Metabolism of Resorcylic Compounds by Bacteria

PURIFICATION AND PROPERTIES OF ORCINOL HYDROXYLASE FROM PSEUDOMONAS PUTIDA 01*

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SUMMARY

Orcinol hydroxylase (EC 1.14.13.6), which catalyzes the first reaction of orcinol catabolism in Pseudomonas putida 01, has been purified to homogeneity, and crystallized. Orcinol hydroxylase catalyzes the hydroxylation of orcinol with equimolar consumption of O_2 and NADH (or NADPH) to 2,3,5-trihydroxytoluene, which is nonenzymically oxidized to a quinone. The visible absorption spectrum of the enzyme shows maxima at 373 and 454 nm and a shoulder at 480 nm. FAD can be dissociated from the protein. Reconstitution of enzymic activity was achieved with FAD, and to a limited extent by FMN. The enzyme has a molecular weight of 63,000 to 68,000 and contains 1 mol of FAD per mol of protein. $K_d$ values for the three substrates orcinol, NADH, and O_2 are 0.03, 0.13, and 0.07 mM, respectively. The molecular activity of the crystalline enzyme is 1560 min^{-1}.

In the absence of orcinol, NADH is only slowly oxidized with formation of H_2O_2. Several analogs of orcinol also serve as substrates for hydroxylation, namely resorcinol, 4-methylresorcinol, and 4-bromo-resorcinol. Other analogs, m-cresol, m-ethylphenol, 4-ethylresorcinol, and phloroglucinol, mimic orcinol as effectors, in that they (a) accelerate electron flow from NADH to the flavin and (b) decrease the apparent $K_m$ for NADH but not to the same extent as the substrates that are hydroxylated. The latter compounds are not hydroxylated. Instead H_2O_2 accumulates as the only product of O_2 reduction. The enzyme therefore behaves either as a hydroxylase or an oxidase. The ratio of hydroxylase to oxidase activities of the enzyme is decreased by an increase in the temperature of incubation; at 60° the reaction with orcinol is almost 50% uncoupled from hydroxylation.

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It has been proposed (1) that Pseudomonas putida 01 catabolizes orcinol by hydroxylation to give 2,3,5-trihydroxytoluene, which is the substrate of the ring cleavage enzyme of the orcinol pathway, a member of the m-pyrocatechase family of enzymes. Subsequent hydrolyses of the product of ring cleavage, 2,4,6-trioxoheptanoate, yield 2 mol of acetate and 1 mol of pyruvate, via the intermediate, acetylpyruvate (Scheme 1).

The participation of the flavin in the over-all reaction is demonstrated by its rapid reduction under anaerobic conditions by NADH in the presence of orcinol, resorcinol, or m-cresol. Subsequent introduction of oxygen restores the oxidized form and yields H_2O_2 when m-cresol is the effector, but not when orcinol is the effector. Transfer of reducing equivalents from the reduced flavoprotein to free FAD may also occur. Reduction of orcinol hydroxylase by NADH in the absence of an effector is 10^4-fold slower than in the presence of an effector.

The minimal structural requirements for effectors appear to be a 1,3-dihydroxy or 1-alkyl-3-hydroxybenzene, but only the former are substrates for hydroxylation.
hydroxylases, such as salicylate (4, 5), p-hydroxybenzoate (6–8), and melilotate hydroxylases (9, 10) isolated from pseudomonads.

This paper documents a modified purification procedure for orcinol hydroxylase as well as several of its physical and catalytic properties. Some of the data have been presented earlier, in preliminary form (3, 11).

**EXPERIMENTAL PROCEDURE**

**Materials**—*Pseudomonas putida* 01 was cultivated, and extracts prepared, as previously described (1). Sources of chemicals were given earlier (2, 11), except for acetylpyridine analog of NAD (Sigma Chemical Co.) and 4-bromoresorcinol (Aldrich Chemical Co.). 4-Methylresorcinol was a gift from Dr. P. J. Chapman and orcinol acetate was provided by Drs. F. Lyne, R. Light, and R. Bentley.

**Microbiological Methods**—*Ps. putida* 01 was isolated from pond mud by enrichment in a mineral salts medium containing orcinol (0.1%) as the sole source of carbon. The strain was maintained on nutrient agar slopes and induced cells were obtained by growth in mineral salts media of the following composition (grams/liter): KH₂PO₄, 5.4; NaOH, 1.8; (NH₄)₂SO₄, 1.2; MgSO₄, 7 H₂O, 0.4; and FeSO₄, 7 H₂O, 0.01. Mg²⁺ and Fe²⁺ in 5 mM HCl were sterilized separately as a concentrated solution and 0.005 volume was added to the cooled sterile medium in a 14-liter Erlenmeyer flask which was incubated with shaking for 16 to 24 hours and added to 10 liters of the same medium containing saturated (NH₄)₂SO₄ solution, pH 6.8, containing 0.3% mercaptoethanol before application to a hydroxylapatite column. The sterile orcinol solutions (10%) were added (0.01 volume) to complete the medium. The sequence of cultivation used to obtain 10-liter batches of cells was as follows: nutrient broth (80 ml) was inoculated from stock cultures and allowed to grow for 16 to 24 hours; the entire culture was used to inoculate 1 liter of the minimal medium contained in a 4-liter Erlenmeyer flask which was incubated with shaking for 16 to 24 hours and added to 10 liters of the same medium in 14-liter New Brunswick fermentors (New Brunswick Scientific Co., New Brunswick, N. J.). Growth was usually complete within 6 to 8 hours at 30°C due to exhaustion of orcinol from the media. Three further additions of orcinol were made at intervals determined by the extent of growth (measured turbidimetrically at 660 nm) the last addition being approximately 1 hour prior to harvesting at 60,000 × g in an air-turbine Sharples supercentrifuge at 26–33°C. The cells were used directly or stored as a paste at 0°C for up to 48 hours after assessing polargraphically their capacity to oxidize orcinol (3).

