Stereochemical aspects of the oxidation of 4-ethylphenol by the bacterial enzyme 4-ethylphenol methylenedehydroxylase

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The O_2 -independent hydroxylase 4-ethylphenol methylenedehydroxylase (4EPMH) from *Pseudomonas putida* JD1 catalysed the complete conversion of 4-ethylphenol into 1-(4-hydroxyphenyl)ethanol together with a small amount of 4-hydroxyacetophenone, but with no formation of the side product 4-vinylphenol reported to be formed when the similar enzyme *p*-cresol methylhydroxylase (PCMH) catalyses this reaction. The enantiomer of 1-(4-hydroxyphenyl)ethanol produced by 4EPMH was *R*(+) when horse heart cytochrome *c* or azurin was used as electron acceptor for the enzyme. PCMHs from various bacterial strains produced the *S*(–)-alcohol. Both enantiomers of 1-(4-hydroxyphenyl)ethanol were substrates for conversion into 4-hydroxyacetophenone by 4EPMH, but the *S*(–)-isomer was preferred. The K_m and k_{cat} were 1.2 mM and 41 s^{–1} respectively for the *S*(–)-alcohol and 4.7 mM and 22 s^{–1} for the *R*(+)-alcohol. In addition to the 1-(4-hydroxyphenyl)ethanol dehydrogenase activity of 4-EPMH, NAD⁺-linked dehydrogenase activity for both enantiomers of the alcohol was found in extracts of *Ps. putida* JD1.

INTRODUCTION

The enzyme 4-ethylphenol methylenedehydroxylase (4EPMH) catalyses the first two steps in the catabolism of 4-ethylphenol by *Pseudomonas putida* JD1 (Darby *et al.*, 1987; Reeve *et al.*, 1989a). The pathway proceeds by hydroxylation of the side chain to give 1-(4-hydroxyphenyl)ethanol followed by dehydrogenation of the alcohol to yield 4-hydroxyacetophenone. The hydroxylation does not require molecular O_2 , but is thought to proceed by dehydrogenation to a quinone methide intermediate, which is then hydrated. The same enzyme can catalyse a second dehydrogenation to convert the alcohol into the ketone (Scheme 1). This sequence is analogous to that for the oxidation of *p*-cresol by *p*-cresol methylhydroxylase (PCMH) (Hopper, 1976; McIntire *et al.*, 1985), and the enzymes themselves are similar, containing flavoprotein and cytochrome *c* subunits. Each enzyme will hydroxylate both *p*-cresol and 4-ethylphenol, although the 4EPMH is more active with the longer-chain alkylphenols.

Hydroxylation of 4-ethylphenol introduces a chiral centre into the molecule and the possibility of stereospecificity in the enzyme-catalysed reaction with regard to the isomer of alcohol that is formed. A study of the stereochemistry of 1-(4-hydroxyphenyl)ethanol produced by PCMH from *Ps. putida* N.C.I.B. 9869 showed that with phenazine methosulphate as the electron acceptor for the enzyme a mixture consisting of 69.5% *S*(–)- and 30.5% *R*(+)-alcohol was produced (McIntire *et al.*, 1984). However, more recently the use of horse heart cytochrome *c* as electron acceptor has been exploited for the accumulation of 1-

(4-hydroxyphenyl)ethanol by the same enzyme, and in this case the alcohol consisted of > 97% of the *S*(–)-isomer (McIntire & Bohmont, 1987).

In the present paper we report the results of similar experiments with the 4EPMH purified from *Ps. putida* JD1. Although this enzyme closely resembles PCMH, we show that the chirality of the alcohol produced is different, with the *R*(+)-isomer being formed.

MATERIALS AND METHODS

Growth of organism

Ps. putida JD1 was maintained and grown as described by Reeve *et al.* (1989a).

