Purification and Properties of Primary and Secondary Alcohol Dehydrogenases from *Thermoanaerobacter ethanolicus*

FRANK O. BRYANT, 1,2 JUERGEN WIEGEL, 1,3 AND LARS G. LJUNGLAHL 1,2,*

Center for Biological Resource Recovery 1 and Departments of Biochemistry 2 and Microbiology, 3 University of Georgia, Athens, Georgia 30602

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*Corresponding author.

*Thermoanaerobacter ethanolicus* (ATCC 31550) has primary and secondary alcohol dehydrogenases. The two enzymes were purified to homogeneity as judged from sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration. The apparent *M*ₙ of the primary and secondary alcohol dehydrogenases are 184,000 and 172,000, respectively. Both enzymes have high thermostability. They are tetrameric with apparently identical subunits and contain from 3.2 to 5.5 atoms of Zn per subunit. The two dehydrogenases are NADP dependent and reversibly convert ethanol and 1-propanol to the respective aldehydes. The *Vₘₚ* values with ethanol as a substrate are 45.6 μmol/min per mg for the primary alcohol dehydrogenase and 13 μmol/min per mg for the secondary alcohol dehydrogenase at pH 8.9 and 60°C. The primary enzyme oxidizes primary alcohols, including up to heptanol, at rates similar to that of ethanol. It is inactive with secondary alcohols. The secondary enzyme is inactive with 1-pentanol or longer chain alcohols. Its best substrate is 2-propanol, which is oxidized 15 times faster than ethanol. The secondary alcohol dehydrogenase is formed early during the growth cycle. It is stimulated by pyruvate and has a low *Kₘ* for acetaldehyde (44.8 mM) in comparison to that of the primary alcohol dehydrogenase (210 mM). The latter enzyme is formed late in the growth cycle. It is postulated that the secondary alcohol dehydrogenase is largely responsible for the formation of ethanol in fermentations of carbohydrates by *T. ethanolicus*.

*Thermoanaerobacter ethanolicus*, a thermophilic anaerobic bacterium, ferments a wide range of hexoses and pentoses as well as starch and xylan (24-26). When concentrations of substrates are less than 1%, ethanol and CO₂ are the main products, and the yields of ethanol per mol of glucose and xylose are 1.95 mol and 1.45 mol, respectively (6, 24). At substrate concentrations higher than 1% the fermentation rate is slow and a shift of products occurs away from ethanol to acetate and lactate. The sensitivity of the bacterium to high substrate and ethanol concentrations is apparently due to the regulation of several key enzymes involved in the fermentation including alcohol dehydrogenases, lactate dehydrogenase, and acetate kinase (6).

An alcohol dehydrogenase has been isolated from *T. ethanolicus* (5). It is NADP dependent, reacts with ethanol but preferentially oxidizes secondary alcohols, and is affected by pyruvate. Subsequently, an additional alcohol dehydrogenase was detected in *T. ethanolicus* (F. O. Bryant and J. Wiegel, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K31, p. 182). It also is NADP dependent, but it is specific for primary alcohols. In this paper, we describe the purification of the two alcohol dehydrogenases from the same cell lysate and compare some of their properties.

**MATERIALS AND METHODS**

**Growth of bacterium.** *T. ethanolicus* (ATCC 31550) was grown in a 400-liter New Brunswick fermentor at 60°C in a 0.8% (wt/vol) glucose medium as described previously (26). Nitrogen gas was continuously bubbled through the medium during growth. The cells were harvested after 24 h when in logarithmic growth, using a Sharples centrifuge. The yields of wet cells were from 0.5 to 1.0 kg/400 liters of medium.

