NADP-dependent alcohol dehydrogenases in bacteria and yeast: purification and partial characterization of the enzymes from *Acinetobacter calcoaceticus* and *Saccharomyces cerevisiae*

Martin R. Wales† and Charles A. Fewson

Author for correspondence: Charles A. Fewson. Tel: +44 41 339 8855 ext. 5902. Fax: +44 41 330 4620.

Department of Biochemistry,
University of Glasgow,
Glasgow G12 8QQ, UK

An NADP-dependent constitutive alcohol dehydrogenase that can oxidize hexan-1-ol was detected in several Gram-positive and Gram-negative eubacteria and in two yeasts. The enzyme was purified to homogeneity from *Acinetobacter calcoaceticus* NCIB 8250 and from *Saccharomyces cerevisiae* D273-10B. The bacterial enzyme appears to be a tetramer of subunit M_r 40 300 and the yeast enzyme appears to be a monomer of subunit M_r 43 500. The N-terminal amino acid sequence of the bacterial enzyme has 34% identity with part of the sequence of a fermentative alcohol dehydrogenase from *Escherichia coli*. The pI value of the bacterial enzyme was 5.7 and the pH optimum was 10.2. Both the bacterial and yeast enzymes were shown to transfer the *pro-R* hydrogen to/from NADP(H). The substrate specificities of the two enzymes were similar to each other, both oxidizing primary alcohols and some diols, but not secondary alcohols. The maximum velocities of both enzymes were with pentan-1-ol as substrate and there was very low activity with ethanol; the maximum specificity constants were found with primary alcohols containing six to eight carbon atoms. Neither enzyme was significantly inhibited by metal-binding agents but some thiol-blocking compounds inhibited them. It appears that these two alcohol dehydrogenases, one prokaryotic and one eukaryotic, are structurally, kinetically and functionally different from members of the major known groups of alcohol dehydrogenases.

Keywords: *Acinetobacter calcoaceticus*, *Saccharomyces cerevisiae*, alcohol dehydrogenase

INTRODUCTION

A great many alcohol dehydrogenases have been purified and characterized; the vast majority of the enzymes studied have been from animals – mostly mammals – or from plants and yeasts (Jörnvall *et al.*, 1987). Much of the interest in these enzymes in recent years has revolved around comparisons of their primary structures. On this basis, alcohol dehydrogenases have been divided into three groups. (1) Those with subunit M_r values of around 40 000 (Jörnvall *et al.*, 1987). A great many enzymes are known in this group and they have been considered to be zinc-dependent although zinc has been unambiguously

identified in comparatively few of them. (2) The non-metalloenzymes with subunit M_r values of around 28 000 (Thatcher & Sawyer, 1980; Jörnvall *et al.*, 1981; Baker, 1990). (3) A group of enzymes, initially all thought to contain iron, with subunit M_r values of around 40 000. Metal-ion dependency does not appear to be a sufficient predictor of structural homology since different enzymes of group 3 have been identified as containing zinc or iron and in one case zinc and magnesium (Clark, 1992).

Many of these, predominantly eukaryotic, enzymes are induced under anaerobiosis and/or by alcohols (usually ethanol) and most of them are NAD-dependent. The range of alcohol dehydrogenases in prokaryotic organisms appears to be much greater than that in eukaryotes, but few of the prokaryotic enzymes other than a handful

† Present address: Celltech Research, 216 Bath Road, Slough SL1 4EN, UK.

of methanol and ethanol dehydrogenases have been studied in much detail (MacKintosh & Fewson, 1987; Wales & Fewson, 1991). We therefore decided to study a bacterial alcohol dehydrogenase which is apparently constitutive, i.e. produced during growth on non-alcohol carbon sources under aerobic conditions, which uses NADP rather than NAD as cofactor, and which has maximal activity with primary alcohols with chain lengths greater than that of ethanol. A few such enzymes have been previously identified. Apparently constitutive NADP-dependent alcohol dehydrogenase activities with relatively high K_m values for ethanol and lower K_m values for hexan-1-ol have been partially purified from *Mycobacterium tuberculosis* (DeBruyn *et al.*, 1981) and *Pseudomonas aeruginosa* (Tassin & Vandecasteele, 1972). Also, such an activity has been identified in a number of different strains of *Acinetobacter* including *A. calcoaceticus* NCIB 8250, 69V, EB104 and HO1-N (Taichert *et al.*, 1976; Fixter & Nagi, 1984; Asperger & Kleber, 1991; Fox *et al.*, 1992). An enzyme from *A. calcoaceticus* HO1-N was recently partially purified and some characterization achieved (Fox *et al.*, 1992); however, none of these enzymes has been purified to homogeneity or adequately characterized.

The present paper describes the identification of apparently constitutive NADP-dependent hexan-1-ol dehydrogenase activities in a wide variety of eubacteria, and the purification and partial characterization of the enzyme from a strain of *A. calcoaceticus*. During the course of this work it became apparent that a very similar enzyme was present in yeasts, and one of these eukaryotic enzymes, from *Saccharomyces cerevisiae*, was purified and characterized sufficiently to allow it to be compared with the prokaryotic enzyme. Some of these results have been published in a preliminary form (Wales & Fewson, 1991).

METHODS

Materials. All chemicals used were of the best quality available and most of them were obtained from the sources described by Allison *et al.* (1985) and MacKintosh & Fewson (1988) except for those listed below.

Acetaldehyde was from Aldrich, allyl alcohol was from Fisons Scientific Equipment, and *cis*-hex-3-en-1-ol and hex-5-en-1-ol were from Lancaster Synthesis. Matrex Gel Blue A and Red A were from Amicon. Phenyl-Sepharose, a prepacked Superose 6 column and ferritin were from Pharmacia. Pig-heart lactate dehydrogenase, rabbit-muscle pyruvate dehydrogenase, horse-spleen ferritin, horse-heart cytochrome *c*, NADP⁺ (disodium salt) and NADPH (disodium salt) were from Boehringer. *Saccharomyces cerevisiae* NAD-dependent alcohol dehydrogenase was from BDH. Materials used in the determination of stereospecificity of hydride transfer to/from NADP(H) were obtained from the sources described by Baker *et al.* (1992) except dihydrofolate and dihydrofolate reductase, which were from Sigma.

Growth of micro-organisms and preparation of extracts. Stock cultures of *Acinetobacter calcoaceticus* NCIB 8250 and *Escherichia coli* ML30 were maintained at 4 °C in a complex medium as described by Allison *et al.* (1985). Stock cultures of *Rhodotorula graminis* KGX 39 (donated by Genex Corporation, Gaithersburg, MD, USA) and *Bacillus subtilis* NCIMB 3610 were

maintained as lyophilized cultures in a filter-sterilized mixture of three parts heat-inactivated horse serum to one part 30% (w/v) glucose. Spores of *Streptomyces rimosus* 4018 (obtained from Miss L. Drynan of this Department) were stored in sterilized distilled water at -80 °C. *Rhodococcus rhodochrous* NCIMB 13259 was maintained at 4 °C on nutrient agar slopes. *Pseudomonas putida* NCIMB 9494 and *P. aeruginosa* NCIMB 10548 were maintained at 4 °C on agar slopes containing a defined medium as described by Murray *et al.* (1972). *Sacch. cerevisiae* D273-10B (Reid & Schatz, 1982) was maintained at 4 °C on peptone/yeast extract/galactose agar slopes.

