Substrate-specificity of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase encoded by TOL plasmid pWW0

Metabolic and mechanistic implications

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The substrate-specificities of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase, encoded by TOL plasmid pWW0 of *Pseudomonas putida* mt-2, were determined. The rates of benzyl alcohol dehydrogenase-catalysed oxidation of substituted benzyl alcohols and reduction of substituted benzaldehydes were independent of the electronic nature of the substituents at positions 3 and 4. Substitutions at position 2 of benzyl alcohol affected the reactivity of benzyl alcohol dehydrogenase: the velocity of the benzyl alcohol dehydrogenase-catalysed oxidation was lower for compounds possessing electron-withdrawing substitutions. In the reverse reaction of benzyl alcohol dehydrogenase, none of the substitutions tested influenced the apparent k_{cat} values. The rates of benzaldehyde dehydrogenase-catalysed oxidation of substituted benzaldehydes were influenced by the electronic nature of the substitutions: electron-withdrawing groups at positions 3 and 4 favoured the oxidation of benzaldehydes. Substitution at position 2 of benzaldehyde greatly diminished the benzaldehyde dehydrogenase-catalysed oxidation. Substitution at position 2 with electron-donating groups essentially abolished reactivity, and only substitutions that were strongly electron-withdrawing, such as nitro and fluoro groups, permitted enzyme-catalysed oxidation. The influence of the electronic nature and the position of substitutions on the aromatic ring of the substrate on the velocity of the catalysed reactions provided some indications concerning the transition state during the oxidation of the substrates, and on the rate-limiting steps of the enzymes. *Pseudomonas putida* mt-2 containing TOL plasmid pWW0 cannot grow on toluene derivatives substituted at position 2, nor can it grow on 2-substituted benzyl alcohols or aldehydes. One of the reasons for this may be the substrate-specificities of the benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase.

INTRODUCTION

Soil and water micro-organisms play an important role in the degradation of organic compounds in the environment, and thereby maintain the carbon cycle in equilibrium. The *Pseudomonas* species have evolved a large variety of catabolic pathways for the degradation of diverse organic compounds. *Pseudomonas putida* mt-2 containing TOL plasmid pWW0 is capable of growing on xylenes or toluene as a sole source of carbon and energy due to the metabolic pathway encoded by this plasmid [1]. This catabolic pathway consists of the two parts: an upper pathway, induced by toluene, xylenes and their alcohol derivatives, which catalyses the conversion of these compounds into their carboxylic acid derivatives, and a lower pathway, which catalyses the degradation of (substituted) benzoic acids to tricarboxylic acid-cycle intermediates through the oxygenative cleavage of the aromatic ring [2–4].

The upper pathway contains three enzymes: xylene monooxygenase, responsible for a methyl group oxidation on toluene/xylenes, benzyl alcohol dehydrogenase (EC 1.1.1.90), which oxidizes (substituted) benzyl alcohols to aldehydes, and benzaldehyde dehydrogenase (EC 1.2.1.28), which oxidizes (substituted) benzaldehydes to (substituted) benzoic acids [5]. These two dehydrogenases have previously been purified and characterized [6]. Both enzymes oxidize their substrates with the concomitant reduction of NAD^+ . Benzyl alcohol dehydrogenase also catalyses a reversible reaction, namely the reduction of benzaldehydes, with the concomitant oxidation of NADH . In the previous study, it had been suggested that the effects of substitutions on the aromatic ring on the activities of the two dehydrogenases were different [6]. In the present paper we have

further characterized the substrate-specificities of these two enzymes.

MATERIALS AND METHODS

Materials

Benzyl alcohol, benzaldehyde and their substituted derivatives were obtained from either Lancaster Synthesis Co. (Morecambe, Lancs., U.K.) or Aldrich Chemie G.m.b.H. (Steinheim, Germany) and were all of the highest purity commercially available (> 98%). The NAD^+ and NADH coenzymes were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 3-(Cyclohexylamino)-1-propanesulphonic acid (Caps), 2-mercaptoethanol and citric acid were obtained from Fluka A.G. (Buchs, Switzerland).

