Alkylphenol Biotransformations Catalyzed by 4-Ethylphenol Methylenehydroxylase

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4-Ethylphenol methylenehydroxylase from *Pseudomonas putida* JD1 acts by dehydrogenation of its substrate to give a quinone methide, which is then hydrated to an alcohol. It was shown to be active with a range of 4-alkylphenols as substrates. 4-*n*-Propylphenol, 4-*n*-butylphenol, chavicol, and 4-hydroxydiphenylmethane were hydroxylated on the methylene group next to the benzene ring and produced the corresponding chiral alcohol as the major product. The alcohols 1-(4'-hydroxyphenyl)propanol and 1-(4'-hydroxyphenyl)-2-propen-1-ol, produced by the biotransformation of 4-*n*-propylphenol and chavicol, respectively, were shown to be *R*(+) enantiomers. 5-Indanol, 6-hydroxytetralin, 4-isopropylphenol, and cyclohexylphenol, with cyclic or branched alkyl groups, gave the corresponding vinyl compounds as their major products.

The enzyme 4-ethylphenol methylenehydroxylase (4EPMH) catalyzes the first step in the degradation of 4-ethylphenol by *Pseudomonas putida* JD1 (2, 6). It is a flavocytochrome *c* and is similar in structure and mode of action to the well-studied enzyme *p*-cresol methylhydroxylase (PCMH). Both of these enzymes act by dehydrogenation of the substrate to give a quinone methide, which is then hydrated to give the hydroxylated product (Fig. 1). Thus, in contrast to many hydroxylases, they are not oxygenases. With 4-ethylphenol as the substrate, the product is the chiral alcohol 1-(4'-hydroxyphenyl)ethanol, and we have shown previously that 4EPMH produces the *R*(+) enantiomer of this alcohol in high enantiomeric excess (7). Formation of the intermediate quinone methide requires that the hydroxyl and alkyl groups of the alkylphenol be positioned *para* to each other. Providing this condition is met, a number of compounds are potential substrates and precursors for the stereospecific formation of chiral alcohols by this enzyme. Here we examine a number of possible substrates and analyze the products of their biotransformation.

Besides 4-ethylphenol, the following compounds all gave a positive rate in the spectrophotometric assay for 4EPMH by using cytochrome *c* as the electron acceptor (6): 4-*n*-propylphenol, 4-*n*-butylphenol, chavicol (4-allylphenol) 5-indanol, 6-hydroxytetralin, 4-isopropylphenol, 4-cyclohexylphenol, and 4-hydroxydiphenylmethane. To confirm that these were true substrates and that activity was not due to small amounts of impurities, oxidation of 5 μ mol of each substrate was measured in a Warburg apparatus with phenazine methosulfate as the acceptor. In most cases, this gave a value close to one molecule of oxygen used for each molecule of substrate added, in accord with auto-oxidation of reduced phenazine methosulfate to give hydrogen peroxide, showing that all of the substrate was transformed. Even where full oxidation was not achieved, it was

sufficient to rule out minor impurities as the cause of apparent activity.

Further confirmation of the biotransformation of these compounds came from examination of the reaction products by gas chromatography-mass spectrometry (GC-MS). The reaction mixtures were extracted three times with an equal volume of diethyl ether, the pooled extracts were dried over anhydrous sodium sulfate, and the ether was evaporated to dryness. GC-MS was performed with a Hewlett-Packard 5890 gas chromatograph attached to a 5971 mass-selective detector. An HP5 (cross-linked 5% phenylmethylsilicone) column (25 m by 0.2 mm; 0.33- μ m film thickness) was used with helium as the carrier gas. A temperature program of 2 min at 70°C rising at 30°C/min to 270°C was used. The results are summarized in Table 1.