**Enzyme Assay**—Hydroxylase activity was routinely assayed by following oxygen consumption polarographically with a Clark O₂ electrode (Yellow Springs Instruments Co., Yellow Springs). The reaction mixtures usually contained 50 mM KH₂PO₄-NaOH buffer, pH 6.8 (2.9 ml), enzyme solution (5 to 100 μl); 25 μM NADH (46 μM); and 25 mM orcinol (20 μl). The temperature was 30°C. Air-saturated buffer was assumed to contain 232 nmol of O₂ per ml at 30°C (12). A unit of activity is that amount of enzyme that catalyzes the consumption of 1 μmol of O₂ min⁻¹.

**Purification of Orincol Hydroxylase**—The purification procedure outlined here is now preferred to that described earlier (3) and is suitable for large quantities of crude extracts of cells. A paste of orcinol-grown *Ps. putida* (750 g wet weight) is suspended in 1500 ml of 20 mM KH₂PO₄-NaOH buffer, pH 6.8, containing 0.3% mercaptoethanol. Batches (50 ml) are subjected three times to the highest output (150 to 200 watts) from a Branson ultrasonic disintegrator for 20 seconds at 3–5°C. The combined extracts are centrifuged at 27,000 × g for 20 min and the supernatants further clarified by ultracentrifugation at 55,000 × g for 90 min.

![SCHEME 1. The catabolism of orcinol by *Pseudomonas putida*: a, orcinol hydroxylase; b, 2,3,5-trihydroxytoluene 1,2-oxygenase; c, acetylpyruvate hydroxylase.](https://example.com/scheme1.png)

**Fig. 1.** Elution profiles of orcinol hydroxylase from DEAE-cellulose, Sephadex G-100, and hydroxylapatite columns. Conditions of chromatography are described under "Experimental Procedure."
addition of (NH₄)₂SO₄ solution until a faint turbidity persists. This solution is clarified by centrifugation and set aside for 2 to 7 days until crystallization occurs.

**Chromatography**—Flavins were chromatographed as described by Kilgour et al. (13). Phenols were checked for purity by chromatography on thin layer silica gel sheets in benzene-acetic acid-water (80:20:20:saturated), and detected by ultraviolet light or Gibbs reagent.

**Gel Filtration**—The molecular weight of orcinol hydroxylase was determined on Sephadex G-75 columns calibrated with pepsin, trypsin, cytochrome c, and alkaline phosphatase as described by Andrews (14).

**Ultracentrifugation**—Sedimentation velocity and equilibrium experiments, with schlieren and interference optics, respectively, were performed with a Spinco model E analytical ultracentrifuge. Sedimentation velocity runs, at 60,286 rpm were carried out with a wedge cell and a normal cell, allowing two complex (5.2 and 10.4 mg/ml) to be analyzed simultaneously. Equilibrium measurements were made by the meniscus depletion method (15) at three concentrations, 0.3, 0.6, and 1.2 mg/ml at 20.522 rpm and analyzed as described by Small and Resnick (16). Molecular weight determinations were made assuming a partial specific volume of 0.73 for orcinol hydroxylase.

**N₂H₄-terminal Analysis**—Dansylation of orcinol hydroxylase was carried out by the method of Gray (17) and the solution was chromatographed as described by Hartley (18).

**Disc Gel Electrophoresis**—The procedures described by Cooksey (19) were used for electrophoresis at pH 8.6 and under denaturing conditions in the presence of urea and sodium dodecyl sulfate (19). Details of individual runs appear in the legends. Protein was detected by staining with Coomassie blue which proved more sensitive than Amido Schwarz for our preparations.

Orcinol hydroxylase activity was detected by immersing the unfixed, unstained gels in an incubation mixture consisting of: 2-(p-iodophenyl)-3-p-nitrophenyl-5-phenyltetrazolium chloride, 10 mg; 25 mM NADH (0.2 ml); 25 mM orcinol (0.1 ml); 25 mM EDTA buffer, pH 8.8. Incubations were carried out in the dark to minimize light-catalyzed dye reduction. Color developed in about 20 min, and intensified over a period of about 18 hours. With crude preparations of the enzyme, several formazan precipitation bands appeared, due to dialysing activities in the sample. These are easily distinguished from orcinol hydroxylase activity because they also appear when the detection mixtures lack orcinol, in which case the orcinol hydroxylase band is washed off.

The molecular weight of orcinol hydroxylase was determined by a modification of the methods of Dunker and Rueckert (20). Enzyme solution (6 mg/ml) was incubated for 90 min at 60° with an equal volume of 5 M urea containing 2 mM iodoacetamide and 1% sodium dodecyl sulfate. Samples and standards were treated with 10% SDS and electrophoresed on starch gels containing 0.5% sulfosalicylic acid, then stained for 3 hours with Coomassie blue. Cytochrome c, trypsin, pepsin, and ovalbumin were used as standards.

**Analytical Methods**—Protein was determined by the method of Lowry et al. (21). Hydrogen peroxide was estimated by the release of O₂ after addition of catalase to reaction mixtures, with a Clark electrode (22). These experiments require rigorous washing of the reaction cuvettes between separate determinations, particularly with aged cuvettes, electrode holders, and Teflon membranes on the electrode. We have observed that residual catalase activity is satisfactorily removed after several rinses in boiling water. Failure to remove the adsorbed catalase results in irreproducible estimates of hydrogen peroxide and the extent of uncoupling. 2,3,5-Trihydroxytoluene and hydroxyquinol concentrations were measured by the O₂ consumed when a partially purified preparation of 2,3,5-trihydroxytoluene 1,2-oxidase (3) catalyzed their oxidation. Absence of further O₂ consumption on the addition of the ring cleavage enzyme for other potential hydroxylated products, e.g. 4-ethylcatechol, was used as evidence that hydroxylation (of 3-ethylphenol) had not occurred and this correlated well with the absence of H₂O₂ formed, and with the inability to detect the catechols by chromatography. Routine kinetic spectrophotometric measurements were made in a Unicam SP 800 ultraviolet visible spectrophotometer which could be adapted for simultaneous polarographic assays (23). Detailed spectral studies were performed with a Zeiss DMR 21 spectrophotometer. Circular dichroism measurements were made with a Cary model 60 spectropolarimeter. Specific conditions used are given in the legends.

