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Novel NADP-linked alcohol–aldehyde/ketone oxidoreductase in thermophilic ethanologenic bacteria

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An NADP-specific alcohol–aldehyde/ketone oxidoreductase was detected in cell extracts of *Thermoanaerobium brockii* and *Clostridium thermohydrosulfuricum*, but not in *Thermobacteroides acetoethylicus* or *Clostridium thermocellum*. The enzyme was purified from *Ta. brockii* by differential procedures that included heat treatment and an affinity-chromatography step on Blue Dextran–Sepharose. The 44-fold-purified enzyme displayed one band (mol.wt. approx. 40000) after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The enzyme had a broad substrate specificity that included linear and branched primary alcohols, linear and cyclic secondary alcohols, linear and cyclic ketones, and acetaldehyde. The NADP-specific alcohol–aldehyde/ketone oxidoreductase was considerably more active towards secondary alcohols than towards other substrates. The enzyme had remarkable stability to heating at 86°C for 70 min, but was rapidly denatured on boiling. Secondary-alcohol dehydrogenase activity displayed a noticeable inflexion point at 50°C in Arrhenius plots and a high Q_{10} value (>2.0). The enzyme was inactivated by the thiol-blocking reagent *p*-chloromercuribenzoate, but was not significantly inhibited by common metal-ion-binding agents. The NADP-linked alcohol–aldehyde/ketone oxidoreductase of *Ta. brockii* appears to have properties distinct from those of previously described primary- and secondary-alcohol dehydrogenases.

NADP-linked alcohol dehydrogenases have been detected previously in *Escherichia coli* (Hatanaka *et al.*, 1971*a*), *Leuconostoc mesenteroides* (DeMoss *et al.*, 1951; Hatanaka *et al.*, 1971*b*), *Clostridium kluyveri* (Hillmer & Gottschalk, 1974), pig liver (Dutler *et al.*, 1971) and yeasts (Barth & Kunkel, 1979). In general, secondary-alcohol dehydrogenase activity is often associated with NADP- or NAD-linked ethanol dehydrogenses, but these alcohol dehydrogenses display a noticeable preference for primary alcohols (Hatanaka *et al.*, 1971*a*,*b*; Bränden *et al.*, 1975). NAD-linked secondary-alcohol dehydrogenase activity was shown to correlate with the production of fusel oils (e.g. propan-1-ol, 2-methylpropan-1-ol, pentan-1-ol and 3-methylbutan-1-ol) during growth of *Saccharomyces cerevisiae* (Singh & Kunkee, 1976). NAD-linked alcohol dehydrogenases have been detected (Bellion & Wu, 1978; Hou *et al.*, 1979*a*) in and purified (Bellion & Wu, 1978; Hou *et al.*, 1979*b*; Patel *et al.*, 1979) from methanol-oxidizing microbes (i.e. methylotrophs) that displayed broad specificity for secondary alcohols, and from obligate aerobic *Pseudomonas* (Niehaus *et al.*, 1978) and *Comamonas* (Barrett *et al.*, 1980) species.

Thermophilic bacteria are of considerable interest for industrial alcohol and enzyme production because these microbes are uniquely suited for direct biomass fermentation to ethanol via reduced-pressure distillation and yield active thermostable enzymes of commercial interest (Zeikus, 1979, 1980); NADP-linked ethanol dehydrogenase was specifically detected during spore formation in the butyrogenic moderate thermophile *Clostridium thermosaccharolyticum* (Hsu & Ordal, 1970), and during growth of the ethanologenic extreme thermophile *Thermoanaerobium brockii* (Lamed & Zeikus, 1980*a*,*b*).

Ta. brockii cell extracts contain both NAD- and NADP-linked ethanol dehydrogenase activities (Lamed & Zeikus, 1980*a*,*b*). However, the NAD-linked ethanol dehydrogenase of this obligate anaerobe was less active and was not stable towards O_2. In the present work we demonstrate that NADP-linked alcohol dehydrogenase is not detected in all thermophilic ethanologenic bacteria, and we report

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the first attempt to purify and characterize an NADP-linked alcohol dehydrogenase from caldoactive bacteria. This enzyme appears novel, since it is extremely thermostable and has a very broad substrate specificity. The enzyme is referred to as an alcohol–aldehyde/ketone oxidoreductase to distinguish it from more common alcohol dehydrogenases because of its wide substrate range.

