A new alcohol dehydrogenase, reactive towards methanol, from *Bacillus stearothermophilus*

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An NAD⁺-dependent alcohol dehydrogenase (ADH) was purified to homogeneity from an aerobic strain of *Bacillus stearothermophilus*, DSM 2334 (ADH 2334), and compared with the ADH from *B. stearothermophilus* NCA 1503 (ADH 1503). When an antibody raised against ADH 2334 was used, no cross-reactivity with ADH 1503 was observed on Western blots; by means of an enzyme-linked-immunoabsorbent-assay ('e.l.i.s.a.') procedure, it was found that ADH 1503 had less than 6% of the antigenic activity of ADH 2334. Amino acid analyses detected very small differences in composition, equivalent to about 40 sequence changes, between the two enzymes. The new enzyme has the same six-amino-acid *N*-terminal sequence as ADH 1503. ADH 2334, but not ADH 1503, is reactive towards methanol; both enzymes can oxidize ethanol, propan-1-ol, butan-1-ol and butan-2-ol. The new enzyme has a distinctive pH optimum at pH 5.5–6 and has significantly lower K_m^{Ethanol} and $k_{\text{cat}}^{\text{Ethanol}}$ values than those of ADH 1503. From steady-state kinetic parameters of the reaction with ethanol, propan-1-ol and butan-1-ol, it was shown that ADH 2334 has an ordered mechanism in both directions, with NAD⁺ being the compulsory first substrate in alcohol oxidation and NADH release being the rate-limiting step. ADH 1503 has an ordered addition of NAD⁺ and alcohol, but NADH release is not rate-limiting.

INTRODUCTION

NAD⁺-dependent alcohol dehydrogenases (ADHs; EC 1.1.1.1) are widely distributed enzymes (Sund & Theorell, 1963), among which very thorough studies of the liver and yeast enzymes have been made (Brändén *et al.*, 1975). Among the bacteria, reports of characterized ADHs are remarkably sparse. However, there are reports of a structurally characterized enzyme, obviously related to the horse and yeast enzymes, from *Bacillus stearothermophilus* (Bridgen *et al.*, 1973; Runswick & Harris, 1978) and of two enzymes from *Zymomonas* (Wills *et al.*, 1981; Neale *et al.*, 1986), one of which is a conventional Zn-dependent enzyme, the other of which is Fe-dependent and may have novel properties.

For industrial-scale growth of micro-organisms there are engineering advantages in the use of methanol as a substrate for cell growth (Antony, 1982). Bacteria usually oxidize methanol by a quinoprotein-dependent methanol oxidase, which yields formaldehyde and 1 mol of ATP from electrons entering the electron-transport chain via cytochrome *c*. NAD⁺-dependent oxidation of methanol yields formaldehyde and NADH which, entering at an earlier point in the electron-transport pathway, gives rise to three molecules of ATP. There should therefore be an energetic advantage, manifest in terms of cell yield, to a micro-organism that assimilates methanol by the NAD⁺-dependent pathway, and it could be economically valuable to genetically engineer a suitable activity into a bacterium.

However, the best-known ADHs have little activity towards methanol (Sund & Theorell, 1963; Brändén *et al.*, 1975), and there are no substantial reports of an

NAD⁺-dependent methanol-oxidizing enzyme in micro-organisms. Such an activity was found by C. W. Jones (unpublished work) and, in a subsequent screening at about 40 species of bacteria, by D. W. Tonge & J. Foundling (unpublished work), who could only detect the enzyme in extracts of the aerobic *Bacillus stearothermophilus* DSM 2334. Other strains of the organism did not possess the activity.

In the present paper we report the purification and properties of the methanol-reactive, NAD⁺-dependent, ADH from *B. stearothermophilus*, var. *non diastaticus* (DSM 2334) and show that it has enzymic, structural and immunological properties different from those of the ADH of *B. stearothermophilus* NCA 1503.

