THE HYDROXYLATION OF p-CRESOL AND ITS CONVERSION TO p-HYDROXYBENZALDEHYDE IN PSEUDOMONAS PUTIDA

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SUMMARY Cell extracts of Pseudomonas putida catalyze the conversion of p-cresol to p-hydroxybenzylalcohol when phenazine methosulfate, an electron acceptor, is added. The reaction will proceed under anaerobic conditions and a mechanism involving dehydrogenation to a heteroquinone followed by hydration is proposed. This contrasts with the known attack on methyl groups by mono-oxygenases. The same requirements are found for the alcohol dehydrogenase and the major product from reaction mixtures is the aldehyde. Of the compounds tested as substrates only those with the appropriate groups in the para orientation were attacked.

INTRODUCTION The degradation of methyl-substituted aromatic compounds by bacteria often involves oxidation of the methyl group to carboxyl prior to ring fission [1,2,3]. This was shown for the metabolism of 2,4-xylenol, by a species of Pseudomonas, for which the initial attack was on the para-methyl group [4]. Another substrate for this system was p-cresol which could also serve as growth substrate. It was suggested that the intermediates in the pathways are the corresponding alcohols and aldehydes. p-Hydroxybenzaldehyde has been identified as an intermediate of p-cresol metabolism in another species of Pseudomonas [3]. In this respect the process resembles the attack by bacteria on alkanes in which the terminal methyl group is converted to carboxyl [5,6]. The alkane oxidation is initiated by a hydroxylation catalyzed by a mono-oxygenase as also is the attack on methoxy groups [7,8] and on some methyl-substituted aromatics [9]. In contrast it is shown in this paper that molecular oxygen is not required for the para-methyl hydroxylase of the 2,4-xylenol-degrading Pseudomonas which requires an electron acceptor rather than a donor.

MATERIALS AND METHODS The organism used was Pseudomonas N.C.I.B. 9866 which has been examined by Dr. M.E. Rhodes-Roberts and identified as a strain of Pseudomonas putida. Growth and preparation of crude cell-free extracts have been described previously [4]. For these experiments cells were grown with 2,4-xylenol as carbon source.

The enzyme was partially purified by loading crude extract (34.5 mg of protein per ml) from 10g wet weight of cells in 20 mM Tris-HCl buffer, pH 7.6, onto a DEAE-column (7x1.4 cm) equilibrated with the same buffer. The column was eluted at 4°C with 35 ml batches of the Tris buffer containing increasing amounts of NaCl. The enzyme was eluted in fractions containing 0.1 M and 0.2 M NaCl. These fractions were pooled and the protein, precipitated by addition of $(NH_4)_2SO_4$ to 70% of saturation, was collected by centrifuging at 20,000 g for 10 min at 4°C. The precipitate was redissolved in 5.0 ml of 50 mM KH_2PO_4-Na_2PO_4 buffer, pH 7.5, applied to a Sephadex G200 column (50x2.5 cm) and eluted with the same buffer. Fractions containing activity were pooled and are referred to as partially purified enzyme (3.0 mg of protein per ml).

Oxygen uptake was followed in a conventional Warburg apparatus or using a Yellow Springs Instrument Model 53 oxygen monitor at 30°C. The enzyme was routinely assayed using the O_2 electrode in a volume of 2.5 ml containing 125 µmol of Glycine-NaOH buffer, pH 9.0; 2.0 µmol of phenazine methosulfate (PMS) and enzyme. The reaction was started by addition of 0.05 ml of 30 mM p-cresol. The rate obtained in this assay was directly proportional to the enzyme concentration.

A spectrophotometric assay was used to demonstrate activity under anaerobic conditions. An anaerobic cuvette contained,in 3.0 ml,150 µmol of Glycine-NaOH buffer, pH 9.0; 3.0 µmol of phenazine methosulfate; 0.15 µmol of dichlorophenolindophenol; enzyme and 1.5 µmol of substrate in the side-arm. The cuvette was alternately evacuated and filled with N_2 five times before tipping. The decrease in absorption at 600nm due to reduction of DCPIP was followed at 30°C in a Unicam SP8000.

Soluble protein was measured colorimetrically [10] using bovine serum albumin as standard.