**Alcohol dehydrogenase assays.** The two alcohol dehydrogenases were assayed spectrophotometrically at 60 or 70°C, as indicated, with ethanol as the substrate by following the reduction of NADP (*E₁₄₅ₐ₀ = 6.22 mM⁻¹ cm⁻¹*). The assay mixture contained 200 mM ethanol and 1.25 mM NADP⁺ in 100 mM Tris hydrochloride (pH 8.9). Assays in the reverse reaction were with 50 mM acetaldehyde and 0.5 mM NADPH in 100 mM Tris hydrochloride (pH 7.6). The primary alcohol dehydrogenase was also assayed with 1-butanol (200 mM) substituted for ethanol, and the secondary enzyme was assayed using 2-propanol (200 mM) instead of ethanol. Stopped cuvettes containing all components except enzyme (final volume, 0.95 ml) were preincubated at the appropriate temperatures before the reaction was started by addition of the enzyme. One unit of activity is the amount of alcohol dehydrogenase that reduces or oxidizes 1 μmol of NADP or NADPH per min, respectively. Specific activity is in units per milligram of protein. Protein was estimated by the rose bengal dye method of Elliott and Brewer (9) with bovine serum albumin as a standard.

**Gel electrophoresis.** Tube gel electrophoresis of active enzymes was performed at pH 8.9 using 10% polyacrylamide running gels and 2.5% polyacrylamide stacking gels (3). Staining of gels for alcohol dehydrogenase activity was performed essentially as described by Ursprung and Leone (21). The gels were incubated in a solution containing 37.5 ml of 0.5 M Tris hydrochloride (pH 9.0); 1.5 ml of either ethanol, 2-propanol, or 1-butanol; 1.5 ml of NADP (50 mg/ml); 3.75 ml of Nitro Blue Tetrazolium (84 mg/ml); and 0.375 ml of phenazine methosulfate (3 mg/ml). Incubations were at room temperature for a period of 10 min to 2 h as required to develop a good activity stain.

**Molecular weight determinations.** Molecular weights of subunits were determined using sodium dodecyl sulfate-gel electrophoresis at pH 7.0 with 10% polyacrylamide and 0.4% bisacrylamide gels (22). Molecular weights of the active enzymes were estimated according to Andrews (1) using an Ultrogel AcA 34 column (100 by 1.5 cm) (LKB Instruments...
Inc., Rockville, Md.). Molecular weight protein standards were obtained from Bio-Rad (Richmond, Calif.).

**Metal determination.** The enzymes were dialyzed against 10 mM Tris hydrochloride (pH 7.6), and the solutions were then analyzed for metals by plasma emission spectroscopy with a Jarrell Ash Plasma Cmp 750 instrument (13). Solutions of metals of known concentrations were used as standards.

**Amino acid analyses.** Protein samples were carboxymethylated according to Crestfield et al. (8). Acid hydrolysis was then performed under vacuum at 110°C in 6 N ultrapure HCl (Alfa Products, Danvers, Md.) for 36, 43, 60, 67, 84, or 91 h. The amino acids were determined with a Beckman model 119CL Amino Acid Analyzer. Serine and threonine concentrations were extrapolated to zero time. Isoleucine and valine concentrations were extrapolated to maximum amounts. The concentrations of all other amino acids were averaged. Tryptophan was determined by $A_{277}$ after subtracting the absorbance due to tyrosine.

**Purification of alcohol dehydrogenases from *T. ethanolicus.*** The two alcohol dehydrogenases were purified from the same cell preparation. All steps of the purification except for the hydroxyapatite chromatography step were performed with Tris hydrochloride (pH 7.6) containing 2 mM dithiothreitol. The purification was performed at 4°C.

**Step 1. Preparation of cell extract.** Frozen cells (480 g) were suspended in 3 volumes of 20 mM Tris buffer and passed twice through a French pressure cell (Amicon Corp., Lexington, Mass.) at 12,000 lb/in². The lysate was centrifuged at 100,000 × g for 60 min. The two alcohol dehydrogenases were found in the supernatant.

