NADP-dependent aliphatic alcohol dehydrogenases in micro-organisms

Martin R Wales

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Department of Biochemistry

University of Glasgow

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ABBREVIATIONS

The abbreviations used are those recommended by The Biochemical Journal [Biochem. J. (1991) **273**, 1-9] with the following additions.

ACP	Acyl carrier protein
ATCC	American Type Culture Collection
AU	Absorbance units
BSA	Bovine serum albumin
DCIP	2,6-Dichloroindophenol
DTT	Dithiothreitol
EMBL	European Molecular Biology Laboratory
NBT	Nitro Blue Tetrazolium
NCI(M)B	National Collection of Industrial (& Marine) Bacteria
OD	Optical density
PMS	*N*-Methylphenazonium methosulphate
PQQ	Pyrroloquinoline quinone
PTH	Phenylthiohydantoin
r.p.m.	revolutions per minute
TEMED	*N,N,N',N'*-Tetramethylenediamine
TFA	Trifluoroacetic acid

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Finally, and most importantly, I would like to thank my parents, without whose support, even starting this project would have been an impossibility.

SUMMARY

SUMMARY

1. NADP-dependent aliphatic alcohol dehydrogenases had been previously identified in various strains of *Acinetobacter calcoaceticus*, including *A. calcoaceticus* NCIB 8250 and *Acinetobacter sp.* HO1-N. During the course of this study similar enzymes were identified in the yeast *Saccharomyces cerevisiae* D273-10B and in all of the other Gram negative and Gram positive eubacteria and yeasts that were examined. This thesis is chiefly concerned with purification and characterisation of these enzymes in *A. calcoaceticus* and *S. cerevisiae* and with studies on the physiological role of the enzyme of *A. calcoaceticus*.

2. Methods were developed to purify NADP-dependent aliphatic alcohol dehydrogenases of *A. calcoaceticus* NCIB 8250, *Acinetobacter sp.* HO1-N, and *S. cerevisiae* D273-10B in a similar, but not identical, fashion by disruption of the organisms in the French pressure cell and preparation of high speed supernatants, followed by ion exchange chromatography on DEAE Sephacel, gel filtration chromatography on Sephacryl S300HR, hydrophobic interaction chromatography on Phenyl Sepharose, then affinity chromatography on Matrex Gel Blue A (*A. calcoaceticus*) or Matrex Gel Red A (*S. cerevisiae*).

3. The enzymes from both strains of *A. calcoaceticus* were tetramers as judged by comparison of subunit (40 000) and native (165 000) M_r values estimated by SDS polyacrylamide gel electrophoresis and gel filtration respectively, whereas the enzyme of *S. cerevisiae* was a monomer (M_r 43 500).

4. The *N*-terminal amino acid sequence of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 was determined and shows significant homology (34 % identity) with that of an alcohol dehydrogenase sequence from *Escherichia coli* but not with any other alcohol dehydrogenases that have been described so far.

5. The absorption spectrum of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 shows no evidence of a bound cofactor or prosthetic group.

6. Assays were developed for NADP-dependent alcohol dehydrogenases from both bacteria and yeast. Alcohol oxidation was followed by monitoring the reduction of $NADP^+$ at 340 nm. The pH optimum of alcohol oxidation of the bacterial enzymes was 10.2 whereas that of the yeast enzyme was 10.7. Aldehyde reduction was followed by monitoring the oxidation of NADPH at 340 nm and 460 nm. The pH optimum of aldehyde reduction was 6.8 for the enzymes of both *A. calcoaceticus* NCIB 8250 and *S. cerevisiae* D273-10B. The pI value of the enzyme of *A. calcoaceticus* NCIB 8250 was 5.7.

7. The apparent equilibrium constant of the enzyme of *A. calcoaceticus* NCIB 8250 was 9.2×10^{-13} M in glycine buffer, and 2.9×10^{-13} M in CAPS buffer, assuming a 1:1 stoichiometry of reaction.

8. NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 was unable to utilise NAD^+ or DCIP or DCIP and PMS as cofactors. It transferred the pro*R* hydrogen to/from the C4 position of the nicotinamide ring of NADP(H). NADP-dependent alcohol dehydrogenase from *S. cerevisiae* D273-10B was unable to use NAD^+ as cofactor and transferred the pro*R* hydrogen to/from the C4 position of the nicotinamide ring of NADP(H) also.

9. The substrate specificities of the enzymes from both organisms were very similar, oxidising only primary alcohols and some 1,x diols but not secondary alcohols. A more detailed study of the substrate specificity of NADP-dependent alcohol dehydrogenase from *A. calcoaceticus* NCIB 8250 showed that it also oxidised benzyl alcohol, some unsaturated primary alcohols and the polyols ribitol and mannitol at relatively low rates. However, it did not oxidise hexadecan-1-ol, certain sugars, erythritol, glyceraldehyde,

cyclohexan-1-ol, hexahydrobenzyl alcohol or some amino acid alcohols.

10. The enzyme of *A. calcoaceticus* NCIB 8250 was not inhibited or activated by a wide range of salts and metal ions, nor was it inhibited by the metal binding agents EDTA, pyrazole, sodium azide, 2'2'bipyridyl or 8'hydroxyquinoline. NADP-dependent alcohol dehydrogenase from *S. cerevisiae* D273-10B was not inhibited by the metal binding agents 2'2'bipyridyl or pyrazole but high (100 mM) concentrations of EDTA or sodium azide partially inhibited the enzyme.

11. The enzyme of *A. calcoaceticus* NCIB 8250 was not inhibited by the thiol acting agents *N*'ethylmaleimide, iodoacetate or iodoacetamide but it was inhibited by *p*-chloromercuribenzoate. NADP-dependent alcohol dehydrogenase from *S. cerevisiae* D273-10B was not inhibited by iodoacetate but was inhibited by *N*'ethylmaleimide, iodoacetamide and *p*-chloromercuribenzoate; hexan-1-ol and NADP both gave protection from inhibition by *p*-chloromercuribenzoate.

12. The maximal velocity of all the NADP-dependent alcohol dehydrogenases for alcohol oxidation was observed with pentan-1-ol as substrate [65 to 90 EU (mg protein)$^{-1}$]. The K'_m values for alcohol oxidation were minimum for hexan-1-ol and octan-1-ol as substrates, K'_m values for hexan-1-ol were 1.2, 0.3 and 6.2 mM for *A. calcoaceticus* NCIB 8250, *Acinetobacter sp.* HO1-N and *S. cerevisiae* D273-10B respectively. The maximum velocities of aldehyde reduction were lower i.e. 27-40 EU (mg protein)$^{-1}$ for butanal. K'_m values for butanal reduction were 3.97 and 0.75 mM for *A. calcoaceticus* NCIB 8250 and *S. cerevisiae* D273-10B respectively. K'_m values for $NADP^+$ were 38 μM and 20 μM whereas those for NADPH were 4.3 μM and 0.8 μM for *A. calcoaceticus* NCIB 8250 and *S. cerevisiae* D273-10B respectively.

13. The enzyme of *A. calcoaceticus* NCIB 8250 was located in the cytoplasm, no activity could be detected in membrane or periplasmic fractions.

14. Continuous culture of *A. calcoaceticus* NCIB 8250 revealed that specific activity of NADP-dependent alcohol dehydrogenase decreased with increasing growth rate regardless of growth condition, that there was a considerable effect of temperature of growth on enzyme activity i.e. activity was higher in extracts of cells grown at higher temperatures, and that there was some correlation between enzyme activity and wax content of cells when cells were grown in media containing low or limiting concentrations of nitrogen but not when cells were grown in media containing low or limiting concentrations of carbon.

15. The activity of the enzyme in crude extracts varied less than two to three-fold when *A. calcoaceticus* NCIB 8250 was grown in batch culture or in continuous culture on various alcohol and non-alcohol carbon sources, or under nitrogen or carbon limitation.

16. Mutants of *Acinetobacter sp.* HO1-N with a decreased ability to degrade wax esters showed little variation in NADP-dependent alcohol dehydrogenase activity with respect to the wild type.

17. No conclusions were reached as to the physiological role of the enzyme although it appeared not to be involved directly in wax ester metabolism.

18. Benzyl alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 transferred the pro*R* hydrogen to/from the C4 position of the nicotinamide ring of NAD(H).