Thin layer chromatography of products was carried out on Kieselgel GF_{254} (E. Merck A.-G., Darmstadt, Germany) as previously described [4]. Phenolic compounds were separated using solvents A: benzene-dioxan-acetic acid (90:25:4 by vol) [11]; B: chloroform-acetone (80:20 by vol) [2]; C: isopropanol-benzene-conc ammonium hydroxide (3:1:1 by vol) [12]. For 2,4-dinitrophenylhydrazones 2% (v/v) tetrahydrofuran in benzene was used. Phenolics were detected by the quenching of fluorescence under u.v. light and by spraying with diazotized p-nitroaniline [13] or with Folin-Ciocalteau phenol reagent followed by exposure to ammonia.

RESULTS

Aerobic Assays: p-Cresol oxidation by crude cell-free extracts in the Warburg was very slow and only slightly stimulated by the presence of NADH, a possible cofactor for a mono-oxygenase hydroxylation. However, the inclusion of phenazine methosulfate, an auto-oxidizable electron acceptor, resulted in a high rate of O_2 uptake (Fig 1).

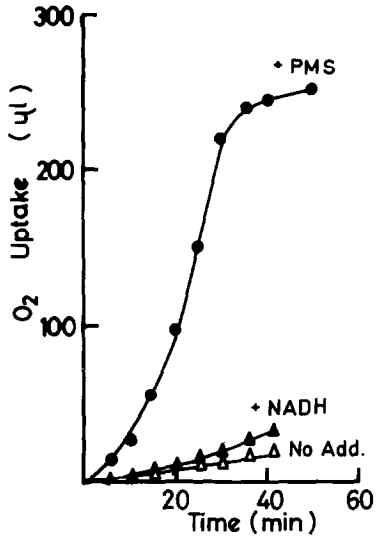


Fig. 1. The oxidation of p-cresol by crude cell-free extracts. Warburg flasks at 30°C contained 1.0 ml of extract; 0.6 ml of 50 mM KH_2PO_4-Na_2HPO_4 buffer, pH 7.0; 0.2 ml of 20% (w/v) KOH in the center well; 0.2 ml of 30 mM substrate in the sidearm and 1.0 µmol of phenazine methosulfate (●) 1.5 µmol of NADH (▲) or no further addition (Δ). Endogenous rates (about 6 µl of O_2 per min) have been subtracted.

The contents of the Warburg flask, containing the phenazine methosulfate, were acidified with HCl and, after removal of precipitated protein by centrifuging, extracted with diethyl ether. The ether was dried over anhydrous Na_2SO_4 and then evaporated to dryness. T.l.c. of the product obtained, in solvent A, showed p-hydroxybenzoate as the major product. In a similar experiment using partially purified enzyme the major product on t.l.c. corresponded to p-hydroxybenzaldehyde when either p-cresol or p-hydroxybenzyl-alcohol was used as substrate. In this experiment using 9.0 µmol of substrate there was an uptake of 1.04 µmol of O_2 per µmol of p-cresol and 0.52 µmol of O_2 per µmol of p-hydroxybenzylalcohol.

Phenazine methosulfate-stimulated O_2 uptake could also be followed in the O_2 monitor with the maximum rate at about pH 9.0 for crude extract and this formed the basis of the routine enzyme assay. There was no activity in crude extract heated to 100°C for 5 min.

Anaerobic Assays: The lack of any direct O_2 requirement for the enzyme reaction was shown by demonstrating activity under anaerobic conditions. In these assays phenazine methosulfate reoxidation was linked to dichlorophenol-indophenol reduction which was followed spectrophotometrically. Addition of p-cresol, 2,4-xylenol or p-hydroxybenzylalcohol to a reaction mixture containing 20 µl of crude extract in an anaerobic cuvette resulted in a rapid decrease in extinction at 600 nm. Both extract and phenazine methosulfate were essential for this activity. There was, however, no reaction when m-cresol was used as substrate.