Two types of product were seen: those with an additional mass of 16, indicative of hydroxylation, and those with a mass of 2 less than the substrate. The products from 4-ethylphenol (Fig. 1), which was used as a substrate here for comparative purposes, have been well characterized previously as 1-(4'-hydroxyphenyl)ethanol for the addition of oxygen and 4-vinylphenol ($-2H$), which has been shown to be a minor product formed by rearrangement of the quinone methide (5). By analogy and from similarities to the mass spectra of the 4-ethylphenol products, the compounds in Table 1 with an additional oxygen atom are the alcohols, and $-2H$ indicates the vinyl compounds. The alcohol from 4-*n*-propylphenol was confirmed as 1-(4'-hydroxyphenyl)propanol by comparison with authentic material.

Chavicol (4-allylphenol) has an unsaturated alkyl group and presents an alternative site for hydration. Another enzyme, vanillyl alcohol oxidase (VAO) from *Penicillium simplicissimum*, which can also hydroxylate alkylphenols with intermediate formation of quinone methides, hydroxylates chavicol to give coumaryl alcohol (3). However, when the product of 4EPMH hydroxylation of chavicol was reduced with hydrogen by using platinum oxide as catalyst, it gave a compound identical to 1-(4'-hydroxyphenyl)propanol, demonstrating that the alcohol in this case is 1-(4'-hydroxyphenyl)-2-propen-1-ol. This

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FIG. 1. Biotransformation of 4-ethylphenol by 4EPMH. The quinone methide intermediate is shown in brackets. The dotted arrow signifies 4-vinylphenol as a minor product.

compound was unstable on prolonged storage, and a major product was identified by GC-MS as 4-hydroxypropylphenone, which could be formed by a 1,2-hydride shift after protonation to give a carbocation.

Constraint of the alkyl side chain as in 5-indanol and 6-hydroxytetralin or branching as in 4-isopropylphenol or 4-cyclohexylphenol appears to affect the relative rates of hydration and rearrangement of the quinone methide, resulting in formation of the vinyl compound as the major product (Table 1 and Fig. 2). The possibility that the vinyl compounds were produced by dehydration of the alcohols during extraction procedures was checked by reducing the product of 5-indanol oxidation before diethyl ether extraction, using hydrogen with platinum oxide as a catalyst. The extracted product had a mass spectrum and retention time identical to those for 5-indanol, confirming that the enzymatic product was the vinyl compound. The constraint on the side chain of 5-indanol as compared with 4-*n*-propylphenol also resulted in a large increase in the K_m value (Table 2).

The broad specificity of 4EPMH was further demonstrated by the hydroxylation of 4-hydroxydiphenylmethane (Table 1).

FIG. 2. Biotransformation of 5-indanol and 6-hydroxytetralin by 4EPMH. The quinone methide intermediate is shown in brackets.

The major product, constituting 85% of the total, was the alcohol, but in this case, the quinone methide cannot rearrange in the same way as the others because of the unsaturated benzene ring, and the only plausible structure for the minor product is the resonance-stabilized quinone methide.

It has been shown previously that 4EPMH with cytochrome *c* as an electron acceptor gives the *R*(+) enantiomer of 1-(4'-hydroxyphenyl)ethanol, with an enantiomeric excess of >98% (7). A similar examination was made of the alcohols produced by hydroxylation of 4-*n*-propylphenol and chavicol by 4EPMH. The oxidations were performed in 250-ml Erlenmeyer flasks on a rotary shaker at 30°C with reaction mixtures containing the following in 50 ml of 10 mM Tris-HCl buffer (pH 7.6): 31 mg of horse heart cytochrome *c*, 4 mg of 4EPMH, 400 mg (wet weight) of a membrane preparation from *P. putida* cells grown on succinate (to reoxidize reduced cytochrome *c*), and 130 μmol of substrate. Products were obtained by extraction with diethyl ether as before. Enantiomers of alcohols were separated as their diacetyl derivatives by high-performance liquid chromatography (HPLC) on a chiral column (7). The enzymatic product from 4-*n*-propylphenol corresponded to the *R*(+) enantiomer, with an enantiomeric excess of 90%, and the specific rotation was +60.4°. The specific rotation reported for the *R*(+) alcohol from 4-ethylphenol was 48.8° (7). Similarly the diacetyl derivative of 1-(4'-hydroxyphenyl)-2-propen-1-ol