CHAPTER 1
INTRODUCTION

1. Introduction

1.1. Biological alcohol oxidation

Many different enzymes can catalyse the oxidation of alcohols. Alcohol oxidoreductases can be classified into three types on the basis of the type of electron acceptor used:

(a) NAD(P)-dependent alcohol dehydrogenases, which catalyse the reaction

$$\text{alcohol} + \text{NAD(P)}^+ \rightleftharpoons \text{aldehyde} + \text{NAD(P)H} + \text{H}^+;$$

(b) NAD(P)-independent alcohol dehydrogenases, which catalyse the reaction

$$\text{alcohol} + 2\text{A}_{(ox)} \rightleftharpoons \text{aldehyde} + 2\text{A}_{(red)} + 2\text{H}^+$$

where $A_{(ox)}$ and $A_{(red)}$ represent the oxidised and the reduced forms of the electron acceptor;

(c) alcohol oxidases, which catalyse the reaction

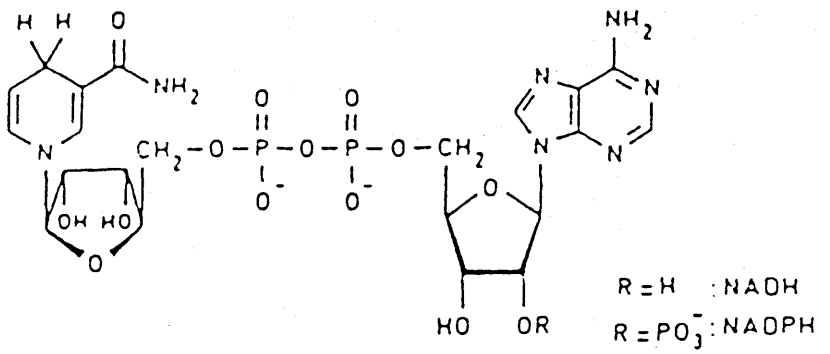
$$\text{alcohol} + \text{O}_2 \rightleftharpoons \text{aldehyde} + \text{H}_2\text{O}_2.$$

Type (a) are the best known type of the three, in both prokaryotes and eukaryotes, and use NAD or NADP as coenzymes. Both of these coenzymes have a low electron transfer potential ($E_0' = -0.32$ v) and are involved in two electron transfers. The reactions these enzymes catalyse are reversible, hydrogen transfer occurring to and from the carbon-4 of the nicotinamide ring of NAD(P)/H. Enzymes using these coenzymes can transfer to, or from, only the pro*R* or the pro*S* position of the prochiral carbon-4 position (Rossman *et al.*, 1975; see Figure 1.1; Section 5.5.2). These enzymes are usually found in the cytoplasm of eukaryotic organisms but are also found in prokaryotes.

The type (b) enzymes identified so far are all quinoproteins using pyrroloquinoline quinone (PQQ), or a haem and PQQ, as electron acceptor and occur predominantly in the periplasmic space of Gram negative bacteria (Vries *et al.*, 1990; Arfman, 1991).

The type (c) enzymes use flavins as electron acceptors, are membrane-bound, and are found in some yeasts and white rot fungi (Veenhuis *et al.*, 1983; Bourbonnais and Paice, 1988).

(a)



(b)

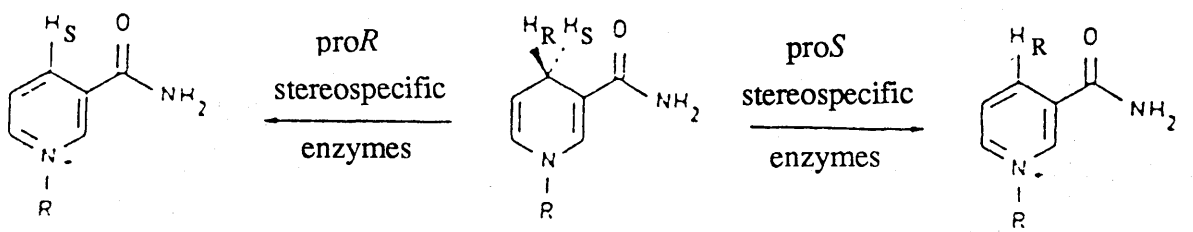


Figure 1.1. NAD(P)/H: structure and stereospecificity of hydride transfer

(a) shows the structure of NAD(P)/H; (b) shows the stereospecificity of hydride transfer to from NAD(P)/H.

Among alcohol oxidoreductases there is a wide diversity at each of the various different levels of protein structure i.e. amino acid composition, primary, secondary, tertiary and quarternary structure. Also, there is variation in their metal content and in their substrate specificity, some enzymes utilise primary aliphatic and/or aromatic alcohols whereas others use polyols, steroids or sugars. Frequently, more than one alcohol dehydrogenase, or type of alcohol dehydrogenase, exist in the same organism and some enzymes are induced either by their substrates or in response to other external stimuli, whereas other enzymes are constitutive.

1.2. Comparison of alcohol oxidation enzymes

The massive diversity of alcohol dehydrogenases, in terms of their structures and functions, makes them fascinating enzymes to study with respect to the evolutionary relationships amongst them. In order to establish the evolutionary relationships that exist amongst these enzymes, it is necessary to obtain adequate structural information (Rossman *et al.*, 1975; Dayhoff *et al.*, 1978).

(a) Direct structural comparison

Much is known about the primary structures of NAD(P)-dependent alcohol dehydrogenases, and three groups have been established on the basis of their amino acid sequences, and are present in both prokaryotes and eukaryotes. The zinc-dependent alcohol dehydrogenases are the best characterised group (Branden *et al.*, 1975; Yokoyama *et al.*, 1990) and have been identified in many organisms. These enzymes are also known as "long chain" alcohol dehydrogenases because of their subunit size (approx. 380 amino acids). Non-zinc alcohol dehydrogenases, also known as "short chain" alcohol dehydrogenases (with subunits of approx. 250 amino acids), which have no metal ion dependence have been identified in mammals, insects and bacteria. More recently, a group of so-called "iron-activated" alcohol dehydrogenases (with subunits of about 380 amino acids) have been identified. These enzymes are mostly bacterial in origin with only one eukaryotic member known.

The primary structures of only two NAD(P)-independent alcohol dehydrogenases are known, both from methylotrophic bacteria (Vries *et al.*, 1990).

The primary sequences of two yeast alcohol oxidases have been deduced from their DNA sequences and they show significant homology with an insect glucose dehydrogenase [see Introduction 1.5 (a)].

Horse liver alcohol dehydrogenase is the only alcohol dehydrogenase for which the crystal structure is known (Branden *et al.*, 1975), although attempts are being made to determine the three dimensional structure of the non-zinc alcohol dehydrogenase of *Drosophila melanogaster* (Villaroya *et al.*, 1989; Ribas de Populana *et al.*, 1991). Detailed mechanistic studies have been carried out on various zinc-dependent alcohol dehydrogenases, most predominantly horse liver alcohol dehydrogenase and yeast alcohol dehydrogenase, where its three dimensional structure has been modelled on that of horse liver.

As well as the multitude of known alcohol dehydrogenases that could be sequenced, existing knowledge about primary structures needs to be put in the context of mechanistic studies and studies at further structural levels. Comparison of alcohol oxidation enzymes in these ways will, no doubt, eventually provide examples of both divergent and convergent evolution of proteins, therefore allowing us to understand how, and why, different groups of alcohol oxidation enzymes have arisen, catalysing basically the same reaction in apparently different ways.

(b) Indirect structural comparison

Immunological cross-reactivity has been used to show evolutionary relationships between some proteins, including alcohol dehydrogenases such as some NAD(P)-independent alcohol dehydrogenases (Vries *et al.*, 1990). Positive results from this method almost always indicate at least 60 % sequence identity although the converse is not always true i.e. a negative result does not always indicate a lack of homology (Chalmers and Fewson, 1989) such as in comparison of benzyl alcohol dehydrogenase of *Acinetobacter calcoaceticus* and the TOL-encoded enzyme of *Pseudomonas putida* which do show sequence homology (30 %) but do not react immunologically (Chalmers

et al., 1991).

Other methods, such as comparison of amino acid compositions or kinetic coefficients, proteolytic maps of pure proteins, or subunit exchange experiments have been used in comparing closely related proteins (see Chalmers and Fewson, 1989 for examples) but are of limited value in considering alcohol dehydrogenases because of the wide diversity of primary structure that exists, particularly amongst the microbial enzymes.

1.3. NAD(P)-dependent alcohol dehydrogenases

1.3.1. Zinc-dependent alcohol dehydrogenases

(a) In eukaryotes

(i) Structure: On the basis of their primary structures, zinc-dependent alcohol dehydrogenases have been identified in mammals (e.g. Branden *et al.*, 1975), birds (e.g. Kaiser *et al.*, 1990), plants (e.g. Matton *et al.*, 1990) and yeasts (e.g. Branden *et al.*, 1975). Primary structures are known for many such enzymes, especially amongst the mammalian alcohol dehydrogenases. Three distinct levels of identity of primary structure can be identified (Table 1.1), presumably due to three distinct gene duplication events. Firstly, enzymes in one class, such as the class 1 dimeric enzymes in man and horse, have > 90 % residue conservation. Secondly, enzymes of different classes show 40-60 % identity, e.g. the plant and animal enzymes. Finally, dimeric alcohol dehydrogenases, tetrameric alcohol dehydrogenases and polyol dehydrogenases show about 25 % identity (Jornvall *et al.*, 1987). In zinc-dependent alcohol dehydrogenases there are 22 strictly conserved residues (Jornvall *et al.*, 1987). Most predominant among them are 11 glycines and this presumably reflects the structural importance of this residue. Two prolines are also conserved, and they may have some secondary structural role. Two cysteines and one histidine are conserved [see section (ii) Zinc ligands], as are five acidic

but no basic residues, and one valine (Jeffrey & Jornvall, 1988).

Horse liver alcohol dehydrogenase (Class 1) may be considered to be the "classical" zinc-dependent alcohol dehydrogenase and has been most studied in terms of its structure and mechanism. Therefore, it will be described as a typical alcohol dehydrogenase and all numbering of amino acid residues etc. in discussing zinc-dependent alcohol dehydrogenases refers to this enzyme (Figure 1.2). It exists as a dimer and is not active in the monomeric state (Branden *et al.*, 1975).

(ii) Zinc ligands: Horse liver alcohol dehydrogenase binds two zinc atoms, one catalytic and one structural. The catalytic zinc is bound in the bottom of a pocket lined predominantly with hydrophobic residues. The three zinc ligands are the sulphur atoms of Cys-46 and Cys-174 and the nitrogen atom of His-67. A fourth ligand is water or a hydroxyl ion, depending on the pH. The structural zinc ion is bound in a lobe formed from residues 93-113 by four sulphur atoms, from Cys-97, Cys-100, Cys-103 and Cys-111 (Branden *et al.*, 1975). This second zinc atom is not present in eukaryotic sorbitol dehydrogenases which have only one zinc atom per subunit (Jeffrey and Jornvall, 1987; Maret and Auld, 1988). The amino acid sequence of sheep liver sorbitol dehydrogenase is known (Figure 1.2) and it has all the catalytic zinc ligands but only one structural zinc ligand (Cys 103; Figure 1.2).