Materials and methods

Chemicals

All chemicals were reagent grade. N_2 and He were purchased from Matheson (Chicago, IL., U.S.A.). All biochemicals were obtained from Sigma (St. Louis, MO, U.S.A.). Blue Dextran, Sephadex and Sepharose were products of Pharmacia (Uppsala, Sweden). The Sephadex calibration standards catalase (mol.wt. 190000), fructose bisphosphate aldolase (mol.wt. 158000) and albumin (mol.wt. 68000) were obtained from Boehringer (Mannheim, W. Germany).

Organisms and growth conditions

Thermoanaerobium brockii strains HTD4 (Zeikus *et al.*, 1979) and HTB (Zeikus *et al.*, 1980), *Clostridium thermohydrosulfuricum* strains 39E (Zeikus *et al.*, 1980) and 567 from DSM (Göttingen, W. Germany) and *Thermobacteroides acetoethylicus* strain HTB2 (Zeikus *et al.*, 1980; Ben-Bassat & Zeikus, 1981) were mass-cultured in 14-litre Microferm fermentors (New Brunswick) that contained 12 litres of a glucose complex medium (TYEG medium) described previously (Zeikus *et al.*, 1979). Fermenter cultures were grown at 65°C and were continuously stirred (100rev./min) and gassed with N_2 (50ml/min). Cells were harvested in late-exponential growth phase (~10h-old cells) and collected by centrifugation at 35000*g* in a Sorvall RC-5 centrifuge (DuPont Instruments) equipped with a KSB continuous-flow system.

Preparation of cell extracts

Cell extracts were prepared for routine alcohol dehydrogenase assays by using the anaerobic procedures and assay conditions described previously for *Ta. brockii* (Lamed & Zeikus, 1980*a*). For purification of NADP-linked alcohol dehydrogenase, *Ta. brockii* cells (2g wet wt.) were suspended in 10ml of 30mM-Tris/HCl buffer, pH7.3, containing 4mM-dithiothreitol and 2μg of deoxyribonuclease/ml. Tis suspension was passed through a French pressure cell at 1400kg/cm^2 and then centrifuged at 20000*g* for 30min. Protein was determined by the method of Bradford (1976) with Bio-Rad (Rockville, NY, U.S.A.) reagents.

Chromatography and electrophoresis

Chromatographic procedures developed by Ryan & Vestling (1974) for purification of NAD(P)-coupled enzymes were followed. Sepharose 4B-CL (100ml) was activated by CNBr and then allowed to react with Blue Dextran (2g) for 16h at 4°C. Approx. 60% of the added Blue Dextran was bound to the Sepharose beads. Standard methods (Laemmli, 1970) that employed 0.3cm × 10cm slab gels (7.5%) were used for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Alcohol dehydrogenase assays

The reaction mixture (1ml total volume) for the standard secondary-alcohol dehydrogenase assay was composed of 0.1M-Tris/HCl buffer, pH7.8 (adjusted to temperature), containing 0.5mM-$NADP^+$, 150mM-butan-2-ol and the amount of enzyme indicated. The reaction was measured at 334nm ($\varepsilon_{334} = 6.1\,mM^{-1}\cdot cm^{-1}$) with an Eppendorf recording spectrophotometer. One unit of enzyme activity represents the amount of enzyme that catalysed the oxidation of 1μmol of butan-2-ol/min at 40°C. This standard assay was used for all enzyme-activity measurements except where indicated in the text. Where indicated, butan-2-ol was replaced in the reaction mixture either by another alcohol or by a ketone or aldehyde, in which case NADPH (0.2mM) oxidation was measured. Primary-alcohol dehydrogenase activity was assayed as described previously (Lamed & Zeikus, 1980*a*).

Results

Enzyme detection in thermophilic ethanologens

The occurrence of NADP-linked alcohol–aldehyde/ketone oxidoreductase was examined in the described species of obligately anaerobic caldoactive bacteria. Table 1 illustrates that the activity was present in strains of *Ta. brockii* and *C. thermohydrosulfuricum*, but not in *Tb. acetoethylicus or C. thermocellum*. Most notably, the secondary-alcohol dehydrogenase activities of the enzyme were 10–20-fold greater than the primary-alcohol dehydrogenase activities. The specific activities did not markedly depend on the primary (e.g. ethanol, propan-1-ol or butan-1-ol) or secondary (e.g. propan-2-ol or butan-2-ol) alcohol used in the assay. NAD-linked alcohol dehydrogenase was not detected in cell extracts when aerobic assay conditions were employed. The activity in *Ta. brockii* and *C. thermohydrosulfuricum* strains was reversible, as demonstrated by detection of NADPH-dependent reduction of acetone in crude cell extracts.

