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The Purification and Properties of *p*-Cresol–(Acceptor) Oxidoreductase (Hydroxylating), a Flavocytochrome from *Pseudomonas putida*

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The enzyme that catalyses the hydroxylation of the methyl group of *p*-cresol was purified from *Pseudomonas putida*. It has mol.wt. 115000 and appears to contain two subunits of equal molecular weight. One subunit is a *c*-type cytochrome and the other is a flavoprotein. Reduction of the cytochrome occurred on addition of substrate. The same enzyme catalyses both *p*-cresol hydroxylation and the further oxidation of the product, 4-hydroxybenzyl alcohol. The stoicheiometry of acceptor reduced per molecule of substrate oxidized is that for two dehydrogenation reactions. The K_m for *p*-cresol is 7.3×10^{-6}M and that for 4-hydroxybenzyl alcohol is 47.6×10^{-6}M. The enzyme, which is assayed with phenazine methosulphate as electron acceptor, was stimulated by particulate material, which probably contains the acceptor *in vivo*.

The hydroxylation of the methyl groups on a number of compounds is often the initial step in their degradation by bacteria. Thus alkanes are converted into the corresponding alcohol, and further oxidation gives the aldehyde and finally a carboxylic acid (Gholson *et al.*, 1963). Similarly methoxy groups on aromatic compounds are attacked by hydroxylation (Ribbons, 1970; Bernhardt *et al.*, 1975). In both of these examples the hydroxylase is a mono-oxygenase with a requirement for a reduced cofactor. This also appears to be the case for oxidation of some methyl-substituted aromatic compounds to carboxylic acids, since reduced nicotinamide nucleotides are essential for their oxidation by cell extracts (Hopper & Chapman, 1971).

Although metabolism of both 2,4-xylenol and *p*-cresol by *Pseudomonas putida* N.C.I.B. 9866 also proceeds by methyl-group oxidation (Chapman & Hopper, 1968), the hydroxylase for the *p*-methyl group requires an electron acceptor rather than a donor and does not appear to be a mono-oxygenase type of enzyme (Hopper, 1976). It was shown that hydroxylation of *p*-cresol can occur under anaerobic conditions with phenazine methosulphate as the electron acceptor and that a similar activity converted the *p*-hydroxybenzyl alcohol formed into *p*-hydroxybenzaldehyde. A mechanism for hydroxylation of *p*-cresol, involving dehydrogenation to the quinone methide followed by hydration, was proposed, and a second similar dehydrogenation would yield the aldehyde. However, it was not clear whether both activities were catalysed by the same enzyme.

In the present paper we describe the purification and some properties of the *p*-cresol–(acceptor) oxidoreductase (hydroxylating) from *Ps. putida* N.C.I.B. 9866.

Materials and Methods

Maintenance and growth of organism

The organism, *Ps. putida* N.C.I.B. 9866, was maintained by growth on nutrient-agar slants for 24h at 30°C, followed by storage at 4°C. It was subcultured monthly

For enzyme purification, cells were grown at 30°C in medium containing (per litre): Na_2HPO_4, 4.33g; KH_2PO_4, 2.65g; NH_4Cl, 2.0g; nitrilotriacetic acid, 0.1g; salts solution (Rosenberger & Elsden, 1960), 4.0ml; 2,4-xylenol, 0.3g. The bacteria were grown in 10-litre batches in a Microferm MF-114 fermenter (New Brunswick Scientific Co., New Brunswick, NJ, U.S.A.), which was aerated at a rate of 4 litres/min and stirred at 150rev./min. Growth was followed by measuring turbidity at 540nm in a Unicam SP.500 spectrophotometer. Further additions of substrate were made when necessary to give higher yields. Cells were harvested in an Alfa Laval LAB 102B continuous-flow centrifuge (Alfa Laval Co., Brentford, Middx., U.K.) and stored at −20°C.

Enzyme assays

The enzyme was routinely assayed by following O_2 uptake, caused by reoxidation of phenazine methosulphate reduced by the enzyme, with a Clark-

type oxygen electrode in a stirred vessel at 30°C (Oxygen monitor model 53; Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.). The pH optimum for the enzyme was 9.0, and so assays were carried out at this pH in a reaction mixture that contained, in 2.5ml of 50mM-glycine/NaOH buffer, pH9.0, 2μmol of phenazine methosulphate, enzyme and 1.5μmol of *p*-cresol. There was little change in the rate on increasing the phenazine methosulphate concentration. A linear relationship between the rate and the amount of enzyme added was observed.