(iii) Cofactor binding: NAD binding has been studied in detail in horse liver alcohol dehydrogenase. The important residues in NAD binding in terms of side chain interactions with the cofactor are: Asp 223 which hydrogen bonds to the adenosine ribose, and is conserved throughout this family; Lys 228 which hydrogen bonds to the 3'-oxygen of the adenosine ribose, and is conserved or replaced by arginine; Ser 48 and His 51 which hydrogen bond nicotinamide ribose, the serine being conserved or replaced by threonine whereas the histidine is conserved or replaced by tyrosine (mostly) or serine; and Arg 369 which hydrogen bonds a phosphate oxygen and is conserved or replaced by lysine. All these residues are conserved or conservatively replaced throughout the zinc-dependent alcohol dehydrogenases (Eklund, 1988). The GxGxxG fingerprint region of the NAD binding domain is also conserved throughout these enzymes (Wierenga *et al.*,

1986).

(iv) Catalytic mechanism: The mechanism of action of horse liver alcohol dehydrogenase is well established, being an ordered mechanism with the coenzyme binding first. It is thought that the oxygen atom of the alcohol binds the zinc, displacing the bound water molecule, forming the enzyme-substrate complex [Figure 1.3 (a)]. The carbonyl bond forms, then the product and cofactor dissociate [Figure 1.3 (b)], the latter being the rate determining step (Fersht, 1985).

(v) Substrate specificity: Horse liver alcohol dehydrogenase has a very broad substrate specificity. The enzyme can oxidise a variety of primary alcohols, activity decreasing with increasing size from ethanol to octan-1-ol, some secondary alcohols (although not propan-2-ol), some aromatic alcohols, some primary and secondary cyclic alcohols, vitamin A and ethanediol (Sund and Theorell, 1963). In addition some isozymes (of Class 1, 2 and 3 enzymes) can oxidise dopamine intermediary alcohols (Mardh and Vallee, 1986), some hydroxysteroid alcohols (McEvily *et al.*, 1988), some hydroxyphenyl glycols (Mardh *et al.*, 1985) and intermediary alcohols of the shunt pathway of mevalonate metabolism (Keung, 1991). Such specificities of different isozymes have led to a number of hypotheses about the functions of the isozymes (See Introduction 1.6).

Class 2, 3 and 4 enzymes have much higher K_m values for ethanol (Bosron *et al.*, 1979; Koivusalo *et al.*, 1989; and Moreno and Pares, 1991, respectively) than the class 1 enzyme (Sund and Theorell, 1963). The Class 3 enzyme oxidises large chain primary alcohols, such as 12-hydroxydodecanoate, optimally at high pH (i.e. pH 9.6) but show a glutathione-dependent formaldehyde dehydrogenase activity that is optimal at physiological pH (pH 7.0) (Koivusalo *et al.*, 1989; Koivusalo and Uotila, 1991). The class 4 enzyme utilises octan-1-ol optimally at high pH (pH 10.0), k'_{cat}/K_m values increasing with increasing chain length from ethanol to octan-1-ol (Moreno and Pares, 1991).

Polyol dehydrogenases show some variation in substrate specificity also. The stereochemical requirements for polyol substrates of sheep liver sorbitol dehydrogenase

are: a hydroxyl group at C1; C2 must be in the *S* conformation; C4 must be in the *R* conformation (Eklund *et al.*, 1985). Therefore it will not use erythritol as a substrate (McCorkindale and Edson, 1954). However, human liver sorbitol dehydrogenase shows significant variation with respect to polyol substrates, using erythritol and (2*R*, 3*S*) butan-2,3-diol for instance (Maret and Auld, 1988). Also, the sheep liver enzyme will use ethanol and butan-1-ol (Eklund *et al.*, 1985; McCorkindale and Edson, 1954) whereas the human liver enzyme will not (Maret and Auld, 1988).

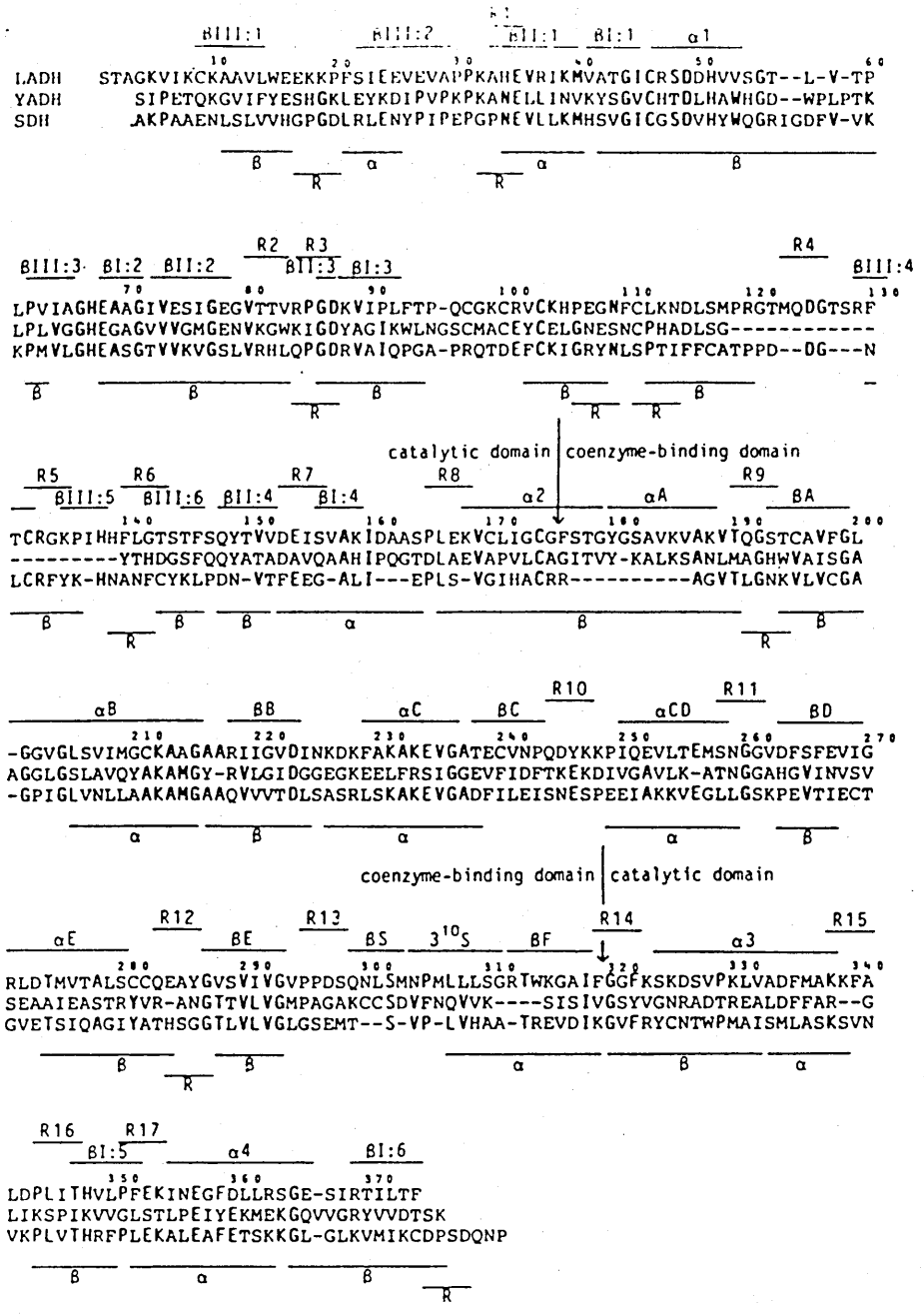
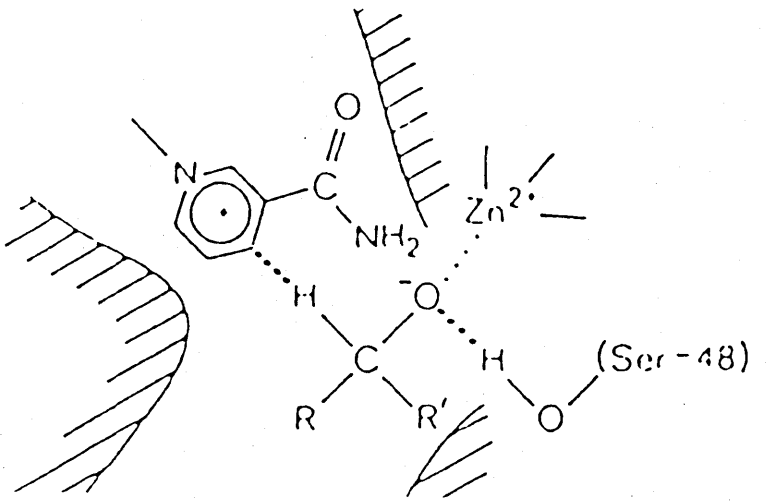


Figure 1.2. Comparison of the primary structures of sheep liver sorbitol dehydrogenase (SDH) , horse liver alcohol dehydrogenase (HLADH) and yeast alcohol dehydrogenase (YADH)

The primary sequences of the three enzymes were aligned as described in Jornvall *et al.* (1984), amino acids being given by their one letter codes. The top lines in each row show the elements of secondary structure in the known conformation of the horse liver enzyme as determined crystallographically. Similarly, the bottom lines in each row indicate the results of predictions of secondary structures from the amino acid sequence of sorbitol dehydrogenase. Taken from Jornvall *et al.* (1984).