MATERIALS AND METHODS

Chemicals

Methanol was obtained as a special preparation containing 0.002% (v/v) ethanol from ICI Agricultural Division, Billingham, Cleveland, U.K. Ethanol ('Ethanol 95') was from James Burroughs, London SE11 5DF, U.K. Propan-1-ol, 'Chromasolv' (> 99.7%, v/v) and butan-1-ol (reagent grade; > 99.5%) were supplied by Riedel-de Haen, Hannover, Germany. NAD⁺ (grade II), NADH (grade II) and yeast ADH were from Boehringer Corp., Lewes, East Sussex, U.K. Octyl-Sepharose CL-4B and 5'-AMP-Sepharose 4B were purchased from Pharmacia Ltd., Milton Keynes, Bucks., U.K. Hydroxyapatite (Bio-Gel HT) was a product of Bio-Rad Laboratories, Watford, Herts., U.K. Methane-sulphonic acid (4 M), trifluoroacetic acid and amino acid calibration

Abbreviations used: ADH 1503, NAD⁺-dependent alcohol dehydrogenase from *Bacillus stearothermophilus*, strain N.C.A. 1503 N.C.I.B. 8924 or EP 00170; ADH 2334, NAD⁺-dependent alcohol dehydrogenase from *Bacillus stearothermophilus*, strain DSM 2334; e.l.i.s.a., enzyme-linked immunoabsorbent assay.

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mixtures were from Pierce, Cambridge, U.K. Dansyl chloride and 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate were supplied by BDH, Poole, Dorset, U.K. Equine liver ADH and amino acid calibration mixtures were obtained from Sigma, Poole, Dorset, U.K.

Bacillus stearothermophilus cells

B. stearothermophilus var. *non diastaticus* (DSM 2334) was obtained from ICI Agricultural Division as a slope culture, which was grown in minimal medium. For purification of the enzyme, the starting material was a cell paste of the organism grown aerobically at 55 °C and pH 7 on a minimal medium similar to that of Epstein & Grossowitz (1969), with the exceptions that sodium succinate (10 mM) was substituted for glucose, KNO₃ was omitted and biotin (1 mg/l), nitriloacetic acid (1 g/l) and CaCl₂ (17 mg/l) were added. The cells were harvested by centrifugation (4000 g, 10 min), washed in 50 mM-Tris/HCl, pH 7.5, and stored as a cell paste at -20 °C.

B. stearothermophilus (NCA 1503; N.C.I.B. 8924; EP 00170), catalogue 106, was a frozen cell paste from the PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wilts., U.K.

The identities of the samples used to prepare crude extracts and the ADH from strain DSM 2334 and of the cell paste of strain NCA 1503 were checked by independent microbial analysis at the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, U.K., who confirmed that both organisms were strains of *Bacillus stearothermophilus* and that our 2334 sample had properties similar to those of the isolate described by Epstein & Grossowitz (1969).

Purification of ADH 2334

In a typical purification, all steps being carried out at 0-4 °C, frozen cell paste (20 g) was suspended in 50 mM-potassium phosphate, pH 6.8 (60 ml), containing 2 mM-EDTA, 2 mM-2-mercaptoethanol, 0.1 mM-phenyl-methanesulphonyl fluoride and DNAase I (3 µg·ml⁻¹) and lysed by two passages through an Aminco pressure cell at 0.5 MPa (500 bar). After clarification by centrifugation at 10000 g for 30 min, the crude supernatant was adjusted to 70% saturation of (NH₄)₂SO₄, clarified by centrifugation as described above, and loaded on to a column of octyl-Sepharose 4B (30 cm × 1.6 cm) equilibrated in 70%-satd. (NH₄)₂SO₄. The column was eluted with a linear gradient (120 ml) from 70% saturation of (NH₄)₂SO₄ to 1% ethanol in 50 mM-potassium phosphate, pH 6.8. Active fractions were pooled, diluted to a conductivity of less than 15 mS and loaded on to a hydroxyapatite column (15 cm × 1 cm) equilibrated in 50 mM-potassium phosphate, pH 6.8, containing 0.1 mM-dithiothreitol, and eluted on a linear gradient of 50 mM-0.5 M-phosphate. Material for structural study was chromatographed on an Ultragel AcA 34 column (40 cm × 2.6 cm) in 10 mM-NH₄HCO₃ and freeze-dried.

ADH 1503

A sample of salt-free enzyme as a dry powder was obtained from Mr. M. J. Runswick, MRC Laboratory, Cambridge, U.K. For enzymic studies it was dispersed into 0.1 M-Na₂CO₃, pH 9.6, containing 1 mM-dithiothreitol, and immediately adjusted to pH 7.