Preparation of cell extracts and membranes

Cells from 1 litre of culture of *Ps. putida* JD1 grown on 4-ethylphenol were harvested, and the cell pellet was resuspended in 7.5 ml of 21 mM-sodium/potassium phosphate buffer, pH 7.0. The cells were disrupted by ultrasonic disintegration at 4 °C with an MSE Soniprobe 150 in 30 s bursts for a total of 4 min. The extract was centrifuged at 22000 *g* for 15 min at 4 °C, giving a supernatant solution that is termed crude extract. The membranes were obtained by centrifuging the crude extract at 120000 *g* for 1 h at 5 °C. The red gelatinous membrane pellet was washed by resuspension in 10 ml of 75 mM-Tris/HCl buffer, pH 7.6,

Scheme 1. Reactions catalysed by 4-ethylphenol methylenedehydroxylase

Abbreviations used: 4EPMH, 4-ethylphenol methylenedehydroxylase; PCMH, *p*-cresol methylhydroxylase.

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centrifuging as before and finally resuspension in 10 ml of the Tris buffer.

Purification of enzymes

4EPMH was purified as described by Reeve *et al.* (1989a). PCMHs were purified by the procedure used by Koerber *et al.* (1985). Azurin was purified by the procedure described by Causer (1985).

Production of 1-(4-hydroxyphenyl)ethanol by whole cells

Cells from a 1-litre culture of *Ps. putida* JD1 grown with 0.05 % 4-ethylphenol as the sole carbon source were harvested and resuspended in 20 ml of 42 mM-sodium/potassium phosphate buffer, pH 7.0. The suspension was placed in a 100 ml Erlenmeyer flask, and more buffer was added to bring the level almost to the top of the flask. 4-Ethylphenol was added to a concentration of 2.5 mM and the flask was incubated, without shaking, at 30 °C. Accumulation of the alcohol was monitored by extracting 2 ml samples, taken every 30 min, with 5 ml of diethyl ether and examining the products in the extract by t.l.c. After 90 min the cells were removed by centrifuging at 13000 *g* for 20 min and the supernatant solution was extracted three times with 100 ml of diethyl ether. The pooled extracts were dried over anhydrous Na₂SO₄ and the ether was removed by evaporation.

Production of 1-(4-hydroxyphenyl)ethanol with the use of purified enzyme

4-Ethylphenol was oxidized enzymically with various electron acceptors for the hydroxylase.

With phenazine methosulphate as acceptor the reaction mixture contained, in 10 ml of 50 mM-Tris/HCl buffer, pH 7.6, 50 µmol of 4-ethylphenol, 20 µmol of phenazine methosulphate and 1 mg of 4 EPMH. The mixture was incubated with shaking at 27 °C in the dark for 90 min. The reaction was stopped by addition of 4 ml of 2 M-HCl, and the mixture was extracted three times with 50 ml portions of diethyl ether. The pooled extracts were dried over anhydrous Na₂SO₄ and the ether was removed by evaporation.

With horse heart cytochrome *c* as acceptor the reaction mixture contained, in 10 ml of 10 mM-Tris/HCl buffer, pH 7.6, 50 µmol of 4-ethylphenol, 0.5 µmol of horse heart cytochrome *c*, 10 units of cytochrome *c* oxidase and 0.5 mg of 4EPMH. The reaction mixture was incubated, with shaking, at 37 °C for 7 h. Products were then extracted with diethyl ether as described above.

With azurin as acceptor the reaction mixture contained, in 10 ml of 50 mM-Tris/HCl buffer, pH 7.6, 50 µmol of 4-ethylphenol, 5 mg of azurin, membrane preparation containing 5.9 mg of protein and 5 mg of 4EPMH. The mixture was incubated, with shaking, at 25 °C for 2 h. The membrane material was then removed by centrifuging at 120000 *g* for 1 h at 4 °C and products were extracted with diethyl ether as described above.