**Step 2. Adsorption on DEAE 23.** The supernatant was mixed with DEAE 23 (Whatman, Inc., Clifton, N.J.) previously equilibrated with 20 mM Tris buffer, and the suspension was stirred overnight. The slurry was poured into a chromatography tube to form a column (30 cm long by 8 cm in diameter) which was washed with 2 liters of 20 mM Tris buffer. The alcohol dehydrogenases were then eluted from the column with 2 liters of 50 mM Tris buffer containing 100 mM NaCl.

**Step 3. Separation of primary and secondary alcohol dehydrogenases on Matrex Gel Red A.** The active fraction from step 2 was applied to a column (6.0 by 5.5 cm) of Matrex Gel Red A dyed affinity material (Amicon Corp.) previously equilibrated with 5 volumes of 20 mM Tris buffer containing 5 mM pyruvate, 0.05 mM NADP, and 0.1 mM MgCl₂. After application of the enzyme, the column was washed with 500 ml of 10 mM Tris buffer containing the components of the equilibrating buffer. The primary alcohol dehydrogenase eluted in the void and wash volume (2,200 ml). The secondary alcohol dehydrogenase, retained on the column, was subsequently eluted with 470 ml of 100 mM Tris buffer containing 100 mM NaCl plus the components of the equilibrating buffer.

**Purification of the primary alcohol dehydrogenase, step 4(P): Ultrogel AcA 34 gel filtration.** The 2,200-ml void-wash solution from the Matrex Gel Red A column was concentrated to 24 ml by ultrafiltration using a PM 30 membrane (Amicon Corp.). The concentrated protein was applied to an Ultrogel AcA 34 column (100 by 2.75 cm) equilibrated with 10 mM Tris buffer. The column was eluted with the same buffer. Fractions of 8 ml containing 20 U or more of ethanol-dependent alcohol dehydrogenase were pooled.

**Step 5(P): Hydroxyapatite chromatography.** The pooled fractions of step 4(P) were applied to a hydroxyapatite (Bio-Rad) column (6 by 2.25 cm) equilibrated with 10 mM Tris buffer. The column was washed with 200 ml of the same buffer, and then the enzyme was eluted with 200 ml of 50 mM potassium phosphate (pH 7.0) containing 2 mM dithiothreitol.

**Step 6(P): DEAE-Sephadex chromatography.** The active fraction from step 5(P) was applied to a DEAE-Sephadex (Sigma Chemical Co., St. Louis, Mo.) column (10 by 1.75 cm) equilibrated with 20 mM Tris buffer (pH 7.6). The column was washed with 200 ml of equilibrating buffer. The primary alcohol dehydrogenase was eluted from the column with 50 mM Tris buffer and concentrated to 12 ml by ultrafiltration on a PM30 membrane.

**Purification of the secondary alcohol dehydrogenase, step 4(S): Ultrogel AcA 34 chromatography.** The secondary alcohol dehydrogenase eluted from the Matrex Gel Red A column described above (step 3) was concentrated to 8 ml by ultrafiltration on a PM10 membrane and applied to an Ultrogel AcA 34 column (100 by 2.75 cm) equilibrated with 10 mM Tris buffer containing 5 mM pyruvate, 0.05 mM NADP, and 0.1 mM MgCl₂. The enzyme was eluted with the same solvent. Fractions of 4 ml were collected, and those containing 50 U or more of 2-propanol-dependent alcohol dehydrogenase were pooled.

**Step 5(S): Hydroxyapatite chromatography.** The pooled fractions of step 4(S) were applied to a hydroxyapatite column (3 by 1.75 cm) equilibrated with 10 mM Tris buffer containing 5 mM pyruvate, 0.05 mM NADP, and 0.1 mM MgCl₂. The column was washed with this buffer. The secondary alcohol dehydrogenase was then eluted using a gradient between 50 and 80 mM potassium phosphate buffer (pH 7.0) containing 2 mM dithiothreitol, 5 mM pyruvate, 0.05 mM NADP, and 0.1 mM MgCl₂. A volume of 580 ml was collected and concentrated to 32 ml on a PM30 membrane.