Enzyme purification

Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase, which are encoded by TOL plasmid pWW0 of *Ps. putida*, were purified as described previously [6] except that the final gel-filtration step was replaced by a passage through a hydrophobic interaction column (75 mm $\times$ 7.5 mm TSK-Phenyl 5-PW hydrophobic interaction column; Bio-Rad Laboratories, Richmond, CA, U.S.A.). The samples were charged on the column, pre-equilibrated with 50 mM-potassium phosphate buffer, pH 7.4, containing 2.5 mM-dithiothreitol (buffer A), and the proteins were eluted from the column, after a wash with 20 ml of buffer A, by a linear gradient of 0–50% (v/v) propan-2-ol in buffer A. Fractions containing benzyl alcohol dehydrogenase or benzaldehyde dehydrogenase were collected and dialysed against

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Table 1. Kinetic parameters of benzyl alcohol dehydrogenase

The kinetic parameters were determined under the standard assay conditions. The concentration of NAD⁺ (1 mM) was 21 times superior to the K_m of the enzyme for NAD⁺. The apparent K_{cat} values were calculated as (apparent maximum velocity)/(concentration of active sites) assuming a subunit molecular mass of 42 kDa and one active site per subunit. Not detected (N.D.) indicates that the compound was not a substrate.

Substrate	Apparent K_m (μM)	Apparent V_{max} ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	Apparent k_{cat} (s^{-1})	Specificity k_{cat}/K_m ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$)
Benzyl alcohol	220	330	220	1
2-Methylbenzyl alcohol	100	280	190	1.9
3-Methylbenzyl alcohol	9.4	200	130	14
4-Methylbenzyl alcohol	84	320	210	2.5
2-Methoxybenzyl alcohol	390	100	70	0.2
3-Methoxybenzyl alcohol	13	190	130	9.9
4-Methoxybenzyl alcohol	37	93	62	1.7
2-Chlorobenzyl alcohol	140	21	14	0.1
3-Chlorobenzyl alcohol	330	140	91	0.3
4-Chlorobenzyl alcohol	19	280	180	9.5
2-Nitrobenzyl alcohol	N.D.			
3-Nitrobenzyl alcohol	300	190	130	0.4
4-Nitrobenzyl alcohol	330	10	6	0.02
2-Fluorobenzyl alcohol	310	60	40	0.1
3-Fluorobenzyl alcohol	150	86	57	0.4
4-Fluorobenzyl alcohol	140	130	88	0.6
3,5-Dimethylbenzyl alcohol	160	100	67	0.4

1 litre of buffer A containing 40 % (v/v) glycerol, and stored at -70°C .

Enzyme purity

The purity of the enzymes was checked by SDS/PAGE on a Pharmacia Phastgel system with 10–15 % polyacrylamide gels. Since the V_{max} value of the purified enzymes has previously been determined [6], the degree of purity of the purified enzymes was also checked by measuring their V_{max} values for their respective substrates.

Enzyme assays

Stock solutions of all substrates were prepared in aq. 50 % (v/v) dimethyl sulphoxide. It has been demonstrated (results not shown) that the presence of small amounts (< 1 %, v/v) of dimethyl sulphoxide did not affect the kinetic parameters of both dehydrogenases with the unsubstituted substrates under standard conditions. The standard assay for benzyl alcohol dehydrogenase was carried out at 25°C in 1 ml of 100 mM-glycine/NaOH buffer, pH 9.4, containing 1.0 μmol of NAD⁺, 1.0 μmol of benzyl alcohol and an appropriate amount of purified benzyl alcohol dehydrogenase. The Michaelis–Menten kinetic parameters of the enzyme were measured under these conditions, except that various amounts of the substrates were added to the reaction mixture. The standard assay for the reduction of benzaldehyde by benzyl alcohol dehydrogenase (the reverse reaction of benzyl alcohol dehydrogenase) was carried out in 1 ml of 100 mM-citric acid/NaOH buffer, pH 5.7, containing 100 nmol of NADH, 500 nmol of benzaldehyde and an appropriate amount of purified benzyl alcohol dehydrogenase. The Michaelis–Menten kinetic parameters were determined under these conditions except that various amounts of the substrates were used. Benzaldehyde dehydrogenase was assayed in 1 ml of 100 mM-Caps buffer, pH 9.6, containing 1.0 μmol of NAD⁺, 0.5 μmol of benzaldehyde and an appropriate amount of purified benzaldehyde dehydrogenase. The Michaelis–Menten kinetic parameters were determined under these conditions except that various amounts of the substrates were used. The reactions of these two enzymes were monitored by following the increase (or the decrease in the case