(a)



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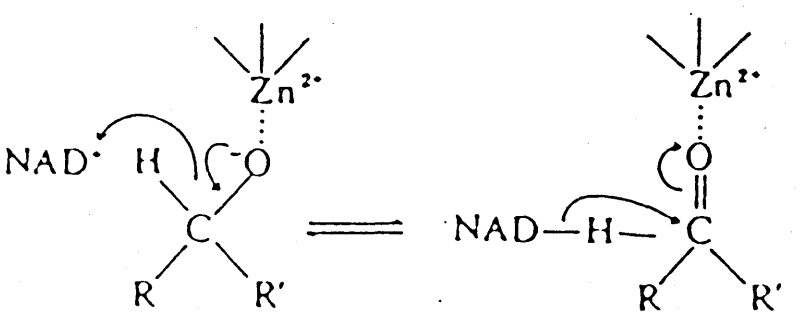


Figure 1.3. The active site of horse liver alcohol dehydrogenase and its reaction mechanism

(a) Representation of the active site of horse liver alcohol dehydrogenase showing zinc and its ligands; (b) a proposed reaction mechanism. Taken from Fersht (1985).

Table 1.1. Levels of homology among eukaryotic zinc-dependent alcohol dehydrogenases

The three levels of sequence homology among eukaryotic zinc-dependent alcohol dehydrogenases based on the amino acid sequence identities among the different sub-groups. Class 1, 2, 3 and 4 alcohol dehydrogenases are all mammalian enzymes. Arrows indicate that further sub-classes exist that have not been represented here, with further levels of homology. Abbreviations: ADH, alcohol dehydrogenase; DH, dehydrogenase. (Jornvall *et al.*, 1987; Kaiser *et al.*, 1990).

<table>
<tr><th colspan="3">Sequence identity (%)</th></tr>
<tr><th>25 %</th><th>60 %</th><th>90 %</th></tr>
<tr><td rowspan="8">Dimeric ADHs</td><td rowspan="4">Class 1</td><td>Human α</td></tr>
<tr><td>Human β</td></tr>
<tr><td>Human γ</td></tr>
<tr><td>→</td></tr>
<tr><td>Class 2 →</td><td>→</td></tr>
<tr><td>Class 3 →</td><td>→</td></tr>
<tr><td>Class 4</td><td></td></tr>
<tr><td>Plant ADHs →</td><td>→</td></tr>
<tr><td>Avian ADHs</td><td></td></tr>
<tr><td>Tetrameric ADHs →</td><td>→</td><td></td></tr>
<tr><td>Tetrameric polyol DHs →</td><td>→</td><td></td></tr>
</table>

(b) Prokaryotic enzymes

Some bacterial alcohol dehydrogenases have been reported to contain zinc [e.g. alcohol dehydrogenase of *Leuconstoc mesenteroides* (Schneider-Bernlohr *et al.*, 1981)] or to require zinc for activity [e.g. alcohol dehydrogenase of *Mycobacterium tuberculosis* (DeBruyn *et al.*, 1981)]. However comparatively few of them have been positively identified as belonging to this group on the basis of their primary structure (Table 1.2).

(i) Structure: Complete amino acid sequences have been obtained for only the NAD-dependent, fermentative alcohol dehydrogenase from *Alcaligenes eutrophus* (Jendrossek *et al.*, 1988), the type 1 NAD-dependent alcohol dehydrogenase from *Zymomonas mobilis* (Keshev *et al.*, 1990) and the NADP-dependent secondary alcohol dehydrogenase from *Thermoanaerobium brockii* (Peretz and Burstein, 1989). The latter is the first NADP-dependent enzyme in this group to have been sequenced so far. In addition, *N*-terminal sequences of three bacterial alcohol dehydrogenases have been found to show significant homologies with horse liver alcohol dehydrogenase. These are: *Bacillus stearothermophilus* 1503 alcohol dehydrogenase (Bridgen *et al.*, 1973; Jeck *et al.*, 1979); the chromosomally-encoded benzyl alcohol dehydrogenase of *Acinetobacter calcoaceticus* NCIB 8250 (Chalmers *et al.*, 1991); and the TOL plasmid-encoded benzyl alcohol dehydrogenase of *Pseudomonas putida* (Chalmers *et al.*, 1991). The first five residues of a methanol-utilising alcohol dehydrogenase of *B. stearothermophilus* 2334 have been determined also (Dowds *et al.*, 1988). All these enzymes are tetramers with subunits of about M_r value 40 000, except the methanol-utilising alcohol dehydrogenase of *B. stearothermophilus* 2334 whose quarternary structure is not known, but which has subunits of M_r value 35 000 (Sheehan *et al.*, 1988).

Over the whole primary structure, the NAD-dependent type 1 alcohol dehydrogenase of *Z. mobilis* (Keshav *et al.*, 1990), NADP-dependent alcohol dehydrogenase of *T. brockii* (Peretz and Burstein, 1989) and NAD-dependent alcohol dehydrogenase of *A. eutrophus* (Jendrossek *et al.*, 1988) show 34 %, 27 % and 15 % identity with horse liver alcohol dehydrogenase respectively (Table 1.3). There is 35 % identity between NADP-dependent alcohol dehydrogenase of *T. brockii* and NAD-

dependent alcohol dehydrogenase of *A. eutrophus*, 25 % between NAD-dependent alcohol dehydrogenase of *A. eutrophus* and type 1 alcohol dehydrogenase of *Z. mobilis* and about 16 % between NADP-dependent alcohol dehydrogenase of *T. brockii* and NAD-dependent type 1 alcohol dehydrogenase of *Z. mobilis* (Table 1.3).

The *N*-terminal amino acid sequences of those bacterial alcohol dehydrogenases of this group for which only these data are known, show about 25 % to 30 % identity with horse liver alcohol dehydrogenase (Table 1.3). NAD-dependent alcohol dehydrogenase of *Bacillus stearothermophilus* shows the most identity (30 %) with horse liver alcohol dehydrogenase (Bridgen *et al.*, 1973), followed by the benzyl alcohol dehydrogenases of *A.calcoaceticus* and *P. putida* (25 %) (Chalmers *et al.*, 1991). Considering the *N*-termini of all the enzymes (including those for which the entire sequence is known), of those residues strictly conserved amongst eukaryote sequences, Glu 49 is completely conserved and Cys 46 almost completely so. Pro 31 is conserved or replaced by isoleucine or leucine. Glu 35 appears to vary the most, being conserved in two, replaced by aspartate in two, glutamine in one and phenylalanine in another. Several other residues are almost completely conserved with respect to horse liver alcohol dehydrogenase such as Lys 10, Ala 10 and 11 and Val 13. This comparison ignores the *N*-terminal amino acid sequence of the methanol-utilising alcohol dehydrogenase of *B. stearothermophilus* 2334 for which too little sequence (only five residues) is available to make valid comparisons (Dowds *et al.*, 1988).

There are no strictly conserved residues in the *N*-terminal sequences of these enzymes other than those mentioned above. *Z. mobilis* type 1 alcohol dehydrogenase and *B. stearothermophilus* 1054 alcohol dehydrogenase are the most similar (45 % identity; Neale *et al.*, 1986), *P. putida* and *A. calcoaceticus* benzyl alcohol dehydrogenases show 36 % identity (Chalmers *et al.*, 1991), *T. brockii* alcohol dehydrogenase and *A. eutrophus* alcohol dehydrogenase share 32 % identity (Peretz and Burstein, 1989), *B. stearothermophilus* 1054 alcohol dehydrogenase shows about 30% identity with *P. putida* benzyl alcohol dehydrogenase (Chalmers *et al.*, 1991) and *A. eutrophus* alcohol dehydrogenase, but the latter two are only 23 % identical to each other (Table 1.3). No

other identities (< 20 %) exist and some pairs of enzymes are considerably less similar to each other e.g. *T. brockii* alcohol dehydrogenase and *P. putida* benzyl alcohol dehydrogenase share only 8 % identity. Overall, *Z. mobilis* type 1 alcohol dehydrogenase seems to be least related to the others.

Of the 22 residues conserved in the eukaryotic enzymes, all but the two prolines are conserved in NAD-dependent type 1 alcohol dehydrogenase of *Z. mobilis* (Keshav *et al.*, 1990) and presumably they do not have an essential secondary structural role to play. Eighteen residues are conserved in NADP-dependent alcohol dehydrogenase of *T. brockii* i.e. 11 glycines, 2 aspartates, 1 proline, 1 cysteine, 1 histidine and 1 glutamate (Peretz and Burstein, 1989). In NAD-dependent alcohol dehydrogenase of *A. eutrophus* only 15 of these 18 are conserved, with 2 glycines and 1 aspartate being replaced by 2 alanines and 1 glutamate, respectively (Jendrossek *et al.*, 1988).

No secondary structural studies have been made on the bacterial enzymes but presumably they resemble horse liver alcohol dehydrogenase with about equal amounts of α helix and β sheet (Branden *et al.*, 1975). All seven enzymes are thought to be tetramers as judged by gel filtration chromatography (Table 1.4).