For kinetic studies a spectrophotometric assay was used by linking phenazine methosulphate oxidation to reduction of 2,6-dichlorophenol-indophenol and following the decrease in A_{600}. A 1cm-path-length cuvette contained, in 3.0ml of 50mM-glycine/NaOH buffer, pH9.0, 2μmol of phenazine methosulphate, 0.3μmol of 2,6-dichlorophenol-indophenol, enzyme and various amounts of substrate.

The NAD^+-linked 4-hydroxybenzyl alcohol dehydrogenase was assayed spectrophotometrically by following the reduction of NAD^+ at 370nm and 30°C. A 1cm-path-length cuvette contained, in 1.0ml of 50mM-glycine/NaOH buffer, pH9.0, 0.5μmol of NAD^+, 0.8μmol of 4-hydroxybenzyl alcohol and enzyme.

Buffers

Tris buffer was prepared by titrating Tris base solution to the required pH with 0.5M-HCl and diluting to the required concentration. Phosphate buffer was prepared by titrating KH_2PO_4 solution to the required pH with 0.5M-NaOH and diluting to the required concentration. Similarly glycine buffer was prepared by titrating solutions of glycine with 0.5M-NaOH.

Polyacrylamide-gel electrophoresis

Electrophoresis of the enzyme was carried out on 6.5% (w/v) and 8.5% (w/v) polyacrylamide gels by the method of Davis (1964) in the apparatus described by Rogers (1965) at pH8.9. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described by Weber & Osborn (1969). Complete disaggregation of the enzyme required incubation for 2h at 37°C in 10mM-phosphate buffer, pH7.0, containing 1% (w/v) sodium dodecyl sulphate and 1% (w/v) mercaptoethanol. This was then dialysed overnight against 10mM-phosphate buffer, pH7.0, containing 0.1% sodium dodecyl sulphate and 0.1% mercaptoethanol. Gels were stained for protein with either Amido Black 10B (C.I. 20470) or Coomassie Brilliant Blue G-250 (C.I. 42655) and cleared by washing with 7% (v/v) acetic acid in water or acetic acid/ethanol/water (2:9:9, by vol.) respectively.

Gels were stained for enzyme activity by placing them in reaction mixtures containing, in 1.75ml of 50mM-glycine/NaOH buffer, pH9.0, 1.5μmol of *p*-cresol or 4-hydroxybenzyl alcohol and 0.4mg of either Nitro Blue Tetrazolium {3,3'-(3,3-dimethoxy-4,4'-biphenylene)-bis-[2-(*p*-nitrophenyl)-5-phenyl-2,4-tetrazolium chloride]} or 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride. These were incubated at room temperature (20°C) in the dark. The Nitro Blue Tetrazolium gave a blue colour with the enzyme, and the latter compound gave a red colour.

Ultracentrifuge studies

These were carried out with a Beckman–Spinco model E ultracentrifuge equipped with Rayleigh interference optics. Samples were previously dialysed for 24h against 50mM-phosphate buffer, pH7.5, at 2°C. Sedimentation-equilibrium experiments were conducted by using the short-column meniscus-depletion method of Yphantis (1964) and were performed at 17980rev./min for 24h at an initial protein concentration of 0.5mg/ml. In these runs the solution compartment in the double-sector cells, equipped with sapphire windows, contained 100μl of enzyme solution on 50μl of fluorocarbon oil (Beckman, Palo Alto, CA, U.S.A.); the reference compartment contained 120μl of diffusate on 40μl of fluorocarbon oil. The attainment of equilibrium was determined experimentally by measuring fringe displacements at given radial positions. A value of 0.725cm^3/g was assumed for the partial specific volume, $\bar{v}$, for the protein.

Enzyme purification of m-cresol

Redistillation of the *m*-cresol failed to remove all of the material that reduced the enzyme (see the Results section). It was removed enzymically by incubating 0.2ml of *m*-cresol with 10ml of 50mM-glycine/NaOH buffer, pH9.0, 9.0ml of partially purified enzyme (7.8mg of protein/ml) and 2ml of 10mM-phenazine methosulphate in a 50ml Erlenmeyer flask at 30°C on an orbital shaker. After 2h the protein was precipitated by addition of 10ml of 25% (w/v) metaphosphoric acid and removed by centrifuging at 12000*g* for 10min. After addition of 0.2g of semicarbazide hydrochloride the solution was extracted with 2×20ml of diethyl ether. The pooled extracts were dried over anhydrous Na_2SO_4 and then evaporated to dryness to yield the *m*-cresol.