of the reverse reaction of benzyl alcohol dehydrogenase) in absorbance at 340 nm with a Uvikon 810 double-beam spectrophotometer (Kontron Instruments, Zurich, Switzerland). In the case of the determination of the Michaelis–Menten constants, the reactions were all determined at least three times. In the case of benzyl alcohol dehydrogenase, the concentration of the substrates varied from 5 times inferior to the K_m value to 5 times superior to this value, generally from 10 μM to 1 mM for the benzyl alcohol dehydrogenase-catalysed oxidation and 1 μM to 200 μM for benzyl alcohol dehydrogenase-catalysed reduction, whereas for benzaldehyde dehydrogenase the concentrations of the substrates were varied from 10 μM to 1 mM, the latter concentrations usually being already inhibitory for benzaldehyde dehydrogenase. The results were plotted as double-reciprocal plots of 1/(velocity) as a function of 1/(substrate concentration). In the case where a substrate or a reaction product had an absorption coefficient at 340 nm greater than 1 % of that of NADH, a correction factor [$\epsilon_{\text{NADH}}/(\epsilon_{\text{NADH}} - \epsilon_{\text{subst.}} + \epsilon_{\text{prod.}})$] was introduced, where $\epsilon_{\text{subst.}}$ and $\epsilon_{\text{prod.}}$ are the molar absorption coefficients of the substrate and the product respectively.

RESULTS

Substrate-specificity of benzyl alcohol dehydrogenase

The specificity of benzyl alcohol dehydrogenase was examined with substrates containing substituents, at positions 2, 3 and 4 of the aromatic ring, of different electronic properties, ranging from electron-donating groups (e.g. methoxy and methyl groups) to electron-withdrawing groups (e.g. fluoride, chloride or nitrate groups; Table 1). Benzyl alcohol dehydrogenase had the highest specificity constant (k_{cat}/K_m) for 3-methylbenzyl alcohol and the lowest K_m values for 3-methyl- and 3-methoxy-benzyl alcohol. This result is in agreement with the proposition by Sala-Trepat & Evans [7] that TOL pWW0-encoded catabolic enzymes exhibit a preferred specificity towards 3-methyl-substituted aromatic compounds. Of the substrates tested, only 2-nitrobenzyl alcohol was not oxidized. Among the substrates tested, the nitro group is the only one whose chemical nature causes it to be planar with the aromatic ring. This property, together with its relatively large

Table 2. Kinetic parameters of benzyl alcohol dehydrogenase as an aldehyde reductase

The kinetic parameters were determined under the standard assay conditions. Values given are averages of at least three different determinations. The apparent k_{cat} values were calculated as described in the legend of Table 1.

Substrate	Apparent K_m (μM)	Apparent V_{max} ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	Apparent k_{cat} (s^{-1})	Specificity k_{cat}/K_m ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$)
Benzaldehyde	26	1000	700	27
2-Methylbenzaldehyde	0.9	530	370	400
3-Methylbenzaldehyde	12	1300	910	75
4-Methylbenzaldehyde	6	960	670	120
2-Methoxybenzaldehyde	16	1000	730	50
3-Methoxybenzaldehyde	3.3	860	600	180
4-Methoxybenzaldehyde	4.4	550	390	90
2-Chlorobenzaldehyde	0.7	710	490	680
3-Chlorobenzaldehyde	2.2	970	680	310
4-Chlorobenzaldehyde	2.1	570	400	180
2-Nitrobenzaldehyde	390	1200	840	2
3-Nitrobenzaldehyde	5.3	830	580	110
4-Nitrobenzaldehyde	3.7	440	310	80
2-Fluorobenzaldehyde	3.0	800	560	190
3-Fluorobenzaldehyde	2.3	750	530	230
4-Fluorobenzaldehyde	12	1000	690	60

Table 3. Kinetic parameters of benzaldehyde dehydrogenase

The kinetic parameters were determined under the standard assay conditions. The apparent k_{cat} values were calculated as described in the legend in Table 1, except that the subunit molecular mass of benzaldehyde dehydrogenase is 57 kDa. Not detected (N.D.) indicates that the compounds were not substrates.