All micro-organisms were grown into stationary phase before being harvested, washed and then either used immediately or stored at -20 °C.

For small-scale growth of *A. calcoaceticus* NCIB 8250, an inoculum (0.1 ml) from a stock culture was transferred to 50 ml of complex medium (Allison *et al.*, 1985) in a 250 ml flask and shaken at 200 r.p.m. for 16 h at 30 °C. Eight millilitres of this culture was then transferred to 400 ml of the same medium in a 2 litre flask and shaken for 16 h at 30 °C before harvesting. Large quantities of *A. calcoaceticus* NCIB 8250 were grown in a complex medium in a 10 litre fermenter fitted with a 20 litre reservoir using essentially the method of Allison *et al.* (1985). The inoculum used was a 400 ml culture prepared as described above. The fermenter (Braun Biostat V; F. T. Scientific Instruments) was operated at 30 °C with an aeration rate of 4 litres of sterile air min⁻¹ and stirring at setting 2.5 (approx. 250 r.p.m.). After growth for 20 h, 9.5 litres culture was removed for harvesting and 9.5 litres fresh medium transferred to the fermenter from the reservoir. After a further 20 h growth, 9.5 litres culture was removed for harvesting and the remaining medium transferred from the reservoir. The culture was grown for a final 20 h, then all the culture was removed and harvested.

Stock culture (0.1 ml) of *E. coli* ML30 was transferred to 50 ml glycerol/salts medium (Holms & Bennett, 1971) and shaken at 200 r.p.m. for 16 h at 37 °C. One millilitre of this culture was then transferred to 250 ml of the same medium and shaken at 200 r.p.m. for 24 h at 37 °C. Four millilitres of this culture was then transferred to 1 litre of the same medium and stirred (using a magnetic stirrer) with aeration (500 ml air min⁻¹) for 24 h at 37 °C.

A loopful of a culture of *P. putida* NCIMB 9494 or *P. aeruginosa* NCIMB 10548 was transferred to 50 ml of complex medium, containing DL-lactate, in a 250 ml flask and shaken at 200 r.p.m. for 16 h at 30 °C. Eight millilitres of this culture was then transferred to 400 ml of the same medium in a 2 litre flask and shaken for 24 h at 30 °C before harvesting.

B. subtilis NCIMB 3610 was plated out on a sterile nutrient agar plate which was then incubated for 24 h at 30 °C. One colony was used to inoculate 10 ml sterile nutrient broth, which was then incubated for 24 h at 30 °C. Five millilitres of this culture was transferred to 500 ml sterile nutrient broth (in a 2 litre flask) and shaken for 16 h at 30 °C.

A loopful of a culture of *Rhodococcus rhodochrous* was transferred to 500 ml sterile nutrient broth and shaken at 200 r.p.m. for 72 h at 30 °C.

Spores of *Strep. rimosus* 4018 suspended in distilled water (0.1 ml) were transferred to 1 litre yeast extract/malt extract medium (Hopwood *et al.*, 1985) in a 2 litre flask and shaken at 200 r.p.m. for 72 h at 30 °C. Cells were harvested and 1 g of pellet was resuspended in 100 ml modified minimal medium (Hobbs *et al.*, 1989) and shaken at 200 r.p.m. for 24 h at 30 °C.

For small-scale growth of *Sacch. cerevisiae* D273-10B, a loopful of culture was transferred to 50 ml peptone/yeast extract/

galactose medium (Reid & Schatz, 1982) in a 250 ml flask and shaken at 200 r.p.m. for 20 h at 30 °C. Four millilitres of this culture was then transferred to 400 ml of the same medium in a 2 litre flask and shaken for 20 h at 30 °C before harvesting. Larger amounts of *Sacch. cerevisiae* D273-10B were obtained by inoculating 80 ml of culture prepared as described above into 4 litre peptone/yeast extract/galactose medium in a 10 litre flask. The culture was grown for 20 h at 30 °C with stirring and aeration (500 ml sterile air min⁻¹).

Rhodotorula graminis KGX 39 was inoculated into 50 ml nutrient broth in a 250 ml flask and shaken at 250 r.p.m. for 24 h at 30 °C. Eight millilitres of this culture was then transferred to 400 ml nutrient broth in a 2 litre flask and shaken for 24 h at 30 °C before harvesting.

In all cases the micro-organisms were suspended in 50 mM Tris/HCl containing 2 mM DTT, pH 8.0, before disruption. Ultrasonic disruption was carried out using a Lucas-Dawes soniprobe. An ice/water slurry was used to cool the soniprobe and the cell suspension. *E. coli*, *P. putida* and *P. aeruginosa* were suspended in buffer so that the equivalent OD₅₀₀ was 50, and broken by 4 × 30 s periods of exposure to 90 W, alternating with 3 × 30 s cooling periods. *Strep. rimosus* (1 g wet wt) was suspended in buffer (6 ml) and broken by 10 × 15 s exposure to 45 W, alternating with 10 × 15 s cooling periods. *B. subtilis* was suspended in buffer so that the equivalent OD₅₀₀ was 50, and broken by 5 × 30 s periods of exposure to 90 W, alternating with 4 × 30 s cooling periods. In all cases the homogenates were centrifuged at top speed in an MSE Microcentaur centrifuge for 10 min at 4 °C and the supernatants were used immediately or stored at -20 °C. For disruption of *A. calcoaceticus*, *Rhodotorula graminis*, *Rhodococcus rhodochrous* and *Sacch. cerevisiae* in the French pressure cell, the bacteria and the yeasts were suspended in three vols 50 mM Tris/HCl containing 2 mM DTT, pH 8.0, and broken by three passages through a pressure cell (FA-073 or FA-003 models; American Instrument Company), which had been pre-cooled in ice, at a pressure of 98 MPa. The homogenates were centrifuged at 100 000 g for 1 h at 4 °C and the supernatants used immediately or stored at -20 °C.

Measurement of enzyme activities. All enzyme assays were done in duplicate at 27 °C. One unit of enzyme activity is defined as 1 μmol of substrate converted per min.

(i) NADP-dependent alcohol dehydrogenase of *A. calcoaceticus*. The oxidation of hexan-1-ol was measured in an assay mixture containing: 800 μl 100 mM glycine/NaOH buffer, pH 10.2 (assay concentration 80 mM), containing 25 mM hexan-1-ol (assay concentration 20 mM); 100 μl 10 mM NADP⁺ (assay concentration 1 mM); enzyme; and water to 1 ml. The rate of NADP⁺ reduction was measured spectrophotometrically at 340 nm.