**RESULTS**

**Appearance of primary and secondary alcohol dehydrogenases during growth of *T. ethanolicus.*** Alcohol dehydrogenase activity was assayed with ethanol, 2-propanol, and 1-butanol as substrates in cell extracts of cells obtained during the growth cycle at 55, 60, and 68°C for 36 h. The results in Fig. 1 were obtained with the fermentation performed at 60°C. They show that the secondary alcohol dehydrogenase appears early during the fermentation and that at the end of the fermentation it drastically decreases, whereas the primary alcohol dehydrogenase slowly in-
creases during the fermentation and peaks at the beginning of the stationary phase. Similar results were obtained with the culture grown at 68°C. In cells grown at 55°C the two alcohol dehydrogenases seem to emerge together, but in similarity with the fermentation at the higher temperatures the secondary alcohol dehydrogenase decreased before the primary enzyme.

**Purification of primary and secondary alcohol dehydrogenases from *T. ethanolicus***. The purification of the alcohol dehydrogenases is summarized in Table 1. During the purification the enzymes were assayed for activity with ethanol, which serves as a substrate for both enzymes; 2-propanol, which is specific for the secondary alcohol dehydrogenase; and 1-butanol, which has high activity with the primary but very low activity with the secondary alcohol dehydrogenase. The enzymes were separated on the Matrex Gel Red A column (step 3) (Fig. 2). The primary alcohol dehydrogenase passed through the column with the void volume. After a wash with 100 mM Tris buffer, the secondary alcohol dehydrogenase was eluted with the Tris buffer containing 100 mM NaCl. Further purification of the alcohol dehydrogenases using Ultrogel AcA 34, hydroxyapatite, and DEAE-Sephadex resulted in electrophoretically homogeneous proteins (Fig. 3). The secondary alcohol dehydrogenase was purified 163-fold to a specific activity of 170 when assayed with 2-propanol as the substrate at 60°C and pH 8.9. Under the same conditions, but with 1-butanol as the substrate, the primary alcohol dehydrogenase had a specific activity of 40.5 after being purified 123-fold. The specific activities with ethanol were 25.7 and 11.4 for the primary and the secondary alcohol dehydrogenases, respectively. It should be noted that the secondary alcohol dehydrogenase reacted slowly with 1-butanol in the activity staining of gel D in Fig. 3, whereas no reaction was observed between the primary alcohol dehydrogenase and 2-propanol (gel G).

**Molecular weight, amino acid composition, and metal content**. The primary and secondary alcohol dehydrogenases eluted from an Ultrogel AcA 34 column as single symmetrical peaks corresponding to *M* 
values of 184,000 and 172,000, respectively. During sodium dodecyl sulfate-polyacrylamide gel electrophoresis the proteins migrated as single bands with apparent molecular weights for the primary and secondary alcohol dehydrogenases of 44,500 and 42,000, respectively. These results indicate that each enzyme exists as a tetramer of apparently identical subunits. The amino acid compositions for the enzymes as calculated per subunit are given in Table 2. Although the contents of the amino acids in the two enzymes are rather similar, it should be noticed that methionine apparently is absent in the primary alcohol dehydrogenase. Estimations of average hydrophobicity according to Bigelow (2) indicate that the secondary enzyme is somewhat more hydrophobic (1,175 cal per residue) than the primary enzyme (1,095 cal per residue). Both enzymes contain high numbers of cysteine residues.

