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Allylic or Benzylic Stabilization Is Essential for Catalysis by Bacterial Benzyl Alcohol Dehydrogenases

Abigale J. Curtis, Megan C. Shirk, and Ray Fall[1]
Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215

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Benzyl alcohol dehydrogenase from *Acinetobacter calcoaceticus* (AC-BADH) and TOL plasmid-encoded benzyl alcohol dehydrogenase from *Pseudomonas putida* (TOL-BADH) have previously been shown to oxidize a variety of aromatic alcohols but not aliphatic substrates. Here, we have expressed the genes for AC-BADH and TOL-BADH in *Escherichia coli,* purified the resulting over-expressed enzymes, and shown that each is an effective catalyst of both benzylic and allylic alcohol oxidation, but not of oxidation of nonallylic analogs. Enzyme specificity (k_{cat}/K_m) for both enzymes was higher with an aliphatic, allylic alcohol (3-methyl-2-buten-1-ol) than with benzyl alcohol. These results suggest that bacterial benzyl alcohol dehydrogenases use the resonance stabilization provided by allylic and benzylic alcohols to promote catalysis.

Benzyl alcohol dehydrogenase from *Acinetobacter calcoaceticus* (AC-BADH) [1] and TOL plasmid-encoded benzyl alcohol dehydrogenase from *Pseudomonas putida* (TOL-BADH) [2] belong to the family of zinc-containing, long-chain alcohol dehydrogenases [3]. The substrate specificity of these bacterial enzymes has been investigated in detail. While AC-BADH and TOL-BADH oxidize a variety of aromatic alcohols, they are generally believed to be poor catalysts for the oxidation of aliphatic alcohols [1,2,4,5]. Based on the comparison of amino acid sequences of AC-BADH and TOL-BADH with that of horse liver alcohol dehydrogenase, for which there is a three-dimensional structure [6], it has been suggested that the preference of these enzymes for aromatic alcohols lies in the structure of the substrate-binding cleft [4]. This cleft may have clusters of aromatic residues to facilitate binding of aromatic alcohols. Failure to oxidize both aliphatic and aromatic alcohols, as occurs with the horse liver and human class II alcohol dehydrogenases [7,8], may be due to structural constraints in the elongated substrate binding pocket [4,7].

Recently, we characterized a *P. putida* isolate that contains an inducible allylic alcohol dehydrogenase that is highly active towards the oxidation of aliphatic, allylic substrates such as 3-methyl-2-buten-1-ol (321-MB), as well as benzyl alcohol [9,10]. In addition, this 321-MB dehydrogenase has molecular and catalytic properties similar those of AC-BADH and TOL-BADH, suggesting it is a member of the bacterial benzyl alcohol dehydrogenase (BADH) family. However, we did not rule out the possibility that the highly purified enzyme was a mixture of two similar alcohol dehydrogenases, one with preference towards allylic alcohols and one with preference for benzylic alcohols.

Here, we extend these findings to the question of whether oxidation of aliphatic, allylic alcohols is a general property of the BADH family of enzymes. We show that AC-BADH and TOL-BADH, when expressed in *Escherichia coli*, catalyze efficient oxidation of 321-MB and other non-aromatic, allylic alcohols. The implications of these findings for understanding the catalytic specificities of bacterial BADHs are discussed.

MATERIALS AND METHODS

Enzyme preparation. A culture of chemically competent *E. coli* strain BL21(DE3) (Stratagene) was transformed with plasmid pDG30, containing the gene for AC-BADH [4], and a transformant was grown to induce expression of the enzyme according to Gillooly et al. [4]. Similarly, *E. coli* XL1 Blue MR5′ (Stratagene) was transformed with plasmid pXB930, containing the *xylB* gene for the TOL-BADH, and TOL-BADH was expressed by growth of the cells in L broth containing 50 μg/ml ampicillin [11]. Plasmid pXB930, obtained from M. Shimao (Tottori University, Department of Biotechnology, Tottori 680, Japan), was constructed by PCR-amplification of the *xylB* gene and upstream ribosome binding site from plasmid pGSH2873 [11], and cloning into plasmid pUC18 (Gibco BRL) using EcoR1 and BamH1 restriction sites (M. Shimao, unpublished). BADH purifications starting with cells from 1 L of culture were carried out as described in detail for the purification of *P. putida* 321-MB dehydrogenase [10]. Briefly, each cell extract was chromatographed on DEAE-Sephacel, BADH eluted with a KCl gradient, and fractions containing at least 20% of peak BADH activity were pooled and applied to a Phenyl Sepharose column that was eluted with a linear gradient of 0-65% (v/v) ethylene glycol. Fractions eluting in