Immunological analyses

Antiserum to ADH 2334 was raised in rabbits. Intramuscular injections (100 µg) in complete Freund's adjuvant were given on day 0, followed by injection in incomplete Freund's adjuvant on day 14, and a final injection at multiple sites on the back on day 28. For e.i.s.a. assay (Engvall & Perlmann, 1972), ADH (10⁻⁵-1 µg) in 0.1 M-Na₂CO₃, pH 9.6, was coated on to wells of a polystyrene microtitre dish. Excess sites were blocked by brief incubation with 4% (w/v) bovine serum albumin. The antigen-antibody complex was formed by incubation of the coated wells with a fixed amount of antiserum (0.1 ml at 1:500 dilution) and detected, using peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:1000 dilution) with *o*-phenylenediamine substrate, by absorbance change at 492 nm.

Polyacrylamide-gel electrophoresis

Analytical SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli (1970) in slab gels. Western blots were transferred to nitrocellulose (Towbin *et al.*, 1979) and detected with the peroxidase-linked-antibody procedure. ADH activity was developed by an alcohol-dependent tetrazolium procedure (Wieme, 1979) after polyacrylamide-gel electrophoresis carried out with solutions and reagents from which SDS was omitted.

ADH activity and kinetics

Alcohol-dependent NADH production was measured by increase in absorbance at 340 nm, using a molar absorption coefficient of 6.22 × 10³ M⁻¹·cm⁻¹, at 37 °C, in 0.1 M-potassium phosphate, pH 7.0, containing 0.5 mM-NAD⁺. In some cases 0.1 M-glycine/KOH was used, and pH-dependence was also measured by using mixtures of 0.05 M-citric acid and 0.1 M-K₂HPO₄ (Dawson *et al.*, 1986). Protein concentrations for specific-activity estimation were measured by the procedure of Lowry *et al.* (1951) and confirmed by amino acid analysis. Data-fitting to the Michaelis-Menton equation was carried out by a non-linear-regression procedure (Ralston, 1981) with the weighting factor α (Mannervik, 1982) estimated from log-log plots of the squares of differences between duplicates against dependent variable value. Coefficients of variation of parameter estimates were usually less than 10%, but up to 20% in a few instances. k_{cat} values were calculated on the basis of a subunit molar mass of 35000 g·mol⁻¹.

Protein chemistry

The *N*-terminal amino acid was determined as the dansyl derivative (Gray, 1972), and the *N*-terminal sequence was analysed by the procedure of Chang (1983). Protein composition was determined after hydrolysis in 4 M-methanesulphonic acid, containing 0.2% (w/v) 3-(2-aminoethyl)indole and conversion of cystine into *S*-sulphocysteine (Simpson *et al.*, 1976), followed by amino acid separation on a JEOL-5AH amino acid analyser operating an accelerated version of the original procedure (Spackman *et al.*, 1958).

RESULTS AND DISCUSSION

Dye-affinity (Neale *et al.*, 1986) and AMP-Sepharose (Comer *et al.*, 1975) chromatography were initially tried

Fig. 1. SDS/polyacrylamide-gel electrophoresis of the two ADHs of *B. stearothermophilus*

Samples (4 μ g) of ADH 1503 (a) and ADH 2334 (b) were stained with Coomassie Blue. The arrows and M_r values refer to the positions of the standard proteins [bovine serum albumin (M_r 66000), ovalbumin (45000) and carbonic anhydrase (29000)].

Fig. 2. Electrophoresis of ethanol dehydrogenases

Samples of crude extract from strain 1503 (a) and pure ADH 2334 (b) were loaded at the top of the gel slab. ADH 2334 has a greater anionic mobility at the alkaline pH of the separation.

as purification procedures for the 2334 enzyme, but in all cases incomplete binding, loss of activity and, occasionally, multiple active species were obtained. The given purification procedure was designed to avoid proteolysis at the early stages. At the first column-chromatography stage, the enzyme was eluted at 15% saturation of $(\text{NH}_4)_2\text{SO}_4$ and was unstable. The incorporation of 1% (v/v) ethanol into the gradient considerably increased stability at this stage and was continued through the succeeding steps. The enzyme was eluted at 250 mM-phosphate on hydroxyapatite chromatography. The final preparation, after 50–100-fold purification to homogeneity, had a specific activity of about 2.1 units $\cdot$ mg $^{-1}$.