**TABLE 1. Purification of primary and secondary alcohol dehydrogenases from *T. ethanolicus***

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Total activity (U)</th>
<th>Substrate (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Cell extract</td>
<td>34,560</td>
<td>6,912</td>
<td>0.2</td>
</tr>
<tr>
<td>2 DEAE 23</td>
<td>7,652</td>
<td>1,148</td>
<td>0.15</td>
</tr>
<tr>
<td>3 Matrex Gel Red A eluate</td>
<td>1,375</td>
<td>1,379</td>
<td>1.0</td>
</tr>
<tr>
<td>100 mM NaCl</td>
<td>212</td>
<td>532</td>
<td>2.51</td>
</tr>
<tr>
<td>4(P). Ultrogel AcA 34</td>
<td>165</td>
<td>231</td>
<td>1.4</td>
</tr>
<tr>
<td>5(F). Hydroxypatite</td>
<td>95</td>
<td>162</td>
<td>1.7</td>
</tr>
<tr>
<td>6(P). DEAE-Sephadex</td>
<td>3.5</td>
<td>90</td>
<td>25.7</td>
</tr>
<tr>
<td>4(S). Ultrogel AcA 34</td>
<td>55</td>
<td>347</td>
<td>6.3</td>
</tr>
<tr>
<td>5(S). Hydroxypatite</td>
<td>26</td>
<td>296</td>
<td>11.4</td>
</tr>
</tbody>
</table>

* Steps designated (P) and (S) refer to primary and secondary alcohol dehydrogenases, respectively.

* Determined with ethanol as the substrate.

![FIG. 2. Separation of primary (1-butanol-active) and secondary (2-propanol-active) alcohol dehydrogenases (ADH) from *T. ethanolicus* on a Matrex Gel Red A column.](http://aem.asm.org/)

![FIG. 3. Electrophoresis of purified alcohol dehydrogenases from *T. ethanolicus* on 10% polyacrylamide gels. To each gel was applied about 20 μg of protein. Gels: A to D, Secondary alcohol dehydrogenase; E to H, Primary alcohol dehydrogenase; A and E, protein stained; B and F, activity stained with ethanol; C and G, activity stained with 2-propanol; D and H, activity stained with 1-butanol.](http://aem.asm.org/)
and 
V...
with 
aldehyde
have
5.54
values
less
ethanol.
This is
in
as
ethanol,
1-butanol,
1-propanol,
1-pentanol,
1-hexanol,
1-heptanol,
2-propanol,
2-butanol,
2-pentanol,
2-heptanol,
3-hexanol,
3-heptanol,
1,2-propanediol,
1,3-propanediol,
1,3-butenediol,
ethylene glycol,
2-methyl-1-propanol
Zn
ethanol
and 
catalyzes
samples
We assume
primary
Secondary
alkaloid
and
alcohol
is oxidized
acatalaldelyde
is
val.
Val
103
21
1,2-propanediol
2,3-butanediol
2,3-butanediol
1-propanol
1-butanol
1-pentanol
1-hexanol
1-heptanol
2-propanol
2-butanol
2-pentanol
2-heptanol
3-hexanol
1,2-propanediol
1,3-propanediol
1,3-butenediol
ethylene glycol
2-methyl-1-propanol
acetaldelyde
acetaldehyde
propionaldehyde
acetone
2,3-butenedione

TABLE 3. Apparent $K_m$ and $V_m$ values for the primary and secondary alcohol dehydrogenases from T. ethanolicus

<table>
<thead>
<tr>
<th>Alcohol dehydrogenase</th>
<th>Ethanol/ NADP (V_m)</th>
<th>Ethanol (K_m)</th>
<th>NADPH (K_m)</th>
<th>1-Butanol/ NADP (V_m)</th>
<th>1-Butanol (K_m)</th>
<th>NADPH (K_m)</th>
<th>2-Propano/ NADP (V_m)</th>
<th>2-Propanol (K_m)</th>
<th>NADPH (K_m)</th>
<th>Acetaldehyde/ NADPH (V_m)</th>
<th>Acetaldehyde (K_m)</th>
<th>NADPH (K_m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>45.6</td>
<td>16.4</td>
<td>0.83</td>
<td>44.6</td>
<td>47.1</td>
<td>0.15</td>
<td>231</td>
<td>16.4</td>
<td>0.35</td>
<td>375</td>
<td>210</td>
<td>1.5</td>
</tr>
<tr>
<td>Secondary</td>
<td>13</td>
<td>49</td>
<td>0.022</td>
<td></td>
<td></td>
<td></td>
<td>26</td>
<td>44.8</td>
<td>0.36</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Assays were at 70°C and pH 8.9 with alcohols and NADP as substrates, and at pH 7.6 with acetaldehyde and NADPH as substrates. $K_m$ values are millimolar and $V_m$ values are units per milligram. All values were obtained from double-reciprocal plots and by replots (19). Both enzymes use a sequential mechanism.