Alcohols with > 10 carbons were 'solubilized' in buffer using a method adapted from Tassin & Vandecasteele (1971). Alcohols were added to 0.111 M glycine/HCl buffer containing 0.7 mg BSA ml⁻¹ to give a final concentration 10/9 times that desired in the buffer when made up to volume (i.e. 0.625 mM or less). The glycine/alcohol solution was sonicated between five and ten times at 120 W, the pH adjusted to 10.2 with NaOH, and the buffer made up to volume with a solution of 0.7 mg BSA ml⁻¹. The enzyme was then assayed as described above except that the sonicated buffer/alcohol solution was used in the reaction mixture. In all cases, the final concentration of alcohol in the buffer was 0.5 mM or less.

The reduction of butanal was measured in an assay mixture containing: 2.4 ml 100 mM bisTris/HCl buffer, pH 6.8 (assay concentration 80 mM); 0.3 ml 300 mM butanal (assay con-

centration 30 mM); 0.2 ml 300 μM NADPH (assay concentration 20 μM); enzyme; and water to 3 ml. The rate of oxidation of NADPH was monitored at 340 nm (excitation)/460 nm (emission) in a Perkin-Elmer LS-5 luminescence spectrophotometer with an RA 100A recorder using a 3 ml fluorimetric cuvette (Hughes and Hughes) with a 1 cm light path. Reaction velocities were calculated initially in arbitrary fluorescence units min⁻¹ (mg protein)⁻¹, and converted into enzyme units (mg protein)⁻¹ using a calibration curve of NADPH against arbitrary fluorescence units prepared each time the fluorimeter was used.

(ii) NADP-dependent alcohol dehydrogenase of *Sacch. cerevisiae* D273-10B. The enzyme was assayed in terms of alcohol oxidation in the same way as described above except that the pH value of the buffer was 10.7. Aldehyde reduction was assayed as described above.

(iii) NAD-dependent alcohol dehydrogenase from *Sacch. cerevisiae*. This enzyme was assayed as described for NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 except that 1 mM NAD⁺ and 250 mM ethanol were used instead of 1 mM NADP⁺ and 25 mM hexan-1-ol, respectively.

Determination of kinetic constants. Steady-state kinetic data were analysed by the Direct-Linear method (Eisenthal & Cornish-Bowden, 1974) using the Enzpack computer program (Williams, 1985). Apparent equilibrium constants were determined essentially as described by Baker & Fewson (1989). Results are given as the mean values ± standard deviations, with the number of experiments used to obtain these values shown in parentheses.

Purification of NADP-dependent alcohol dehydrogenase from *A. calcoaceticus* NCIB 8250. All steps were carried out at 4 °C. The extract was prepared with the French pressure cell as described above and dialysed against 50 mM Tris/HCl/2 mM DTT, pH 8.0.

Step 1: ion-exchange chromatography on DEAE-Sephacel. Dialysed extract (approx. 35 ml) was applied at 35 ml h⁻¹ to a column of DEAE-Sephacel (2.6 × 14 cm) pre-equilibrated with 50 mM Tris/HCl/2 mM DTT, pH 8.0. Once loaded, and after allowing 15 min for protein binding, the column was washed with at least five column volumes of the same buffer at 35 ml h⁻¹ until the A₂₈₀ of the effluent was < 0.05. Then, elution was carried out with a linear 0.03 M KCl gradient in 50 mM Tris/HCl/2 mM DTT, pH 8.0 (500 ml), at a flow rate of 35 ml h⁻¹. Fractions of 5.83 ml were collected and those with peak activity were pooled and concentrated to < 15 ml in dialysis tubing using CM-cellulose. The dialysis tubing was cleaned of CM-cellulose every hour.

Step 2: gel-filtration chromatography through Sephacryl S300HR. The concentrated DEAE-Sephacel pool was applied at 25 ml h⁻¹ to a column of Sephacryl S300HR (2 cm × 84 cm) and then the enzyme was eluted with 50 mM Tris/HCl/2 mM DTT, pH 8.0 at 25 ml h⁻¹. Fractions of 6.25 ml were collected and those with peak activity were pooled.

Step 3: hydrophobic-interaction chromatography on phenyl-Sepharose CL-4B. (NH₄)₂SO₄ was added to the Sephacryl S300HR pool to a final concentration of 5% (w/v), then it was applied at 27 ml h⁻¹ to a column of phenyl-Sepharose (2.6 cm × 10 cm). After allowing 15 min for the protein to bind, the column was washed at 27 ml h⁻¹ with at least five column volumes of 50 mM Tris/HCl/2 mM DTT/5% (w/v) (NH₄)₂SO₄, pH 8.0, until the A₂₈₀ of the effluent was < 0.05. The enzyme was eluted with 40% (v/v) ethanediol in 50 mM Tris/HCl/2 mM DTT, pH 8.0. Fractions of 6.75 ml were collected and those with peak activity were pooled.

Step 4: dye-affinity chromatography through Matrex Gel Blue A. The phenyl-Sepharose pool was applied at 25 ml h⁻¹ to a column of Matrex Gel Blue A (2.6 cm × 6 cm). After allowing 15 min for the protein to bind, the column was washed at 25 ml h⁻¹ with at least five column volumes of 50 mM Tris/HCl/2 mM DTT, pH 8.5, until the A_{280} of the effluent was < 0.02. The enzyme was eluted with 50 mM Tris/HCl/2 mM DTT/1 mM NADP⁺/5 mM hexan-1-ol, pH 8.5 and collected over ice in a reservoir.

Step 5: concentration of Matrex Gel Blue A eluate by ion-exchange on DEAE-Sephacel. As the eluate from the Matrex Gel Blue A column was collected in the reservoir, it was immediately loaded onto a column of DEAE-Sephacel (1 cm × 4 cm) at 10 ml h⁻¹. Once loaded, the column was washed with at least two column volumes of 50 mM Tris/HCl/2 mM DTT, pH 8.0, before elution with 50 mM Tris/HCl/2 mM DTT/0.3 M KCl, pH 8.0. Fractions of 1 ml were collected and those with peak activity were pooled.

The concentrated pool of pure enzyme was exhaustively dialysed against 50 mM Tris/HCl/2 mM DTT, pH 8.0, before being stored frozen at -20 °C.

Purification of NADP-dependent alcohol dehydrogenase from *Sacch. cerevisiae* D273-10. The enzyme was purified as described for the enzyme from *A. calcoaceticus* NCIB 8250 except that: (i) The preparation of extract, initial dialysis, first ion-exchange, gel filtration and hydrophobic-interaction chromatographic steps were carried out in buffers containing 1 mM benzimidazole and 1 mM EDTA. (ii) Elution from the ion-exchange column was carried out with a linear 0–1.0 M KCl gradient. (iii) Pooled active fractions from the ion-exchange column were concentrated by precipitation with (NH₄)₂SO₄ to a final saturation of 75%. (iv) The column of Sephacryl S300HR was 94 cm long. (v) Sample was applied to the phenyl-Sepharose column in 50 mM Tris/HCl/2 mM DTT only [i.e. in the absence of (NH₄)₂SO₄]. (vi) The dye-affinity chromatography step was carried out on Matrex Gel Red A (not Blue A) as follows. MgCl₂ (100 mM) was added to the phenyl-Sepharose pool to give a final concentration of 5 mM. The pool was applied at 20 ml h⁻¹ to a column of Matrex Gel Red A (2.6 × 7.5 cm) and the column was washed with at least four column volumes of 50 mM Tris/HCl/2 mM DTT/50 mM MgCl₂, pH 8.5. Enzyme was eluted with 50 mM Tris/HCl/2 mM DTT/5 mM MgCl₂/0.2 mM NADP⁺, pH 8.5. Fractions of 5 ml were collected and those with peak activity were pooled. No further concentration step was necessary. Pure enzyme was dialysed and stored as described for the enzyme from *A. calcoaceticus*.