**Spectral Assays of Phenol Reduction and Oxidation—Cuvettes** (1-cm light path, 1-ml volume), fitted with a Suba-seal rubber serum cap (William Freeman and Co., Ltd. Technical Sales Division, Staines, Middlesex, England) were charged with buffer and enzyme previously equilibrated with a nitrogen atmosphere. N₂ gas was bubbled through the reaction mixture for 3 to 5 min by means of two hypodermic syringes before substrate additions and spectral measurements were made. Substrate solutions were previously rendered O₃ free before injection of microliter quantities with Hamilton syringes. Oxygen was delivered to the reaction mixtures by addition of aerated 20 mM phosphate buffer, pH 6.8. Measurements were made at room temperature, 24-27°.

**Difference Spectra of Orcinol Hydroxylase-Enzyme-Effect Complex Versus Enzyme—Each pair of similar cuvettes in tandem contained orcinol hydroxylase and buffer separately.** Difference spectra were recorded when addition of effectors to the enzyme (sample beam) and to the buffer (reference beam), and similar additions of water had been made to the remaining two cuvettes.

**RESULTS**

**Purification and Crystallization of Orcinol Hydroxylase**—A summary of the purification procedure outlined under "Experimental Procedure" is given in Table I and elution profiles are shown in Fig. 1. The loss of enzymic activity from the DEAE-cellulose chromatography may appear large but this is due to the complete separation of the ring cleavage enzyme from orcinol hydroxylase. The specific activities recorded for crude and protamine sulfate-treated extracts are optimistic by a factor of 2, because the polarographic assay measures the two sequential reactions of the catabolic pathway catalyzed by orcinol hydroxylase and 2,3,5-trihydroxytoluene oxygenase, and the activity of the latter enzyme is in excess. Each of these enzymes catalyzes the fixation of 1 mol of oxygen per mol of substrate (Scheme 1).

Orcinol hydroxylase readily crystallizes from (NH₄)₂SO₄ solutions as yellow plates.

**Stability of Orcinol Hydroxylase and pH Optimum**—Orcinol hydroxylase activity is stable in crude extracts for days at 4°, but thiold reagents, such as mercaptoethanol, are required to stabilize purified preparations. Oracinol, EDTA, and FAD singly or in combination were not as effective as stabilizers. The enzyme was most stable at pH 7.

This pH optimum for stability is broadened by the presence of 0.3% mercaptoethanol. The pH optimum for activity is difficult to evaluate because the values do not take into account the non-enzymic oxidation rate of the product of reaction, which increases 10-fold between pH values of 6 and 8. At pH 6.8 in the presence of 0.3% mercaptoethanol suspensions of crystals and solutions at 5 to 10 mg of protein per ml of orcinol hydroxylase lose about

### Table I

| Step | Volume | Protein | Specific activity | Yield |
|------|--------|---------|------------------|-------|
| Crude extract | 1710 | 35.7 | 0.28 | 100 |
| 55,000 x g Supernatant after protamine sulfate | 1500 | 30.3 | 0.34 | 90 |
| DEAE-cellulose eluate | 822 | 8.3 | 1.26 | 9 |
| (NH₄)₂SO₄ and Sephadex G-100 | 124 | 2.9 | 12.7 | 26 |
| (NH₄)₂SO₄ and hydroxyapatite | 150 | 1.05 | 24 | 22 |
FIG. 2. Schlieren patterns from a sedimentation velocity study of orcinol hydroxylase. The lower cell contained 10.4 mg of orcinol hydroxylase and the upper cell contained 5.2 mg of orcinol hydroxylase dissolved in 20 mM KH$_2$PO$_4$-NaOH buffer, pH 6.8, and 0.3% mercaptoethanol. Frames recorded are for 2, 14, 37, and 68 min (left to right). Rotor speed, 60,286 rpm. Temperature, 4.2°C.

FIG. 3. Plot of log c (c = fringe displacement) against the square of the distance from the axis of rotation ($r^2$) for orcinol hydroxylase in 20 mM KH$_2$PO$_4$-NaOH buffer, pH 6.8, containing 0.1% 2-mercaptoethanol. Fringe patterns were obtained 20 hours after attaining a speed of 26,522 rpm. The initial protein concentration was 0.6 mg per ml. Temperature, 10°C.

50% activity in 8 to 10 weeks, at 4°C. Comparable measurements in the absence of mercaptoethanol were not made because large losses of enzymic activity occurred during purification without this supplementation.

Homogeneity—Orcinol hydroxylase obtained from hydroxylapatite columns yields two diffuse bands after polyacrylamide disc gel electrophoresis at pH 8.6 in Tris-HCl buffer. In sodium dodecyl sulfate gels a single band appears. The enzyme appears homogeneous upon ultracentrifugation giving a single symmetrical schlieren pattern (Fig. 2) and linear relationships between log $C$ versus $r^2$ from the sedimentation equilibrium data (Fig. 3). The latter measurements were made between 0.3 and 1.2 mg of protein per ml and gave a molecular weight value of approximately 65,000. Analysis of the NH$_2$-terminal amino acid of this preparation showed that isoleucine was the only NH$_2$-terminal amino acid present in the sample.

From the purification data it can be calculated that the orcinol hydroxylase content of Pseudomonas putida accounts for about 0.6% of the total protein of cells grown on orcinol as the sole carbon source, bearing in mind that the sensitivity of the assay is reduced 2-fold when the ring cleavage enzyme has been removed.

FIG. 4. Absorption spectra of orcinol hydroxylase and FAD in 20 mM KH$_2$PO$_4$-NaOH buffer, pH 6.9. —, orcinol hydroxylase (0.073 mM). - - -, FAD (0.073 mM).

Molecular Weight Determination—The molecular weight of orcinol hydroxylase was estimated as 65,000 by dodecyl sulfate disc gel electrophoresis and as 63,000 by gel filtration chromatography, respectively, values in reasonable agreement with those obtained from sedimentation equilibrium measurements.