$^b$ $K_m$ with ethanol as the alcoholic substrate.

$^c$ $K_m$ with 1-butanol as the alcoholic substrate.

$^d$ $K_m$ with 2-propanol as the alcoholic substrate.

Zinc, but no other metal, was found in both alcohol dehydrogenases by using plasma emission spectroscopy. Samples of the primary enzyme had from 3.2 to 4.6 atoms per subunit, and those of the secondary enzyme had from 3.2 to 5.54 atoms per subunit. We assume that the two enzymes each have 4 Zn per subunit.

Enzymatic properties. Apparent $K_m$s for NADP, NADPH, ethanol, 1-butanol, 2-propanol, and acetaldehyde are listed in Table 3. With both enzymes the $K_m$s for NADPH vary with the alcohol. This is very noticeable with the secondary alcohol dehydrogenase: with ethanol the $K_m$ for NADPH is 22 $\mu$M, whereas with 2-propanol it is 350 $\mu$M.

Both enzymes convert acetaldehyde to ethanol with NADPH as the reductant. The primary alcohol dehydrogenase catalyzes this reaction very efficiently ($V_m$ = 375 U/mg) in comparison with the secondary alcohol dehydrogenase ($V_m$ = 26 U/mg). However, it should be noted that the $K_m$ values for acetaldehyde and NADPH with the primary enzyme are much higher than with the secondary alcohol dehydrogenase.

Substrate specificity. Both alcohol dehydrogenases in T. ethanolicus are NADP dependent. The rates with NAD are less than 0.5% of those with NADP. The relative activities with various substrates as compared with ethanol or acetaldehyde for the alcohol dehydrogenases are listed in Table 4.

The primary alcohol dehydrogenase oxidizes 1-propanol, 1-butanol, 1-pentanol, and 2-methyl-1-propanol at initial rates about equal to or higher than that of ethanol. Secondary alcohols and 1,3- and 1,4-butanediol are not oxidized, and 1,3- and 1,2-propanediol as well as ethylene glycol are rather poor substrates. Propionaldehyde is reduced at a rate higher than that of acetaldehyde, but ketones such as aceton or 2,3-butenedione are not reduced.

The secondary alcohol dehydrogenase is less specific than the primary enzyme. Besides ethanol, it oxidizes 1-propanol, 1-butanol, and 2-methyl-1-propanol. In the reverse reaction, propionaldehyde is reduced faster than acetaldehyde. However, the best substrates are secondary alcohols; 2-propanol is oxidized at a rate 15 times faster than that of ethanol. Similarly, aceton and 2,3-butenedione are reduced faster than acetaldehyde. Neither alcohol dehydrogenase is active with methanol, methoxyethanol, 2-aminoethanol, tert-butanol, 1,4-butenediol, lactate, formaldehyde, or pyruvate.