Absorption spectra

Visible-absorption spectra were measured in a Unicam SP.1800 double-beam recording spectrophotometer.

Iron determination

Iron analysis was carried out directly on pure enzyme in 23mM-phosphate buffer, pH7.1, with an EEL 240 atomic absorption spectrometer (Evans Electroselenium Ltd., Halstead, Essex, U.K.). Standard curves were prepared by using an iron standard supplied by BDH, Poole, Dorset, U.K.

Protein determinations

The biuret method of Gornall *et al.* (1949) was used to determine protein, except where very low concentrations were present, when the method of Lowry *et al.* (1951) was used. Standard curves were prepared by using bovine serum albumin. The elution of protein from columns was followed by measuring A_{280} of the fractions.

Materials for enzyme purification

Pre-swollen micro-granular DEAE-cellulose DE-52 (W. R. Balston, Maidstone, Kent, U.K.) was equilibrated with 20mM-Tris/HCl buffer, pH7.6.

Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) was swollen in 50mM-phosphate buffer, pH7.5.

Bio-Gel HTP hydroxyapatite (Bio-Rad Laboratories, Richmond, CA, U.S.A.) was equilibrated with 1mM-phosphate buffer, pH6.8.

Chemicals

o-Cresol, *m*-cresol, *p*-cresol, 3,4-xylenol, phenazine methosulphate, Nitro Blue Tetrazolium and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride were obtained from BDH. 4-Ethylphenol was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A., as was 2,4-xylenol, which was redistilled before use. 4-Hydroxybenzyl alcohol was obtained from K & K Laboratories, Plainview, NY, U.S.A. 2,6-Dichlorophenol-indophenol and Amido Black 10B were obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K. Coomassie Brilliant Blue G-250 was obtained from Serva Feinbiochemica G.m.b.H., Heidelberg, Germany. Bovine serum albumin (Cohn fraction V) was from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Fumarase, glyceraldehyde 3-phosphate dehydrogenase and phosphorylase *a* were obtained from Boehringer Corp., London W.5, U.K. NAD^+ was obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.

Experimental and Results

Stimulatory factor in crude extracts

During the preliminary purification studies it was noticed that, whereas heat-treatment of crude extract at 70°C for 1min resulted in more than a 50% loss of hydroxylase activity, the enzyme was completely stable to this treatment after DEAE-cellulose chromatography of crude extract. Crude extract (6ml, 50mg of protein/ml) was loaded on a DEAE-cellulose column (5cm×2cm) and eluted first with 25ml of 20mM-Tris/HCl buffer, pH7.6; 5ml fractions were collected. The hydroxylase activity was then eluted with buffer containing 0.2M-KCl and was coincident with a red-coloured protein. Although the enzyme was active alone, the rate was markedly stimulated by earlier fractions containing material washed straight through the column. Despite the stimulation, phenazine methosulphate was still required for O_2 uptake. A maximum stimulation of 2.7-fold was given in an O_2-monitor assay containing 50μl of enzyme fraction (10.4mg of protein/ml) and 100μl of an earlier fraction (9.7mg of protein/ml). However, the stimulatory effect was lost after treatment at 70°C for 1min, although the activity of the enzyme fraction remained unchanged.

The fractions causing stimulation were cloudy, suggesting the presence of particulate material, and indeed stimulation by particulate material could be demonstrated. Crude extract (12ml) was centrifuged at 160000*g* for 1h at 2°C. The pellet was washed with 50mM-phosphate buffer, pH7.5, and, after further centrifuging as before, was resuspended in 1ml of the buffer.

These washed particles gave a 2.5-fold stimulation of purified enzyme.

Purification of enzyme

The enzyme was routinely purified from 2,4-xylenol-grown cells. Activity was also present in succinate-grown cells, but at only 50% of that in cells grown on 2,4-xylenol or *p*-cresol. The enzyme was stable at room temperature, and in the purification described here the column chromatography was for convenience performed at this temperature. Other purifications in which all steps were carried out at 4°C gave similar results.

Step 1: *preparation of crude extract.* Frozen cells (100g wet wt.) were thawed in 200ml of 20mM-Tris/HCl buffer, pH7.6, and disrupted by passing the cold suspension twice through a precooled French pressure cell with a pressure difference at the orifice of 138MPa. Unbroken cells and large particulate material were removed by centrifuging at 10000*g* for 20min at 2°C and the supernatant solution was retained.