Flavin Content of Orcinol Hydroxylase—The visible absorption spectrum of orcinol hydroxylase is shown in Fig. 4 and compared with that of FAD. The yellow color of the enzyme is abolished by dithionite but reappeared on aeration. The identification of the enzyme-bound flavin as FAD was achieved by a combination of chromatography and absorption spectroscopy of the chromophore dissociated from the enzyme. The cofactor co-chromatographed with FAD in Solvents 1 and 3 of Ref. 6, although faint spots were usually observed with $R_f$ values similar to FMN; other minor components were also observed; the absorption maxima and minima of the protein-free cofactor coincided with those of FAD. A molar extinction at 450 nm (assuming a molecular weight of 65,000) of orcinol hydroxylase was estimated as 10,500, which indicated that 1 mol of FAD is bound per mol of orcinol hydroxylase (a value of 0.94 is obtained using the molar extinction of 11,300 for FAD). Reconstitution of enzymic activity by the addition of flavin nucleotides to the apoenzyme is shown in Table II. Under these conditions most of the activity of the apoenzyme was recon-
denatured protein. Activities were assayed polarographically yielded a colorless apoenzyme solution (33 µg/ml) free from saturated (NH₄)₂SO₄ solution (6 ml) adjusted to pH 2.8 with 0.5 N HCl and kept at 0° for 16 hours. The supernatant was used for the identification of the flavin. The precipitate was sedimented and resuspended in 20 mM phosphate, pH 6.8 (1.5 ml). Re-centrifugation yielded a colorless apoenzyme solution (33 µg/ml) free from denatured protein. Activities were assayed polarographically after incubation with the flavin nucleotides (0.04 mM) for 3 hours at 0°.

### Table II

| Enzyme      | Flavin addition | Specific activity µmol min⁻¹ mg of protein⁻¹ |
|-------------|-----------------|-------------------------------------------|
| Holoenzyme  |                 | 18                                        |
| Apoenzyme   |                 | 2.8                                       |
| Apoenzyme   | FMN             | 3.5                                       |
| Apoenzyme   | FAD             | 14.7                                      |

![Fig. 5. Simultaneous assay of orcinol hydroxylation by oxygen consumption and quinone formation at pH 6.8 and 8.0 by orcinol hydroxylase. The reaction mixture contained: 100 mM KH₂PO₄-NaOH buffer, pH 6.8 or 8.0 (as indicated, 2.9 ml) and orcinol hydroxylase (10 µl). Additions of 25 mM NADH (20 µl) and 25 µM orcinol (20 µl) were made as indicated. The rapid pH-dependent nonenzymic formation of the quinone of 2,3,5-trihydroxytoluene is shown by the absorbance traces at 490 nm. The final addition of NADH shows the reduction of the quinone formed and further consumption of oxygen, until the reaction mixtures become anaerobic. (This nonenzymic oxidation of NADH by O₂ in the presence of 2,4,5-trihydroxytoluene has been demonstrated in the absence of orcinol hydroxylase.) Temperature, 30°.](image)

stirred by the addition of FAD; FMN was a poor substitute for FAD.

**Stoichiometry**—Initial attempts to determine the stoichiometric relationships of the hydroxylation reaction were thwarted by the rapid nonenzymic oxidation of the product of orcinol hydroxylation, 2,3,5-trihydroxytoluene, to a quinone (3, 11). This occurred too rapidly even at pH 6.8, as shown in Fig. 5, which compares the time course of the reaction at pH 6.8 and at pH 8.0. The amount of oxygen consumed is in excess of that required for a nonoxygenase (mixed function oxidase) reaction, due to the subsequent nonenzymic oxidation of the product. Formation of the quinone is shown by the increase in absorbance at 490 nm.

We have obtained indirect evidence that orcinol hydroxylase catalyzes a reaction in which equimolar consumption of orcinol, O₂, and NADH occurs. In the presence of limiting quantities of NADH, excess orcinol, and large quantities of enzyme, approximately 1.5 µmol of O₂ were consumed per µmol of NADH supplied and the reaction mixtures typically turned brick-red (λ_max 485 nm) indicating that the quinone accumulated. By incorporating large quantities of 2,3,5-trihydroxytoluene 1,2 oxygenase, purified to the DEAE-cellulose stage (3), into these reaction mixtures, quinone formation was not detected by its visible absorption spectrum; the amount of O₂ consumed however increased to approximately 2 mol of O₂ per mol of NADH supplied (Table III), a result expected for the two sequential reactions of the orcinol pathway (Scheme 1). As expected, approximately 2 mol of O₂ are similarly consumed per mol of orcinol supplied (excess NADH) when the ring cleavage enzyme is present.

**Aromatic Substrate-Dependent Specificity of Orinol Hydroxylase**—The difficulties encountered in directly determining the stoichiometry of orcinol hydroxylation led to an examination of the reactions with analogs of orcinol. It was known that resorcinol and m-cresol also stimulate NADH oxidation by orcinol hydroxylase and that one of the presumed analogous products, 3-methylcatechol (from m-cresol) was more stable to nonenzymic oxidation by oxygen than 2,3,5-trihydroxytoluene (3). An attempt to establish the stoichiometry with m-cresol revealed that O₂ and NADH were consumed in equimolar quantities, but in excess of that required for a simple hydroxylation. An equimolar amount of hydrogen peroxide was shown to be formed (11). 3-Methyl catechol, the expected product from m-cresol hydroxylation, was not detected chromatographically, nor by the dioxygenase assay, for which it is known to be a substrate (1, 3) and hydrogen peroxide was formed in amounts equivalent to the NADH and oxygen consumed (Table IV). Hydroxyquinol was detected as a product of the reaction with resorcinol as effector by (a) chromatography, (b) formation of its quinone, and (c) oxidation by the ring cleavage enzyme, 2,3,5-trihydroxytoluene oxygenase (and hydroxyquinol 1,2-oxygenase purified from Ps. putida ORC). Fig. 6 shows the course of reaction when resorcinol is the aromatic substrate; during the initial stages of the reaction both O₂ concentration and A₄₈₀ nm rapidly decreased, but after 4 min the A₄₈₀ nm values began to rise again, probably due to the nonenzymic formation of hydroxybenzoquinone from hydroxyquinol, the product of resorcinol oxidation. Sequential additions of catalase and 2,3,5-trihydroxytoluene 1,2-oxygenase increased

![Table III](image)

| Experiment | Orinol supplied | NADH supplied | O₂ consumed | NADH consumed |
|------------|-----------------|---------------|-------------|---------------|
| 1          | 1.0             | 0.2           | 0.285       | ND           |
| 2          | 1.0             | 0.3           | 0.42        | 0.45         |
| 3          | 1.0             | 0.2           | 0.38        | 0.18         |
| 4          | 1.0             | 0.3           | 0.68        | 0.26         |
| 5          | 0.1             | 0.5           | 0.18        | ND           |
| 6          | 0.2             | 0.5           | 0.38        | ND           |

* ND, not determined.  
* b ---, irrelevant.