Table 1.2. Bacterial zinc-dependent alcohol dehydrogenases

Enzyme	Organism from	Abbreviation
Fermentative alcohol dehydrogenase	*Alcaligenes eutrophus*	AEADH
Type 1 alcohol dehydrogenase	*Zymomonas mobilis*	ZMADH1
Secondary alcohol dehydrogenase	*Thermoanaerobium brockii*	TBADH
Alcohol dehydrogenase	*Bacillus stearothermophilus*1503	BSADH1503
Methanol utilising alcohol dehydrogenase	*Bacillus stearothermophilus* 2334	BSADH2334
Benzyl alcohol dehydrogenase	*Acinetobacter calcoaceticus*	ACBADH
TOL-encoded benzyl alcohol dehydrogenase	*Pseudomonas putida*	TOLBADH

Table 1.3. Identities between bacterial zinc-dependent alcohol dehydrogenases and horse liver alcohol dehydrogenase

The percentage amino acid identities between individual zinc-dependent alcohol dehydrogenases and between each of these enzymes and horse liver alcohol dehydrogenase. The abbreviations used are those quoted in Table 1.2, except HLADH, horse liver alcohol dehydrogenase.The sequences were determined by protein sequencing or deduced from the DNA sequence: AEADH, DNA-deduced (Jendrossek *et al.*, 1988); ZMADH1, DNA-deduced (Keshav *et al.*, 1990); TBADH, complete protein sequence (Peretz and Burstein, 1989); BSADH1503, *N*-terminal sequence and some internal sequence (Bridgen *et al.*, 1973; Jeck *et al.*, 1979); ACBADH and TOLBADH, *N*-terminal sequences (Chalmers *et al.*, 1991).

	HLADH	AEADH	ZMADH1	TBADH	BSADH1503	ACBADH	TOLBADH
HLADH	100						
AEADH	15	100					
ZMADH1	34	25	100				
TBADH	27	35	16	100			
BSADH1503	30	<20	45	<20	100		
ACBADH	25	<20	<20	<20	<20	100	
TOLBADH	25	23	<20	<20	30	36	100

(ii) The presence of zinc and its ligands: The presence of zinc has been unambiguously identified only in *Z. mobilis* type 1 alcohol dehydrogenase, by atomic absorption spectrophotometry (Wills *et al.*, 1981; Table 1.4). However, as in the eukaryotic enzymes, two of the catalytic zinc ligands are preserved in *T. brockii* alcohol dehydrogenase (Peretz and Burstein, 1989), *Z. mobilis* type 1 alcohol dehydrogenase (Keshav *et al.*, 1990) and *A. eutrophus* alcohol dehydrogenase (Jendrossek *et al.*, 1988), and these are Cys 46 and His 57. Unlike horse liver alcohol dehydrogenase, there is no evidence for a structural zinc atom in any of the bacterial enzymes fully sequenced. *T. brockii* alcohol dehydrogenase lacks a chunk of 18 amino acids, from positions 96-113 in horse liver alcohol dehydrogenase which contain the structural zinc ligands (Cys 97, 100, 103, 111; Peretz and Burstein, 1989). *Z. mobilis* type 1 alcohol dehydrogenase contains the structural zinc ligands but contains only one zinc molecule per subunit (Wills *et al.*, 1981). *A. eutrophus* alcohol dehydrogenase has retained only one of these ligands, Cys 97 (Jendrossek *et al.*, 1988). *T. brockii* alcohol dehydrogenase and *A. eutrophus* alcohol dehydrogenase can be considered analogous to sheep liver sorbitol dehydrogenase, a related polyol dehydrogenase which has all the catalytic zinc ligands but only one structural one, Cys 103 and has only one zinc atom/subunit (Jeffrey & Jornvall, 1988).

The *N*-terminal amino acid sequence of *B. stearothermophilus* 1054 has Cys 46 and His 57 also (Jeck *et al.*, 1979). No evidence is available as to the presence or absence of any zinc ligands in *A. calcoaceticus* benzyl alcohol dehydrogenase but *P.putida* benzyl alcohol dehydrogenase lacks Cys 46 and His 57. The inability of chelating agents to inhibit the TOL-plasmid encoded benzyl alcohol dehydrogenase of *P. putida* and *A. calcoaceticus* benzyl alcohol dehydrogenase (A.J. Scott and C.A. Fewson, unpublished results; MacKintosh and Fewson, 1988b) suggests that the zinc is somehow protected, or that the enzymes do not in fact contain a zinc ion at the active site.

(iii) Cofactor binding: Most of the coenzyme binding regions in horse liver alcohol dehydrogenase are conserved or conservatively replaced in *A. eutrophus* alcohol dehydrogenase, *Z. mobilis* type 1 alcohol dehydrogenase and *T. brockii* alcohol

dehydrogenase.

Considering those residues whose side chains bind NAD, in *Z. mobilis* type 1 alcohol dehydrogenase His 51, Asp 223, Lys 228 and Arg 369 are all conserved (Keshav *et al.*, 1990). Ser 48 is conservatively replaced by Thr. Also, the GxGxxG fingerprint region is conserved (Keshav *et al.*, 1990). In *A. eutrophus* alcohol dehydrogenase His 51 and Arg 223 are conserved, Ser 48 is replaced by threonine, Lys 228 by arginine and Arg 369 by lysine, all conservative substitutions (Jendrossek *et al.*, 1988). The fingerprint region of the NAD binding domain is altered somewhat, being AxGxxG, which has structural implications for the enzyme in that the turn between the end of a β sheet and the α helix of the βαβ fold of the NAD binding domain will be less tight (Jendrossek *et al.*, 1988).

T. brockii alcohol dehydrogenase is an NADP-dependent enzyme, therefore some variation with horse liver alcohol dehydrogenase in this region is to be expected. Ser 48 and His 51 are both conserved and Arg 369 is replaced by lysine as in *A. eutrophus* alcohol dehydrogenase (Peretz and Burstein, 1989). The main changes concern those residues interacting with the adenosine moiety. Asp 223 is replaced by the much smaller, uncharged glycine (198 in *T. brockii* alcohol dehydrogenase) presumably because of the extra phosphate group on the 2'-oxygen of ribose, i.e. the Asn would not allow proper orientation of the NADP (Peretz and Burstein, 1989). Also Asn 225 is replaced by arginine, the positive charge presumably interacting with the extra phosphate of NADP (Peretz and Burstein, 1989). Arg 228 is replaced by cysteine which presumably has a different function, if any, in cofactor binding (Peretz and Burstein, 1989). NADP-dependent enzymes often contain a GxGxxA region (Scrutton *et al.*, 1990), however, although TBADH uses NADP it contains a GxGxxG region (Peretz & Burstein, 1989).

(iv) Substrate specificities: All of the aliphatic alcohol dehydrogenases in this group for which sequence data is available oxidise lower primary alcohols (Table 1.4). Also, *A. eutrophus* alcohol dehydrogenase can oxidise butan-2,3-diol and acetaldehyde (Steinbuchel and Schlegel, 1984) and the *T. brockii* and *B. stearothermophilus* 1054 (Bridgen *et al.*, 1973) enzymes can oxidise lower secondary alcohols, the *T. brockii*

alcohol dehydrogenase showing a kinetic preference for secondary alcohols (Lamed and Zeikus, 1981). *B. stearothermophilus* 2334 alcohol dehydrogenase is the only one of these enzymes to oxidse methanol (Sheehan *et al.*, 1988). The two benzyl alcohol dehydrogenases oxidise aromatic alcohols only (MacKintosh and Fewson, 1988b; Shaw and Harayama, 1990).

Table 1.4. Properties of zinc-dependent alcohol dehydrogenases

The abbreviations used are those quoted in Table 1.2.The metal ion present is given only if it has been unambiguously identified. Only those substrates oxidised, as opposed to being reduced, are listed. The data used in the table were taken from the references listed: AEADH, Steinbuchel and Schlegel (1984), Jendrossek *et al.* (1988); ZMADH1 Wills *et al.* (1981), Neale *et al.* (1986); TBADH, Lamed and Zeikus (1981), Peretz and Burstein (1989); BSADH1503, Bridgen *et al.* (1973), Jeck *et al.* (1979); BSADSH2334, Dowds *et al.* (1988); ACBADH, MacKintosh and Fewson (1988ab); TOLBADH, Chalmers *et al.* (1990).

Enzyme	Metal	Coenzyme	Substrates	Function	Structure
AEADH	?	NAD(P)	ethanol, butan-2,3-diol acetaldehyde	fermentative	4 x 40 000
ZMADH1	Zn	NAD	ethanol butan-1-ol	fermentative	4 x 40 000
TBADH	?	NADP	lower primary and secondary alcohols except methanol	fermentative	4 x 38 000
BSADH1503	?	NAD	methanol, lower primary and secondary alcohols	fermentative	4 x 40 000
BSADH2334	?	NAD	lower primary and secondary alcohols except methanol	fermentative	? x 35 000
ACBADH	?	NAD	aromatic alcohols	catabolic	4 x 40 000
TOLBADH	?	NAD	aromatic alcohols	catabolic	4 x 40 000

1.3.2. Non-zinc, "short-chain" alcohol dehydrogenases

The first member of this group identified on the basis of primary sequence was an insect alcohol dehydrogenase, that of *Drosophila melanogaster* (Thatcher and Sawyer, 1980). Since then a wide variety of dehydrogenases and other proteins has been identified as belonging to this group, in both prokaryotes and eukaryotes.

In eukaryotes non-zinc "short-chain" alcohol dehydrogenases have been identified in several species of *Drosophila* (Villaroya *et al.*, 1989). Other eukaryotic proteins belonging to this group on the basis of statistical comparison of their sequences (Baker, 1990) include: human placental 17β-hydroxysteroid dehydrogenase (Peltoketo *et al.*, 1988); human placental 15β-prostaglandin dehydrogenase (Krook *et al.*, 1990); and mouse adipocyte p27 protein (Navre and Ringold, 1988).