Enzyme purification from Ta. brockii

The NADP-linked alcohol–aldehyde/ketone

oxidoreductase was purified 44-fold from *Ta. brockii* cells in the five steps summarized in Table 2. Crude cell extracts were diluted 2-fold in water and heated at 90°C for 5 min. This resulted in a large precipitate, which was removed by centrifugation at 20000*g* for 30 min. The purification at this step was about 3-fold, with only a 7% loss in activity. The heat-treated enzyme was adjusted to 20 mM-Tris/HCl buffer, pH 8.0, and applied (20 ml) to a Whatman DE-52 DEAE-cellulose column (1.5 cm × 16 cm) previously equilibrated wth 20 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-dithiothreitol. The enzyme was eluted with a linear gradient of NaCl. Fig. 1 shows the purification of the enzyme by DEAE-cellulose chromatography; it was eluted as a single symmetrical activity peak that contained both secondary- and primary-alcohol dehydrogenase activity. NAD-linked alcohol dehydrogenase was not detected in the activity fractions when assays employed 0.5 μM- or 5 mM-NAD^+. The relative activity proportions for ethanol dehydrogenase, butan-2-ol dehydrogenase, acetaldehyde reductase and acetone reductase remained constant at each purification step.

The 7-fold-purified enzyme obtained from DEAE-cellulose chromatography was adjusted to pH 7.3 and applied directly (30 ml) to a Blue Dextran–Sepharose column (1.0 cm × 2.5 cm) previously equilibrated with 50 mM-Tris/HCl buffer, pH 7.3, containing 1 mM-dithiothreitol. The adsorbed en-

Table 1. *Comparison of NADP-dependent alcohol–aldehyde/ketone oxidoreductase activity in ethanologenic thermophilic bacteria*

The reaction mixture (1 ml total volume) contained 0.1 M-Tris/HCl buffer, pH 7.8, 0.5 mM-$NADP^+$, 150 mM-ethanol or -butan-2-ol, and 5–20 μl of cell extract. For full details see the text.

Species	Specific activity (μmol/min per mg at 40°C)	
	Ethanol	Butan-2-ol
Thermoanaerobium brockii		
Strain HTD4	0.20	3.0
Strain HTB	0.18	2.0
Clostridium thermohydrosulfuricum		
Strain 39E	0.20	5.5
Strain 567	0.05	0.70
Clostridium thermocellum	<0.01	<0.01
Thermobacteroides acetoethylicus	<0.01	<0.01

Table 2. *Purification procedures used for NADP-linked alcohol–aldehyde/ketone oxidoreductase of Ta. brockii*

Activity was determined by the standard secondary-alcohol dehydrogenase assay. For full experimental details see the text.

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein at 40°C)	Yield (%)	Purification (fold)
1. Crude extract	10	300	750	2.5	—	—
2. Heat treatment	20	100	700	7	93	3
3. DEAE-cellulose	30	31	530	17	70	7
4. Blue Dextran–Sepharose	4	6	500	78	67	41
5. Sephadex G-200	20	4	450	110	60	44

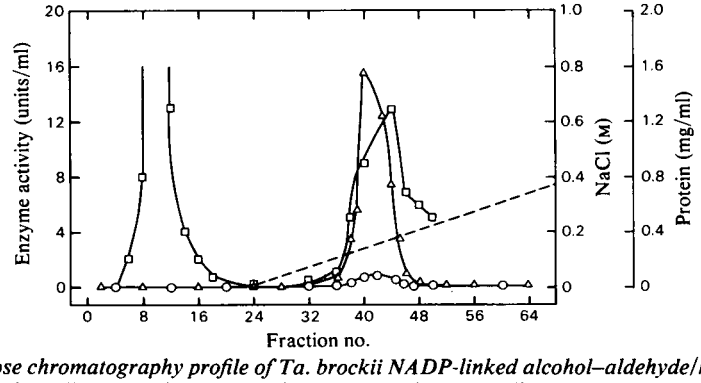

Fig. 1. *DEAE-cellulose chromatography profile of Ta. brockii NADP-linked alcohol–aldehyde/ketone oxidoreductase*

Heat-treated *Ta. brockii* cell extract (3.0 ml, purification step 2) was applied to a 1.5 cm × 16 cm DEAE-cellulose column previously equilibrated in 20 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-dithiothreitol. The column was washed with this solution and eluted by a linear NaCl gradient (----) in the same buffer. Fractions (2.5 ml) were collected and assayed for protein (□), propan-1-ol dehydrogenase (○) and propan-2-ol dehydrogenase (△) activities.

zyme was washed with 10ml of the above solution, and was then eluted with 2mM-$NADP^+$ in 50mM-Tris/HCl buffer, pH7.3, containing 1mM-dithiothreitol. The enzyme was eluted as one sharp activity peak. These fractions were pooled and applied to a Sephadex G-200 column (1.4cm × 80cm) previously equilibrated with 50mM-Tris/HCl buffer, pH8.0, containing 1mM-dithiothreitol. The activity peak in this gel-filtration step was eluted with a $K_{av.}$ centred at 0.32 $[K_{av.} = (V_t - V_0)/(V - V_0)]$; V_t (the total solute volume) and V_0 (the void volume) were determined with dinitrophenol and Blue Dextran. On the basis of these results and a molecular-weight calibration curve, the molecular weight of the enzyme was estimated to be 150000.