Step 2: *heat-treatment.* Portions (40ml) of the crude extract were heated rapidly, with continuous stirring, to 70°C, kept at this temperature for 1min and then quickly cooled in ice/water. The precipitated protein was removed by centrifuging at 15000*g* for 15min at 2°C, washed by resuspending in 100ml of 20mM-Tris/HCl and again centrifuged. The pre-

cipitate was discarded and the supernatant solutions were pooled.

Step 3: *DEAE-cellulose chromatography* 1. The heat-treated extract was loaded on a DEAE-cellulose column (16cm×2.5cm) at room temperature. This was then washed with 120ml of 20mM-Tris/HCl buffer, pH7.6. The enzyme was eluted with buffer containing 0.2M-KCl and 10ml fractions were collected. Fractions 9–15 inclusive were pooled.

Step 4: *DEAE-cellulose chromatography* 2. The pooled fractions were dialysed for 4h at 2°C against 2 litres of 20mM-Tris/HCl buffer, pH7.6, and then loaded on a DEAE-cellulose column equilibrated with this buffer. The column was eluted at room temperature with a linear gradient constructed from 400ml of the buffer and 400ml of buffer containing 0.3M-KCl, and 7.5ml fractions were collected. The enzyme coincided with a red-coloured band which appeared in fractions 47–62. Fractions 49–60 inclusive were pooled.

Step 5: *Sephadex G-200 gel filtration.* The pooled fractions were dialysed for 4h against 2 litres of 20mM-Tris/HCl buffer, pH7.6, and concentrated by loading on a small DEAE-cellulose column (3cm×2cm) and elution in a small volume of buffer containing 0.3M-KCl. The enzyme solution was then loaded on a Sephadex G-200 column (58cm×2.5cm) and eluted with 50mM-phosphate buffer, pH7.5, at room temperature. Fractions (5ml) were collected and fractions 22–31 inclusive were pooled.

Step 6: *hydroxyapatite chromatography.* The pooled fractions were applied to a hydroxyapatite column (11cm×2.5cm), which was then eluted at room temperature with a linear gradient constructed from 400ml of 0.05M-phosphate buffer, pH6.8, and 400ml of 0.5M-phosphate buffer, pH6.8, and 5ml fractions were collected. Fractions 29–31, orange–red in colour, were pooled and stored at 2°C or, for periods longer than a few days, at −20°C.

The results from such a purification are shown in Table 1.

4-Hydroxybenzyl alcohol dehydrogenase activity

Crude extracts contained a 4-hydroxybenzyl alcohol dehydrogenase activity which, like the *p*-cresol hydroxylase, could be linked to phenazine methosulphate reduction and assayed in the same way. The rate with *p*-cresol was about 1.5 times that with the alcohol, and this ratio was maintained through the purification of the hydroxylase.

• Crude extracts of 2,4-xylenol- or *p*-cresol-grown cells also contained an NAD^+-linked 4-hydroxybenzyl alcohol dehydrogenase (sp. activities 2.5 and 2.0μmol of substrate/min per mg of protein respectively).

Polyacrylamide-gel electrophoresis

After electrophoresis on polyacrylamide gels the enzyme could be seen as a red band. Although on one occasion a single band was present after gels had been stained with Amido Black for protein, more often a major band was followed closely by a band of much lower intensity and by a third band, even less intense. However, all three bands gave a positive reaction when gels were stained for activity by linking the reaction to reduction of tetrazolium compounds, and the same gradation of activity was seen. The same pattern appeared with either *p*-cresol or 4-hydroxybenzyl alcohol as substrate. The secondary bands were seen on gels run at room temperature or at 2°C.

Molecular-weight determinations

A mol.wt. of 115000 was obtained by using the ultracentrifuge by the sedimentation-equilibrium (meniscus-depletion) method.

Electrophoresis of the enzyme, after incubation with sodium dodecyl sulphate and mercaptoethanol, on polyacrylamide gels containing sodium dodecyl sulphate gave a single band when stained for protein with Coomassie Brilliant Blue. A comparison of its mobility relative to Bromophenol Blue with the

Table 1. *Summary of the purification of p-cresol methylhydroxylase from Ps. putida N.C.I.B.* 9866

The starting material was 100g of cells grown on 2,4-xylenol. Values in parentheses are those calculated on the assumption that heat-treatment removes the stimulatory material but results in no loss of enzyme. One unit of enzyme is the amount that catalyses the uptake of ½μmol of O_2/min at 30°C in the O_2-monitor assay. This is equivalent to oxidation of 1μmol of substrate/min.

