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Incorporation of [^{18}O]Water in the Formation of *p*-Hydroxybenzyl Alcohol by the *p*-Cresol Methylhydroxylase from *Pseudomonas putida*

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In the hydroxylation of the methyl group of *p*-cresol by an enzyme from *Pseudomonas putida* the oxygen atom is derived from water. Although a second reaction by the same enzyme converts the product, *p*-hydroxybenzyl alcohol, into the aldehyde, the alcohol is an enzyme-free intermediate.

The catabolism of *p*-cresol and 2,4-xylenol by *Pseudomonas putida* N.C.I.B. 9866 involves the initial oxidation of the *para*-methyl group to a carboxy group via the alcohol and aldehyde (Chapman & Hopper, 1968). The first step is hydroxylation of the methyl group, and the enzyme that catalyses this reaction has been purified and shown to be a flavocytochrome *c* (Hopper & Taylor, 1977). Unlike the methyl hydroxylases that attack the terminal groups of alkanes (McKenna & Coon, 1970) or methoxy groups on aromatic compounds (Bernhardt *et al.*, 1975), this enzyme does not appear to be an oxygenase and requires an electron acceptor rather than a donor. Phenazine methosulphate can be used as an artificial electron acceptor and the enzyme can be assayed by following oxygen uptake due to reoxidation of the reduced acceptor. The major product from *p*-cresol under these conditions is *p*-hydroxybenzaldehyde, since the enzyme will also attack *p*-hydroxybenzyl alcohol, and a route involving two dehydrogenase steps has been proposed (Hopper, 1976). In this scheme the *p*-hydroxybenzyl alcohol is produced by hydration of the quinone methide formed by the first dehydrogenation. The oxygen of the alcohol group is, therefore, derived from water. In the work described in the present paper this is confirmed by the use of $H_2{}^{18}O$.

Materials and Methods

Preparation of enzyme

The *p*-cresol methylhydroxylase was purified from *Pseudomonas putida* N.C.I.B. 9866 grown on 2,4-xylenol, as described by Hopper & Taylor (1977), and has a specific activity of 7.5 μmol of substrate transformed/min per mg of protein.

Incubations with enzyme

All reaction mixtures were made up in Warburg flasks containing 2 μmol of phenazine methosulphate in 2.8ml of 0.1M-glycine/NaOH buffer, pH9.0, with 0.2ml of 20% (w/v) KOH in the centre well. Flasks were wrapped in aluminium foil to keep out light. Reaction mixtures 1 and 2 also contained 0.8mg of purified *p*-cresol methylhydroxylase and 100 μmol of *p*-cresol and the water in reaction mixture 2 was enriched with 17.4% $H_2{}^{18}O$. Reaction mixture 3 contained 0.8mg of enzyme, inactivated by boiling for 5min before addition, and 30 μmol of *p*-hydroxybenzyl alcohol and the water was enriched with 17.4% $H_2{}^{18}O$.

The reaction mixtures were incubated at 30°C and the O_2 uptake was followed in a conventional Warburg apparatus. Reactions were stopped by addition of 2.0ml of 25% (w/v) metaphosphoric acid. Precipitated protein was removed by centrifuging at 12000*g* for 10min.

Isolation of products

Reaction mixtures were extracted five times with 5ml of diethyl ether and the pooled extracts were each dried over anhydrous Na_2SO_4 and then evaporated to dryness under a stream of N_2. The products were separated by t.l.c. as bands on plates, coated to a thickness of 0.25mm with Kieselgel GF_{254} (E. Merck A.-G., Darmstadt, Germany), in benzene/dioxan/acetic acid (90:25:4, by vol.) (Pastuska, 1961). The bands corresponding to *p*-hydroxybenzyl alcohol when the plates were viewed under u.v. light were scraped off and eluted three times with 5ml of diethyl ether. The pooled extracts were each centrifuged and the supernatants evaporated to dryness. This yielded approx. 2mg of solid from reaction mixtures 1 and 2 and 3mg from reaction mixture 3.

Mass spectra

Mass spectra of the products were determined on an A.E.I. MS 30 instrument by a direct-insertion-probe sampling technique. The probe temperature was 50°C, and an ionization potential of 70eV was used.

Chemicals

p-Cresol and phenazine methosulphate were obtained from BDH Chemicals, Poole, Dorset, U.K. *p*-Hydroxybenzyl alcohol was obtained from K & K Laboratories, Plainview, NY, U.S.A. Normalized $H_2{}^{18}O$, from YEDA Research and Development Co., Rehovoth, Israel, was generously given by Dr. R. E. Cripps, Shell Bioscience Laboratories, Sittingbourne, Kent, U.K.