The purified enzyme displayed one major band when examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (see Fig. 2). The subunit molecular weight of the enzyme was estimated to be 40000 by migration comparisons with known protein standards (catalase, mol.wt. 57000; hen ovalbumin, mol.wt. 43000; creatine kinase, mol.wt. 40000; deoxyribonuclease I, mol.wt. 31000). Further examination of catalytic properties of the NADP-linked alcohol–aldehyde/ketone oxidoreductase employed purified enzyme. The purified enzyme required NADP(H) as electron carrier and did not recognize NAD(H) at 0.3mM-NADH or 5mM-NAD^+.

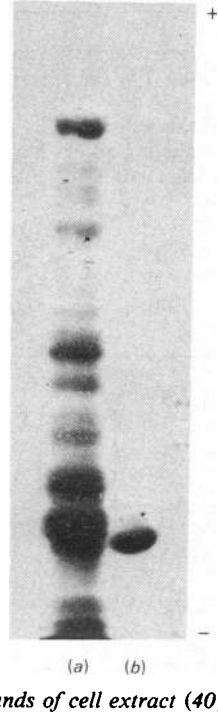

Fig. 2. *Protein bands of cell extract (40μg of protein, a) and purified NADP-linked alcohol–aldehyde/ketone oxidoreductase (4μg of protein, b) of Ta. brockii after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis*

For full experimental details see the text. The direction of migration was downward.

Enzyme substrate specificity

Purified NADP-linked alcohol–aldehyde/ketone oxidoreductase of *Ta. brockii* displayed activity towards acetaldehyde, a variety of primary and secondary alcohols, and linear and cyclic ketones (Table 3). Activity of this reversible enzyme was highest for secondary alcohols and decreased in order of acetaldehyde, linear ketones, cyclic ketones and primary alcohols. Interestingly, pent-1-en-3-ol, in which both residues are larger than methyl, was a very good substrate. However, β-substitution by a second hydroxy group as in pentane-2,4-diol significantly decreased observed activity. The activity of purified enzyme with primary alcohols was not affected by branching, as in 2-methylpropan-1-ol and 3-methylbutan-1-ol. The effect of ring size and ring substitution was studied in cyclic ketones. The enzyme was active on five- to seven-membered ring ketones but not with cyclo-octanone as substrate. The enzyme activity observed decreased from 2-methylcyclohexanone through to 4-methylcyclohexanone. Double bonds in the β-position (e.g. allyl alcohol) did not decrease enzyme activity. Methanol, glycerol and L-lactic acid did not serve as substrates.

The effect of substrate concentration on the activity of the NADP-linked alcohol–aldehyde/ketone oxidoreductase towards primary alcohol, secondary alcohol, ketone, and aldehyde is shown in Fig. 3. Saturation kinetics were observed for butan-2-ol or acetaldehyde as the substrate concentration was increased from 0 to 3mM, but a nearly linear relationship was obtained with ethanol. The apparent K_m values obtained from linear double-reciprocal plots were 0.12mM for butan-2-one, 0.31mM for butan-2-ol and 0.7mM for acetaldehyde. Half-maximal saturation was reached at about 6mM-methanol. Enzyme activity was not inhibited at high (100–150mM) ketone, alcohol or aldehyde concentrations. The apparent K_m values for $NADP^+$ and NADPH were in the micromolar range, which was too low to be accurately determined by the spectrophotometric assay employed.

Effect of temperature on activity

The effect of temperature on butan-2-ol dehydrogenase and propan-2-ol dehydrogenase activities was determined in phosphate buffer, pH7.1, because the pH of this buffer is not drastically altered by changes in temperature. Fig. 4 represents an Arrhenius plot that summarizes the results. A

Table 3. *Substrate specificity of NADP-linked alcohol–aldehyde/ketone oxidoreductase from Ta. brockii*
Reaction mixtures (1 ml) contained 0.1 M-Tris/HCl buffer, pH 7.8, 0.5 mM-$NADP^+$ or 0.2 mM-NADPH, 150 mM of the indicated alcohol/ketone or 75 mM-acetaldehyde and 1 μg of purified enzyme. For full experimental details see the text. Enzyme activity was not affected by decreasing or increasing substrate concentration 2-fold.