Electrophoresis. SDS-PAGE was carried out according to the method of Laemmli (1970) using 2–100 µg protein per track. Slab gels (19.0 cm × 9.5 cm × 0.15 cm) were stained after electrophoresis using Coomassie Brilliant Blue G250 as described by MacKintosh & Fewson (1988). Dye-stained gels were scanned using an LKB 2202 Ultrosan laser densitometer.

Determination of M_r , pI and K_{eq} values, protein concentrations and conductivities. These were determined essentially as described by Baker & Fewson (1989).

Protein sequencing. The N-terminus of the NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 was sequenced by Dr J. N. Keen at the SERC Protein Sequencing Unit, Department of Biochemistry and Molecular Biology, University of Leeds, UK, on two separate occasions. Run 1 was carried out using an Applied Biosystems 477-A liquid-phase sequencer equipped with on-line phenylthiohydantoin (PTH) analysis. Run 2 was carried out by solid-phase sequencing as

described by Findlay *et al.* (1989). This involves automated solid-phase Edman degradation with PTH-amino acids being identified off-line by reverse-phase (C18) microbore HPLC.

Stereospecificity of hydride transfer. The method for the synthesis of tritiated (4S)-[4-³H]NADPH is based on the fact that glucose-6-phosphate dehydrogenase of *Leuconostoc mesenteroides* specifically transfers a hydride ion (H⁻, or ³H⁻ in the case of tritium) from glucose 6-phosphate to the *si*-face of the dihydronicotinamide ring of NAD⁺ or NADP⁺ (Arnold *et al.*, 1976). [³H]Glucose 6-phosphate was produced by phosphorylation of [1-³H]glucose by ATP using *Sacch. cerevisiae* hexokinase. The linked assay was carried out in a mixture (500 µl) containing 5 mM potassium phosphate buffer, 60.5 µM [1-³H]glucose (specific activity 6 Ci mmol⁻¹; 222 GBq mmol⁻¹), 500 µM ATP, 7.4 units hexokinase, 60.5 µM NADP⁺ and 53.3 units of glucose-6-phosphate dehydrogenase. The rate of NADP⁺ reduction was followed at 340 nm and the reaction was assumed to be at equilibrium when there was no further change in A_{340} . Protein was then removed from the mixture by ultrafiltration using a Centricon-10 microconcentrator. The (4S)-[4-³H]NADPH was used immediately.

The method for the synthesis of tritiated (4R)-[4-³H]NADPH is based on the fact that dihydrofolate reductase specifically removes the *pro*-R-hydrogen from NADPH (Blakely *et al.*, 1963). The filtered reaction mixture containing (4S)-[4-³H]NADPH was incubated with 90 mM dihydrofolate and 0.03 units dihydrofolate reductase. The rate of NADPH oxidation was followed by measuring the change in A_{340} and the protein removed. Then the tritiated NADP⁺ was purified by HPLC using a Machery & Nagel Nucleosil 7-C18 reverse-phase column (25 cm × 4.6 mm) with pre-column (4 cm × 4.6 mm) run isocratically with 2% (v/v) methanol/0.1% (v/v) trifluoroacetic acid to elute NADP⁺. Fractions containing NADP⁺ were collected and lyophilized.

Tritiated (4R)-[4-³H]NADPH was produced in a mixture (500 µl) containing 10 mM potassium phosphate, pH 8.0, 8.8 µM [³H]NADP⁺, 50 µM glucose 6-phosphate and 27 units glucose-6-phosphate dehydrogenase. The reaction was followed at 340 nm and the protein removed. The resulting (4R)-[4-³H]NADPH was used immediately.

The incubation of tritiated NADPH with NADP-dependent alcohol dehydrogenase was carried out in the same way on NADP-dependent alcohol dehydrogenases from both *A. calcoaceticus* NCIB 8250 and *Sacch. cerevisiae* D273-10B. To a reaction mixture (1 ml) containing 10 mM potassium phosphate buffer, pH 7.0, 25 mM butan-1-ol and NADPH (assay concentration 20 µM) was added either (4R)-[4-³H]NADPH (assay concentration 0.7 µM) or (4S)-[4-³H]NADPH (assay concentration 0.5 µM). The reaction was initiated by addition of NADP-dependent alcohol dehydrogenase (3 µg) and followed by measuring the change in A_{340} . When the reaction had reached equilibrium, protein was removed. NADP⁺ and butan-1-ol were separated by HPLC. Samples (< 500 µl) were injected onto a Machery & Nagel Nucleosil 7-C18 reverse-phase HPLC column (25 cm × 4.6 mm) with pre-column (4 cm × 4.6 mm) pre-equilibrated with 0.1% (v/v) trifluoroacetic acid. NADP⁺ was eluted isocratically in 2% (v/v) methanol/0.1% (v/v) trifluoroacetic acid over 15 min, then butan-1-ol was eluted in 100% (v/v) methanol in 0.1% (v/v) trifluoroacetic acid over 20 min; in both cases solutions were pumped through the column at 1 ml min⁻¹ and fractions were collected at 1 min intervals and counted for incorporation of ³H. The effluent was monitored between 190 nm and 370 nm, NADP⁺ being identified by its distinctive absorbance pattern. Butan-1-ol was identified by its co-elution with unlabelled butan-1-ol on a Perkin-Elmer 8700 series gas chromatograph

Table 1. NADP-dependent hexan-1-ol dehydrogenase activities identified in micro-organisms

The results were obtained in the present work and by Tassin & Vandecasteele (1972) [1], DeBruyn *et al.* (1981) [2] and Fox *et al.* (1992) [3]. ND, Not determined.

Organism	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]	K _{m(app)} (hexan-1-ol) (mM)
<i>Acinetobacter calcoaceticus</i> NCIB 8250	104	1.16
<i>Acinetobacter calcoaceticus</i> HO1-N [3]	290	0.29
<i>Escherichia coli</i> ML30	11.0	0.88
<i>Pseudomonas putida</i> NCIMB 9494	5.4	0.46
<i>Pseudomonas aeruginosa</i> NCIMB 10548	7.7	1.11
<i>Pseudomonas aeruginosa</i> 196A [1]	57.8 (ADH1)	0.79
	10.2 (ADH2)	1.20
<i>Bacillus subtilis</i> NCIMB 3610	2.8	7.50
<i>Rhodococcus rhodochrous</i> NCIMB 13259	6.6	2.86
<i>Streptomyces rimosus</i> 4018	3.6	12.1
<i>Mycobacterium tuberculosis</i> var. <i>bovis</i> [2]	43.0	ND
<i>Saccharomyces cerevisiae</i> D273-10B	12.7	3.35
<i>Rhodotorula graminis</i> KGX 39	74.0	2.16

with a Chromosorb (Perkin-Elmer) 104 column at 175 °C. Radioactivity (³H) was measured using an LKB model 1209 Rackbeta liquid-scintillation counter. Samples of up to 500 µl were added to 5 ml of Ecoscint A (National Diagnostics) and mixed thoroughly.

