hydroxybenzoate with either NADH or NADPH as a cosubstrate. Furthermore, similar apparent V_{max} values were obtained in all cases.

In comparison with other aromatic flavohydroxylases, a couple of unusual properties can be identified for the newly isolated *m*-hydroxybenzoate hydroxylase. The unique position of substrate hydroxylation has already been mentioned. The feature that this hydroxylase can utilize either NADH or NADPH with about equal efficiency is also unique among all flavohydroxylases. It should be noted that two *m*-hydroxybenzoate hydroxylases have been previously identified. One of them is a 3-hydroxybenzoate 4-hydroxylase species, detected in *Aspergillus niger* (Premkumar et al., 1969) and *Pseudomonas testosteroni* (Michalover & Ribbons, 1973), which catalyzes the normal ortho position hydroxyl insertion. A 3-hydroxybenzoate 6-hydroxylase species has also been isolated from *P. aeruginosa* (Groseclose & Ribbons, 1973), which, unlike the present *P. cepacia m*-hydroxybenzoate hydroxylase, was reported to have a M_r of 85000 per flavin site and showed preference for NADH over NADPH. None of these earlierly detected *m*-hydroxybenzoate hydroxylases have been subject to further investigations.

The *P. cepacia m*-hydroxybenzoate hydroxylase is also quite distinct from salicylate hydroxylase from the same cell system. These two enzymes are distinguishable immunochemically. Although both have about the same minimal molecular weight, native salicylate hydroxylase is an identical dimer whereas *m*-hydroxybenzoate hydroxylase appears to be monomeric. *m*-Hydroxybenzoate hydroxylase is also much less acidic than salicylate hydroxylase. In spite of a ~2-fold larger molecular weight, native salicylate hydroxylase has a much larger mobility (0.43) than *m*-hydroxybenzoate hydroxylase (0.13) in nondenaturing (7.5%) gel electrophoresis under conditions described under Experimental Procedures. At pH 7.6, salicylate hydroxylase binds tightly to DEAE-Sephadex, but *m*-hydroxybenzoate hydroxylase is very weakly retarded on the column. When subjected to chromatofocusing chromatography, salicylate hydroxylase was eluted at pH <5 (Ya-Wun Yang and S.-C. Tu, unpublished results) while *m*-hydroxybenzoate hydroxylase was eluted at pH 6.7. Finally, from the point of view of gene expression, these two enzymes and a *p*-hydroxybenzoate hydroxylase species all appear to be differentially induced in *P. cepacia*. In the accompanying paper, spectral properties, stereospecificity for NADH oxidation, and kinetic mechanism for this unusual 3-hydroxybenzoate 6-hydroxylase are described.

Registry No. NADH, 58-68-4; NADPH, 53-57-6; MHB, 99-06-9; FAD, 146-14-5; salicylate hydroxylase, 9059-28-3; 3-hydroxybenzoate 6-hydroxylase, 51570-26-4; 4-hydroxybenzoate 3-hydroxylase, 9059-23-8.

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