Purification step	Volume (ml)	Total protein (mg)	Total activity units)	Specific activity (units/mg)	Recovery (%)	Purification
(1) Crude extract	230	10180	4910 (1520)	0.466 (0.149)	100	1
(2) Heat-treatment	310	1550	1520	0.98	31 (100)	2.1 (6.6)
(3) First DEAE-cellulose chromatography	72	654	1256	1.92	26 (83)	4.1 (12.9)
(4) Second DEAE-cellulose chromatography	89	199	1009	5.06	20.5 (66)	10.9 (34)
(5) Sephadex G-200 gel filtration	52	108	573	5.31	11.7 (38)	11.4 (35.6)
(6) Hydroxyapatite chromatography	45	71	534	7.52	10.9 (35)	16.1 (50.5)

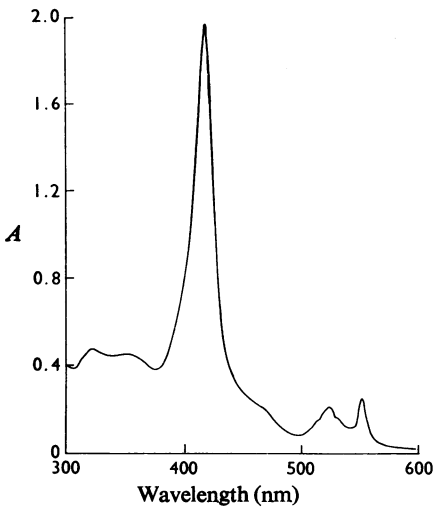

Fig. 1. *Absorption spectrum of pure enzyme*
The spectrum of the purified enzyme (1.58mg/ml) was recorded against a blank of 50mM-phosphate buffer, pH6.8.

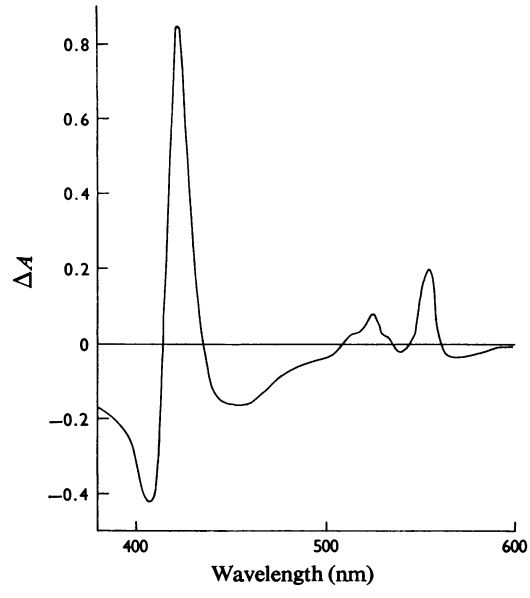

Fig. 2. *Difference spectrum of reduced against oxidized enzyme*
Enzyme solution (2.0ml; 1.42mg of protein/ml) was oxidized by addition of 3μl of H_2O_2 (50%, w/w). A spectrum was recorded with 1.0ml in both the sample and reference cuvettes (1cm light-path) to establish the baseline. A few crystals of sodium dithionite were then added to the sample cuvette and the spectrum was again recorded. A similar spectrum was obtained on addition of 0.01ml of 10mM-*p*-cresol or -4-hydroxybenzyl alcohol instead of sodium dithionite.

mobilities of protein standards, glyceraldehyde 3-phosphate dehydrogenase (0.77), mol.wt. 37000, fumarase (0.64), mol.wt. 48500, bovine serum albumin (0.54), mol.wt. 68000, and phosphorylase *a* (0.37), subunit mol.wt. 94000, gave a mol.wt. of approx. 56000. Without the prior incubation the major band corresponded to a protein of higher molecular weight than the phosphorylase standard (94000), and only a trace of the lower-molecular-weight band was seen.

Absorption spectra

The visible-absorption spectrum of the pure enzyme (Fig. 1) is typical of a haemoprotein and gave $\lambda_{max.}$ for the α-, β- and γ-peaks of 552, 542 and 417nm respectively. Marked changes occurred in the spectrum when the enzyme was oxidized by addition of 3μl of H_2O_2 (50%, w/w). The α- and β-peaks were replaced by a single broad peak and there was a decrease in the γ-peak, which shifted to 411nm. A more sensitive method of looking at these changes is by means of difference spectra. Such a spectrum for the enzyme is shown in Fig. 2. The same spectrum was observed when the enzyme was reduced chemically with sodium dithionite or by substrate addition. In a separate experiment the difference spectrum was produced by *p*-cresol or 4-hydroxybenzyl alcohol at concentrations close to that of the enzyme itself (about 1.0μM), but there was no reduction by *o*-cresol, phenol or toluene at concentrations of 0.14mM. An unexpected result was the reduction by *m*-cresol, since this is not a substrate for the enzyme. However, a concentration 75 times that of *p*-cresol was required to give the same degree of reduction. This is consistent with the presence of *p*-cresol as a 1–2% impurity in the *m*-cresol, and after enzymic purification of the *m*-cresol it was no longer active.

