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# CHARACTERIZATION OF AN ALCOHOL DEHYDROGENASE FROM THERMOANAEROBACTER ETHANOLICUS ACTIVE WITH ETHANOL AND SECONDARY ALCOHOLS

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SUMMARY Thermoanaerobacter ethanolicus contains a NADP-dependent alcohol dehydrogenase. Ethanol is a substrate but secondary alcohols such as 2-propanol, 2-butanol and 2-pentanol are oxidized at a faster rate. The enzyme has a molecular weight of 172,000 and consists of 4 subunits. Each subunit contains 4 zinc atoms. With ethanol the kinetics are sigmoidal; however, in the presence of pyruvate a Michaelis-Menten kinetic pattern is approached. At 70°C and pH 9 the purified enzyme oxidizes ethanol with NADP at a rate of 13 μmol $min^{-1}$ $mg^{-1}$, whereas it reduces acetaldehyde with NADPH two times that rate.

## INTRODUCTION

Thermoanaerobacter ethanolicus (ATCC 31550), which grows between 38 and 78°C, ferments a range of carbohydrates (1). When growing in media containing 10 g per liter of glucose or less, the main product is ethanol, whereas in media with a higher concentration of glucose, acetate and lactate are also produced (2). To evaluate the reason for this shift in product formation we have embarked on a study of enzymes involved in the production of ethanol. In this report we describe the purification and some properties of an alcohol dehydrogenase from T. ethanolicus. The enzyme oxidizes ethanol and 1-propanol with NADP as an electron acceptor. However, secondary alcohols are oxidized at a faster rate than the primary alcohols. The substrate specificity indicates that the presence of a hydrophobic group, preferably a methyl group, is needed next to the carbinol group.

## MATERIALS AND METHODS

T. ethanolicus was grown at 50°C as previously described (1) using a 400 l New Brunswick fermentor. Cells were harvested using a Sharpless centrifuge and were stored frozen at -20°C until used. The alcohol dehydro-

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genase was assayed at 70°C by following the reduction of $NADP^+$ at 340 nm ($\varepsilon = 6.22 \times 10^3\ M^{-1}\ min^{-1}$). The assay solution contained 200 mM ethanol, 1.25 mM $NADP^+$ and 50 mM Tris·HCl, pH 9.0. One unit of enzyme is defined as the amount that reduces 1 µmol of $NADP^+$ per min, and specific activity is expressed as units per mg of protein. Protein was assayed according to Elliott and Brewer (3). Disc gel electrophoresis was performed according to Brewer and Ashworth (4), and SDS-acrylamide gel electrophoresis according to Weber et al (5). The Mr was determined using gel filtration with an Ultrogel AcA 34 column (1.5 cm x 100 cm) (LKB Bromma, Sweden) with glucose-6-phosphate dehydrogenase (Mr 240,000), yeast alcohol dehydrogenase (Mr 150,000), hexokinase (Mr 102,000) and ovalbumin (Mr 45,000) as standards. Metal analyses were done with plasma emission spectroscopy (6). In addition, zinc was assayed by using a Perkin Elmer 403 atomic absorption spectrometer. Activity staining of gels from disc electrophoresis was done according to Ursprung and Leone (7). Amino acid analyses were performed after carboxymethylation according to Crestfield et al (8) and acid hydrolysis at 110°C for 20, 45, 69 and 123 h.

Purification of alcohol dehydrogenase from T. ethanolicus - 50 g of frozen cells were suspended in 150 ml 10 mM Tris·HCl, pH 7.6. The suspension was passed twice through a French pressure cell at 12,000 $lb/in^2$ and then centrifuged at 100,000 x g for 60 min. The supernatant solution was heat-treated by storage at 70°C for one h. The precipitated material was removed by centrifugation for 30 min at 15,000 x g. The heat treatment was followed by fractionation using ethanol. This was done at 25°C. Ethanol was first added to a concentration of 60% (v/v). The precipitate was removed by centrifugation and additional ethanol was added to 70%. The 70% ethanol-precipitate containing the enzyme was collected by centrifugation at 15,000 x g for 10 min. The pellet was immediately dissolved in 100 ml of 10 mM Tris·HCl, pH 7.6, containing 2 mM dithiothreitol (DTT), 5 mM pyruvate, 0.05 mM NADP, and 0.08 mM $MgSO_4$. This buffer solution was used in all subsequent steps of purification. Next the solution was applied to a Matrex gel red A (Amicon Corp., Lexington, Mass.) column (2.75 cm x 15 cm). The enzyme was eluted using the above buffer solution with a 2 l gradient from zero to 1.0 M NaCl. The enzyme eluted at about 0.4 M NaCl. Fractions containing the enzyme were pooled, and the solution was concentrated to 10 ml by ultrafiltration using a PM 10 membrane (Amicon Corp., Lexington, Mass.). The solution was now applied to an Ultrogel AcA 34 column (2.75 cm x 100 cm). The active fractions from this column were directly applied to a DEAE Sephadex A-50 (Pharmacia, Uppsula, Sweden) 2.75 cm x 30 cm column from which the enzyme was eluted using the buffer solution with a 2 l gradient from zero to 0.2 M of NaCl. The purified enzyme eluted at about 0.1 M NaCl.