Results and Discussion

Purified *p*-cresol methylhydroxylase and its substrate were incubated with phenazine methosulphate as electron acceptor in an unenriched reaction mixture and in a reaction mixture enriched with $H_2{}^{18}O$. As a control to monitor non-enzymic exchange of $H_2{}^{18}O$ with the expected reaction product, *p*-hydroxybenzyl alcohol, a third reaction mixture also enriched with $H_2{}^{18}O$ was set up containing *p*-hydroxybenzyl alcohol and heat-inactivated enzyme. The O_2 consumption by the mixtures, due to autoxidation of phenazine methosulphate reduced by the enzyme, was monitored, and after the uptake of 500 μl of O_2 by reaction mixtures 1 and 2 the reactions were stopped. This was equivalent to oxidation of 20–25% of the added *p*-cresol. There was no O_2 uptake by reaction mixture 3.

Each reaction mixture contained material corresponding to *p*-hydroxybenzyl alcohol on t.l.c., and this identification was confirmed by the mass-spectral data (Fig. 1). The mass of the molecular ion at *m/e* 124 corresponded to that calculated for $C_7H_8O_2$ and the spectra were essentially the same as that published for *p*-hydroxybenzyl alcohol by Budzikiewicz *et al.* (1965), a characteristic feature being an ion at *m/e* 107 due to loss of OH. Other fragmentation pathways involve successive loss of H_2O and CO giving ions at *m/e* 106 and 78; successive loss of H, CO and H_2O giving ions at *m/e* 123, 95 and 77 (phenyl ion); successive loss of H_2, H and CO giving ions at *m/e* 122, 121, 93 and 65 (cyclopentadienyl cation).

Thus *p*-hydroxybenzyl alcohol has been demonstrated as a free intermediate in the oxidation of *p*-cresol by this enzyme under aerobic conditions. Previously *p*-hydroxybenzaldehyde was identified as the product, and the alcohol was only found when anaerobic conditions were used with an amount of electron acceptor less than that required for complete oxidation (Hopper, 1976). It is not clear whether the second reaction, converting the alcohol into aldehyde, is of physiological importance since the organism also contains an NAD^+-linked alcohol dehydrogenase (Hopper & Taylor, 1977).

The mass spectrum of the *p*-hydroxybenzyl alcohol from reaction mixture 2, enriched with $H_2{}^{18}O$, gave an additional ion at *m/e* 126 (M^++2)

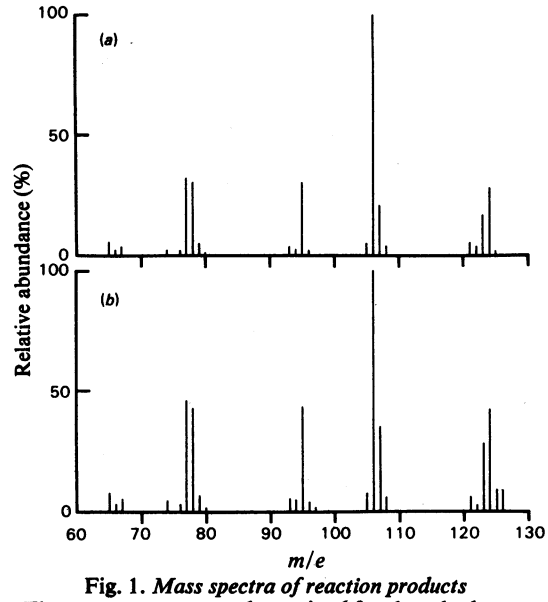

Fig. 1. *Mass spectra of reaction products*
The mass spectra were determined for the *p*-hydroxybenzyl alcohol isolated from (*a*) reaction mixture 1 (unenriched) and (*b*) reaction mixture 2 (enriched with $H_2{}^{18}O$).

(Fig. 1*b*) indicative of the incorporation of $H_2{}^{18}O$ as predicted (Hopper, 1976), and the labelled *p*-hydroxybenzyl alcohol represents 16.6% of the total, which is in good agreement with the 17.4% enrichment of $H_2{}^{18}O$ in the reaction mixture. The location of the label in the alcohol group is shown by the lack of a significant peak at *m/e* 109 for loss of OH from this group (Fig. 1*b*). There was no evidence for isotope exchange between *p*-hydroxybenzyl alcohol and water as judged from the mass spectrum of the product from reaction mixture 3, which was essentially the same as shown in Fig. 1(*a*). This accords with the report by Samuel (1962) that alcohols do not exchange with water in neutral or basic solution and phenols undergo exchange with difficulty.

These results confirm that in the reaction catalysed by *p*-cresol methylhydroxylase the oxygen in the hydroxyl group is derived from water. Similar enzymes have also been isolated from another strain of *Ps. putida* (Keat & Hopper, 1978), and this type of reaction has been proposed, again from isotope-labelling experiments, for a step in the pathway for the catabolism of α-conidendrin by *Pseudomonas multivorans* (Toms & Wood, 1970)

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