For a larger-scale accumulation of the alcohol the reaction mixture contained, in 50 ml of 10 mM-Tris/HCl buffer, pH 7.6, 5 mg of 4EPMH, 31 mg of cytochrome *c* and membrane preparation containing 91 mg of protein. 4-Ethylphenol (15 mg) was added and the mixture was shaken at 25 °C. Disappearance of 4-ethylphenol was monitored by extraction of 0.5 ml samples of the reaction mixture with 1.0 ml of diethyl ether, and analysis of the extract was by t.l.c. with the system described in the next subsection. Further additions of 4-ethylphenol were made to a total of 150 mg. The reaction mixture was extracted three times with 100 ml portions of diethyl ether, which were then dried over anhydrous Na₂SO₄ and the ether was evaporated to give a white solid. This was washed with 20 ml of light petroleum (b.p. 40–60 °C) to remove any residual 4-ethylphenol and with 20 ml

of chloroform to remove 4-hydroxyacetophenone. The yield was 117 mg of the alcohol.

Purification and stereochemical analysis of 1-(4-hydroxyphenyl)ethanol

The 1-(4-hydroxyphenyl)ethanol produced was purified by preparative t.l.c. on precoated silica-gel GHLF plates (Analtech, Newark, NJ, U.S.A.) with chloroform/methanol (20:1, v/v) as solvent. Products were detected by their quenching of fluorescence when plates were viewed under u.v. light. The band corresponding to the alcohol was scraped off the plate and extracted with 5 ml of diethyl ether. The silica gel was removed by centrifuging and the ether was evaporated to dryness.

The enantiomeric composition of the 1-(4-hydroxyphenyl)ethanol was determined by separation of the isomers by h.p.l.c. on a 4.9 mm × 250 mm 'Pirkle column', which contained D-phenylglycine covalently bound to a 5 µm-particle-size spherical silica base (Hichrom Ltd., Reading, Berks., U.K.). The alcohol was analysed as its 1-(4-methoxyphenyl)ethyl acetate derivative. This was prepared as described by McIntire *et al.* (1984) by methylation of the phenolic hydroxy group with diazomethane and acetylation of the side-chain hydroxy group by incubation with acetic anhydride in pyridine at 60 °C for 4 h. The *R*(+)- and *S*(-)-isomers were resolved by using this column with hexane/propan-2-ol (97:3, v/v) as solvent at a flow rate of 1 ml/min. The compounds were detected with a u.v. monitor at a wavelength of 254 nm.

H.p.l.c. of products

Products were separated by h.p.l.c. with the use of a Pye-Unicam PU4003 pump and controller with a PU4025 u.v. detector linked to an Apple computer for data collection. The column packing was Partisil 10 ODS3 and a solvent of acetonitrile/water (31:69, v/v) was used at a flow rate of 1 ml/min. Peaks were detected at 276 nm. Retention times were: 4-ethylphenol, 9.53 min; 1-(4-hydroxyphenyl)ethanol, 3.95 min; 4-hydroxyacetophenone, 4.83 min; 4-vinylphenol, 8.57 min.

Preparation of *S*(-)-1-(4-hydroxyphenyl)ethanol

Racemic 1-(4-hydroxyphenyl)ethanol was prepared by the reduction of 4-hydroxyacetophenone with NaBH₄. The separation of *S*(-)-1-(4-hydroxyphenyl)ethanol from this racemate was achieved by stereospecific esterification with the use of a lipase from *Pseudomonas fluorescens* (Biocatalysts Ltd., Pontypridd, Mid-Glamorgan, Wales, U.K.). This enzyme esterifies the *R*(+)-isomer at a faster rate than the *S*(-)-isomer and after a suitable incubation produces an unequal mixture of esters of *R*(+)- and *S*(-)-alcohols with pure non-esterified *S*(-)-alcohol. Racemic alcohol (200 mg) was dissolved in 10 ml of vinyl acetate, and 0.5 g of lipase was added. The mixture was incubated at 28 °C on an orbital shaker for 22 h. The lipase was then removed by filtration and the excess vinyl acetate was removed on a rotary evaporator. The residue, a mixture of ester and alcohol, was separated by flash chromatography on a silica-gel 60 column (21 cm × 2 cm) with chloroform/methanol (20:1, v/v) as solvent. Fractions were examined by t.l.c., and those containing the free alcohol were pooled and the solvent was removed on a rotary evaporator. This yielded 80 mg of material, which, when converted into the 1-(4-methoxyphenyl)ethyl acetate derivative and examined by h.p.l.c. on a chiral column gave a peak only for *S*(-)-1-(4-hydroxyphenyl)ethanol. The product had $[\alpha]_D^{20} -49.1^\circ$ (*c* 0.36 in ethanol), measured with a Bendix NLP 143D automatic polarimeter.