RESULTS

Occurrence of NADP-dependent hexan-1-ol dehydrogenase activities in micro-organisms

In an attempt to identify any non-fermentative constitutive NADP-dependent alcohol dehydrogenases, various micro-organisms were grown aerobically in batch culture to stationary phase on growth media with non-alcohol carbon sources. Organisms grown in this way were assayed for soluble NADP-dependent hexan-1-ol dehydrogenase activity using the method described for the prokaryotic enzyme in Methods, and the approximate K_{m(app)} value of any such activity found was determined (Table 1). Such an activity was found in all of the Gram-negative eubacteria, Gram-positive eubacteria and yeasts tested. In all cases, NADP-dependent ethanol dehydrogenase activity, assayed as described for the *A. calcoaceticus* enzyme except that 0.5 M ethanol was included in the reaction mixture instead of hexan-1-ol, was less than 20% of the NADP-dependent hexan-1-ol dehydrogenase activity. No such activity was identified in the halophilic archaeobacterium, *Haloferax mediterranei*.

Purification of the NADP-dependent alcohol dehydrogenases from *A. calcoaceticus* and *Sacch. cerevisiae*

Typical purifications of NADP-dependent alcohol dehydrogenases from *A. calcoaceticus* NCIB 8250 and *S. cerevisiae* D273-10B are shown in Table 2. Dialysis of crude

extract prior to ion-exchange chromatography was necessary in both purifications in order to ensure consistent binding of the enzyme to the first column. Both enzymes eluted at similar salt concentrations from the ion-exchange columns: the prokaryotic enzyme at 100 mM KCl and the eukaryotic enzyme at 60 mM KCl. Not surprisingly, in view of their different quaternary structures (see below), the enzymes behaved very differently on gel filtration. Both enzymes seem quite hydrophobic, binding to phenyl-Sepharose in the presence of little or no (NH₄)₂SO₄ and eluting rapidly in the presence of buffer containing 40% (v/v) ethanediol. Different dye-affinity columns were used to purify the different enzymes to homogeneity although both enzymes bound to each of the dyes under similar conditions. However, the enzymes showed different elution patterns from the respective dyes. The bacterial enzyme eluted in a large volume, necessitating an immediate concentration step, whereas the yeast enzyme eluted in a much smaller volume.

The enzymes were purified to homogeneity as determined by SDS-PAGE and scanning of the resulting gels. Both purifications were reproducible. The purification of the prokaryotic enzyme resulted in 100–400 µg of enzyme with a specific activity of 58 ± 5 units (mg protein)⁻¹ among ten independent preparations. The eukaryotic enzyme purification resulted in 300–340 µg of enzyme with specific activities of 34.0 and 30.8 enzyme units (mg protein)⁻¹ in two independent preparations. Both purifications gave 10–25% recovery and took about a week to complete.

Relative molecular mass

The native M_r of the prokaryotic enzyme, estimated by gel-filtration chromatography through a calibrated FPLC Superose 6 column, was 158 500 (151 500, 165 500)

Table 2. Purification of NADP-dependent alcohol dehydrogenases

Full details of the purification are given in Methods.

	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity [units (mg protein) ⁻¹]	Yield (%)	Purification (-fold)
(a) From <i>A. calcoaceticus</i> NCIB 8250						
Dialysed extract	45.5	141	1442	0.10	100	1
DEAE-Sephacel	56.0	139	86.8	1.61	99	16
CM-Cellulose	11.8	129	90.7	1.42	92	14
Sephacryl S300HR	63.0	70	19.5	3.59	50	36
Phenyl-Sephacel	20.0	68	4.8	14.2	48	142
Blue A/DEAE-Sephacel	4.7	28	0.47	59.6	20	596
Dialysis	6.0	24	0.42	57.1	17	571
(b) From <i>Sacch. cerevisiae</i> D273-10B						
Dialysed extract	124	50.6	6200	0.008	70	0.7
DEAE-Sephacel	76	49.9	449	0.111	68	9.0
(NH ₄) ₂ SO ₄ precipitation	11.5	38.5	381	0.101	53	8.4
Sephacryl S300HR	46	31.8	59.8	0.532	44	44
Phenyl-Sephacel	39	16.6	6.63	2.50	23	208
Red A	34	18.9	0.44	43.0	26	3763
Dialysis	40	13.1	0.34	38.5	15	3369

1 10 20 30 38
 AADH X N N T **F H A Y A A L E A G A A L V P Y Q F D A G - E L Q X H Q V E V X V E Y**
 EADH V D K I **F R A - A A L A A A D A R I P L A K M A V A E S G M G I V E D K V I K**
 31 40 50 60 68

Fig. 1. N-terminal amino acid sequence of the NADP-dependent alcohol dehydrogenase from *A. calcoaceticus* NCIB 8250. The N-terminal amino acid sequence (AADH) was determined for two separate samples of enzyme containing approx. 12 pmol and 35 pmol of protein (see Methods). The N-terminal amino acid sequence of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 is compared with that of the fermentative alcohol dehydrogenase of *E. coli* (EADH; Goodlove *et al.*, 1989). Identical residues are shown in bold type. The residue number (with reference to the N-terminus) is shown above (for AADH) or below (for EADH); X, unknown residue; -, gap inserted.

whereas that of the eukaryotic enzyme was 46 200 (47 800, 45 700). The subunit M_r of the prokaryotic enzyme, estimated using calibrated SDS-PAGE gels, was $40\,300 \pm 2000$ ($n = 10$ samples of enzyme) whereas that of the eukaryotic enzyme was $43\,500 \pm 700$ ($n = 5$ samples of enzyme). From a comparison of the subunit and native M_r values, the prokaryotic enzyme appears to be a tetramer and the eukaryotic enzyme a monomer.

Absorption spectrum of the prokaryotic enzyme

The absorption spectrum of NADP-dependent alcohol dehydrogenase ($60\ \mu\text{g ml}^{-1}$) from *A. calcoaceticus* had a maximum at 280 nm; there was very little absorbance between 300 and 340 nm and none above 340 nm, indicating that, unlike some alcohol dehydrogenases, the

enzyme is not associated with bound cytochrome, haem, PQQ or flavin as prosthetic group.

N-terminal amino acid sequence of the prokaryotic enzyme

The sequence of the first 38 amino acids of NADP-dependent alcohol dehydrogenase from *A. calcoaceticus* was determined (PIR accession number E37334) and then compared with other protein sequences. The only other alcohol dehydrogenase, or related enzyme, to which any significant homology was observed was a fermentative alcohol dehydrogenase from *E. coli* (see Fig. 1 and Discussion).

Attempts at determining the N-terminal amino acid

sequence of the eukaryotic enzyme were unsuccessful, perhaps because it is blocked.