TABLE 1. Results of GCMS analysis of reaction products

Substrate	M_r	m/z of product in:	
		Major GC peak	Minor GC peak ^a
4-Ethylphenol	122	138 (+O)	120 (-2H)
4- <i>n</i> -Propylphenol	136	152 (+O)	134 (-2H)
4- <i>n</i> -Butylphenol	150	166 (+O)	148 (-2H)
Chavicol	134	150 (+O)	
5-Indanol	134	132 (-2H)	
6-Hydroxytetralin	148	146 (-2H)	
4-Isopropylphenol	136	134 (-2H)	152 (+O)
4-Cyclohexylphenol	176	174 (-2H)	
4-Hydroxydiphenylmethane	184	200 (+O)	182 (-2H)

^a Peak is < 20% of the major peak.

TABLE 2. Steady-state kinetic constants for 4EPMH with various substrates

Substrate	K_m (μM)	k_{cat} (s ⁻¹)	$10^{-4} \times (k_{cat}/K_m)$ (M ⁻¹ s ⁻¹)
<i>p</i> -Cresol ^a	3,010 ± 33	29.4	0.98
4-Ethylphenol	79.8 ± 13.3	205	257
4- <i>n</i> -Propylphenol	112.6 ± 16	231	205
4- <i>n</i> -Butylphenol	39.5 ± 2.1	65	165
5-Indanol	1,187 ± 62	51	4.31
4-Hydroxydiphenylmethane	145 ± 9	0.47	0.324

^a Taken from reference 6.

produced from chavicol gave a single peak by HPLC on a chiral column, showing that again only one enantiomer is produced (enantiomeric excess of >90%). This was confirmed as the *R*(+) enantiomer by showing that the diacetyl derivative of the alcohol after reduction of the double bond behaved identically on HPLC to the *R*(+) enantiomer of diacetyl 1-(4'-hydroxyphenyl)propanol.

Thus, the stereospecificity for the addition of water to the quinone methide seen in 4-ethylphenol hydroxylation was maintained for these two higher homologues. The same stereospecificity of 4-ethylphenol hydroxylation is seen for the VAO from *Penicillium simplicissimum*, mentioned previously, whereas PCMH gives the *S*(-) alcohol (4). VAO and the flavin subunit of PCMH belong to the same family of oxidoreductases that have a conserved flavin-binding domain. From studies of the active sites of these enzymes, it has been proposed that this difference in enantiomeric selectivity is due to the relative positions of residues that could activate water in the enzymatic process (1, 8, 9). Because 4EPMH is similar in structure and mechanism to PCMH and might also belong to this flavoprotein family, it will be interesting to see its active site conforms to this proposed pattern.

Steady-state kinetic experiments were performed with several of these substrates in assays using cytochrome *c* as an electron acceptor as described previously (7). Assays were performed in triplicate, and the data were analyzed with R. J. Leatherbarrow's nonlinear regression analysis computer program ENZFITTER (Elsevier-Biosoft, Cambridge, United Kingdom, 1987) to calculate the kinetic constants and their standard errors (Table 2). 4-Ethylphenol is the best substrate, with the specificity constant decreasing with increasing chain length. A very bulky side group as in 4-hydroxydiphenylmethane greatly lowers the specificity constant, and the constraint of

the side chain in 5-indanol as compared with 4-*n*-propylphenol has a marked effect on the K_m .

Because it acts as a dehydrogenase rather than a mono-oxygenase, 4EPMH does not suffer the disadvantage of requiring a source of reduced pyridine nucleotides for catalysis of biotransformations, and we have shown that it has the potential for production of chiral alcohols in high enantiomeric excess from alkylphenols.

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