Substrate	Apparent $V_{max.}$ (μmol/min per mg of protein at 40°C)
Primary alcohols	
CH_3OH	0
CH_3–CH_2OH	3.2
CH_3–CH_2–CH_2OH	3.6
CH_3–CH_2–CH_2–CH_2OH	4.1
CH_3–CH_2–CH_2–CH_2–CH_2OH	0.9
Branched primary alcohols	
CH_3–$CH(CH_3)$–CH_2OH	3.1
CH_3–CH_2–$CH(CH_3)$–CH_2OH	3.0
Secondary alcohols	
CH_3–$CH(OH)$–CH_3	59.0
CH_3–CH_2–$CH(OH)$–CH_3	78.0
CH_2–CH=$CH(OH)CH_2$–CH_3	65.0
CH_3–$CH(OH)$–CH_2–$CH(OH)$–CH_3	2.6
Cyclic secondary alcohol	
Cyclohexanol	12.2
Aldehyde	
CH_3–CHO	7.8
Linear ketones	
CH_3–CO–CH_3	10.4
CH_3–CO–CH_2–CH_3	7.6
CH_3CO–CH_2–CH_2–CH_3	4.2
Cyclopropyl methyl ketone	0.5
Cyclic ketones	
Cyclopentanone	6.0
Cyclohexanone	6.2
2-Cyclohexanone	4.9
2-Methylcyclohexanone	6.0
3-Methylcyclohexanone	2.0
4-Methylcyclohexanone	0.5
Cycloheptanone	3.0
Cyclo-octanone	<0.1
4-Norbornanone	4.9

notable deflexion point at near 50°C was observed with both alcohols. The effect of temperature on butan-2-ol dehydrogenase activity was associated wtih an unusually high Q_{10} value of 2.9 in the 25–50°C temperature range.

The thermostability of *Ta. brockii* NADP-linked alcohol–aldehyde/ketone oxidoreductase is shown in Fig. 5. These experiments were conducted with dilute enzyme (16 μg/ml) in the presence of air and without the addition of a thiol-protecting agent. Enzyme activity was not influenced by heating at 65°C for 70 min. The enzyme displayed good stability towards heating at 86°C, but it was only moderately stable at 91°C and it denatured rapidly on boiling. Enzyme thermostability and activity was not altered by the addition of 2 mM-EDTA or by enzyme freezing and thawing.

General enzyme properties

The effect of pH on *Ta. brockii* alcohol–aldehyde/ketone oxidoreductase activity was examined in Tris/HCl buffers at 40°C. The pH optimum for acetaldehyde reduction was near 7.8. Enzyme activity at pH 7.0 and 9.0 was inhibited by 50%. The pH optimum for propan-2-ol oxidation was broad and occurred at pH 7.8–9.0. The secondary-alcohol dehydrogenase activity was inhibited by 30% at pH 7.0.

Enzyme activity was not significantly influenced (±5%) by the presence of EDTA (5 mM) or the following metal chlorides in the reaction mixture: $MgCl_2$ (10 mM), $CaCl_2$ (10 mM), $CuCl_2$ (1 mM) and $ZnCl_2$ (1 mM). *p*-Chloromerucibenzoate (0.05 mM), a thiol-blocking reagent, instantly inactivated the

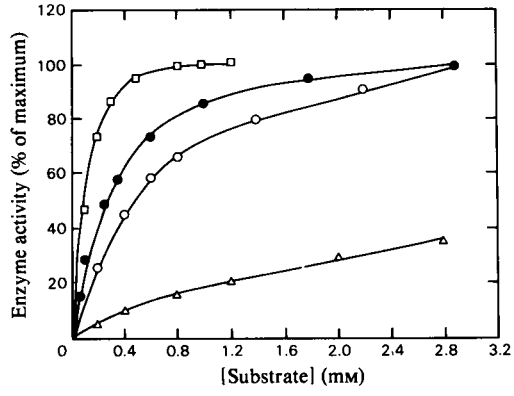

Fig. 3. *Dependence of Ta. brockii NADP-linked alcohol–aldehyde/ketone oxidoreductase on substrate concentration*

Reaction conditions were as follows: 40°C; 1 ml of reaction mixture that contained 0.1 M-Tris/HCl buffer, pH 7.8, 0.5 mM-$NADP^+$ or 0.2 mM-NADPH, the substrate and 2.5 μg of purified enzyme. For full experimental details see the text. Maximal activity represents (in μmol/min per mg of protein): ●, butan-2-ol, 78; □, butan-2-one, 7.6; △, ethanol, 3.2; ○, acetaldehyde, 7.8.