1. P. J. Chapman and D. W. Ribbons, unpublished results.
FIG. 6 (left). Partial uncoupling of electron flow from NADH to oxygen catalyzed by orcinol hydroxylase in the presence of the "partial substrate" resorcinol. The reaction mixture contained: 100 mM KH$_2$PO$_4$-NaOH buffer, pH 6.8 (2.9 ml) and additions of 25 mM NADH (20 l), orcinol hydroxylase (50 l) and 25 mM resorcinol (20 l) were made as indicated. When the reaction had proceeded nearly to completion as judged by the O$_2$ consumption, catalase (50 l) was added, resulting in a burst of oxygen evolution. When this had ceased 2,3,5-trihydroxytoluene oxygenase (100 l) was added and a rapid consumption of oxygen occurred. Temperature, 30°.

FIG. 7 (right). Uncoupling of electron flow from hydroxylation in orcinol hydroxylase with the effector m-ethylphenol. The reaction mixture for the simultaneous assay of O$_2$ consumption and NADH utilization contained: 100 mM KH$_2$PO$_4$-NaOH buffer, pH 6.8 (2.9 ml) and additions of 25 mM NADH (20 l); orcinol hydroxylase (50 l); and 25 mM m-ethylphenol (20 l) were made as indicated. When the reaction had proceeded nearly to completion, catalase (50 l) was added and after the rapid evolution of oxygen had ceased, 2,3,5-trihydroxytoluene oxygenase was added (100 l). Temperature, 30°.

TABLE IV

Role of aromatic compounds as substrates and effectors

Reaction mixtures contained: 20 mM KH$_2$PO$_4$-NaOH buffer, pH 6.8 (2.8 ml); 25 mM solution of aromatic compound (10 l); 25 mM NADH (20 l); and orcinol hydroxylase (5 to 100 l of a 6 mg/ml solution). Oxygen consumption and NADH consumption were measured simultaneously as described in Figs. 1 and 2. Hydroxylated products were estimated by the addition of 2,3,5-trihydroxytoluene 1,2-oxygenase, and H$_2$O$_2$ by the addition of catalase. The experiments with orcinol, resorcinol, 4-methylresorcinol, and 4-bromoresorcinol as the effectors were the only ones that were allowed to progress to completion before the addition of ring cleavage enzyme and catalase.

| Aromatic compound supplied | Enzyme volume | Hydroxylated product formed | H$_2$O$_2$ formed |
|---------------------------|---------------|----------------------------|-----------------|
| **A. Orcinol.**           |               |                            |                 |
| Orcinol                   | 5             | 94%                        | 1.5             |
| Resorcinol                | 5             | 94%                        | N.D.            |
| Resorcinol                | 20            | 32%                        | N.D.            |
| Phloroglucinol            | 50            | 88%                        | N.D.            |
| m-Ethylphenol             | 30            | 96%                        | 97              |
| 4-Ethylresorcinol         | 30            | 97%                        |                 |
| **B. Orcinol.**           |               |                            |                 |
| Orcinol                   | 10            | 88%                        | N.D.            |
| Resorcinol                | 25            | 43%                        | 49              |
| m-Cresol                  | 25            | 0%                         | 95              |
| 4-Bromoresorcinol         | 100           | N.D.                       | 5-10            |
| 4-Methylresorcinol        | 100           | N.D.                       | 5-10            |

* N.D., not determined.

and depleted the O$_2$ concentration, respectively, indicating that both H$_2$O$_2$ and hydroxyquinol had been formed (Fig. 6). The combined measurements of H$_2$O$_2$ and hydroxyquinol formed however did not account for all of the O$_2$ initially consumed, probably due to the nonenzymic formation of the quinone in the reaction mixtures.

A survey of a number of analogs of orcinol for substrate and effector function with orcinol hydroxylase is shown in Tables IV and V. m-Cresol, m-ethylphenol, 4-ethylresorcinol, and phloroglucinol completely uncouple electron flow from hydroxylation with consequent formation of hydrogen peroxide. Fig. 7 shows a reaction with m-ethylphenol as effector, yielding only hydrogen...
peroxide as a product of oxygen reduction. Only orcinol, 4-methylresorcinol, 4-bromoresorcinol, and to a lesser extent resorcinol have been found as effectors that also undergo hydroxylation (Table IV). Compounds which did not stimulate oxygen reduction by NADH included: orsellinic acid, pyrogallol, 3,5-dihydroxybenzoate, 2,3-dihydroxybenzoate, 2,4-dihydroxybenzoate, and those compounds listed in Table VII.

**Effectors and Nucleotide Specificity, and their Kinetic Constants—** Apparent $K_m$ values for the aromatic substrates (effectors), electron donors, and oxygen, for orcinol hydroxylase are given in Tables V and VI. Although these values were not obtained in the presence of saturating concentrations of the other substrates, other experiments have shown that the apparent $K_m$ values for orcinol are not appreciably affected between NADII concentrations of 50 and 830 $\mu M$; similarly, the apparent $K_m$ for NADH is not altered between concentrations of 200 and 500 $\mu M$. $V_{\text{max}}$ values obtained are not recorded because they have only comparative value within a particular group of experiments. The most notable feature of these results is that in the presence of orcinol, 4-methylresorcinol, and 4-bromoresorcinol, *i.e.* the substrates that do not show extensive uncoupling of electron flow from hydroxylation, the apparent $K_m$ values for NADII are relatively low. With other aromatic effectors, apparent $K_m$ values for NADH are much greater. The $K_D$ values (see later, Figs. 14 and 15) for the enzyme-effector complexes compare favorably with these kinetic values obtained for orcinol, resorcinol, and m-cresol (Table V). The apparent $K_m$ value for orcinol is not significantly altered by the use of different electron donors (Table VI) and NADH is the preferred nucleotide for the enzyme.