[1] Corresponding author. Fax: 303-492-1149. E-mail: fall@terra.colorado.edu.

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the main peak of BADH, containing at least 20% of peak activity, were pooled, stored at −70°C, and used in the work described here.

Enzyme assay. During purification the enzymes were assayed at 25°C in a reaction mixture (1.5 ml) containing 100 mM Bis-Tris Propane/200 mM hydrazine, pH 9.4, 667 μM 321-MB or benzyl alcohol (from a stock solution of DMSO), and 2.67 mM NAD^+ [10]. Substrate specificity and kinetic parameters for AC-BADH and TOL-BADH were determined essentially as described by MacKintosh and Fewson [12] (100 mM Bicine, pH 9.2, 2 mM NAD^+) and by Shaw et al. [13] (100 mM glycine, pH 9.4, 1 mM NAD^+), respectively, except that hydrazine was omitted. Reagent grade alcohols were obtained commercially. Kinetic data were analyzed using the Enzfitter program (Sigma Chemical Co.). Horse liver alcohol dehydrogenase was obtained from Sigma Chemical Co. and assayed as described in their assay protocol.

Analytical methods. Protein concentrations were determined by the method of Bradford [14] using bovine serum albumin as the standard. The purity of the enzymes during the purification process was monitored by using SDS-polyacrylamide gel electrophoresis (Novex).

RESULTS AND DISCUSSION

AC-BADH and TOL-BADH were expressed separately in *E. coli*, and during their purification by ion exchange and hydrophobic interaction chromatography alcohol dehydrogenase activity with benzyl alcohol (BzOH) and 3-methyl-2-buten-1-ol (321-MB) co-purified in each case. An example of a Phenyl Sepharose purification profile for AC-BADH is shown in Figure 1. Some AC-BADH eluted before an ethylene glycol gradient was applied and during the initial stages of the gradient, followed by a large peak of activity at about 60% (v/v) ethylene glycol. The main peak of BADH appeared to be the fraction of enzyme binding to higher affinity phenyl binding sites on the chromatographic resin. In all fractions with detectable AC-BADH, dehydrogenase activity was seen with either BzOH or 321-MB as substrate. Interestingly, when assayed in the presence of hydrazine to minimize the reverse reaction (i.e. aldehyde reduction) [12], AC-BADH activity with 321-MB, an allylic substrate, was over two times that with BzOH, a benzylic substrate (peak fractions, Figure 1). Similar results were seen during the purification of TOL-BADH. Since BADH activity is not expressed by the host *E. coli* strains (data not shown), and since AC-BADH and TOL-BADH were each expressed from a single cloned gene, these results show that these bacterial BADHs are effective catalysts for oxidation of both allylic and benzylic substrates.

The substrate specificities of the purified enzymes were studied using several types of primary alcohols (Table 1). Substrates tested included benzylic, non-benzylic, allylic, and non-allylic alcohols. The enzymes showed activities for the benzylic and allylic substrates and no significant activities for the remaining alcohols. For example, while 321-MB was an excellent substrate for both AC-BADH and TOL-BADH, these enzymes