Effects of pH on enzyme activities

The optimal pH for alcohol oxidation by the prokaryotic enzyme was pH 10.2 and that for the eukaryotic enzyme was pH 10.7. The pH optimum of aldehyde reduction by both enzymes lies at around pH 6.8, although the reaction rate was high (> 95% of maximal rate) over a broad range of pH (pH 6.4–7.15).

The isoelectric point of the enzyme from *A. calcoaceticus* was at pH 5.7 as judged by chromatofocussing on an FPLC Mono P column.

Cofactor specificity and stereospecificity of hydride transfer

Neither enzyme detectably (< 1%) reduced NAD⁺ when assayed with 1 mM NAD⁺ instead of 1 mM NADP⁺ in the reaction mixture.

The stereospecificity of hydride transfer was determined by incubating the enzymes in the presence of (4*S*)-[4-³H]NADPH or (4*R*)-[4-³H]NADPH and butanal. The criteria used to determine the stereospecificity were as follows. If incubating the enzyme with (4*S*)-[4-³H]NADPH and butanal resulted in most of the radioactivity being identified in butan-1-ol the enzyme would have transferred the ³H-labelled *pro-S* hydride. However, if the radioactivity was found in NADP⁺ the enzyme would have transferred the unlabelled *pro-R* hydride.

Similarly, if incubating the enzyme with (4*R*)-[4-³H]NADPH and butanal resulted in most of the radioactivity being identified in butan-1-ol, the enzyme would have transferred the ³H-labelled *pro-R* hydride. However, if the radioactivity was identified in NADP⁺ then the enzyme would have transferred the unlabelled *pro-S* hydride.

When the prokaryotic enzyme was incubated with butanal and (4*S*)-[4-³H]NADPH the ratio of counts incorporated into the resulting NADP⁺ and butan-1-ol was 92.5%:7.5%, respectively, i.e. the prokaryotic enzyme was stereospecific for the *pro-R* hydride of NADPH. This was confirmed by incubation of the enzyme with butanal and (4*R*)-[4-³H]NADPH, the ratio of counts incorporated into the resulting NADP⁺ and butan-1-ol being 22.7%:77.3%, respectively.

Similarly, when the eukaryotic enzyme was incubated with butanal and (4*S*)-[4-³H]NADPH the ratio of counts incorporated into the resulting NADP⁺ and butanal was 92.5%:7.5%, respectively, i.e. this enzyme also was stereospecific for the *pro-R* hydride of NADPH. This was confirmed by incubation of the enzyme with butanal and (4*R*)-[4-³H]NADPH, the ratio of counts incorporated into the resulting NADP⁺ and butan-1-ol being 30.0%:70.0%, respectively.

Effects of metal-binding agents, salts, metal ions and common metabolites on the activities of the enzymes

Pre-incubation of the prokaryotic enzyme (5 µg ml⁻¹) with EDTA (100 mM), sodium azide (100 mM), pyrazole (50 mM), 2,2'-bipyridyl (20 mM) or 8-hydroxyquinoline [10 mM, in 10% (v/v) acetone] at 27 °C for 20 min had no significant effect on the activity when the enzyme was subsequently assayed in the presence of the potential inhibitor at the same concentration as that used in the pre-incubation. Incubation of the eukaryotic enzyme (5 µg ml⁻¹) at 27 °C for 20 min with pyrazole (50 mM) or 2,2'-bipyridyl (20 mM) had no significant effect on enzyme activity. However, incubation of this enzyme with 10 mM EDTA or 10 mM sodium azide did result in 20 and 40% inhibition, respectively.

No significant effect (i.e. less than 10% variation in enzyme activity) was observed when the prokaryotic enzyme (5 µg ml⁻¹) was pre-incubated at 27 °C for 5 min with 1 mM concentrations of each of the following salts and common metabolites and then assayed for activity in the presence of the salt or metabolite at the same concentration as that used in the pre-incubation: NaCl; KCl; (NH₄)₂SO₄; ZnCl₂; CuCl₂; MgSO₄; MgCl₂; MnCl₂; FeSO₄; FeCl₃; ATP-Mg²⁺; ADP-Mg²⁺; acetyl-CoA; succinate.

Effects of thiol-blocking agents

Pre-incubation of the prokaryotic enzyme (5 µg ml⁻¹) at 27 °C for 20 min with the thiol-blocking agents iodoacetate (10 mM), iodoacetamide (10 mM) or *N*-ethylmaleimide (10 mM) had no effect on activity when the enzyme was subsequently assayed in the presence of the potential inhibitor at the same concentration as that used in the pre-incubation. However, incubation of the enzyme with 10 µM 4-chloromercuribenzoate completely inhibited the enzyme, but no protection from this inhibition was observed in the presence of hexan-1-ol (5 mM) and/or NADP⁺ (1 mM).

Incubation of the eukaryotic enzyme (5 µg ml⁻¹) at 27 °C with iodoacetate (10 mM) had no effect on enzyme activity. However, incubation of the enzyme with iodoacetamide (1 mM), *N*-ethylmaleimide (1 mM) or 4-chloromercuribenzoate (10 µM) completely inhibited the enzyme. No protection of the inhibition resulting from incubation with 10 mM iodoacetamide or 10 mM *N*-ethylmaleimide was observed in the presence of hexan-1-ol (10 mM) or NADP⁺ (1 mM). However, some protection of the partial inhibition resulting from 1 µM 4-chloromercuribenzoate occurred in the presence of 1 mM NADP⁺, and almost complete protection occurred in the presence of 10 mM hexan-1-ol.

Substrate specificity and kinetic coefficients

The prokaryotic enzyme. The NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 is specific for primary aliphatic alcohols (Table 3). There

Table 3. Relative activities of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 with various alcohols as substrates

The enzyme was assayed as described in Methods except that the substrates listed below were used in place of hexan-1-ol; all substrates were at 50 mM except for *cis*-hex-3-en-1-ol, which was at 10 mM. The relative activity of the enzyme towards each substrate is given with respect to the activity with hexan-1-ol, which was 57.3 units (mg protein)⁻¹.

Substrate	Activity as % of activity with hexan-1-ol
Ethanol	8.5
Ethanediol	8.5
Propan-1-ol	28.0
Allyl alcohol	28.0
Butan-1-ol	36.3
Butan-1,4-diol	29.0
Pentan-1-ol	133
Ribitol	4.0
Hexan-1-ol	100
Mannitol	6.5
<i>cis</i> -Hex-3-en-1-ol	18.7

was no activity with: the aliphatic alcohols methanol, propan-2-ol, butan-2,3-diol, pentan-2-ol, hex-5-en-1-ol or octan-2-ol; the cyclic alcohols hexahydrobenzyl alcohol, phenyl ethanol or cyclohexanol; the amino acid alcohols serine, threonine or histidinol; or the sugars glyceraldehyde, erythritol, glucose, fructose or galactose. Primary aliphatic alcohols with more than about ten carbons are practically insoluble in water (Hodgman *et al.*, 1959) and were made accessible as substrates to the enzyme by pre-sonication of alcohol in the presence of BSA. When hexan-1-ol was treated by this method the activity measured was only a third to a quarter of that measured when the enzyme was assayed normally (Table 4). A decrease in activity was observed with increasing chain-length of the primary alcohol substrate at the concentrations assayed. The activity of the enzyme with tetradecan-1-ol as substrate was very low, and no activity was observed with hexadecan-1-ol (Table 4).