**Inhibitors of Oracinol Hydroxylase—** A preliminary survey of possible inhibitors of orcinol hydroxylase is given in Table VII. These results show that several analogs of orcinol which are not effectors significantly inhibit the reaction, as do high concentrations of orcinol and chlorogluconic. Effectors with only two substituents in the benzene ring do not show "substrate" inhibition at high concentrations.

**Reduction of Oracinol Hydroxylase by NADH—** The preparation of large amounts of pure orcinol hydroxylase has allowed us to examine the reduction of flavin in the enzyme by NADH. In the absence of an effector and oxygen, the rate of reduction of the FAD of orcinol hydroxylase by NADH is low (Fig. 8) and appears biphasic; the final spectrum obtained is similar to that for the enzyme reduced by dithionite. The molecular activity calculated for the initial rate of reduction by NADH is approximately 0.05 min$^{-1}$ as compared with the value of 1600 min$^{-1}$ for the over-all hydroxylation reaction when orcinol is the effector. Addition of oxygen to the reaction mixtures restores the oxidized flavin spectrum. Similar anaerobic experiments in the presence of orcinol, resorcinol, m-cresol, or m-ethylphenol gave instantaneous bleaching of the flavin spectrum. Fig. 9 shows experiments where the flavin is titrated to its reduced form by NADH in the presence of (a) orcinol, (b) resorcinol, and (c) m-cresol.

**Reoxidation of Reduced Oracinol Hydroxylase—** Fig. 10 shows the spectral changes that occur when aerated buffer is introduced to orcinol hydroxylase that had been reduced previously by NADH, in the presence of orcinol or m-cresol as described in Fig. 9. With m-cresol, addition of successive increments of O$_2$ to the reduced enzyme produced increases of $A_{454}$ of only one-half the magnitude (Curves 1, 2, and 3) produced by similar quantities of O$_2$ with orcinol as substrate. After the addition of catalase the $A_{454}$ increased (Curves 8 to 6) by approximately those values observed when orcinol was present. Therefore, in confirmation of the data...
FIG. 9. Anaerobic titration of orcinol hydroxylase by NADH in the presence of (a) orcinol, (b) resorcinol, and (c) m-cresol. Each cuvette contained: orcinol hydroxylase (0.8 ml containing 32 pM of enzyme in 20 mM KH₂PO₄-NaOH buffer, pH 6.8). Curve 0 shows the spectra of the enzyme after flushing with N₂ in the presence of effector (0.32 mM). (a) orcinol, Curves 1, 2, 3, 4, and 5 show the spectral changes that occurred after the sequential additions of 2 μl of 7 mM NADH to the cuvette. (b) resorcinol, Curves 1, 2, 3, 4, and 5 were drawn after sequential additions of 3, 2, 2, 2, and 2 μl of 7 mM NADH. (c) m-Cresol, Curves 1, 2, 3, 4, and 5 were obtained after sequential additions of 3, 2, 2, 3, and 3 μl of 7 mM NADH. Temperature, 26°.

of Table IV, it appears that H₂O₂ is formed by addition of O₂ to reduced orcinol hydroxylase in the presence of m-cresol, but not orcinol. The increment seen upon the addition of catalase supports this notion (Curves 3 and 4, Fig. 10, right).

Reoxidation of Reduced Oracinol Hydroxylase by Other Electron Acceptors—Reduced orcinol hydroxylase is able to transfer reducing equivalents to a variety of electron acceptors, other than oxygen. These include free FAD, ferricyanide, cytochrome c, acetylpyridine-NAD, and tetrazolium salts. Fig. 11 shows the rapid reduction of free FAD by NADH in the presence of orcinol hydroxylase and orcinol, which also compares the slow nonenzymic rate of FAD reduction by NADH.

Circular Dichroism of Oracinol Hydroxylase—The circular dichroism spectrum of orcinol hydroxylase is shown in Fig. 16. In the presence of the substrate orcinol the circular dichroism spectrum is altered by a slight shift and decrease in positive values to longer wavelengths (368 ~ 371 nm), whereas the negative band became less intense at 455 nm (Fig. 16).
I I
520 480 440 400 360
WAVELENGTH (nm]

Fig. 11. Reduction of added FAD by reduced orcinol hydroxylase (left) compared with the nonenzymic reduction of FAD by NADH (right). Temperature, 26°C. Left, cuvette contained orcinol hydroxylase (1 ml, 2.7 mg of protein in 20 mM KH₂PO₄-NaOH buffer, pH 6.8) Curve 1. Then 25 mM orcinol (10 µl) and 25 mM FAD (2 µl) were added after flushing the enzyme with N₂ (Curve 2). Four sequential additions of 25 mM NADH (2 µl) were added and gave instantaneous bleaching of the flavin spectra (Curves 5, 4, 6, and 6). The reduction of total flavin present is less than that anticipated from the amount of NADH added. This may be due to a time-dependent transfer of reducing equivalents from the enzyme to free FAD, or to incomplete removal of O₂ from the cuvette. Right, cuvette contained 20 mM KH₂PO₄-NaOH buffer, pH 6.8 (1 ml), 25 mM orcinol (10 µl), and 25 mM FAD (4 µl), and was flushed with N₂ (Curve 1). The slow reduction of the flavin spectrum is shown in Curves 2 (5 min), 3 (11 min), 4 (15 min), and 5 (20 min) after the addition of 25 mM NADH (10 µl).

Effect of Temperature on Hydroxylase and Oxidase Activities—Fig. 17 shows the effect of temperature on the catalytic activities of orcinol hydroxylase. Clearly at elevated temperatures orcinol hydroxylase loses some of its regulatory properties for effector-dependent oxidation of NADH. NADH oxidase activity becomes a substantial contributor for the reduction of oxygen at higher temperatures, with concomitantly less hydroxylation of orcinol. Similar observations were made for the hydroxylase and oxidase activities when resorcinol was the effector, i.e. elevated temperatures of incubation reduce hydroxylase in favor of oxidase activity. The addition of urea (0.1 to 2.0 M) to reaction mixtures did not give similar results, but showed a progressive loss of activity as the urea concentration was raised.