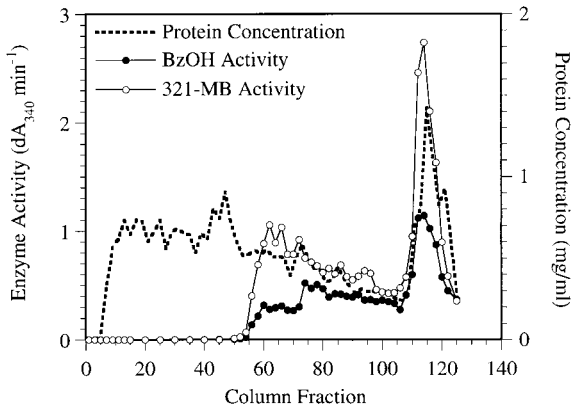


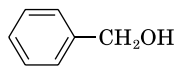
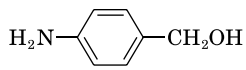
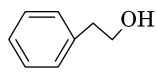
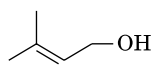
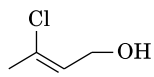
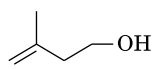
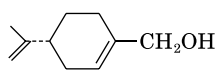
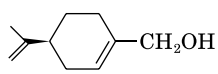
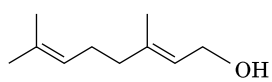
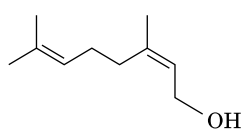
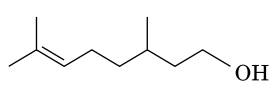
FIG. 1. Copurification of BzOH and 321-MB activities of AC-BADH. AC-BADH, expressed in *E. coli* and initially purified by DEAE Sephacel chromatography, was further purified by chromatography on a Phenyl Sepharose hydrophobic interaction column as described under Materials and Methods. AC-BADH was eluted with a linear gradient of 0 to 65% ethylene glycol beginning at fraction 75, and fractions were assayed with both benzyl alcohol (BzOH) and 3-methyl-2-buten-1-ol (321-MB).

showed only a trace or no activity with 3-methyl-3-buten-1-ol, a non-allylic analog of 321-MB. Similarly, while geraniol was a good substrate for both enzymes, its non-allylic analog, citronellol, was a poor substrate at best, and the non-benzylic alcohol, 2-phenylethanol, was not a substrate for either enzyme, consistent with previous work [1,2]. Both enzymes oxidized a variety of allylic, isoprenoid substrates, including R- and S-perillyl alcohols where the allylic double bond is endocyclic (Table 1), although at rates lower than that seen with preferred substrates such as 321-MB and 4-aminobenzyl alcohol. Racemic perillyl alcohol is a known substrate of AC-BADH [5], and as shown here is also a good substrate for TOL-BADH. Not all short-chain allylic alcohols were substrates for these enzymes. 3-Chloro-2-buten-1-ol was a poor substrate for both enzymes; this is of interest because this alcohol is the preferred substrate for the allylic alcohol dehydrogenase from *P. putida* [10]. And, as reported earlier for AC-BADH [1], allyl alcohol is not a substrate for either enzyme (data not shown).

Kinetic parameters for AC-BADH and TOL-BADH are shown in Table 2. The data obtained with BzOH as substrate generally agree with published values [5,13], although values for k_{cat} are 5-10 fold lower, likely due to the absence of hydrazine in the assay. In our laboratory, when hydrazine was included in the assay, the activities increased 3-fold, in which case our k_{cat} values would more closely correspond to published data. Hydrazine was excluded from the assay because it complicates analysis of the kinetics of the reaction. With 321-MB as substrate both enzymes exhibited higher k_{cat} values than obtained with BzOH as substrate. K_M values for 321-MB with both enzymes were slightly lower than for BzOH. The specificity constants (k_{cat}/

TABLE 1

Relative Activities of AC-BADH and TOL-BADH with Various Substrates[a]

Substrate		Activity relative to benzyl alcohol (%)	
		AC-BADH	TOL-BADH
Benzyl alcohol		100	100
4-Aminobenzyl alcohol		270	280
2-Phenylethanol		0.6	<0.5
3-Methyl-2-buten-1-ol		210	180
3-Chloro-2-buten-1-ol		9.1	3.3
3-Methyl-3-buten-1-ol		0.8	<0.5
R-(+)-perillyl alcohol		61	49
S-(−)-perillyl alcohol		85	68
Geraniol		63	68
Nerol		25	77
Citronellol		2.6	2.2

[a] Substrate concentrations were fixed at 200 μM, and activities were measured as described under Materials and Methods. Detection limit: <0.5% relative activity. Data shown are the averages of triplicate determinations. AC-BADH, *A. calcoaceticus* benzyl alcohol dehydrogenase; TOL-BADH, *P. putida* TOL plasmid-encoded benzyl alcohol dehydrogenase.