The alkaline pyridine haemochromagen of the enzyme was prepared by adding 0.04ml of 5M-NaOH and 0.3ml of pyridine to 0.9ml of enzyme solution, containing 0.79mg of protein. After addition of a few crystals of sodium dithionite, the spectrum of the solution in a 1cm-path-length semimicro-cuvette was recorded. This gave α-, β- and γ-peaks at 550, 520 and 414.5nm respectively, with an A_{550} of 0.16.

Haem/protein ratio

From the millimolar absorption coefficient of 31.18 for the α-peak of the alkaline pyridine ferrohaemochrome of cytochrome *c* (Bartsch, 1971) and a mol.wt. of 115000, the ratio of haem to protein in the enzyme was calculated as 0.93:1.

Table 2. *Iron content of hydroxylase*
Iron was determined by direct analysis of enzyme solution by atomic absorption spectroscopy. Two different enzyme solutions were measured, undiluted or diluted with 23mM-phosphate buffer, pH7.1. A correction of 0.06p.p.m. of Fe has been made for the buffer.

Enzyme (mg of protein/ml)	Concentration (μM)	Iron (p.p.m.)	Content (μM)	Fe/enzyme molar ratio
1.46	12.7	0.64	11.4	0.90
0.73	6.35	0.39	6.9	1.09
2.4	20.8	1.1	19.6	0.94
1.2	10.4	0.57	10.2	0.98

The ratio was also calculated from measurements of the iron content of the enzyme by atomic absorption spectroscopy (Table 2).

Flavin component of enzyme

A solution of purified enzyme (1ml, containing 1.58mg of protein) was extracted with 4ml of ice-cold acetone containing 0.5M-HCl. The precipitate obtained from centrifuging the solution at 28000*g* for 10min at 2°C was a yellow colour. This was resuspended in 1ml of 8.1M-Tris/HCl buffer, pH8.5, but only dissolved on addition of a small amount of strong NaOH to give a pH of 12. The solution had the visible-absorption spectrum of a flavin (Fig. 3), with a peak at 450nm which was abolished on reduction with dithionite.

If it is assumed that the absorption of the flavin is not altered in the protein complex then the flavin/protein ratio calculated by using ε 11.3×10^3 litre·mol^{-1}·cm^{-1} for FAD was 1.03:1, or by using 12.8×10^3 litre·mol^{-1}·cm^{-1} for FMN was 0.91:1.

Clearly the acetone/HCl treatment failed to dissociate the flavin from the protein. A number of other treatments, after resuspension of the protein in 50mM-phosphate buffer, pH7.5, including heating at 100°C for 10min, incubation for 10min at 50°C with an equal volume of ethanol, and bringing to 90% of saturation with $(NH_4)_2SO_4$ followed by a lowering of the pH to 3 with 0.2M-HCl, also failed to release flavin. The flavin remained attached to protein after the incubation with mercaptoethanol and sodium dodecyl sulphate before electrophoresis.

When the acetone was removed from the flavoprotein supernatant solution it left a cloudy solution. This was neutralized, and to 0.9ml was added 0.04ml of 5M-NaOH and 0.3ml of pyridine. The spectrum, on addition of sodium dithionite, was that of an alkaline ferrohaemochrome, but this compound rapidly reoxidized. To maintain the sample reduced for long enough to record the spectrum, the cuvette contents were flushed with N_2