Substrate	Apparent K_m (μM)	Apparent V_{max} ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	Apparent k_{cat} (s^{-1})	Specificity k_{cat}/K_m ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$)
Benzaldehyde	460	17	16	0.036
2-Methylbenzaldehyde	N.D.			
3-Methylbenzaldehyde	630	26	25	0.04
4-Methylbenzaldehyde	530	6.2	5.9	0.01
2-Methoxybenzaldehyde	N.D.			
3-Methoxybenzaldehyde	940	9	8.6	0.01
4-Methoxybenzaldehyde	1100	16	15	0.02
2-Chlorobenzaldehyde	N.D.			
3-Chlorobenzaldehyde	1300	32	30	0.03
4-Chlorobenzaldehyde	700	60	57	0.08
2-Nitrobenzaldehyde	480	5	4.8	0.01
3-Nitrobenzaldehyde	810	190	180	0.23
4-Nitrobenzaldehyde	310	130	130	0.44
2-Fluorobenzaldehyde	2000	21	20	0.01
3-Fluorobenzaldehyde	170	64	61	0.04
4-Fluorobenzaldehyde	310	17	16	0.05

volume, may cause a steric hindrance, and hence prevent oxidation of the alcohol. It is also probable that this compound forms a hydrogen bond between an oxygen atom of the nitro group and the alcohol hydrogen atom, which could render this molecule resistant to oxidation by benzyl alcohol dehydrogenase. No obvious relationships between the electronic nature of these substituents and the K_m values for the substituted substrates were found. The K_m values for 3-methyl-, 4-chloro- and 3-methoxybenzyl alcohol were the lowest among the substrates examined.

Substrate-specificity of benzyl alcohol dehydrogenase in the reverse reaction

The influence of aromatic ring substitution on the reduction of benzaldehydes by benzyl alcohol dehydrogenase was also studied. The substrate-specificity of the reverse reaction of benzyl alcohol

dehydrogenase was broader than that in the forward reaction (Table 2). For 2-substituted compounds, the enzyme has high K_m values for the two most voluminous substituent groups (methoxy and nitro), but very low K_m values for the methyl-, fluoro- and chloro-substituted compounds. These smaller groups at position 2 may not cause a structural hindrance, as the K_m values of benzyl alcohol dehydrogenase for 2-methyl- and 2-chloro-benzaldehyde in the reverse reaction were much lower than for benzaldehyde.

Substrate-specificity of benzaldehyde dehydrogenase

The substrate-specificity of benzaldehyde dehydrogenase was quite different from that of benzyl alcohol dehydrogenase (Table 3). The K_m values of benzaldehyde dehydrogenase were generally 10 times greater than those of benzyl alcohol dehydrogenase, and

the K_m value for 2-fluorobenzaldehyde was exceptionally high. The apparent k_{cat} values of benzaldehyde dehydrogenase were generally lower than those of benzyl alcohol dehydrogenase. Benzaldehyde dehydrogenase was only capable of oxidizing 2-substituted substrates if the substituent was highly electro-negative, such as a fluoro or nitro group, and at a very low rate. This is contrary to the effects of these two substitutions on the oxidation of benzyl alcohols by benzyl alcohol dehydrogenase, which are discussed above. The highest value of the specificity constant of benzaldehyde dehydrogenase was for 4-nitrobenzaldehyde, although that of 3-nitrobenzaldehyde was almost as high. 3-Methyl- and 4-methyl-benzaldehyde, which are the natural substrates expected from the specificity of the TOL catabolic pathway, were not particularly good substrates for benzaldehyde dehydrogenase.

Inhibition of benzaldehyde dehydrogenase by 2-substituted benzaldehydes

As shown in Table 3, 2-chlorobenzaldehyde, 2-methylbenzaldehyde and 2-methoxybenzaldehyde were not substrates of benzaldehyde dehydrogenase. The effects of these three compounds as inhibitors of benzaldehyde dehydrogenase were examined. All three compounds inhibited the enzyme, but in different ways. 2-Methylbenzaldehyde was not a potent inhibitor. It exerts a non-competitive inhibition, with a K_i value of approx. 2000 μM . This compound is thus not efficiently recognized by benzaldehyde dehydrogenase. 2-Methoxybenzaldehyde and 2-chlorobenzaldehyde were competitive inhibitors with K_i values of 60 and 130 μM respectively. These compounds thus compete with benzaldehyde for the substrate-binding site of benzaldehyde dehydrogenase but are not oxidized by the enzyme.