K_M) for these enzymes indicate that each has greater catalytic efficiency with 321-MB than BzOH (Table 2).

The ability of AC-BADH and TOL-BADH to oxidize a range of both benzylic and acyclic, allylic alcohols was unexpected, as these enzymes have been shown to be relatively specific for aromatic alcohols, or cyclic alcohols such as perillyl, cinnamyl and coniferyl alcohol [1,2,4,5]. This is in contrast to the archetypal example in the group 1 family of alcohol dehydrogenases, horse-liver alcohol dehydrogenase (HLADH), which is capable of oxidizing straight and branched chain primary alcohols, secondary alcohols, cyclohexanol, and BzOH [15]. We found that HLADH, like the bacterial BADHs exhibited significant activity when assayed with 321-MB. HLADH exhibited the following relative rates of activity with 100 μM alcohol substrates: BzOH (100); 321-MB (1795). Thus a general feature of all these enzymes, and the 321-MB dehydrogenase of *P. putida* [10], is that they are all effective catalysts of 321-MB oxidation.

A structural feature shared by these enzymes is the active site cleft to which a substrate binds. The arrangement of amino acids that line the inside and outside of the cleft in HBADH influences substrate specificity [7]. In the case of AC-BADH and TOL-BADH, it seems likely that the hydrophobic core of the substrate-binding cleft is such that it facilitates interaction with the aromatic ring of the benzylic substrates

TABLE 2

Kinetic Parameters for AC-BADH and TOL-BADH[a]

Enzyme	Substrate	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
AC-BADH	BzOH	46	63	1.4×10^6
	321-MB	17	41	2.4×10^6
TOL-BADH	BzOH	130	18	1.4×10^5
	321-MB	110	37	3.4×10^5

[a] AC-BADH, *A. calcoaceticus* benzyl alcohol dehydrogenase; TOL-BADH, *P. putida* TOL plasmid-encoded benzyl alcohol dehydrogenase.

[4]. A small allylic substrate like allyl alcohol is apparently of insufficient hydrophobicity to bind to this core, while a larger, non-polar allylic alcohol like 321-MB exhibits binding that is comparable to that of aromatic substrates. The importance of the allylic double bond in binding of 321-MB to the enzyme is suggested by the finding that its non-allylic analog, 3-methylbutan-1-ol, is also a very poor substrate for AC-BADH with a K_m (390 μM) that is about 23–fold higher and a k_{cat} (2.6 s^{-1}) that is about 5-fold lower than obtained with 321-MB; 3-methylbutan-1-ol was such a poor substrate for TOL-BADH that kinetic data were not collected.

Why are benzylic and allylic substrates preferred by these enzymes? It is well known that benzylic and allylic systems, where π electrons can overlap an adjacent p orbital to form symmetric hybrid orbitals, can provide resonance stabilization to adjacent cationic, anionic or radical centers [16]. In the BADH and other alcohol dehydrogenase reactions a partial positive charge develops on the reactive carbinol carbon center during hydride transfer to NAD^+ [17]. Perhaps in BADH enzymes benzylic and allylic substrates not only enhance substrate binding, but promote catalysis by lowering the activation barrier to this reaction.

As a greater insight into the substrate specificity of these enzymes develops, we can better understand how these enzymes function and how they might be useful in future mechanistic and biotechnological applications. Gillooly and Fewson [5] have already shown that site-directed mutagenesis of AC-BADH can alter the active site of the enzyme to greatly enhance its specificity for perillyl alcohol. By similar means it might be possible to manipulate and explore the ability of these bacterial enzymes to oxidize allylic alcohols, and to use them to resolve racemic mixtures of allylic alcohols, or similarly their corresponding aldehydes by the facile reductive reaction catalyzed by these enzymes [12,13].

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