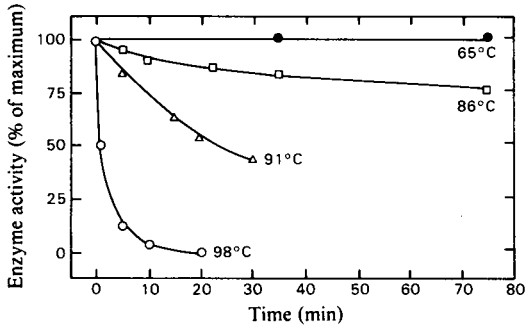

Fig. 4. *Thermal stability of Ta. brockii secondary alcohol dehydrogenase*

Purified alcohol dehydrogenase (16 μg of protein/ml of 25 mM-potassium phosphate buffer, pH 7.1) was heated for the time and temperature indicated for butan-2-ol dehydrogenase activity. For full experimental details see the text.

enzyme. Enzyme activity in the presence of *p*-chloromercuribenzoate was restored by the addition of 5 mM-dithiothreitol and $ZnCl_2$. In this case, $MgCl_2$ or $CaCl_2$ did not substitute for $ZnCl_2$ in restoring enzyme activity.

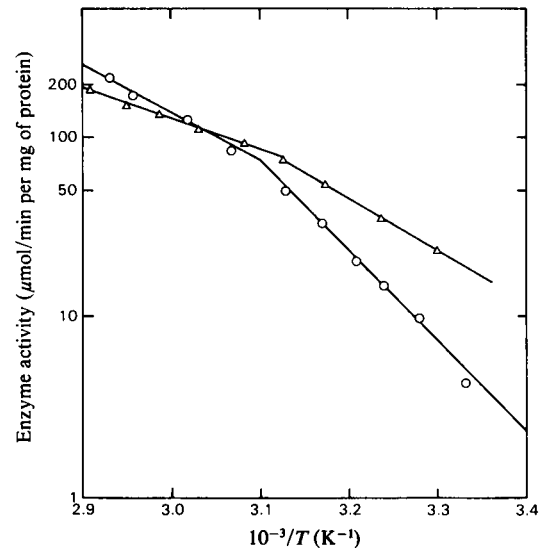

Fig. 5. *Temperature dependence of Ta. brockii secondary-alcohol dehydrogenase*

The reaction mixture contained (1 ml total volume) 50 mM-potassium phosphate buffer, pH 7.3, 0.5 mM-$NADP^+$, 150 mM-propan-2-ol (△) or butan-2-ol (○) and 0.5 μg of the purified enzyme; the temperature was as indicated. For full experimental details see the text.

Discussion

The NADP-linked alcohol–aldehyde/ketone oxidoreductase of *Ta. brockii* was easily purified to near homogeneity in five simple steps. The heat-treatment and affinity-chromatography steps are especially attractive for large-scale purification of the enzyme. A similar affinity-chromatography procedure has been used for the large-scale purification of horse liver alcohol dehydrogenase (Roy & Nishikawa, 1979). The results suggest that the NADP-linked alcohol–aldehyde/ketone oxidoreductase activity detected in crude extracts of *Ta. brockii* and *C. thermohydrosulfuricum* derives from a single enzyme that maintained a constant pattern of activity during purification. The ease of purification and high activity of the alcohol–aldehyde/ketone oxidoreductase in *Ta. brockii* or *C. thermohydrosulfuricum*, together with the enzyme's broad substrate specificity, reversibility and stability, all suggest that this enzyme may have industrial, synthetic and analytical uses not common to previously described alcohol dehydrogenases.

The general molecular properties of NADP-linked alcohol–aldehyde/ketone oxidoreductase from *Ta. brockii* are similar to those of described alcohol dehydrogenases. The subunit molecular weight of

40000 is very similar to that of yeast (Jörnvall, 1973) and horse liver (Jörnvall, 1970) alcohol dehydrogenases. Gel-filtration studies on this thermophilic enzyme suggest a tetrameric structure in the native state, which is also the case for yeast alcohol dehydrogenase (Jörnvall, 1970). The *Ta. brockii* enzyme was inactivated by *p*-chloromercuribenzoate, which suggests that it contains an essential thiol group, and this is in accord with known alcohol dehydrogenases (Bränden *et al.*, 1975; Patel *et al.*, 1979). The indication here that Zn^{2+} ions are involved in the enzyme active site are not conclusive and need further study. The metal ions in the active site of the *Ta. brockii* enzyme must be very tightly bound, because enzyme activity was not significantly decreased at 86°C in the presence of EDTA (an effective Zn^{+} chelator). In this respect the *Ta. brockii* enzyme appears more stable than other described alcohol dehydrogenases (Sund & Theorell, 1963; Bränden *et al.*, 1975; Patel *et al.*, 1979).