A number of different prokaryotic proteins have been identified as belonging to this group on the basis of the same statistical analysis of their primary structures as was carried out on eukaryotic proteins (Baker 1990): dihydrodiol dehydrogenases of *P. pseudoalcaligenes* and *P. putida* (Baker, 1990); ribitol dehydrogenase from *Klebsiella aerogenes* (Morris *et al.*, 1974); glucose dehydrogenase from *Bacillus megaterium* (Jornvall *et al.*, 1984); glucitol-6-phosphate dehydrogenase of *E. coli* (Yamada and Saier, 1987); bile acid 7-hydroxysteroid dehydrogenase of *Eubacterium sp.* strain VPI 12708 (Baron *et al.*, 1991); the *fixR* protein of *Bradyrhizobium japonicum* (Fisher *et al.*, 1987); the *act3* gene product from *Streptomyces coelicolor* (Hallam *et al.*, 1988); and the *nodG* protein of *Rhizobium melitoti* (Debelle and Sharma, 1986; Fisher *et al.*, 1987). In addition, Baron *et al.* (1991) have identified other hydroxysteroid dehydrogenases belonging to this group i.e. 7α-hydroxysteroid dehydrogenase of *E. coli* (Yoshimoto *et al.*, 1991) and further 7α-hydroxysteroid dehydrogenase isozymes in *Eubacterium sp.* strain VPI 12708 (Coleman *et al.*, 1988; White *et al.*, 1988).

All of these proteins are between 244 amino acid residues (mouse adipocyte p27 protein) and 266 amino acid residues (human placental 15β-prostaglandin dehydrogenase) in length (which approximates to a M_r value of 28 000 to 30 000), except

human placental 17β-hydroxysteroid dehydrogenase which is 327 amino acids long.

Some secondary structural analyses, using Chou and Fasman (1978) type algorithms, have been carried out on the primary sequence of *D. melanogaster* (Thatcher and Sawyer, 1980; Benyajatti *et al.*, 1981; Ribas de Populana *et al.*, 1991), and the predicted structures are in agreement with those determined by circular dichroism (Ribas de Populana *et al.*, 1991). Benyajatti *et al.*(1981) noted that the coenzyme binding domain of the enzyme appeared to be at the *N*-terminal half of the protein, based on a predicted $\beta\alpha\beta$ fold similar to that found in the coenzyme binding domain of dehydrogenases (Rossman *et al.*, 1975). Secondary structure prediction has been attempted on bile acid 7-hydroxysteroid dehydrogenase of *Eubacterium sp.* strain VPI 12708 by Baron *et al.* (1991) using the algorithm of Garnier *et al.* (1978). Like *D. melanogaster*, the *N*-terminal half of the protein was predicted to consist of an alternate β sheet/ α helix arrangement similar to that found in the coenzyme binding domain of dehydrogenases. These predictions are supported by the primary structural data, the GxGxxG fingerprint motif for NAD binding (Wierenga *et al.*, 1986) existing near the *N*-terminal end of the primary sequence (Scrutton *et al.*, 1990). Furthermore, the *N*-terminal half of these proteins are all more conserved than the *C*-terminal half which implies that the former is involved in coenzyme binding which is the same throughout these proteins as far as is known, whereas the latter is involved in the, more variable , substrate specificity.

There is considerable variation in substrate specificities of eukaryotic enzymes in this group. *Drosophila spp.* alcohol dehydrogenases oxidise ethanol. 17β-Hydroxysteroid dehydrogenase catalyses interconversion of phenolic and neutral 17-keto and 17-hydroxysteroids (Pollow *et al.*, 1975; Tseng *et al.*, 1977). No details of the substrate specificity of 15-hydroxyprostaglandin dehydrogenase have been published other than its oxidation of 15-hydroxyprostaglandin. It is unclear what is the function of protein mouse adipocyte p27 protein, but transcription of its gene is associated with protein kinase C mediated inhibition of adipocyte differentiation (Navre and Ringold, 1988). The prokaryotic enzymes, if anything, are even more diverse; substrate

specificities ranging from primary alcohols to polyols to hydroxysteroids, as well as the structural rhizobium proteins which are involved in intercellular communications.

1.3.3. "Iron-activated" alcohol dehydrogenases

This is a recently discovered family of alcohol dehydrogenases with six members to date, five bacterial and one from yeast (Table 1.5). In addition, glycerol dehydrogenase of *Escherichia coli* has been reported to be a member of this group (Clark, 1992). The first sequence to be published for an enzyme in this group was alcohol dehydrogenase 2 of *Z.mobilis* which is NAD-dependent (Conway *et al.*, 1987). Since then the amino acid sequence of alcohol dehydrogenase 4 from the yeast *Saccharomyces cerevisiae* (Williamson and Paquin, 1987), an NADP-dependent butanol-ethanol dehydrogenase from *Clostridium acetobutylicum* (Youngleson *et al.*, 1989), a NAD-dependent propanediol oxidoreductase from *E.coli* (Conway and Ingram, 1989), a fermentative alcohol dehydrogenase also from *E. coli* (Goodlove *et al.*, 1990; Clark 1992); and a novel NAD-dependent methanol dehydrogenase from a thermotolerant *Bacillus sp., B. methanolicus* (Arfman *et al.*, 1989; Arfman, 1991) have been deduced from their gene sequences.

(a) Structure

S. cerevisiae alcohol dehydrogenase 4 and *Z. mobilis* alcohol dehydrogenase 2 show the greatest identity, 53 % between them, with sequence identities between any two other enzymes in the group being around 40 %. *E. coli* fermentative alcohol dehydrogenase and *S. cerevisiae* alcohol dehydrogenase 4 being the most distantly related with 32 % identity between them [Table 1.6 (a)].

Comparison of all six published sequences shows that 54 residues are strictly conserved (Arfman, 1991). As in the other groups, glycine is the single most conserved residue, 13 of them being conserved. Of the others, 14 branched chain residues are conserved as are 7 acidic and 6 basic residues, 5 prolines, 4 residues containing hydroxyl groups, 1 residue containing a thiol group and 2 aromatic residues. Five regions of these

enzymes have been identified as being fully conserved [Arfman, 1991; Table 1.6 (b)].

Attempts have been made to predict the secondary structure of *C. acetobutlylicum* NADP-dependent alcohol dehydrogenase, *S. cerevisiae* alcohol dehydrogenase 4 and *Z. mobilis* alcohol dehydrogenase 2 using a Chou and Fasman (1978) type analysis. These enzymes show a preference to form α helices, whereas the other two groups of NAD(P)-dependent alcohol dehydrogenase are predicted to have equal amounts of α helix and β sheet (Youngleson *et al.*, 1989). Extended α helical structures are predicted between His 196 and Cys 247, and hydropathy plots indicate a major hydrophilic portion between residues 230 and 240, implying that it is a surface region of the protein. This region lies immediately prior to the postulated metal binding region and may play a role in catalysis (Youngleson *et al.*, 1989).

All enzymes in this group have subunits consisting of about 380 amino acids with M_r values of around 40 000. (Table 1.7), except *E. coli* fermentative alcohol dehydrogenase (see Table 1.7 and Section 5.5.1). The native M_r values of the enzymes vary: *S. cerevisiae* alcohol dehydrogenase 4 and *E. coli* fermentative alcohol dehydrogenase are dimers (Drewke and Ciriacy, 1988; Goodlove *et al.*, 1990); *Z. mobilis* alcohol dehydrogenase 2 is a tetramer (Neale *et al.*, 1986); and *Bacillus methanolicus* methanol dehydrogenase is a decamer (Arfman *et al.*, 1989), all values as judged by gel filtration chromatography. The native M_r values of the other alcohol dehydrogenases of this group are not known. *S. cerevisiae* alcohol dehydrogenase 4 is expressed only when a transposable element, Ty, is inserted in the yeast genome (Williamson and Paquin, 1987).

Table 1.5. "Iron-activated" alcohol dehydrogenases

Enzyme	Source	Abbreviation
Type 2 alcohol dehydrogenase	*Zymomonas mobilis*	ZMADH2
Butan-1-ol-ethanol dehydrogenase	*Clostridium acetobutylicum*	CAADH
Propanediol oxidoreductase	*Escherichia coli*	ECPOR
Fermentative alcohol dehydrogenase	*Escherichia coli*	ECADHE
Methanol dehydrogenase	*Bacillus methanolicus*	MDH
Type 4 alcohol dehydrogenase	*Saccharomyces cerevisiae*	SCADH4

Table 1.6. Sequence similarities amongst "iron-activated" alcohol dehydrogenases

Taken fom Arfman (1991).

(a) Identities between "iron-activated" alcohol dehydrogenases

Percentage amino acid identities between individual "iron-activated" alcohol dehydrogenases.The abbreviations used are those quoted in the legend to table 1.5.