Enzyme assays

The assay for 4EPMH and the conditions for the steady-state

kinetic studies were as described by Reeve *et al.* (1989a). Horse heart cytochrome *c* was used as the reoxidizing substrate for the kinetics and its concentration was varied in the range 4–50 μM . The concentration of the *R*(+)-alcohol was varied in the range 2–8 mM and that of the *S*(-)-alcohol in the range 0.5–5 mM with an enzyme concentration of 8.8 nM. Initial rates were measured and the data were analysed to obtain V_{max} and K_m values and their standard errors by using a non-linear-regression-analysis computer program, Enzfitter (Leatherbarrow, 1987).

NAD⁺-linked alcohol dehydrogenase was assayed spectrophotometrically by monitoring the reduction of NAD⁺ at 370 nm (ϵ 2.86 $\text{mm}^{-1}\cdot\text{cm}^{-1}$) at 25 °C. This wavelength was chosen to avoid complications due to the absorbance of 4-hydroxyacetophenone at 340 nm and possible different rates of its further metabolism in various crude extracts. The reaction mixture contained, in 1 ml of 0.1 M-glycine/NaOH buffer, pH 9.8, 2 μmol of NAD⁺, 1 μmol of 1-(4-hydroxyphenyl)ethanol and cell extract. For assay in the reverse direction 1 ml of 0.1 M-sodium/potassium pyrophosphate buffer, pH 7.5, contained 0.34 μmol of NADH, 0.5 μmol of 4-hydroxyacetophenone and cell extract.

O₂ uptake by whole cells

Oxidation of 1-(4-hydroxyphenyl)ethanol by whole cells was measured at 30 °C in a conventional Warburg apparatus. Flasks contained, in 1.8 ml of 42 mM-sodium/potassium phosphate buffer, pH 7.1, 2.5 mg dry wt. of cells and 3 μmol of substrate, with 0.2 ml of 20% (w/v) KOH in the centre well.

RESULTS AND DISCUSSION

Products from enzymic oxidation of 4-ethylphenol

The course of the reaction catalysed by 4-EPMH was monitored by taking samples from a reaction mixture, containing 4-ethylphenol as substrate, at 15 min intervals, extracting the products with diethyl ether and examining them qualitatively and quantitatively by h.p.l.c. The enzyme was continually reoxidized during the course of the experiment by cytochrome *c*, which in turn was reoxidized by a cell membrane preparation. The 4-ethylphenol concentration fell rapidly and after 150 min none was detected. Its decrease was mirrored by the concomitant increase in concentration of 1-(4-hydroxyphenyl)ethanol. There was a slow formation of 4-hydroxyacetophenone, but this amounted to less than 5% of the initial substrate after 150 min. No 4-vinylphenol, a side product from rearrangement of the quinone methide, was detected. This contrasts with the reaction catalysed by PCMH, where only one-third of the substrate disappeared, probably owing to the accumulation of 4-vinylphenol, which acted as a competitive inhibitor of the reaction (McIntire & Bohmont, 1987).