Large differences in enzyme activity towards substrate groups were found for *Ta. brockii* NADP-linked alcohol–aldehyde/ketone oxidoreductase. However, enzyme activities were similar towards a few representative compounds from each substrate group. The reaction rates for oxidation–reduction activities of *Ta. brockii* enzyme can be grouped as follows: highest activity with secondary alcohols, moderate activity with ketones and lower activity with primary alcohols.

These observed differences in substrate activity may be a reflection of changes in rate-limiting steps for each class of substrates. Detailed studies are required to elucidate the mechanism of this specificity.

The wide substrate range for *Ta. brockii* NADP-linked alcohol–aldehyde/ketone oxidoreductase although unique, bears some resemblance to NAD(P)-linked enzyme activities of alcohol-catabolizing micro-organisms. The NAD-linked alcohol dehydrogenases of methylotrophic bacteria (Bellion & Wu, 1978) recognized both primary and secondary alcohols; however, the reversibility of the reaction was not examined. An NADP-linked aldehyde dehydrogenase isolated from an ethanol-catabolizing *Gluconobacter* species recognized a wide range of aldehydes, but the enzyme was not unidirectional (Adachi *et al.*, 1980). Interestingly, the NAD-linked alcohol dehydrogenase of methanol-grown yeasts recognized only secondary alcohols (Patel *et al.*, 1979). Two distinct NAD-dependent alcohol dehydrogenases were detected in *Comamonas terrigena*, one specific for D-alkan-2-ols and primary alcohols and the other specific for L-alkan-2-ols and secondary alcohols (Barrett *et al.*, 1980). The substrate range and reversibility of *Ta. brockii* NADP-linked alcohol–aldehyde/ketone oxidoreductase appears well related to the suggested metabolic functions of the enzyme during growth (Lamed & Zeikus, 1980*b*; A. Ben-Bassat, R. J. Lamed & J. G. Zeikus, unpublished work). Most notably, *Ta. brockii* produced ethanol during glucose fermentation and catabolizes ethanol as sole energy source in co-culture with an H_2-consuming methanogen, and the addition of ketones to glucose-fermenting cells results in the formation of stoicheiometric amounts of the respective alcohols.

The thermal stability of *Ta. brockii* NADP-linked alcohol–aldehyde/ketone oxidoreductase is quite extraordinary. Alcohol dehydrogenases in general are relatively temperature-labile enzymes. Thus yeast alcohol dehydrogenase is somewhat unstable even at 25°C (Takamori *et al.*, 1967), and commercial use of horse liver alcohol dehydrogenase is not recommended above 30°C because 60% of activity is lost on heating at 43°C for 50 min (Jones & Beck, 1976). The dependence of *Ta. brockii* secondary-alcohol dehydrogenase activity on temperature was biphasic. However, the Q_{10} values observed were above 2.0 (below and above the 50°C break-point). The reason(s) for the observed biphasic Arrhenius plots for the *Ta. brockii* enzyme are not known, but they could be related to the differential effect of temperature on binding parameters in the enzyme–substrate–NADP(H) complex (Zuber, 1979). Also, the dependence of enzyme activity on temperature may indicate a conformational change of the enzyme at 50°C; this effect has been reported for some other thermophilic enzymes (Singelton & Amelunxen, 1973; Zuber, 1979). The thermostability of *Ta. brockii* NADP-linked alcohol–aldehyde/ketone oxidoreductase was comparable with that of malate dehydrogenase and isocitrate dehydrogenase purified from a different caldoactive bacterium, *Thermus flavus* (Saiki *et al.*, 1978). The temperature-dependence of *Ta. brockii* alcohol dehydrogenase is most notable when compared with that described for thermophile lactate dehydrogenases. Comparison of temperature effects on purified lactate dehydrogenases from thermophilic and mesophilic bacteria suggested that thermophilic enzymes had lower catalytic efficiencies and lower Q_{10} values than those in mesophilic species (Zuber, 1979). This does not appear to be the case for the first alcohol dehydrogenase characterized from caldoactive bacteria, and hence a detailed enzyme kinetic study comparing the *Ta. brockii* enzyme with that of a mesophilic anaerobic species would be of further interest to understanding function of enzymes from extremely thermophilic bacteria.