Thermostability and effect of temperature. The alcohol dehydrogenases have high thermostability. For instance, no

**TABLE 2. Amino acid compositions of the primary and secondary alcohol dehydrogenases from T. ethanolicus**

<table>
<thead>
<tr>
<th>Residue</th>
<th>Primary Composition (residues/subunit)</th>
<th>Secondary Composition (residues/subunit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>47</td>
<td>37</td>
</tr>
<tr>
<td>Arg</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>Asx</td>
<td>30</td>
<td>33</td>
</tr>
<tr>
<td>Cys</td>
<td>23</td>
<td>18</td>
</tr>
<tr>
<td>Glx</td>
<td>34</td>
<td>31</td>
</tr>
<tr>
<td>Gly</td>
<td>39</td>
<td>46</td>
</tr>
<tr>
<td>His</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>Ile</td>
<td>27</td>
<td>33</td>
</tr>
<tr>
<td>Leu</td>
<td>41</td>
<td>29</td>
</tr>
<tr>
<td>Lys</td>
<td>36</td>
<td>37</td>
</tr>
<tr>
<td>Met</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Phe</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>Pro</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>Ser</td>
<td>27</td>
<td>14</td>
</tr>
<tr>
<td>Thr</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>Tyr</td>
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<td>3</td>
</tr>
<tr>
<td>Trp</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>Val</td>
<td>30</td>
<td>41</td>
</tr>
</tbody>
</table>

$^a$ The composition is given in residues per subunit with assumed $M_s$ of 44,500 and 42,000 for primary and secondary alcohol dehydrogenase, respectively.

**TABLE 4. Relative rates of oxidation of alcohols or reduction of aldehydes or ketones as catalyzed by alcohol dehydrogenases from T. ethanolicus**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Primary Relative activity of alcohol dehydrogenase</th>
<th>Secondary Relative activity of alcohol dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1-Propanol</td>
<td>103</td>
<td>72</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>105</td>
<td>6</td>
</tr>
<tr>
<td>1-Pentanol</td>
<td>138</td>
<td>0</td>
</tr>
<tr>
<td>1-Hexanol</td>
<td>94</td>
<td>0</td>
</tr>
<tr>
<td>1-Heptanol</td>
<td>81</td>
<td>0</td>
</tr>
<tr>
<td>2-Propanol</td>
<td>0</td>
<td>1,500</td>
</tr>
<tr>
<td>2-Butanol</td>
<td>0</td>
<td>133</td>
</tr>
<tr>
<td>2-Pentanol</td>
<td>0</td>
<td>186</td>
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<tr>
<td>2-Heptanol</td>
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<td>5</td>
</tr>
<tr>
<td>3-Hexanol</td>
<td>0</td>
<td>221</td>
</tr>
<tr>
<td>1,2-Propanediol</td>
<td>22</td>
<td>268</td>
</tr>
<tr>
<td>1,3-Propanediol</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>1,3-Butenediol</td>
<td>0</td>
<td>46</td>
</tr>
<tr>
<td>Ethylene glycol</td>
<td>19</td>
<td>3</td>
</tr>
<tr>
<td>2-Methyl-1-propanol</td>
<td>106</td>
<td>32</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Propionaldehyde</td>
<td>112</td>
<td>125</td>
</tr>
<tr>
<td>Acetone</td>
<td>0</td>
<td>194</td>
</tr>
<tr>
<td>2,3-Butenedione</td>
<td>0</td>
<td>153</td>
</tr>
</tbody>
</table>

$^a$ Standard assays at 60°C as given in the text were used. The alcohols were used at 200 mM, and the aldehydes and ketones were 50 mM. The purified primary and secondary alcohol dehydrogenases had specific activities of 25.7 and 12 U/mg, respectively, with ethanol as the substrate. The rates with ethanol and acetaldehyde were then set to 100.
loss of activity was observed in the secondary enzyme during an incubation for 2 h at 70°C in 10 mM Tris hydrochloride (pH 7.6).

The enzymes were assayed for activity at different temperatures by the standard assays. The results are shown in the Arrhenius graphs of Fig. 4. The secondary enzyme has its optimum activity with ethanol as the substrate at 70°C, and with 2-propanol occurs at about 80°C. The energies of activation (Ea) with the respective substrate are about 9,000 cal (37.7 kJ) and 10,000 cal (41.8 kJ) per mol. For the primary enzyme, the apparent temperature optimum with 1-butanol and ethanol is at 85°C and the energy of activation is about 17,000 cal (71.1 kJ) per mol.