Products from Anaerobic Incubations: To obtain sufficient product from an anaerobic reaction for thorough identification a large scale incubation was carried out in a Thunburg tube. The reaction mixture contained, in 5.0 ml of the partially purified enzyme solution and 1.0 ml of 0.5 M Glycine-NaOH buffer, pH 9.0, 2.0 µmol of phenazine methosulfate and 60 µmol of dichlorophenolindophenol. 1.0 ml of 30 mM p-cresol was tipped after the tube had been alternately evacuated and filled with N_2 five times. The mixture was incubated for 2 h at 30°C and then the protein precipitated by the addition of 10 ml of 10% (w/v) metaphosphoric acid. The precipitate was removed by centrifuging and the supernatant extracted three times with 20 ml of diethyl ether after further acidification with HCl to pH 1. The ether was dried over anhydrous Na_2SO_4 and then evaporated to dryness. T.l.c. of the products, in solvent A, showed a major spot coincident with p-hydroxybenzaldehyde and also a trace of p-hydroxybenzylalcohol. The aldehyde was purified by passing a solution of the products in 5.0 ml of water through a column (4x0.6 cm) of Dowex 1 (Cl form) and subjecting an ether extract of the effluent to preparative t.l.c. The aldehyde band was eluted with diethyl ether which was evaporated to dryness. The product gave a single spot on analytical t.l.c. which corresponded to authentic p-hydroxybenzaldehyde as did its I.R. spectrum in a Nujol mull. The 2,4-dinitro-phenylhydrazones of both the product and p-hydroxybenzaldehyde gave the same Rf on t.l.c. and had identical absorption spectra.

Table 1.

Relative rates of enzyme activity with various substrates.

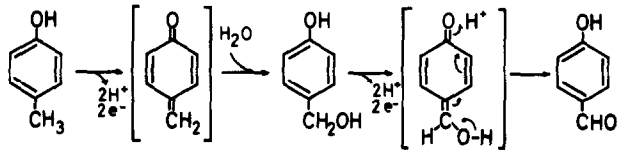
Substrate	Rate (%)
p-Cresol	100
2,4-Xylenol	100
p-Hydroxybenzylalcohol	66
3,4-Xylenol	61
p-Ethylphenol	54

When a higher ratio of p-cresol to dichlorophenolindophenol was used in the reaction mixture (4:1) the yield of p-hydroxybenzylalcohol increased. This too was separated by preparative t.l.c.,using solvent A, and was coincident with authentic alcohol after t.l.c. in solvents A,B,and C.

Specificity: Various compounds were tested as substrates for the partially purified enzyme in the O_2 monitor. Those compounds which were substrates all had methyl or substituted methyl groups para to hydroxyl (Table 1). There was no activity with o- or m-cresol, o- or m-hydroxybenzylalcohol, p-hydroxybenzaldehyde, p-methoxyphenol, 2,3-xylenol, 2,5-xylenol, 2,6-xylenol or 3,5-xylenol.

DISCUSSION The hydroxylation of the p-methyl group of p-cresol or 2,4-xylenol by this organism does not appear to involve molecular oxygen in a mono-oxygenase type of reaction. NADH failed to stimulate significantly oxidation of p-cresol by cell extracts in the Warburg and it was shown previously that no greater stimulation is given with NADPH [4]. The demonstration of activity and the identification of p-hydroxybenzylalcohol and p-hydroxybenzaldehyde as products under anaerobic conditions rules out direct O_2 involvement in the initial hydroxylation. The O_2 uptake in the presence of phenazine

SCHEME 1



Scheme 1. Suggested route for conversion of p-cresol to p-hydroxybenzaldehyde

methosulfate is accounted for by the reoxidation of this compound after reduction by the reaction. The requirement for an electron acceptor, in this case phenazine methosulfate, rather than a donor is typical of a hydroxylase which derives oxygen from H_2O [14,15,16]. A suggested reaction sequence, therefore, is a dehydrogenase attack on the p-cresol to give a hetero-p-benzoquinone. This type of compound is unstable and susceptible to attack by H_2O [17] which would yield p-hydroxybenzylalcohol (Scheme 1). A further dehydrogenase would form p-hydroxybenzaldehyde. The type of benzoquinone suggested could only be formed with the appropriate groups para to each other on the benzene ring and it is significant that compounds with other orientations were not substrates.

The stoicheiometry of O_2 uptake in the Warburg is consistent with two dehydrogenase reactions, each resulting in a requirement for $\frac{1}{2}O_2$, converting p-cresol first to p-hydroxybenzylalcohol and then the aldehyde. The enzyme preparation was active with both of these compounds, which accounts for the isolation of both aldehyde and alcohol from reaction mixtures. It is possible that both activities are catalyzed by the same protein but confirmation of this must await further purification of the enzyme.

Extracts of the Pseudomonas of Dagley and Patel [3] were also capable of converting p-cresol to p-hydroxybenzaldehyde and it is interesting that there was evidence for the involvement of a cytochrome system in p-cresol oxidation. Although in the present experiments phenazine methosulfate was used as the electron acceptor presumably the enzyme, in vivo, is linked to an elect-ron transport system.

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