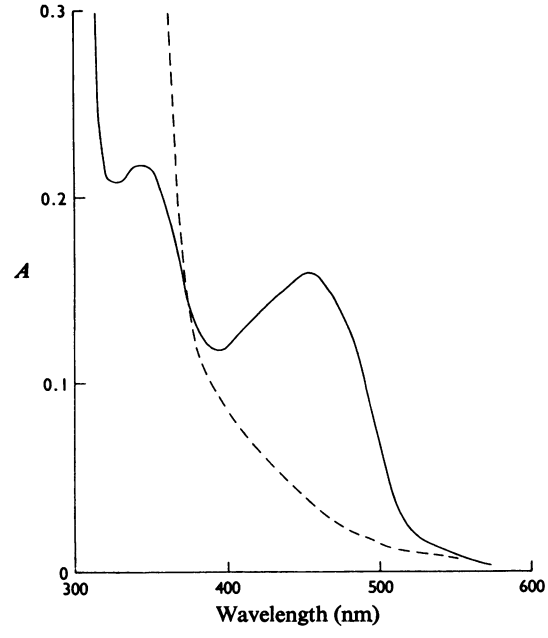

Fig. 3. *Spectrum of protein after acetone/HCl extraction of hydroxylase*
The protein precipitate, after extraction of 1ml of purified enzyme (1.58mg of protein/ml) with 4ml of ice-cold acetone containing 0.5M-HCl, was redissolved in 0.1M-Tris base, pH12, and the spectrum recorded (——). The spectrum after addition of a few crystals of sodium dithionite is also shown (----).

for 1min and the cuvette was fitted with a rubber cap through which a solution of sodium dithionite was injected. The spectrum was very similar to that of the derivative of the purified enzyme, with peaks at 550, 520 and 414nm.

If, instead of formation of the pyridine haemochromagen, the cloudy solution was centrifuged at 28000*g* for 10min at 2°C, a red–brown precipitate was obtained. Although, from spectral evidence, after its solution in alkali, this contained haem, there was also some remaining in the supernatant. In a similar experiment, the measurement of protein by the method of Lowry *et al.* (1951) showed that half the protein was precipitated as flavoprotein and the remainder was divided between the haem–protein precipitate and the supernatant.

Stoicheiometry of reaction

The stoicheiometry of acceptor reduced per molecule of substrate oxidized was measured by using the spectrophotometric assay with 2,6-dichlorophenol-indophenol as acceptor (Table 3). O_2 was

Table 3. *Reaction stoicheiometry*

The spectrophotometric assay mixture, containing 79 μg of pure enzyme in a 1 cm-light-path cuvette, was flushed for 1 min with N_2 and sealed immediately with a rubber cap. Substrate was injected through the cap and the decrease in A_{600} due to the reduction of 2,6-dichlorophenol-indophenol was measured with a Gilford 2400S spectrophotometer. The ε_{mM} for 2,6-dichlorophenol-indophenol of 20.6 at pH 7.0 (Armstrong, 1964) was corrected to 22.5 litre·mmol^{-1}·cm^{-1} for pH 9.0.

Substrate added (μmol)	ΔA_{600}	Acceptor reduced (μmol)	Acceptor/substrate molar ratio
p-Cresol			
0.05	0.735	0.109	2.14:1
0.10	1.41	0.208	2.08:1
4-Hydroxybenzyl alcohol			
0.05	0.375	0.055	1.11:1
0.10	0.72	0.107	1.07:1

excluded from the reaction mixture to prevent reoxidation of 2,6-dichlorophenol-indophenol.

Kinetic studies

Because very low substrate concentrations were needed to measure the K_m of the enzyme, the more sensitive spectrophotometric assay was used rather than the O_2-monitor assay. Initial rates were measured at various concentrations for a range of substrates (Hopper, 1976). For *p*-cresol, 2,4-xylenol and 4-hydroxybenzyl alcohol concentrations ranged from 0.01 to 0.07 mM, and 10 μg of pure enzyme was used in each assay. From double-reciprocal plots of the results, the K_m values for these three substrates were 7.3×10^{-6} M, 17.3×10^{-6} M and 47.6×10^{-6} M respectively. For 3,4-xylenol the range was 0.02–0.14 mM, giving a K_m of 72×10^{-6} M, and using a range of 0.02–0.7 mM for 4-ethylphenol gave a K_m of 360×10^{-6} M.

Discussion

A comparison of the molecular weight of the purified enzyme obtained by ultracentrifuge studies (115000) and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (approx. 56000) suggests that the enzyme consists of two subunits of about equal molecular weight. These are quite tightly linked, as evidenced by the procedures required for their separation. The single band seen on the sodium dodecyl sulphate-containing gels suggests that the two minor bands, seen on normal gels and whose presence was variable, possibly represent aggregates of the enzyme.