DISCUSSION

The influence of an aromatic ring substitution on the velocity of a chemical reaction occurring on another side-chain group was first treated quantitatively by Hammett [8], who proposed the following equation:

$$\log(k/k_0) = \rho \cdot \sigma$$

where k_0 is the rate constant when the 3- and 4-substituent groups are H, k is the rate constant for a compound that possesses a substituent group R at positions 3 or 4, ρ is a constant for a given reaction under a certain set of conditions, and σ is a constant for a group R that represents its electrical effects when attached to an aromatic ring. The $\log(k_{cat})$ values of benzyl alcohol dehydrogenase for different substrates were plotted as a function of their σ values, obtained from refs. [9] and [10] (Fig. 1a). It is evident that the electronic nature of the substituents does not influence the k_{cat} values of benzyl alcohol dehydrogenase, as the slope is nearly zero. The reverse reaction was also independent of the electronic nature of the substitution (Fig. 1b). These results indicate that the rate-limiting steps in the oxidation of benzyl alcohol, and in the reduction of benzaldehydes, are independent of the electronic properties of the substrates. This conclusion, in turn, suggests that hydride transfer, which is accompanied by the transient development of a charge, is not the rate-limiting step in the forward and reverse reactions of benzyl alcohol dehydrogenase. This rate-limiting step may be the release of the reduced NAD^+ , as is the case for other alcohol dehydrogenases, such as horse liver alcohol dehydrogenase [11] and lactate dehydrogenase [12], whereas in the reduction of benzaldehydes it may be the release of the alcohol, as is the case for horse liver alcohol dehydrogenase [13].

The treatment of the k_{cat} values for diverse substrates of benzaldehyde dehydrogenase with the Hammett equation (Fig.

Fig. 1. Correlation of k_{cat} with σ for benzyl alcohol dehydrogenase

(a) Variation of $\log(\text{apparent } k_{cat})$ with σ for benzyl alcohol dehydrogenase in the dehydrogenation reaction. (b) Variation of $\log(\text{apparent } k_{cat})$ with σ for benzyl alcohol dehydrogenase in the aldehyde reductase reaction. Positive values of σ represent electron-withdrawing group, negative values represent electron-donating groups.

2a) demonstrated that the electronic nature of the substituents influences the velocity of the enzyme-catalysed reaction, with a slope of approx. 1.1. The electron-withdrawing groups greatly accelerated the reaction, whereas electron-donating groups apparently decreased benzaldehyde dehydrogenase activity. To determine whether steric hindrance plays a role in these oxidation rates, the k_{cat} values of 3-substituted compounds were plotted separately from those of the 4-substituted ones. As can be seen in Figs. 2(b) and 2(c), the two plots are essentially the same, e.g. with very similar slopes, indicating that the steric hindrance at position 3 is similar to that at position 4, which is known to be low [6].

From the amino acid sequence of benzaldehyde dehydrogenase (M. Lebens, cited in ref. [14]), it has been deduced that this enzyme is a member of the aldehyde dehydrogenase family [14]. Notably, the conserved amino acid residues postulated as being the nucleophile in the active site, namely Cys-302 [15], and Glu-268 [16] (numbering from the human cytosolic aldehyde dehydrogenase), were conserved in benzaldehyde dehydrogenase [14]. The proposed reaction mechanism of aldehyde dehydrogenases consists in the nucleophilic attack of a reactive cysteine residue on the carbonyl carbon atom of a substrate, followed by the transfer of the hydride of the substrate to NAD^+ , and by the formation of a thioester between the cysteine and the substrate, which then undergoes hydrolysis to form an acid product. In the initial nucleophilic attack, the reaction may involve an increase in the electron density on the aldehyde group of the substrate, and the presence of an electron-withdrawing group on the

Fig. 2. Correlation of k_{cat} with σ for benzaldehyde dehydrogenase

(a) Variation of $\log(\text{apparent } k_{cat})$ with σ for benzaldehyde dehydrogenase. (b) Variation of $\log(\text{apparent } k_{cat})$ with σ of 4-substituted benzaldehydes. (c) Variation of $\log(\text{apparent } k_{cat})$ with σ of 3-substituted benzaldehydes.

aromatic ring may favour the reaction by decreasing the net negative charge at the reacting carbon atom. In the subsequent nucleophilic attack of a water molecule or HO^- ion on the thioester intermediate, an aromatic ring substitution would influence the reaction in the same direction: electron-withdrawing groups would decrease the electron density at the carbonyl group and thereby favour the nucleophilic attack, whereas electron-donating groups would have the opposite effect. The enzymes that have been shown to follow this reaction mechanism (human liver aldehyde dehydrogenase [16], glyceraldehyde dehydrogenase [17] etc.) also hydrolyse esters. This esterase activity is believed to be the result of a similar nucleophilic attack, though whether the esterase and dehydrogenase activities are the result of the same catalytic sites in the molecule or two different ones is still open to debate [18,19]. Benzaldehyde dehydrogenase has also been shown to exhibit an esterase activity [6].