Unlike some other alcohol dehydrogenases (Koivusalo & Uotila, 1991; Pocker & Li, 1991), the enzyme did not oxidize formaldehyde (50 mM) and acetaldehyde (50 mM) in the presence or absence of coenzyme A or glutathione at pH 10.2 (in 80 mM glycine/NaOH buffer) or at pH 7.2 (in 50 mM Tris/HCl buffer).

The $K_{m(\text{app})}$ values for primary alcohols decreased as the chain-length of the primary alcohol increased from ethanol to hexan-1-ol/octan-1-ol (Table 5a). The $V_{\text{max}(\text{app})}$ values increased as chain-length increased from ethanol to pentan-1-ol, then decreased with increasing chain-length to octan-1-ol. The NADP-dependent alcohol dehydrogenase partially purified from strain HO1-N of *A. calcoaceticus* by Fox *et al.* (1992) also had very little

Table 4. Ability of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 to oxidize alcohols with long chain-lengths

All of the alcohols used were pre-sonicated in BSA (0.7 mg ml⁻¹) and assayed as described in Methods. The apparent concentration is based on the concentration of the alcohol that was sonicated. Values in parentheses refer to activities with no sonication with BSA at these alcohol concentrations. ND, Not determined.

Alcohol	Apparent concn (mM)	Activity [nmol min ⁻¹ (mg protein) ⁻¹]
Hexan-1-ol	500	8.80 (31.0)
	250	6.60 (21.4)
	100	3.00 (10.1)
Octan-1-ol	500	7.45
	250	5.05
	100	2.50
Decan-1-ol	500	6.50
	250	3.05
	100	1.20
Dodecan-1-ol	500	3.40
	250	3.65
	100	2.65
Tetradecan-1-ol	500	0.35
	250	0.25
	100	0.10
Hexadecan-1-ol	500	ND
	250	0.00
	100	0.00

activity with substrates containing more than 12 C-atoms, but the $K_{m(\text{app})}$ value seemed to decrease as the chain-length increased.

Butanal was used as a typical aldehyde rather than hexanal or a higher-chain aldehyde because of the very low solubilities of the latter (Hodgman *et al.*, 1959). The $K_{m(\text{app})}$ value for butanal was much lower than that for butan-1-ol (3.97 mM and 52.7 mM, respectively). In addition, the $K_{m(\text{app})}$ value for NADPH was lower than that for NADP⁺ (4.3 μ M and 38 μ M, respectively) although the $K_{m(\text{app})}$ of both cofactors was very much lower than that of either alcohol or aldehyde. The $V_{\text{max}(\text{app})}$ was slightly lower for butanal than for butan-1-ol [39.2 units (mg protein)⁻¹ and 57.1 units (mg protein)⁻¹, respectively] (Table 5a).

$K_{m(\text{app})}$ and $V_{\text{max}(\text{app})}$ values of the enzyme for hexan-1-ol were also determined at pH 7.2 in 80 mM Tris buffer (otherwise the enzyme was assayed as before). This pH value was chosen because it was as close to the pH optimum of aldehyde reduction (pH 6.8) as the alcohol oxidation reaction could be reasonably assayed at. The $K_{m(\text{app})}$ and $V_{\text{max}(\text{app})}$ values were 12.1 mM (68% confidence limits, 11.4–17.2) and 41.0 units (mg protein)⁻¹ (68% confidence limits, 38.1–56.4), re-

Table 5. Kinetic constants of selected substrates of NADP-dependent alcohol dehydrogenases

The $K_{m(\text{app})}$ and $V_{\text{max}(\text{app})}$ values for the substrates were determined by measuring the initial reaction rates at various non-saturating concentrations of that substrate in the presence of a fixed, high concentration of the other substrate (1 mM NADP⁺, 20 μ M NADPH, 20 mM hexan-1-ol or 20 mM butanal as appropriate) at pH 10.2 (for the prokaryotic enzyme) or pH 10.7 (for the eukaryotic enzyme). The standard assay mixtures were used except that the concentration of one of the substrates was varied. Aldehyde reduction was assayed fluorimetrically at pH 6.8. The second substrates used in determination of kinetic constants for the cofactors are given in parentheses. The kinetic constants were calculated by the Direct Linear method. Where only one value was determined the 68% confidence limits are given in parentheses. Where two values were determined for a kinetic constant the average is given with the actual values in parentheses. Where more than two values were determined the mean is given with the standard deviation and the sample size in parentheses. ND, Not determined.

Substrate	$K_{m(\text{app})}$ (mM)	$V_{\text{max}(\text{app})}$ [units (mg protein) ⁻¹]
(a) Alcohol dehydrogenase from <i>A. calcoaceticus</i> NCIB 8250		
Ethanol	727 (750, 703)	20.8 (21.7, 19.9)
Butan-1-ol	52.7 (51.6, 53.7)	57.1 (58.2, 57.6)
Pentan-1-ol	5.80 (5.82, 5.78)	66.7 (65.9, 67.4)
Hexan-1-ol	1.22 (0.20, $n = 4$)	58.8 (2.44, $n = 4$)
Heptan-1-ol	1.09 (0.94–1.30)	58.0 (53.7–65.4)
Octan-1-ol	1.10 (1.01, 1.21)	37.9 (38.8, 37.1)
Benzyl alcohol	15.0 (8.3–18.3)	57.5 (55.0–65.8)
Butanal	3.97 (3.22, 4.71)	39.2 (39.3, 39.0)
NADP	38.0×10^{-3}	ND
(hexan-1-ol)	(39.7×10^{-3} , 36.2×10^{-3})	
NADPH	4.33×10^{-3}	ND
(butan-1-ol)	(3.77×10^{-3} , 4.88×10^{-3})	
(b) Alcohol dehydrogenase of <i>Sacch. cerevisiae</i> D273-10B		
Ethanol	4790 (3440–9900)	48.4 (38.4–68.2)
Butan-1-ol	78.6 (77.1, 80.0)	69.8 (76.9, 62.7)
Pentan-1-ol	14.1 (12.7, 15.4)	86.7 (93.8, 79.7)
Hexan-1-ol	6.8 (5.9, 7.6)	55.6 (66.1, 45.1)
Octan-1-ol	6.2 (5.4, 7.0)	54.9 (68.2, 41.6)
Butanal	0.75 (0.71, 0.79)	27.1 (24.7, 29.4)
NADP	20.2×10^{-3}	ND
(hexan-1-ol)	(15.8×10^{-3} , 24.6×10^{-3})	
NADPH	7.9×10^{-4}	ND
(butanal)	(6.7×10^{-4} , 9.0×10^{-4})	

spectively. This $K_{m(\text{app})}$ value is tenfold higher than, and the $V_{\text{max}(\text{app})}$ value 70% of, that obtained at pH 10.2, which suggests that these variations are because of ionizations in the free enzyme as opposed to the enzyme–substrate complex (Fersht, 1985).