DISCUSSION

Orcinol hydroxylase, the first enzyme in the pathway that enables Pseudomonas putida to catabolize orcinol to acetate and pyruvate (Scheme 1), catalyzes a typical monooxygenase (mixed function oxidase) reaction to yield 2,3,5-trihydroxytoluene which is rapidly and nonenzymically oxidized to a quinone (1, 11). The enzyme has been purified to homogeneity and crystallized as yellow plates. The specific activity of the crystalline preparations, which varied between 18 and 24 pmol of O₂ consumed, min⁻¹ mg of protein⁻¹, is higher than that previously reported (3, 11) because previous determinations had used limiting concentrations of NADH in the assay mixtures. This represents a maximum molecular activity of 1500 min⁻¹. The physical properties of orcinol hydroxylase are summarized in Table VIII. It appears to consist of a single polypeptide chain and to be monomeric at a variety of concentrations, containing 1 mol of FAD.

The activity of the apoenzyme was mostly reconstituted by FAD and only slightly by FMN (Table II). With these, and its catalytic properties, orcinol hydroxylase resembles three other flavoprotein monooxygenases, salicylate hydroxylase (4, 5, 24), p-hydroxybenzoate hydroxylase (6-8, 25), and melilotate hydroxylase (9, 10) which were also obtained from several strains of Pseudomonas, and shown to contain FAD. Thus, salicylate hydroxylase from Ps. putida studied by Katagiri and co-workers (4, 24) and the p-hydroxybenzoate hydroxylases from Ps. putida A3-12 (6), Ps. putida M-6 (25), Pseudomonas fluorescens (8), and...
FIG. 15. Spectrophotometric determination of the binding constant for orcinol hydroxylase and m-cresol. The data given were provided from difference spectra obtained under similar conditions to those described in Fig. 13 (bottom). Inset, reciprocal plots of m-cresol concentration and \( A(A_{497} - A_{417}) \).

FIG. 16. Circular dichroism spectra of orcinol hydroxylase alone and in the presence of orcinol. Enzyme (0.114 mM) in 20 mM KH₂PO₄-NaOH buffer, pH 6.8; path length, 1 cm; temperature, 0°C. Enzyme alone (---); enzyme plus 0.42 mM orcinol (----).

FIG. 17. Effect of temperature on uncoupling of electron flow from hydroxylation with orcinol hydroxylase. Enzyme activity was measured polarographically at various temperatures. Reaction mixtures contained: 100 mM KH₂PO₄-NaOH buffer, pH 6.8; 25 mM orcinol or resorcinol (20 μl); 25 mM NADH (40 μl); orcinol hydroxylase (100 μl, 100 μg of protein). The amount of hydrogen peroxide formed was determined by adding 50 μl of twice crystallized beef liver catalase (Sigma Chemical Co.) and measuring the amount of oxygen released, polarographically.

Pseudomonas desmolytica (7) (typed as Pseudomonas acidovorans by Stanier et al. (26)) all contain 1 mol of FAD per molecular weight of protein of 83,500, 93,600, 58,000, and 68,000, respectively, and show no evidence of subunit structure. However, salicylate hydroxylase from an unidentified soil bacterium (5) is a dimer of identical subunits each possessing 1 mol of FAD per molecular weight subunit of about 45,000 and melilolate hydroxylase (10) has recently been shown to be a tetramer of similar subunits of molecular weight 65,000, each containing 1 mol of FAD. Neujahr and Gaal (27) have also described a flavoprotein hydroxylase from the yeast, Trichosporon cutaneum. It, like the enzymes from pseudomonads possesses 1 mol of FAD, although it has a much higher molecular weight (146,000). Additionally, it shares with orcinol hydroxylase the ability to hydroxylate resorcinol, one of several substrates the yeast uses for growth. All five enzymes, and also the flavoproteins 3-hydroxybenzoate 6-hydroxylase (28) and 3-hydroxybenzoate 4-hydroxylase (29) catalyze the introduction of a hydroxyl group into the benzenoid ring, o- or p- to an existing hydroxyl substituent. This occurs with a concomitant decarboxylation for salicylate hydroxylase.

Direct determination of the stoichiometry of the orcinol hydroxylase reaction has been particularly troublesome due to the rapid nonenzymic oxidation of the product, 2,3,5-trihydroxytoluene by oxygen, and reduction of the quinone so formed by NADH. In addition, 2,3,5-trihydroxytoluene may serve as an effector of NADH oxidation by the enzyme in a manner observed for p-hydroxybenzoate hydroxylase where the product, protocatechuate is an effector of electron flow, as is gentisate for 3-hydroxybenzoate-6-hydroxylase (28). Indirect evidence of the expected quantitative relationships of oxygen and NADH consumption is, however, available. Thus, when reactions are limited by NADH, the molar ratio of substrate consumption is orcinol-NADH-O₂ (1:1:1.5). When 2,3,5-trihydroxytoluene 1,2-oxygenase is present such that the ring fission rate is in excess of the nonenzymic rate of oxidation of 2,3,5-trihydroxytoluene, these ratios change to 1:1:2; the 2nd mol of O₂ being consumed by the dioxygenative reaction, and the quinone is not formed.

The catalytic properties of orcinol hydroxylase closely resemble those of salicylate, p-hydroxybenzoate, and melilolate hydroxylases. The rate of enzyme-catalyzed oxidation of NAD(P)H by molecular oxygen (or other electron acceptors) is elevated by at least 4 orders of magnitude in the presence of orcinol or some of its structural analogs. The enzyme probably catalyzes the quantitative hydroxylation of orcinol in vivo, although small and variable amounts of hydrogen peroxide are formed in vivo, possibly due to the product accumulating in reaction mixtures and acting as an effector. Orinol hydroxylase does not hydroxylate m-cresol, phloroglucinol, m-ethylphenol, or 4-ethylresorcinol. Instead the only detected product of oxygen reduction for the last four effectors is hydrogen peroxide. 4-Methylresorcinol and 4-bromo-resorcinol are good substrates for the hydroxylation reaction but resorcinol is intermediate in its role as a substrate or as an effector, in that substantial quantities of both hydroxyquinol and hy-

| Table VIII |
| Summary of properties of orcinol hydroxylase |
| Molecular weight | 65,000 |
| Sedimentation equilibrium | 68,000 |
| Dodecyl sulfate gel electrophoresis | 65,000 |
| Sephadex G-75 | 63,000 |
| Sedimentation coefficient | 4.2 |
| FAD content | 0.94 mol/65,000 daltons |
| NH₂-terminal analysis | Isoleucine |
| Conclusion | Single polypeptide chain of MW ~ 65,000 containing 1 mol of FAD |
drogen peroxide are formed as products of oxygen reduction. These latter three substrates, and orcinol, have dual roles of acting as (a) substrates for hydroxylation, and (b) as effectors for the facilitated oxidation of reduced nicotinamide nucleotides by the FAD in the enzyme. In addition, orcinol also regulates the synthesis of this inducible enzyme, a property not shown for the other effectors.