Stereochemical characterization of 1-(4-hydroxyphenyl)ethanol produced by 4EPMH

The stereochemistry of the 1-(4-hydroxyphenyl)ethanol isolated from the enzymic hydroxylation of 4-ethylphenol was examined, after formation of the 1-(4-methoxyphenyl)ethyl acetate derivative, by h.p.l.c. on a 'Pirkle column'. On the type of column used it is reported that the *R*(+)- and *S*(-)-isomers are separated with the *R*(+)-isomer being eluted before the *S*(-)-isomer (McIntire *et al.*, 1984). This was confirmed by running a sample of the chemically synthesized racemic alcohol and then running a mixture of this and some *S*(-)-1-(4-hydroxyphenyl)ethanol produced enzymically with PCMH from *Ps. putida* N.C.I.B. 9869, with cytochrome *c* as electron acceptor (McIntire & Bohmont, 1987). Separation of the mixture showed

Fig. 1. Separation of *R*(+)- and *S*(-)-1-(4-methoxyphenyl)ethyl acetate by h.p.l.c. on a 'Pirkle column'

(a) Separation of (*RS*)-1-(4-methoxyphenyl)ethyl acetate; (b) 1-(4-methoxyphenyl)ethyl acetate derived from the reaction of 4-EPMH with 4-ethylphenol; (c) mixture of samples (a) and (b); (d) 1-(4-methoxyphenyl)ethyl acetate derived from the reaction of *Ps. putida* N.C.I.B. 9869 PCMH A with 4-ethylphenol; (e) mixtures of samples (a) and (d). Samples were analysed on a 'Pirkle column' with hexane/propan-2-ol (97:3, v/v) as solvent at a flow rate of 1 ml/min and a detector wavelength of 254 nm. Peaks between 5 and 10 min are a result of the procedures for the formation of the derivative.

enhancement of the second peak compared with the synthetic alcohol alone (Fig. 1).

The enantiomeric compositions of the 1-(4-hydroxyphenyl)ethanol produced by pure enzyme with a variety of

Table 1. Enantiomeric composition of 1-(4-hydroxyphenyl)ethanol produced by 4-EPMH linked to a variety of electron acceptors and whole cells of *Ps. putida* JD1

1-(4-Hydroxyphenyl)ethanol was prepared from 4-ethylphenol with the use of whole cells of *Ps. putida* JD1 and purified 4-EPMH coupled to a variety of electron acceptors, and its enantiomeric composition was determined by h.p.l.c. on a 'Pirkle column'.

Hydroxylating system	1-(4-Hydroxyphenyl)ethanol		Enantiomeric excess (%)
	<i>R</i> (+)-Enantiomer (%)	<i>S</i> (-)-Enantiomer (%)	
<i>Ps. putida</i> JD1 whole cells	96	4	92
4EPMH + cytochrome <i>c</i>	> 99	< 1	> 98
4EPMH + azurin	98	2	96
4EPMH + phenazine methosulphate	55	45	10

electron acceptors and by whole cells of *Ps. putida* JD1 are given in Table 1. The h.p.l.c. trace for the product with cytochrome *c* as acceptor and its enhancement of one peak of racemic alcohol are shown in Fig. 1. The finding that 4EPMH produces the *R*(+)-enantiomer of the alcohol, when either horse heart cytochrome *c* or bacterial azurin was used as reoxidant, contrasts with the stereochemistry of the same reaction catalysed by PCMH from *Ps. putida* N.C.I.B. 9869, which gave > 97% of the *S*(-)-enantiomer (McIntire & Bohmont, 1987). Indeed, when a number of PCMHs isolated from several bacterial species were tested in the same way with cytochrome *c* as acceptor they all produced a high enantiomeric excess of the *S*(-)-isomer (Table 2). This included the PCMH from *Ps. putida* JD1 itself when grown on *p*-cresol (Reeve *et al.*, 1988).