## RESULTS

Purification and physical properties - A purification scheme for the alcohol dehydrogenase is given in Table I. During the purification it was noticed that the enzyme was very unstable. However, the enzyme was stable in the presence of a sulfhydryl compound, pyruvate, NADP and $MgSO_4$. These reagents were added to the buffer used during the purification and storage of the enzyme. Of sulfhydryl compounds that were tested, dithiothreitol was found to be the best. The heat treatment and the ethanol fractionation

Table I. Purification of alcohol dehydrogenase from T. ethanolicus

| Step | Protein mg | Units | Specific Activity |
|---|---|---|---|
| Crude extract | 1506 | 345 | 0.229 |
| Heat treatment | 1098 | 359 | 0.327 |
| 60-70% ethanol fraction | 384 | 374 | 0.974 |
| Matrex Red A | 102 | 276 | 2.70 |
| Ultrogel ACA 34 | 35.7 | 226 | 6.33 |
| DEAE-cellulose | 12.8 | 158 | 12.4 |

are rough procedures for enzyme purification. The ability of the alcohol dehydrogenase to withstand these treatments may be due to it being from a thermophilic microorganism. However, the ethanol fractionation should be speedily performed to minimize loss of the enzyme. The yield of the enzyme with the purification procedure outlined in Table I varied between 15 to 45%.

The enzyme was judged pure since a single protein band was observed in gels from disc electrophoresis and that this protein band stained for activity. In addition, a single protein band was observed after electrophoresis in SDS-gels. This latter procedure gave a Mr of 43,000, whereas a Mr of 176,000 was found by gel filtration on a standardized Ultrogel AcA 34 column. The enzyme apparently is a tetramer consisting of subunits with identical Mr. It has high thermal stability. No loss of activity occurred on incubating the enzyme at 70°C for 2 h, and after 2 h at 80°C 77% of the activity remained.

Chemical composition - The amino acid composition of the enzyme given in Table II is similar to other alcohol dehydrogenases with the exception of a high number of cysteine residues of which there apparently are 19 per subunit. The enzyme appears to contain 4 zinc atoms per subunit. Atomic absorption spectrometric analyses yielded 4.5, and plasma emission spectroscopy 3.2-3.9 atoms of zinc per subunit. Aluminum, barium, cadmium, chromium, cobalt, copper, iron, manganese, molybdenum and nickel were present in less than stoichiometric proportions. Calculation of the amount of metals was

Table II. Amino acid composition of alcohol dehydrogenase from T. ethanolicus[a]

| | | | |
|---|---|---|---|
| Lysine | 100 | Alanine | 136 |
| Histidine | 32 | Valine | 100 |
| Arginine | 40 | Methionine | 8 |
| Aspartate | 128 | Isoleucine | 84 |
| Threonine | 64 | Leucine | 112 |
| Serine | 56 | Tyrosine | 16 |
| Glutamate | 112 | Phenylalanine | 44 |
| Proline | 60 | Cysteine | 76 |
| Glycine | 168 | Tryptophan | n.d.[b] |

[a]The Mr of the enzyme was assumed to be 172,000.

[b]n.d. = not determined.

based on a subunit Mr of 43,000 and protein assays with a Rose Bengal dye method (3). The plasma emission spectroscopy analyses revealed the presence of phosphorus in the enzyme. This may be due to tightly bound NADP which is not removed during extensive dialysis, as was indicated by absorption of the reduced enzyme at 340 nm.

Substrate specificity - The enzyme appears specific for NADP. The activity with NAD is less than 0.5% of that with NADP. Table III shows that the enzyme oxidizes several alcohols and also reduces different aldehydes and ketones. The rates with secondary alcohols are greater than that of primary alcohols. It is apparent that to be a substrate the carbinol group must

Table III. Relative rates of oxidation of alcohols or reduction of oxidized substrate by alcohol dehydrogenase from T. ethanolicus.

| Substrate[a] | Rate | Substrate | Rate |
|---|---|---|---|
| Methanol | 0 | 2-Amino ethanol | 0 |
| Ethanol | 100 | Ethyleneglycol | 3 |
| 1-Propanol | 74 | 1,2-Propanediol | 290 |
| 1-Butanol | 4 | 1,3-Propanediol | 0 |
| 1-Hexanol | 0 | 1,4-Butanediol | 0 |
| 2-Propanol | 1600 | Lactate | 0 |
| 2-Butanol | 124 | Acetaldehyde | 100 |
| 2-Pentanol | 182 | Acetone | 190 |
| 2-Heptanol | 0 | 2,3-Butanedione | 220 |
| 3-Hexanol | 21 | Formaldehyde | 0 |
| Methoxy ethanol | 0 | Pyruvate | 0 |