	ZMADH2	SCADH4	MDH	ECPOR	ECADHE	CAADH
ZMADH2	100					
SCADH4	53	100				
MDH	44	46	100			
ECPOR	42	39	40	100		
ECADHE	33	32	35	34	100	
CAADH	37	37	36	36	46	100

(b) Consensus sequences of "iron-activated " alcohol dehydrogenases

The amino acid residue numbers quoted refer to residues in *B. methanolicus* methanol dehydrogenase

Region of identity	**Sequence**
94 to 103	GGGSXXDXXK
190 to 201	GXDAXXHXXEXY
254 to 268	NXXXGXXHXXXHXXG
355 to 365	AXXDXCXXXNP

(b) Cofactor binding

Analysis of all six sequences by Arfman (1991) reveals that a sequence, starting at position 13 in the *Bacillus methanolicus* methanol dehydrogenase sequence, resembled to some extent the NAD(P) binding fingerprint (Wierenga *et al.*, 1986), although calculated alignments are too low to conclude beyond doubt that this region is involved in NAD(P) binding. Youngleson *et al.* (1989) stated that no such sequence could be identified in their analysis of *C. acetobutylicum* alcohol dehydrogenase, *Z. mobilis* alcohol dehydrogenase 2 or *S. cerevisiae* alcohol dehydrogenase 4. A theoretical secondary structure prediction using the methods of Novotny and Auffrey (1984) predicted that βαβ structures which resemble coenzyme binding folds may be found in *S. cerevisiae* alcohol dehydrogenase 4, *B. methanolicus* methanol dehydrogenase and *E. coli* fermentative alcohol dehydrogenase but not the other proteins of this group (Arfman, 1991).

(c) Metal binding

Although this group has become known as the "iron-activated" alcohol dehydrogenases the presence of iron has been identified unambiguously (by atomic absorption spectrophotometry) only in *Z. mobilis* alcohol dehydrogenase 2 (Neale *et al.*, 1986). Furthermore, *S. cerevisiae* alcohol dehydrogenase 4 and *B. methanolicus* methanol dehydrogenase do not contain iron, but atomic absorption spectrophotometry revealed that both enzymes possess one zinc atom per subunit and the latter enzyme contains 1-2 magnesium atoms also (Drewke and Ciriacy, 1988; Vonck *et al.*, 1991; Arfman, 1991). Histidine and cysteine residues have been implicated as metal ion binding ligands (Jornvall *et al.*, 1987) and three of the four conserved histidines lie between residues 250 and 280 in these enzymes, so they may be the iron binding ligands (Youngleson *et al.*, 1989; Arfman, 1991).

(d) Substrate specificity

The enzymes are all oxidise lower primary alcohols (Table 1.7). *Z. mobilis* alcohol dehydrogenase 2 is highly substrate specific, oxidising only ethanol; ethanol activates both its own oxidation and acetaldehyde reduction (Neale *et al.*, 1986). *C. acetobutylicum* alcohol dehydrogenase is the only NADP-dependent enzyme in this

group and oxidises ethanol, propan-1-ol and butan-1-ol (Youngleson *et al.*, 1989). The substrate specificity of *S. cerevisiae* alcohol dehydrogenase differs from that of the three zinc-dependent alcohol dehydrogenases in the organism in that it does not use butan-1-ol as substrate, only ethanol, propan-1-ol and allyl alcohol (Williamson and Paquin, 1987). *B. methanolicus* methanol dehydrogenase is unusual in that it is the only methanol oxidising enzyme in this group (Arfman et *al.*, 1989). Propanediol dehydrogenase of *E.coli* oxidises 1,x diols in preference to ethanol (Sridhara *et al.*, 1969) but its fermentative alcohol dehydrogenase oxidises ethanol, propan-1-ol and butan-1-ol as well as having a CoA-linked acetaldehyde dehydrogenase activity (see Section 5.5.1).

Table 1.7. Properties of "iron-activated" alcohol dehydrogenases

The metal ion present is given only if it has been unambiguously identified. Only those substrates oxidised are listed. The abbreviations used are taken from Table 1.5. The data are taken from the references listed: ZMADH2, Wills *et al.* (1981), Neale *et al.* (1986); CAADH, Youngleson *et al.* (1988); ECPOR, Sridhara *et al.* (1969), Conway and Ingram (1989); ECADHE, Goodlove *et al.* (1990); Clark (1992); MDH, Arfman *et al.*, (1989), Arfman (1991); SCADH4, Williamson and Paquin (1987), Drewke and Ciriacy (1988).

Enzyme	Metal	Coenzyme	Substrates	Function	Structure
ZMADH2	Fe	NAD	ethanol	fermentative	4 x 40 100
CAADH	?	NADP	ethanol, propan-1-ol butan-1-ol	fermentative	? x 43 000
ECPOR	?	NAD	propan-1,2-diol ethanediol, glycerol, ethanol	fermentative and catabolic	? x 40 600
ECADHE	?	NAD	ethanol, propan-1-ol butan-1-ol	fermentative	2 x 96 000
MDH	Zn and Mg	NAD	lower primary alcohols, propan-1,3-diol	fermentative	10 x 43 000
SCADH4	?	NAD	ethanol, propan-1-ol allyl alcohol	catabolic	2 x 40 000

1.3.4. Other NAD(P)-dependent alcohol dehydrogenases

Many other NAD(P)-dependent alcohol dehydrogenases have been purified and partially characterised, although to varying extents, particularly enzymes from micro-organisms. Most of these enzymes have a relatively narrow substrate and inducer specificity but there is considerable variety in the alcohols that different enzymes can oxidise.

An attempt has been made to classify enzymes by their substrate specificity (I.U.B.-I.U.P.A.C., 1979). However, enzymes with very different structural properties can utilise similar substrates (see Introduction 1.3.1, 1.3.2 and 1.3.3 for examples), therefore such groupings do not reflect genuine structural and evolutionary relationships amongst the enzymes.

The major types of substrate specificity are:

(a) Preference for primary aliphatic alcohols.

Often fermentative enzymes show a preference for primary alcohols (for examples see Tables 1.4 and 1.7). However, few enzymes oxidise only primary aliphatic alcohols but an example of an enzyme with such a substrate specificity is NADP-dependent primary alcohol dehydrogenase of *Thermoanaerobacter ethanolicus* (Bryant *et al.*, 1988).

(b) Oxidation of secondary aliphatic alcohols only

Examples of this class include: secondary alcohol utilising alcohol dehydrogenases of bacteria that use C1 compounds (Patel *et al.*, 1978); NADP-dependent secondary alcohol dehydrogenase of *Thermoanaerobacter ethanolicus* (Bryant *et al.*, 1988); and some yeast NAD-dependent secondary alcohol dehydrogenases (Patel *et al.*, 1979).

(c) Preference for secondary aliphatic alcohols

. Examples include the thermostable secondary alcohol dehydrogenase of *P. fluorescens* (Hou *et al.*, 1983) and an alcohol dehydrogenase of *Comamonas terrigena* which prefers *L*-stereoisomers of secondary alcohols (Barrett *et al.*, 1981).

(d) Oxidation of primary and secondary alcohols

Examples include NAD-dependent alcohol dehydrogenase of the thermophilic archeabacterium *Sulfolobus solfataricus* (Rella *et al.*, 1987) and an alcohol dehydrogenase from the white rot fungus *Sporotrichium pulverulentum* (Rudge and Bickerstaff, 1986).

(e) Preference for aromatic alcohols

Some of these enzymes also oxidise aliphatic alcohols whereas others do not. Examples include coniferyl alcohol dehydrogenase of *Rhodococcus erythropolis* (Jaeger *et al.*, 1981) and ρ-hydroxybenzyl alcohol dehydrogenase of *Rhodopseudomonas acidophila* (Yamanka and Minoshima, 1984).

More than one type of alcohol dehydrogenase can be present in the same organism e.g. humans, yeasts (Sections 1.3.1 and 1.3.2; Section 1.9) and bacteria such as acinetobacters (Section 1.8). In micro-organisms especially, the ability to express several types of alcohol dehydrogenases may increase the number of potential carbon sources, or, in the case of fermentative enzymes, allow them to grow anaerobically, therefore permitting them to live under a wider variety of environmental conditions.

1.4. NAD(P)-independent alcohol dehydrogenases

(a) Structure

The methanol dehydrogenases of the Gram negative methylotrophic bacteria are the best studied NAD(P)-independent alcohol dehydrogenases. The primary structures for methanol dehydrogenase from *Paracoccus denitrificans* (Harms *et al.*, 1987) and *Methylobacterium organophilum* (Machlin and Hanson, 1988) have been deduced from their DNA sequences. Both enzymes contain 599 amino acid residues per subunit with a M_r of about 66 000. There is extensive homology between the two enzymes at the DNA level (up to a maximum of 82 % in sections), and an even more pronounced homology between the amino acid sequences. Enzymes from both species are synthesised with

signal peptides which are apparently cleaved between alanine and asparagine residues. There is a 32-residue segment near the *C*-terminus of the *Methylobacterium organophilum* enzyme which may anchor it to the outer face of the inner bacterial membrane (Machlin and Hanson, 1988). No homologies have been reported between these enzymes and any NAD(P)-dependent alcohol dehydrogenases.

Most methanol dehydrogenases are thought to be dimers of two identical (α) subunits with M_r values of between 60 000 and 67 000, each containing one molecule of quinoprotein (Anthony, 1986). However, the methanol dehydrogenases of *Methylobacterium extorquens* AM1 and other bacteria are reported to be tetramers with two α subunits of M_r value 66 000 to 67 000 and two much smaller, identical β subunits of M_r value 8 500 (Anderson and Lidstrom, 1988; Nunn and Anthony, 1988; Nunn *et al.*, 1989) but the role of the β subunits is unknown. Other quinoprotein alcohol dehydrogenases have been reported with more diverse tertiary and quarternary structures. For example, alcohol dehydrogenase of *P. aeruginosa* is a monomer of M_r value 101 000 (Groen *et al.*, 1984), and a membrane bound alcohol dehydrogenase in *Acetobacter polyoxygenes* has two different subunits of M_r 72 000 and 44 000 (Tayama *et al.*, 1989).

(b) Cofactors

The vast majority of NAD(P)-independent alcohol dehydrogenases identified so far have been quinoproteins, located in the periplasmic space of Gram negative bacteria (Vries *et al.*, 1990). There is some evidence for the presence of a quinoprotein in a multienzyme complex which is involved in methanol oxidation in the Gram positive organism *Nocardia sp.* strain 239 and which has activities for NADH, formaldehyde and methanol oxidation (Duine *et al.*, 1984).