PCMH and 4EPMH are very similar in size, subunit structure and prosthetic groups, and each will hydroxylate both *p*-cresol and 4-ethylphenol. However, differences in their specificities towards these substrates have been reported, and we can now add to these this difference in the stereochemistry of their reaction with 4-ethylphenol.

Table 2. Enantiomeric composition of 1-(4-hydroxyphenyl)ethanol produced by PCMH from a variety of bacterial species

1-(4-Hydroxyphenyl)ethanol was prepared from 4-ethylphenol with the use of purified PCMHs from a variety of bacterial species. In all experiments cytochrome *c* was the electron acceptor. The enantiomeric composition was determined by h.p.l.c. on a 'Pirkle column'.

Bacterium	1-(4-Hydroxyphenyl)ethanol		Enantiomeric excess (%)
	<i>R</i> (+)-Enantiomer (%)	<i>S</i> (-)-Enantiomer (%)	
<i>Ps. putida</i> N.C.I.B. 9866	15	85	70
<i>Ps. alcaligenes</i> N.C.I.B. 9867	8	92	84
<i>Ps. putida</i> N.C.I.B. 9869 PCMH A	11	89	78
<i>Ps. putida</i> N.C.I.B. 9869 PCMH B	10	90	80
<i>Ps. testosteroni</i>	15	85	70
<i>Ps. putida</i> JD1	12	88	76

The *R*(+)-isomer also accumulated when whole cells of *Ps. putida* JD1 were incubated with 4-ethylphenol under conditions where the restricted O₂ supply limited the oxidation of later aromatic intermediates (Table 1), suggesting that this is the enantiomer of physiological importance. With intact cells, however, the possibility arises of the production of racemic alcohol with the selective further oxidation of only one isomer. This can be discounted from the experiments with the pure enzyme, as very little 4-hydroxyacetophenone accumulated, and under conditions that approach those in the whole cell, with azurin as acceptor reoxidized by a membrane preparation (Reeve *et al.*, 1989b), again only the *R*(+)-alcohol was found.

It seems, however, that the stereochemistry of the reaction can be modified by the nature of the reoxidant, because with phenazine methosulphate as acceptor a mixture of *R*- and *S*-alcohols in an almost 1:1 ratio was produced (Table 1). The possibility that a chemical reaction with this acceptor was responsible for racemization of the enzymically produced alcohol rather than lack of stereospecificity of the enzyme under these conditions was tested by incubating some *R*(+)-1-(4-hydroxyphenyl)ethanol, produced with cytochrome *c* as acceptor, with 2 mM-phenazine methosulphate in 50 mM-Tris/HCl buffer, pH 7.6, at 27 °C for 90 min in the dark. The alcohol was reisolated and analysed. No racemization had occurred. A similar modification of stereochemistry by phenazine methosulphate was seen for the same reaction catalysed by PCMH, where the *S*(-)-enantiomer was produced with cytochrome *c* as the acceptor (McIntire & Bohmont, 1987) but with phenazine methosulphate a mixture of *S*- and *R*-isomers was obtained (McIntire *et al.*, 1984).

Further oxidation of 1-(4-hydroxyphenyl)ethanol by 4EPMH

4EPMH will also catalyse the conversion of the alcohol into 4-hydroxyacetophenone (Reeve *et al.*, 1989a), and was thought to be necessary for this step in the catabolic pathway. With the finding that the enzyme is highly stereospecific for the production of alcohol, it was of interest to test whether similar specificity was shown for alcohol metabolism. Racemic 1-(4-hydroxyphenyl)ethanol was incubated with 4EPMH, and the proportion of *R*- and *S*-isomers remaining was measured at intervals. The reaction mixture contained, in 10 ml of 10 mM-Tris/HCl buffer, pH 7.6, 0.5 μmol of cytochrome *c*, 20 units of cytochrome *c* oxidase, 1 mg of 4EPMH and 70 μmol of *RS*-1-(4-hydroxyphenyl)ethanol. This was incubated with shaking at 25 °C. Samples (2 ml) were removed at intervals and the alcohol was extracted and analysed. Surprisingly it was the *S*(-)-isomer that disappeared at a higher rate, such that after 4 h the *R*-/*S*-isomer percentage ratio was 86:14. Similar results were obtained with phenazine methosulphate or azurin as electron acceptor.