It is not possible from these studies to determine if the elevated rate of flavin reduction observed is due only to a change in the apparent $K_m$ for pyridine nucleotide or also to a change in the $k_{red}$ (of flavin reduction), or both. However, more detailed studies of the reaction mechanisms of $p$-hydroxybenzoate hydroxylase (6-8, 25, 30, 31), salicylate hydroxylase (4, 5, 32, 33), and melilotate hydroxylase (9, 10, 34), by steady state kinetic analyses, and stopped flow techniques indicate that the $k_{red}$ is an altered parameter. The apparent $K_m$ values for the nucleotide in the presence of different analogs are also considerably different, as we have observed for orcinol hydroxylase (Table V).

The absorption spectrum of orcinol hydroxylase is perturbed by the presence of orcinol (Fig. 12), resorcinol, and m-cresol (Fig. 18); the effector-induced changes in the flavin spectrum are remarkably similar to those observed for $p$-hydroxybenzoate (8, 25, 30) and salicylate hydroxylases (5) and provide estimates of $K_D$ values which are in good agreement with the apparent $K_m$ values (Table V and Figs. 14 and 15). Orcinol has also been shown to substantially change the circular dichroism spectrum of orcinol hydroxylase (Fig. 16). The fluorescence spectrum of orcinol hydroxylase is also quenched in the presence of orcinol. The flavoprotein hydroxylases possess a built-in regulatory property, that prevents indiscriminate oxidation of NAD(P)H and transfer of reducing equivalents to the flavin, a property shown also by pteridine (35), P-450 (36-38), and dioxygenase hydroxylases (39). Once the flavin in the enzyme has been reduced, then alternative routes exist for the reduction of molecular oxygen to either hydroxylated product and water, or hydrogen peroxide, and these are presumably determined by the orientation and reactivity of the reduced enzyme effector $O_2$ complex, when reoxidation of the flavin occurs. The reduced species of the flavin hydroxylases all appear to be readily oxidized by molecular oxygen, irrespective of the method used to reduce the enzyme, e.g. by reduced nucleotides, EDTA, and light or dithionite.

Many of the flavoprotein hydroxylases are particularly versatile in the catalytic activities they possess, and those “isofunctional” enzymes isolated from different strains of related bacteria possess quantitatively different but often overlapping specificities for both the substrates (effectors) and the products of reaction (hydroxylation or hydrogen peroxide). Thus $p$-hydroxybenzoate hydroxylases isolated from the fluorescent pseudomonads, Ps. fluorescens (8) and Ps. putida (25) and the nonfluorescent species Ps. insidiosa (acidovorans) (7, 30) exhibit common capabilities for the transformation of $p$-hydroxybenzoate, 2,4-dihydroxybenzoyl to their 3-hydroxylated products, but this is not shared by them for the transformation of benzoate and 2,3,4-trihydroxybenzoate. Similar differences in the substrate or effector specificities have been shown for the orcinol hydroxylase that another strain of Ps. putida (not that used in this study) elaborates during growth on orcinol. Further versatility of substrate hydroxylation is shown by the 3-hydroxybenzoate 6- and 4- hydroxylases from Pseudomonas aeruginosa and Pseudomonas testosteroni respectively (26, 29). These enzymes hydroxylate several 3-hydroxybenzoates substituted in the 2,4,5- and 6-positions of the benzene nucleus, albeit with different efficiencies. Likewise the phenol hydroxylase from Trichosporon cutaneum catalyzes the sequential hydroxylation of phenol, and the products, catechol, as well as the three cresols, and all of the isomeric fluoro- and chlorophenols (37). Resorcinol, a partial substrate of orcinol hydroxylase is equally as good a substrate for phenol hydroxylase as phenol. Phenol hydroxylase however is restricted to the use of NADPH as reductant (27). This enzyme then is possibly used during growth of the yeast on resorcinol, as is the orcinol hydroxylase of the mutant of Ps. putida 01 (strain 010C), being recruited by mutation to constitutivity. Substrate specificity and analog inhibitor studies with orcinol hydroxylase indicate that a necessity for competitive binding to orcinol hydroxylase is a 1,3-substitution of either (a) two hydroxyl groups, or (b) an alkyl and a hydroxyl group. However, for hydroxylation to occur, case (a) is a prerequisite, and limited substitution in the 4- and 5-positions is allowed, e.g. 4- or 5-methylresorcinol, but not 4-ethylresorcinol are hydroxylated, the latter is an effector only. Those compounds that possess 1,3,5-substitution patterns, e.g. orcinol and phenol, also show “substrate” inhibition at high concentrations.

The effect of elevated temperatures on the catalytic activity of orcinol hydroxylase is to reduce its efficiency for hydroxylation of substrates, such that reoxidation of reduced enzyme-effector complexes by oxygen is altered in that different ratios of products are formed (Fig. 17). The increase in oxidase activity over hydroxylase activity is paralleled by increases in the apparent $K_m$ values for NADII (0.13 mM at 30° to 1.7 mM at 50°), and reduction of tritide transfer from 4R-4P[NADH (Ref. 11 and footnote 3), but the significance of these observations is not clear, and the phenomena may be unrelated. However, the hydroxylase and oxidase activities are related in the proposals made by Palmer and Massey (40) for the alternative routes of flavoprotein oxidation. Thus, the evolutionary origin of flavoprotein hydroxylases from the oxidases is suggested, because physical (temperature) or chemical (41) modification of these proteins results in different products of catalysis.

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