Fig. 3. SDS/polyacrylamide-gel electrophoresis of crude extracts of *B. stearothermophilus*

Strain 1503 extract (70 μ g samples) was run on an SDS-containing gel and either stained with Coomassie Blue (c) or probed after nitrocellulose transfer (a) with the anti-2334 antibody. Strain 2334 extracts (7 μ g and 70 μ g for b and d respectively) are similarly displayed on the stained gel (d) or as a Western blot (b). The single band on the latter track corresponds in position to that of pure ADH 2334 (Fig. 1b), as indicated by the position of the marker proteins (e) bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen and soya-bean trypsin inhibitor.

The purified enzyme demonstrated a single band on SDS/polyacrylamide gels, with an M_r of 35000 (Fig. 1a), and the dansyl procedure indicated a single *N*-terminal amino acid, namely methionine. On gels containing the native enzyme, a single band of activity was present; the electrophoretic mobility of this band was very different from that of the enzyme present in strain 1503 (Fig. 2). When methanol was used instead of ethanol to develop the activity gels, pure strain-2334 enzyme was readily observed, but no active band could be found in crude extracts of strain 1503. The extent of the difference between the two enzymes was further investigated by immunological procedures. On Western blots, crude extracts (Fig. 3d) of strain 2334 displayed a single band of M_r 35000 when probed with anti-(2334 ADH) antibody (Fig. 3b), but no reaction took place between the antibody and large amounts of crude extract of strain 1503 (Fig. 3a). An e.l.i.s.a. procedure was used to confirm that immunological differences exist between the 2334 enzyme and ADH 1503 as purified by Runswick & Harris (1978). Dose-response curves for the two enzymes are shown in Fig. 4. Under the assay conditions, 2334 enzyme saturated the fixed amount of antibody with a half-saturation point at about 0.016 μ g. The response for

Fig. 4. Antigenic responses of ADH 2334 and ADH 1503

The given amounts of ADH 2334 (○) and ADH 1503 (●) were challenged by anti-2334 antibody and complex-formation was measured at 492 nm by the e.l.i.s.a. procedure.

the 1503 enzyme was displaced by about 2 decades and was not fully saturated. Assuming that saturation is feasible, then it may be calculated that ADH 1503 has less than 6% of the antigenic activity of ADH 2334 towards anti-2334 antibody. These experiments demonstrate that the two enzymes from *B. stearothermophilus* have different electrophoretic mobilities and substrate specificities, and that they are immunologically very distant.

The amino acid composition of each protein was determined by replicate analyses and averaged as described in Table 1. Our amino acid composition for ADH 1503 is in general agreement with the previous analysis, although the differences in lysine content may represent a discrepancy. As determined in the present work, differences between the compositions of ADH 2334 and ADH 1503 are barely detectable. Four amino acid contents were significantly different at or above the 95% confidence level, and only the accumulation of these differences makes it possible to accept that the two proteins differ in composition. For proteins of similar molecular mass the procedure described by Cornish-Bowden (1983) allows a prediction of the number of sequence differences from composition data: 38 ± 15 sequence difference differences are predicted between ADH 1503 and ADH 2334.

The *N*-terminal sequence of ADH 2334 determined by the dimethylaminoazobenzene isothiocyanate procedure was:

Met-Lys-Ala-Ala-Val-Val-

This sequence is identical with that of ADH 1503 (Bridgen *et al.*, 1973), and the first five residues are identical with those of *Zymomonas* ADH-1 (Neale *et al.*, 1986).

Table 1. Amino acid composition of ADHs from *B. stearothermophilus*

The quoted values from the present work are the means of two complete analyses for each protein. Each analysis was calculated from replicate measurement of 24 h, 48 h or 72 h hydrolysates against amino acid calibration standards from two suppliers. Corrections in each analysis were determined independently; serine and threonine values were adjusted for the usual small losses by assuming first-order kinetics; valine and isoleucine values were corrected for incomplete liberation by extrapolation to infinite time; methionine (including sulphoxides) was unstable, with losses of about 8% in 24 h, and was accordingly corrected. The significance (*P*) of differences between the mean values was assessed by a *t* test, and differences at 95% or 99% confidence are indicated by * or **.