The specificity of the enzyme for each enantiomer was then determined by steady-state kinetics. A sample of pure *R*(+)-alcohol was prepared by scaling up the reaction for the conversion of 4-ethylphenol into alcohol by 4EPMH. This gave 117 mg of *R*(+)-1-(4-hydroxyphenyl)ethanol with $[\alpha]_D^{20} + 48.8^\circ$ (*c* 0.37 in ethanol). The *S*(-)-isomer was prepared from the racemic mixture by stereospecific esterification with the use of a lipase. The results of the kinetic experiments are given in Table 3. The *K_m* values for both substrates were high and the *k_{cat}* values were low when compared with those for 4-ethylphenol, but the results confirm the preference of the enzyme for the *S*(-)-isomer, with a 7-fold difference in the *k_{cat}*/*K_m* ratios for the two enantiomers.

Other 1-(4-hydroxyphenyl)ethanol dehydrogenases

As 4EPMH was thought to be involved in the oxidation of 1-(4-hydroxyphenyl)ethanol in intact bacteria, this difference in specificity for the isomers of the alcohol might have been reflected

Table 3. Steady-state kinetic parameters for the oxidation of *R*(+)- and *S*(-)-1-(4-hydroxyphenyl)ethanol and 4-ethylphenol by 4-EPMH

Enzyme assays were carried out at 25 °C in 10 mM-Tris/HCl buffer, pH 7.6 (*I* 0.01), with cytochrome *c* concentrations in the range 4–50 μ M and various *R*(+)-1-(4-hydroxyphenyl)ethanol (2–8 mM) and *S*(-)-1-(4-hydroxyphenyl)ethanol (0.5–5 mM) concentrations. The concentration of hydroxylase was 8.8 nM.

Substrate	K_m (mM)	k_{cat} (s^{-1})	$10^{-4} \times k_{cat}/K_m$ ($M^{-1} \cdot s^{-1}$)
<i>R</i> (+)-1-(4-Hydroxy-phenyl)ethanol	4.7 ± 0.6	22 ± 1.3	0.48
<i>S</i> (-)-1-(4-Hydroxy-phenyl)ethanol	1.2 ± 0.1	41 ± 1.3	3.30
4-Ethylphenol*	0.055 ± 0.002	194 ± 3.0	350

* Reeve *et al.* (1989a).

in the rates at which they are oxidized by whole cells. However, both isomers were oxidized at the same rate (0.04 μ mol of O_2 /min per mg dry wt.) in Warburg respirometers.

This result, together with the high K_m of 4EPMH for the alcohol and its preference for the *S*(-)-enantiomer, suggested the possibility of a second enzyme for oxidation of the alcohol, and led to a renewed search for an alternative 1-(4-hydroxyphenyl)ethanol dehydrogenase in this organism. Wider ranges of buffers and substrate concentrations were used than in previous investigations, and this time activity was found in crude extracts of 4-ethylphenol-grown cells with NAD^+ as the acceptor but not with $NADP^+$. There was activity towards both alcohols, at a pH optimum of 9.8, with specific activities of 0.62 and 0.57 unit/mg for the *R*(+)-isomer and *S*(-)-isomer respectively. The

specific activity for the reverse reaction, which had a pH optimum of 6.2, was 0.061 unit/mg. Thus it seems that the physiological role of 4EPMH may be restricted to the first step of 4-ethylphenol degradation, namely the hydroxylation of the methylene group. However, the NAD^+ -linked dehydrogenase activity was also present at high levels in succinate-grown cells, whereas most of the enzymes of this pathway are inducible and so the relationship of this activity to the 4-ethylphenol pathway remains uncertain.

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