Table 6. Apparent turnover numbers and specificity constants for selected substrates of NADP-dependent alcohol dehydrogenases

Apparent turnover numbers ($k_{\text{cat}(\text{app})}$) and specificity constants ($k_{\text{cat}(\text{app})}/K_{m(\text{app})}$) were determined using the mean values for kinetic coefficients given in Table 5 assuming that there was one active site per enzyme subunit, that the subunit M_r values of the prokaryotic and eukaryotic enzymes were 40 300 and 43 500, respectively, and that $V_{\text{max}(\text{app})} = k_{\text{cat}(\text{app})} \times [E_t]$, where $[E_t]$ is the concentration of active sites.

Substrate	$k_{\text{cat}(\text{app})}$ (s ⁻¹)	$k_{\text{cat}(\text{app})}/K_{m(\text{app})}$ (s ⁻¹ M ⁻¹)
(a) Alcohol dehydrogenase from <i>A. calcoaceticus</i> NCIB 8250		
Ethanol	13.9	1.90×10^1
Butan-1-ol	38.3	7.26×10^2
Pentan-1-ol	44.7	7.71×10^3
Hexan-1-ol	39.4	3.23×10^4
Heptan-1-ol	38.9	3.57×10^4
Octan-1-ol	25.4	2.31×10^4
Butanal	26.3	6.62×10^3
(b) Alcohol dehydrogenase from <i>Sacch. cerevisiae</i> D273-10B		
Ethanol	35.1	7.3
Butan-1-ol	50.6	6.4×10^2
Pentan-1-ol	62.9	4.5×10^3
Hexan-1-ol	40.3	5.8×10^3
Octan-1-ol	39.8	6.4×10^3
Butanal	19.6	2.6×10^4

The highest turnover number of the enzyme was for pentan-1-ol as substrate. The highest specificity constants of the enzyme were for hexan-1-ol and heptan-1-ol as substrates (Table 6a).

The eukaryotic enzyme. This enzyme utilized primary alcohols with from two to at least eight carbons (Table 5b). No activity was observed with the secondary alcohol pentan-2-ol. The $K_{m(\text{app})}$ values for primary alcohols decreased from ethanol to hexan-1-ol and octan-1-ol. The $V_{\text{max}(\text{app})}$ values increased as chain-length increased from ethanol to pentan-1-ol, then decreased slightly (Table 5b). The $V_{\text{max}(\text{app})}$ value determined for the aldehyde butanal was only half that of its equivalent alcohol butan-1-ol. However, the $K_{m(\text{app})}$ value for butanal was much lower than that of butan-1-ol. Similarly, the $K_{m(\text{app})}$ for NADPH was much lower than for NADP⁺, although the $K_{m(\text{app})}$ of both cofactors was much lower than that of the alcohol or the aldehyde.

The highest turnover number of the enzyme was for pentan-1-ol as substrate (Table 6b). The highest specificity constants for primary alcohols were for hexan-1-ol and octan-1-ol as substrates, but butanal had a 100-fold higher specificity constant than the primary alcohols.

DISCUSSION

This work was originally aimed at characterizing the NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* but we found that not only was an apparently similar enzyme present in all the eubacteria that we examined, but it was also present in the two yeasts, *Rhodotorula graminis* and *Sacch. cerevisiae* (Table 1). Although four different alcohol dehydrogenases from *S. cerevisiae* have been purified and characterized, and their amino acid sequences deduced from their gene sequences (Brändén *et al.*, 1975; Young & Pilgrim, 1985; Williamson & Paquin, 1987), all of these enzymes are inducible, NAD-dependent and optimally use C₂–C₄ alcohols as substrates. As such, they seem very different from the constitutive NADP-dependent hexan-1-ol dehydrogenase activity identified in the present work. The identification of such constitutive alcohol dehydrogenase activities in a wide variety of both prokaryotic and eukaryotic organisms may well open the door to a whole new group of these enzymes in terms of their structures and of their physiological role.

Comparison of the N-terminal amino acid sequence obtained for the enzyme from *A. calcoaceticus* with those of the other alcohol dehydrogenases described in the literature reveals no significant sequence identities except for a 34% identity with residues 31–68 of a fermentative alcohol dehydrogenase from *E. coli* (Fig. 1). The *E. coli* enzyme has been identified as a member of the group 3 alcohol dehydrogenases but it is somewhat unusual in that it is much larger than the other enzymes in this group, being a dimer of subunit M_r value 96 000 and shows both ethanol and CoA-linked acetaldehyde dehydrogenase activity (Goodlove *et al.*, 1989). A further complication is that although it is the C-terminal half of the protein that shows homology with group 3 alcohol dehydrogenases, when the C-terminal third of the gene is removed the resulting protein shows only the ethanol dehydrogenase activity (Clark, 1992). These confusing, and apparently contradictory, findings make it difficult to assess the significance of the identity between the *Acinetobacter* enzyme and the *E. coli* enzyme.

The subunit M_r values of both the *Acinetobacter* and the *Saccharomyces* enzymes are consistent with these enzymes belonging to either the group 1 or group 3 alcohol dehydrogenases. However, the apparent monomeric structure of the eukaryotic enzyme is somewhat unusual among alcohol dehydrogenases, most being dimers or tetramers. There is little evidence for the existence of a metal ion in either of these enzymes as judged by their lack of inhibition by metal-binding agents. However, it may be that harsher conditions would be needed to inhibit the enzyme in this kind of experiment, e.g. heating in the presence of chelating agents (DeBruyn *et al.*, 1981). Both enzymes appear to use only NADP⁺ as cofactor and both transfer the *pro-R* hydrogen to/from NADP(H). The three structural groups of alcohol dehydrogenases show different stereospecificities. The group 2 enzymes transfer the *pro-S* hydrogen, whereas both groups 1 and 3 transfer the *pro-R* hydrogen (Glasfeld & Benner, 1989).

The eukaryotic enzyme shows similar substrate specificities to the prokaryotic enzyme, oxidizing primary alcohols with NADP⁺, not NAD⁺, as cofactor. In terms of its kinetic coefficients for alcohol oxidation the yeast enzyme is less efficient than the bacterial enzyme; $K_{m(\text{app})}$ values are two- to eightfold lower, $V_{\text{max}(\text{app})}$ values are slightly higher, and consequently specificity constants are up to fivefold lower. Aldehyde reduction is kinetically favoured over alcohol oxidation in the reaction catalysed by both enzymes, given that their $K_{m(\text{app})}$ values for aldehyde are much lower than those for the corresponding alcohol but it is unclear whether this has any physiological significance. There are some differences in the effects of thiol-blocking agents on the enzymes, the yeast enzyme being susceptible to a wider variety of such compounds.

Overall, there are enough structural and kinetic similarities between the eukaryotic and prokaryotic enzymes to suggest that the enzymes may be homologous and that they may have the same physiological role. Furthermore, the existence of NADP-dependent aliphatic alcohol dehydrogenase activities in a variety of different micro-organisms grown under conditions unlikely to induce such activities implies that these enzymes may be ubiquitous in the microbial world, carrying out some common function as yet undefined.

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