DISCUSSION

Relatively recently, several thermophilic anaerobic bacteria have been discovered that may be of potential use for industrial production of ethanol by fermentation (23, 27, 28). A major advantage of these bacteria over *Saccharomyces* spp. or *Zymomonas* spp. which are conventionally used for ethanol fermentations is that the bacteria ferment a wide variety of carbohydrates, including pentoses and some polysaccharides. Unfortunately, the thermophilic bacteria have low ethanol tolerance and are sensitive to high substrate concentrations.

The present work was undertaken to obtain a better understanding of the physiology of *T. ethanolicus*, one of the more promising thermophilic bacteria for ethanol fermentation (17, 23, 27). The demonstration that this bacterium has two different alcohol dehydrogenases clearly indicates that the ethanol fermentation in this organism is not the straightforward process originally imagined. It is impossible to assign exactly the physiological roles of the two alcohol dehydrogenases. However, the secondary alcohol dehydrogenase forms early during the growth cycle, it has a relatively low K_m for acetaldehyde (44.8 mM) in comparison with that of the primary alcohol dehydrogenase (K_m = 210 mM), and its activity is enhanced by pyruvate (5). These facts support the idea that the secondary enzyme is responsible for ethanol formation, at least in the earlier stages of the fermentation. The role of the primary alcohol dehydrogenase, which is formed late in the fermentation, may perhaps be of converting alcohols to aldehydes which can be used as alternative carbon sources for *T. ethanolicus*. However, it is not known whether *T. ethanolicus* can use alcohols as substrates. The K_m values of NADP for both alcohol dehydrogenases are low in comparison with the K_m of NADPH, and clearly, if alcohols are available, they may be used to maintain a high NADPH/NADP ratio and a low redox state in the bacterium. The low K_m for NADP with ethanol as a substrate suggests that the NADP concentration in the bacterium could regulate the production of ethanol.

The two alcohol dehydrogenases of *T. ethanolicus* have rather broad substrate spectra. The primary enzyme oxidizes straight- or branched-chain alcohols from ethanol to heptanol (alcohols with longer carbon chains were not tested). It also oxidizes 1,2-propanediol and ethylene glycol, but at lower rates than unsubstituted alcohols. Other diols, methoxyethanol, or 2-aminoethanol are either very poor substrates or not oxidized at all. The best substrate for the secondary alcohol dehydrogenase is 2-propanol. Although this enzyme oxidizes 1-propanol and 1-butanol (the latter at a very low rate), it seems to work best with alcohols that have the structure R-CHOH-CH_3, in which R can be hydrogen, as in ethanol, or a straight or a branched carbon chain which may contain substitutions, e.g., 1,3-butandiol. Together the two alcohol dehydrogenases apparently allow *T. ethanolicus* to metabolize a wide variety of alcohols. However, this has not been fully investigated.

*T. ethanolicus* is not unique in having two different alcohol dehydrogenases. *Zymomonas mobilis* (18) has two alcohol dehydrogenases; both are active with ethanol, but only one has significant activity with butanol. Secondary alcohols are not used as substrates (14). Recently, two different strains of *Clostridium beijerinckii* were found to have different alcohol dehydrogenases, one for each strain (11). Multiple forms have also been demonstrated in several other microorganisms including *Penicillium* sp. (16), *Escherichia coli* (10), *Acinetobacter* sp. (20), and yeasts (12). A thermostable NAD-dependent alcohol dehydrogenase has been isolated from *Bacillus stearothermophilus* (4, 7). The thermophilic anaerobes *Thermoanaerobium brockii* and *Clostridium thermohydrosulphuricum* (15) contain an NADP-dependent alcohol dehydrogenase that in many aspects is very similar to the secondary alcohol dehydrogenase in *T. ethanolicus*.

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LITERATURE CITED