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References

Adachi, O., Matsushita, K., Shinagawa, E. & Ameyama, M. (1980) *Agric. Biol. Chem.* **44**, 155–164

Barrett, C., Dodgson, K., White, G. & Payne, W. (1980) *Biochem. J.* **187**, 703–709

Barth, G. & Kunkel, W. (1979) *Z. Allg. Mikrobiol.* **19**, 381–390

Bellion, E. & Wu, G. T. (1978) *J. Bacteriol.* **135**, 251–258

Ben-Bassat, A. & Zeikus, J. G. (1981) *Arch. Microbiol.* in the press

Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254

Bränden, C. I., Jörnvall, H., Eklund, H. & Furugren, A. (1975) *Enzymes 3rd Ed.* **11**, 103–190

DeMoss, R. D., Bard, R. C. & Gunsalus, I. C. (1951) *J. Bacteriol.* **62**, 499–511

Dutler, H., Coon, M. J., Kull, A., Vogel, H., Waldvogel, G. & Prelog, V. (1971) *Eur. J. Biochem.* **22**, 203–212

Hatanaka, A. O., Adachi, T., Chiyonobu, T. & Ameyama, M. (1971*a*) *Agric. Biol. Chem.* **35**, 1142–1143

Hatanaka, A. O., Adachi, O., Chiyonobu, T. & Ameyama, M. (1971*b*) *Agric. Biol. Chem.* **35**, 1304–1306

Hillmer, H. & Gottschalk, G. (1974) *Biochim. Biophys. Acta* **334**, 12–23

Hou, C. T., Patel, R. N., Laskin, A. I., Baranabe, N. & Marezak, I. (1979*a*) *Appl. Environ. Microbiol.* **38**, 135–142

Hou, C. T., Patel, R. N., Laskin, A. I., Baranabe, N. & Marezak, I. (1979*b*) *FEBS Lett.* **101**, 197–183

Hsu, E. J. & Ordal, Z. J. (1970) *J. Bacteriol.* **103**, 369–376

Jones, J. B. & Beck, J. F. (1976) in *Techniques of Chemistry* (Jones, J. B., Sih, C. J. & Perlman, D., eds.), vol. 10, pp. 107–402, John Wiley and Sons, New York

Jörnvall, H. (1970) *Eur. J. Biochem.* **13**, 565–576

Jörnvall, H. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2295–2298

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Lamed, R. & Zeikus, J. G. (1980*a*) *J. Bacteriol.* **141**, 1251–1257

Lamed, R. & Zeikus, J. G. (1980*b*) *J. Bacteriol.* **144**, 569–578

Niehaus, W., Frielle, T. & Kingsley, E. (1978) *J. Bacteriol.* **134**, 177–183

Patel, R., Hou, C. T., Laskin, A., Derelanko, P. & Felix, A. (1979) *Eur. J. Biochem.* **101**, 401–406

Roy, S. K. & Nishikawa, A. H. (1979) *Biotechnol. Bioeng.* **21**, 775–785

Ryan, L. D. & Vestling, C. S. (1974) *Arch. Biochem. Biophys.* **160**, 279–284

Saiki, T., Iijima, S., Tohda, K., Beppu, T. & Arima, K. (1978) in *Biochemistry of Thermophiles* (Friedmann, S. M., ed.), pp. 287–303, Academic Press, New York

Singelton, R. & Amelunxen, R. E. (1973) *Bacteriol. Rev.* **37**, 320–342

Singh, R. & Kunkee, R. K. (1976) *Appl. Environ. Microbiol.* **32**, 666–670

Sund, H. & Theorell, H. (1963) *Enzymes 2nd Ed.* **7**, 25–83

Takamori, S., Furuya, E., Suzuki, H. & Katagiri, M. (1967) *Nature (London)* **215**, 417–419

Zeikus, J. G. (1979) *Enzyme Microb. Technol.* **1**, 243–252

Zeikus, J. G. (1980) *Annu. Rev. Microbiol.* **34**, 423–456

Zeikus, J. G., Hegge, P. W. & Anderson, M. A. (1979) *Arch. Microbiol.* **122**, 41–48

Zeikus, J. G., Ben-Bassat, A. & Hegge, P. W. (1980) *J. Bacteriol.* **143**, 432–440

Zuber, H. (1979) in *Strategies of Microbial Life in Extreme Environments* (Shilo, M., ed.), pp. 393–415, Verlag Chemie, Weinheim and New York