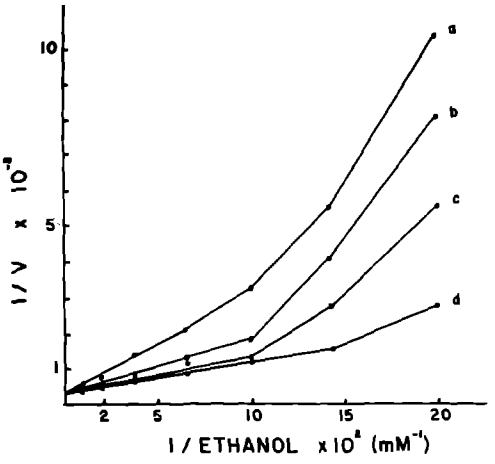


Fig. 1. Double reciprocal plot with ethanol as the variable substrate and with NADP concentrations of a) 25 µM, b) 50 µM, c) 500 µM, and d) 1.0 mM. Reactions were at 70°C, pH 9.0 in 50 mM Tris.

have a small hydrophobic group (e.g., methyl, ethyl, or iso-propyl) attached to it. Thus methanol, ethanols with a hydrophilic group on the second carbon, 1.3-propanediol and 1.4-butanediol do not serve as substrates.

Kinetic properties - Apparent $K_m$-values were determined at 70°C in 50 mM Tris·HCl, pH 9.0. They were for NADP, 0.02 mM; ethanol, 49 mM; NADPH, 0.4 mM; acetaldehyde, 48 mM. The specific activity of the purified enzyme at assay conditions for the oxidation of ethanol with NADP is about 13; the specific activity of the reverse reaction is approximately 26 µmol $min^{-1}$ $mg^{-1}$. The enzyme does not follow Michaelis-Menten kinetics. This is shown in Fig. 1 which is a double reciprocal plot. The lines curve upward which signifies a sigmoidal binding pattern. In Fig. 2 it is seen that in the presence of pyruvate the sigmoidal pattern is less marked.

## DISCUSSION

During the preliminary phase of this study using crude cell-free extracts we observed a reduction of NADP but not of NAD with ethanol. Similarly, acetaldehyde oxidized NADPH but not NADH. We concluded that T. ethanolicus contains only the NADP-dependent alcohol dehydrogenase, which is the subject of this paper. If so, the enzyme functions physiologi-

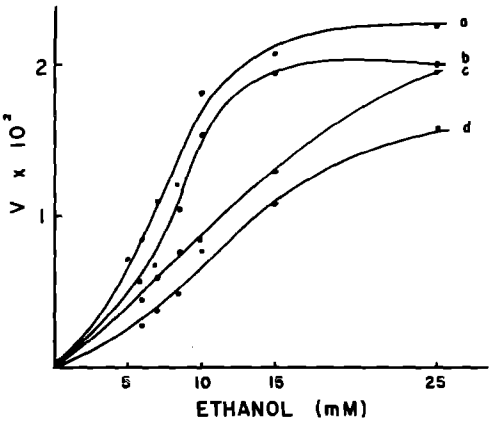


Fig. 2. The effect of pyruvate on the substrate saturation curve with ethanol. Pyruvate concentrations were a) 50 µM, b) 20 µM, c) 10 µM, and d) 0 µM. Reactions were at 70°C, pH 9.0 in 50 mM Tris and 25 µM NADP.

cally as an acetaldehyde reductase, although it reduces 2-ketones faster than acetaldehyde and oxidizes secondary alcohols faster than ethanol. Support for this suggestion is the fact that the main product of T. ethanolicus when grown on glucose is ethanol (1) and that the enzyme catalyzes the reduction of acetaldehyde at a substantial rate.

The substrate spectrum for the T. ethanolicus alcohol dehydrogenase appears to be unique. Certain yeasts (9) and methylotrophic bacteria (10) contain NAD-dependent, and Escherichia coli (11) contains a NADP-dependent secondary alcohol dehydrogenase, but these do not use ethanol or propanol as substrates. The fungus Rhizopus jaranicus contains two NAD-dependent alcohol dehydrogenase of which one oxidizes both primary and secondary alcohols. However, the activity with the primary alcohols was the highest (12). Similarly, yeast alcohol dehydrogenase oxidizes primary alcohols faster than secondary alcohols as does horse liver alcohol dehydrogenase (13).

Liver, and probably also yeast alcohol dehydrogenase contains two atoms of zinc per subunit (14). In the liver enzyme one of the zinc atoms is involved in the catalytic process; it is liganded by two cysteine and one histidine residue. The second zinc atom is coordinated with four

cysteine residues and it may be essential for the structural stability of the enzyme. The T. ethanolicus enzyme appears to contain four zinc atoms per subunit. These may also be bound through cysteine residues, which is indicated by the high number of such residues in the enzyme.

The T. ethanolicus alcohol dehydrogenase exhibits a sigmoid kinetic pattern which is altered toward Michaelis-Menten kinetics in the presence of pyruvate. Although much work remains to establish the reason for this kinetic pattern, a possible interpretation appears to be that the enzyme is allosteric and that pyruvate functions as an effector (15). That pyruvate, which is not a substrate, stabilizes the enzyme supports this interpretation.

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