Amino acid	ADH ... Source ...	Content (mol of amino acid/mol of protein subunit)			
		2334 Present work	<i>P</i>	1503	
				Present work	Runswick & Harris (1978)
Trp		2.9		3.6	—
Lys		30.4		27.0	21.4
His		7.7	*	8.8	8.8
Arg		9.6		11.3	11.8
Cys		6.6		6.9	5.4
Asx		27.0		25.5	24.2
Thr		8.9	*	11.8	12.6
Ser		10.4		10.6	10.3
Glx		34.6		33.1	33.7
Pro		19.0		16.8	16.4
Gly		35.6		36.8	31.6
Ala		33.7	*	31.4	32.4
Val		36.5		40.2	39.5
Met		4.6		4.0	4.5
Ile		20.5		18.1	17.8
Leu		26.8		25.2	22.3
Tyr		10.0		10.3	11.3
Phe		5.4	**	7.5	8.7

A comparative study was carried out to test whether the two enzymes have different enzymic properties. The pH-dependence of the reactions is shown in Fig 5. ADH 1503 has a broad pH optimum at about 8, similar to the optimal pH for the enzyme from yeast (Sund & Theorell, 1963) and some bacteria (Bellion & Wu, 1978; Neale *et al.*, 1986). ADH 2334 was active over a wider range and had a novel pH optimum at 5.5–6.0 in two buffers (Fig. 5) and at different concentrations of the same buffer; there was no pH optimum in the alkaline region.

Each enzyme was shown to be active towards ethanol, propan-1-ol, butan-1-ol, propan-2-ol and octan-1-ol. Systematic studies of the activity towards methanol, ethanol, propan-1-ol and butan-1-ol were made (Table 2); no deviations from linearity on double-reciprocal plots were observed.

Against methanol, ADH 2334 gave a substantial rate of reaction, the K_m value being 200-fold greater than that for ethanol. As ethanol is always present in methanol samples, it can be difficult to rule out an 'artefactual' contribution to a methanol-dependent rate. Methanol-

Table 2. Kinetic parameters for the reaction of ADH with primary alcohols

For the *B. stearothermophilus* enzymes the data were collected in 0.1 M-potassium phosphate, pH 7.0, at 37 °C: $K_m^{Alcohol}$ values were measured at NAD concentration of 0.5 mM; K_m^{NAD} was measured at 50 mM, 10 mM and 10 mM for ADH 1503 with ethanol, propanol and butanol respectively, and at 5 mM, 0.5 mM and 0.4 mM for ADH 2334 with the same respective alcohols. Control experiments demonstrated that the enzymes were saturated at the fixed alcohol concentrations, and hence K_m^{NAD} are indistinguishable from true values; $K_m^{Alcohol}$ values are apparent. Data from the horse liver and yeast enzymes were taken from Dalziel & Dickinson (1966) and Dickinson & Monger (1973), except for the methanol results, which were obtained in the present work.

Enzyme	Parameter value											
	Methanol			Ethanol			Propan-1-ol			Butan-1-ol		
	Parameter ... Units ...	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	K_m (μM)	K_m^{NAD}/k_{cat} (μM·s)	k_{cat} (s ⁻¹)	K_m (μM)	K_m^{NAD}/k_{cat} (μM·s)	k_{cat} (s ⁻¹)	K_m (μM)	K_m^{NAD}/k_{cat} (μM·s)
<i>B. stearothermophilus</i> 2334		0.06	20	1.3	82	30	1.3	18	29	1.1	12	36
<i>B. stearothermophilus</i> 1503		< 0.01	—	6.0	450	14	4.6	370	16	2.4	160	20
Horse liver		0.048	30	2.7	180	1.1	3.2	61	1.2	2.9	11	1.1
Yeast		< 0.03	—	450	2170	0.24	66	2920	2.3	25	3200	10

Fig. 5. pH-dependence of ADH activities

Reaction velocities of ADH 2334 (a) and ADH 1503 (b) were measured in 0.1 M-sodium phosphate (○), 0.1 M-glycine/KOH (△) and citrate/phosphate (●) buffer at 50 mM-ethanol and 0.5 mM-NAD⁺.