Benzaldehyde dehydrogenases from TOL plasmid pWW53 and benzaldehyde dehydrogenases I and II from *Acinetobacter calcoaceticus* N.C.I.B. 8250 are all far more efficient, by several orders of magnitude, than that from TOL plasmid pWW0 [17]. They also all had the highest specificity constant for benzaldehyde, out of the substrates tested, which is not the case for the pWW0-encoded enzyme. 2-Substituted compounds are poor substrates for all these enzymes, and, in the case of benzaldehyde dehydrogenase II from *A. calcoaceticus* N.C.I.B. 8250, are also inhibitors of the enzyme, as is the case for benzaldehyde dehydrogenase from pWW0 [18]. Studies on yeast aldehyde dehydrogenase and rat liver mitochondrial aldehyde dehydrogenases I and II have demonstrated that the larger the substituent size at position 2 the lower the activity. In the case of yeast alcohol dehydrogenase, the lower activity is principally due to a decrease in the V_{max} , whereas in the case of the rat mitochondrial enzymes both the V_{max} and K_m are affected [19]. Benzaldehyde dehydrogenase differs slightly from these aldehyde dehydrogen-

ases in being non-permissive towards 2-chlorobenzaldehyde, and in oxidizing, albeit slowly, 2-nitrobenzaldehyde, which is not a substrate of the mitochondrial enzymes [19]. Benzaldehyde dehydrogenase I from *A. calcoaceticus* N.C.I.B. 8250 and pWW0-encoded benzaldehyde dehydrogenase had a higher specificity constant for 3-methylbenzaldehyde rather than 4-methylbenzaldehyde, whereas the opposite is true for benzaldehyde dehydrogenase II from *A. calcoaceticus* N.C.I.B. 8250 and the pWW53-encoded dehydrogenase [17,18].

The substrate-specificities of benzyl alcohol dehydrogenase from *Ps. putida* MT53 containing TOL plasmid pWW53 [17] and benzyl alcohol dehydrogenase from *A. calcoaceticus* N.C.I.B. 8250 [18] have been investigated. Benzyl alcohol dehydrogenase from TOL plasmid pWW53 had higher K_m and lower V_{max} values for methylbenzyl alcohols, which caused the enzyme to be generally less efficient than benzyl alcohol dehydrogenase from TOL plasmid pWW0. Both enzymes had the highest specificity constant for 3-methylbenzyl alcohol, though the value for the pWW0 enzyme was 14 times higher than that for the pWW53 enzyme. Benzyl alcohol dehydrogenase from *A. calcoaceticus* N.C.I.B. 8250, however, had the greatest affinity for 4-methylbenzyl alcohol. The V_{max} of benzyl alcohol dehydrogenase from *A. calcoaceticus* N.C.I.B. 8250 was generally higher than that from pWW0 and relatively independent of the position of the methyl group; however, the position of the methyl group influenced the K_m values of the enzyme.

Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase from *A. calcoaceticus* N.C.I.B. 8250 seem to have a preference for 4-methyl-substituted aromatic alcohols, although the subsequent reactions in the pathway would produce 4-methylcatechol, which is processed by the *meta*-cleavage pathway, but is not a substrate of the 2-oxoadipate pathway [20]. It has been shown in the case of *A. calcoaceticus* ADP1 that this organism does not encode a *meta*-cleavage pathway [21]. Benzaldehyde dehydrogenase and benzyl alcohol dehydrogenase from two different TOL plasmids, in contrast, prefer the substitution at the 3-position, and the plasmid-containing bacterial cells are capable of growing on 3- or 4-substituted toluenes, benzyl alcohols, benzaldehydes and benzoates. Thus the specificity of a pathway does not necessarily correspond to the specificities of the component enzymes.

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