One of the two subunits of the hydroxylase has the properties of a *c*-type cytochrome. The spectrum of the pyridine haemochromagen is typical for this class of cytochrome (Bartsch, 1971), and a single haem group per molecule of enzyme was calculated from both the spectrum and the iron determination. A property of cytochrome *c* is that the haem is covalently bound to protein and should not be extracted with acetone/HCl (Bendall *et al.*, 1971). It was surprising, therefore, that the haem of the hydroxylase was apparently extracted by this procedure. However, it is clear from protein measurements that the extraction was removing the complete cytochrome subunit rather than just the haem. This also explains the similarity of the spectrum of the pyridine haemochrome of extracted haem with that of pure enzyme and cytochrome *c*. The derivative of the extracted subunit was, however, much more readily reoxidized than that of pure enzyme. The involvement of the cytochrome in the enzymic process was demonstrated by its reduction on addition of substrate (Fig. 2).

The other subunit appears to be a flavoprotein. This was initially suggested from the enzyme spectrum (Fig. 1), which showed a shoulder on the Soret peak at about 450 nm, and from the flavin trough at 450 nm on the reduced–oxidized difference spectrum (Fig. 2). This was confirmed from the spectrum of the protein remaining after acetone/HCl extraction (Fig. 3). This fraction contained half of the original protein, which is consistent with it being one of two subunits of equal molecular weight. The spectrum is typical of a flavoprotein with a peak at 450 nm which disappears on reduction. The position of the second peak at 345 nm is lower than that for free flavin or many flavoproteins (near 380 nm), but this property does vary, and that for the flavoprotein subunit of the flavocytochrome *c* of *Chlorobium thiosulfatophilum* is also quite low at 350 nm (Yamanaka, 1976). From the A_{450} the presence of a single molecule of flavin per molecule of enzyme can be calculated. This appears to be tightly bound and may well be covalently bound, as for the *Chlorobium* protein and a similar one from *Chromatium vinosum* (Bartsch *et al.*, 1968; Walker *et al.*, 1974). No further attempts were made to release and identify the flavin.

The possibility that the same enzyme is responsible for both *p*-cresol hydroxylation and 4-hydroxybenzyl alcohol dehydrogenation has now been confirmed. It is demonstrated by the same ratio of activities throughout purification, the same activity bands on polyacrylamide gels and the reduction of the cytochrome by both compounds but not by other similar compounds that are not substrates. This is also consistent with the proposed mechanism of the reaction by dehydrogenation of *p*-cresol to the quinone methide, and the stoicheiometry of acceptor reduced per molecule of substrate is that for two

dehydrogenase reactions with *p*-cresol as substrate and one with 4-hydroxybenzyl alcohol. This confirms the earlier results for only partially purified enzyme, where O_2 uptake was measured in a Warburg apparatus (Hopper, 1976). Although the enzyme is capable of converting 4-hydroxybenzyl alcohol into 4-hydroxybenzaldehyde, its physiological importance in catalysing this reaction is not clear, since the cells also contain an NAD^+-linked 4-hydroxybenzyl alcohol dehydrogenase. Thus the higher K_m of the hydroxylase for 4-hydroxybenzyl alcohol and the lower $V_{max.}$ will not necessarily result in transient accumulation of the alcohol during growth on *p*-cresol. The low K_m values for *p*-cresol and 2,4-xylenol are increased on introduction of a second methyl group adjacent to the first to give 3,4-xylenol, and particularly by replacement of methyl by the bulkier ethyl group in 4-ethylphenol.

The enzyme is presumably linked *in vivo* to an electron-transport system, replaced in the present experiments by phenazine methosulphate. It is likely that the particulate-stimulating factor in crude extracts contains the natural acceptor, although the system is not complete, as there was no O_2 uptake with the two fractions alone. The apparent stimulation is probably due to a more ready transfer of electrons from this acceptor to phenazine methosulphate. Other hydroxylases that derive the hydroxyl group from water rather than molecular O_2 also appear to be associated with electron-transport systems. An example is the cytochrome *c*-linked nicotinate hydroxylase in *Pseudomonas ovalis*, although, unlike our enzyme, there is no evidence that this is a flavoprotein, and it is firmly bound to membrane (Jones & Hughes, 1972; Jones, 1973). Another cytochrome *c* hydroxylase is lupanine hydroxylase from *Pseudomonas lupanini* (Rogozinski, 1975), and, for *p*-cresol itself, Dagley & Patel (1957) reported evidence for involvement of a cytochrome system in their strain of *Pseudomonas* oxidizing this substrate, although there was no detailed investigation of the enzyme concerned.

When the effect of the stimulating material in crude extracts is taken into account in calculating purifications (values in parentheses in Table 1), the *p*-cresol methylhydroxylase is pure after about 50-fold purification and thus represents about 2% of the soluble cell protein.

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