dependent activity was confirmed by examination of the progress curves. At pH 7, reaction rates with low concentrations of ethanol fell off very rapidly. With the pure methanol sample, the initial rates at pH 7 remained quasi-linear over a larger range than for any of the other alcohols. Of the other enzymes tested, horse liver ADH had substantial activity towards methanol, slightly lower in efficiency, in terms of k_{cat} and $k_{cat}/K_m^{Ethanol}$, than ADH 2334. Methanol dehydrogenase activity of ADH 1503 or yeast ADH could not be demonstrated unequivocally; the trace reaction rates observed were not saturated at up to 100 mM-methanol. It has been shown that yeast ADH will produce formaldehyde from methanol (Mani *et al.*, 1970), but the reaction is so slow that its exact measurement by NADH production requires that the methanol substrate contains less than 0.001 % (v/v) ethanol.

Steady-state data with ethanol, propan-1-ol and butan-1-ol were extended in order to determine the mechanism of the reaction with these alcohols. The classical studies of Dalziel and his school (Dalziel & Dickinson, 1966; Dickinson & Monger, 1973) demonstrate that k_{cat} and K_m^{NAD}/k_{cat} values are very powerful and sensitive indicators of the reaction mechanism of alcohol dehydrogenase and, accordingly, data for those parameters are included in Table 2. For ADH 2334, K_m^{NAD}/k_{cat} values, with ethanol, propan-1-ol or butan-1-ol substrate, are indistinguishable, hence there is a compulsory order of substrate addition, with NAD⁺ leading. Furthermore, the invariance of the k_{cat} values for these three primary alcohols requires that the rate-limiting step does not involve an alcohol, or the product of an alcohol, and must therefore be at NADH release. In ADH 1503 K_m^{NAD}/k_{cat} values for the three alcohols are also indistinguishable, but the k_{cat} values decline along the

Fig. 6. Substrate inhibition of ADH 2334

Dixon plots show the velocity of reaction at an $[NAD^+]$ of 0.5 mM and limiting concentrations of ethanol (○), propan-1-ol (●) and butan-1-ol (△).

series ethanol, propan-1-ol, butan-1-ol. For this enzyme, NAD^+ is added as a compulsory first reactant in alcohol oxidation, but $NADH$ release is not a rate-limiting step.

These formal diagnostic tests were confirmed by observations of substrate inhibition. ADH 2334 is inhibited substantially (> 15%) above 50 mM, 5 mM and 2 mM concentrations of ethanol, propan-1-ol and butan-1-ol respectively. The data (Fig. 6) require a partial inhibition mechanism (Dalziel & Dickinson, 1966), including formation of an enzyme- $NADH$ -alcohol species. It can only occur if the binary enzyme- $NADH$ complex dominates the steady-state population of enzyme species. For ADH 1503, no substrate inhibition was observed with any of the alcohols, and this is consistent with the evidence that enzyme- $NADH$ dissociation is not rate-limiting.

The kinetic mechanism for ADH 2334 is identical with that of the horse liver enzyme, and some circumstantial features, including the reactivity towards methanol, and the similarities in k_{cat} and K_m values with ethanol, propan-1-ol and butan-1-ol, are also similar for these two enzymes. Together with the knowledge that the parent organism is an obligate aerobe, it is therefore apparent that ADH 2334 is an enzyme adjusted to operate in the direction of alcohol oxidation. ADH 1503 had higher K_m and k_{cat} values for primary alcohols and an alkaline pH optimum, properties characteristic of yeast ADH-1. The parent organism can grow aerobically or anaerobically, and there is no evidence as to how ADH 1503 is regulated.

The case of the two ADHs from *B. stearothermophilus* may be similar to the occurrence in yeast of ADH-1 and ADH-2, which have dissimilar enzymic properties in spite of only 23 sequence differences (Russell *et al.*, 1983); unfortunately there are no comparative immunological data for those proteins.

The selection of an alcohol dehydrogenase with the required substrate specificity was carried out by suitable screening of natural microbial sources. Although engineering of the enzyme from a given organism (Murali

& Creaser, 1986) may allow substrate specificity to be modified, it seems likely that many ADHs with novel and useful properties are still awaiting discovery in the largely unknown bacterial sources.

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