Also, NAD(P)-independent alcohol dehydrogenases interact with special cytochromes in the electron transport chain, the conversion of methanol to formaldehyde being tightly linked to energy generation by electron transfer from quinoprotein to oxygen via cytochrome c_L, additional electron carriers and a terminal oxidase (Vries *et al.*, 1990).

(c) Effector molecules

A heat stable, low molecular weight and oxygen labile factor X has been reported to activate methanol dehydrogenase of *Hyphomicrobium* X when assayed under physiological conditions and this factor may be an *in vivo* activator of the enzyme (Dijkstra *et al.*, 1985). In addition, a modifier protein has been reported to decrease the affinity of methanol dehydrogenase of *Methylobacterium extorquens* and *Methylophilus methylotrophus* for formaldehyde (Ford *et al.*, 1985; Page and Anthony, 1986). An activator protein has been identified in a number of thermotolerant methanol-utilising *Bacillus* strains and it has has been purified from *Bacillus* strain C1 and characterised (Arfman , 1991). The activator protein is a dimer of subunit M_r value 27 000, and in the presence of the activator protein and Mg^{2+} ions, methanol dehydrogenase displays biphasic kinetics towards alcohol substrates and NAD (Arfman *et al.*, 1991). From these data it has been suggested that, in the presence of the activator protein and Mg^{2+} ions, the enzyme possesses a high affinity active site for alcohols and NAD as well as having a low affinity, activator protein and Mg^{2+} independent, active site (Arfman ., 1991).

(d) Substrate specificity

Most quinoprotein methanol dehydrogenases oxidise a broad range of primary alcohols and some oxidise secondary alcohols or lower aldehydes also (Patel *et al.*, 1978; Arfman, 1991) and these enzymes have been implicated in methanol utilisation in both methylotrophic and other bacteria. Although the most studied NAD(P)-independent alcohol dehydrogenases oxidise methanol, this is not always the case. Some enzymes have been purified which do not oxidise methanol but, other than this, show a similar range of substrate specificities to methanol dehydrogenases (Groen *et al.*, 1984; Tayama *et al.*, 1989).

1.5. Alcohol oxidases

Alcohol oxidases have been identified predominantly in a number of methylotrophic yeasts and moulds (Veenhuis *et al.*, 1983). These enzymes differ from alcohol

dehydrogenases in that they catalyse the irreversible oxidation of alcohol to aldehyde, oxygen being converted to hydrogen peroxide in the process. Also, alcohol oxidases are flavoproteins, are located in peroxisomes and are present in high amounts during growth on methanol.

(a) Structure

The primary structures of methanol oxidase of *Hansenula polymorpha* (Ledeboer *et al.*, 1985) and two alcohol oxidases of *Pichia pastoris* (Koutz *et al.*, 1989) have been deduced from their DNA sequences. The two *P. pastoris* enzymes are very similar, sharing 97 % identity (Koutz *et al.*, 1989). Comparison of the *P. pastoris* enzymes with methanol oxidase of *H. polymorpha* reveals 76 % amino acid identity (Cavener and Krasney, 1991). The only reported homology between these enzymes and any other enzyme is with the flavoenzyme glucose dehydrogenase of *Drosophila melanogaster*, 26 % identity being observed between any of the alcohol oxidases and the *Drosophila* enzyme (Cavener and Krasney, 1991). The alcohol oxidases do not have any obvious *N*-terminal signalling sequence for secretion unlike glucose dehydrogenase which has an extra 57, predominantly hydrophobic, amino acid residue extension at its *N*-terminus (Cavener and Krasney, 1991).

Most alcohol oxidases so far identified are octamers of subunit M_r 80 000, although in a few cases much smaller enzymes have been reported (Veenhuis *et al.*, 1983).

(b) Cofactors

Each of the identical subunits of alcohol oxidases contains one non-covalently bound FAD as its prosthetic group and flavin semiquinones have been reported in some methanol oxidases e.g. in *H. polymorpha* (Tayrien *et al.*, 1980) The FAD binding region is thought to be near the *N*-terminus, and some residues in this domain are highly conserved. Secondary structural analyses of this region reveals that it corresponds to a βαβ segment of the known FAD binding domain (Cavener and Krasney, 1991) and is therefore similar to the Rossman fold of other nucleotide binding domains (Thieme *et al.*, 1981; Wierenga *et al.*, 1986).

(c) Substrate specificity

Most known alcohol oxidases oxidise methanol and other lower primary alcohols (ethanol, propan-1-ol and butan-1-ol). In some cases, an ability of the enzyme to utilise unsaturated lower primary alcohols has been reported (Sahm and Wagner, 1973; Kato *et al.*, 1976;). Little or no activity is observed with lower secondary alcohols or benzyl alcohol. Reported K_m values for methanol are all in the low mM range but direct comparison of kinetic constants cannot be made due to the variety of different ways in which different enzymes have been assayed (Veenhuis *et al.*, 1983). Also, two veratryl alcohol oxidases which are thought to belong to this group have been purified from the white rot fungus *Pleurotus sajor-caju* and partially characterised (Bourbonnais and Paice, 1988). These enzymes oxidise some aromatic alcohols but not aliphatic alcohols (Bourbonnais and Paice, 1988). They are thought to be involved in lignin degradation, generating H_2O_2 necessary for activity of lignin peroxidase, the first enzyme in the lignin degradation pathway (Bourbonnais and Paice, 1988; Kirk and Farrell, 1987).

Few kinetic studies have been carried out on the second substrate of the enzyme, oxygen. It is thought that alcohol oxidases in methylotrophic yeasts have a low affinity for oxygen and *in vivo* the enzyme probably works outside the range of O_2 concentrations for which it has maximal activity. The hydrogen peroxide produced in the reaction must be decomposed immediately otherwise it inhibits the enzyme by oxidising its thiol groups. *In vivo*, catalase decomposes the hydrogen peroxide and catalase levels generally parallel methanol oxidase levels (Sahm, 1975).

1.6. Physiological roles of alcohol oxidation enzymes

Alcohol oxidation enzymes have been implicated in many roles in a wide variety of both prokaryotic and eukaryotic organisms.

Enzymes that play a fermentative role act as aldehyde reductases, at the same time regenerating NAD(P) which is vital for further substrate conversion and for ATP generation and many such enzymes have been identified (see Tables 1.4 and 1.7). The

"iron-activated" and the zinc-dependent alcohol dehydrogenases of *Z. mobilis* provide interesting examples of this type of enzyme because only one of them is synthesised at any one time, and which one it is depends on the availability of zinc and iron (MacKenzie *et al.*, 1989). Such an adaptation allows this bacterium to survive in a wider range of environments than it otherwise could. Many alcohol dehydrogenases are involved in degradative roles. Enzymes play a direct role in breaking down different alcohols. Examples of enzymes that oxidise methanol and/or degrade other primary alcohols include the alcohol oxidases of methylotrophic yeasts (Introduction 1.5), propanediol oxidoreductase of *E. coli* (Sridhara *et al.*, 1969) and ethanol dehydrogenase of *A. calcoaceticus* (Fixter and Nagi, 1984). Aromatic alcohol degrading enzymes induced by growth on aromatic alcohols are present in many micro-organisms such as pseudomonads and acinetobacters (Shaw and Harayama, 1990; Chalmers *et al.*, 1990). A number of degradative polyol and sugar dehydrogenases exist, such as ribitol dehydrogenase of *K. aerogenes* (Morris *et al.*, 1974) and glucose dehydrogenase of *B. megaterium* (Jornvall *et al.*, 1984). Hydroxysteroid dehydrogenases have been identified in both prokaryotes, such as bile acid 7-hydroxysteroid dehydrogenase of *Eubacterium sp.* strain VPI 12708 (Baron *et al.*, 1991), and eukaryotes, such as human placental 17β-hydroxysteroid dehydrogenase (Peltoketo *et al.*, 1988).

Also, enzymes may be involved further down the degradation pathways of compounds. For example benzyl alcohol dehydrogenase has been implicated in the metabolism of compounds related to mandelate (see Fewson, 1992 for review) and veratryl alcohol oxidase is involved in the biodegradation of lignin (Introduction 1.5). Aliphatic alcohol dehydrogenases have been implicated in metabolism of long chain alkanes in a number of bacteria such as in acinetobacters (Introduction 1.8).

Alcohol dehydrogenases are involved in a wide range of biosynthetic and other metabolic roles also. An alcohol dehydrogenase plays a role in vision by reducing retinal to retinol (Sund and Theorell, 1963). Human liver class 1 alcohol dehydrogenases have been implicated in a number of metabolic processes on the basis of their expression, substrate specificity and kinetics: oxidation/reduction of glycols in noradrenaline

metabolism (Mardh *et al.*, 1985); steroid metabolism (McEvily *et al.*, 1988); and oxidation/reduction of dopamine alcohols/aldehydes in dopamine metabolism (Mardh and Vallee, 1986). Liver class 2 and class 3 enzymes are inhibited by testosterone and its metabolically active metabolite 5α-dihydrotestosterone and seem to have some function in steroid biosynthesis also (Mardh *et al.*, 1986). All three classes of enzyme have been implicated in the shunt pathway of mevalonate metabolism, where they are thought to oxidise geraniol, farnesol and their pyrophosphate derivatives (Keung, 1991).

In plants, a number of fermentative alcohol dehydrogenases have been identified in different tissues (Wolyn and Jelenkovic, 1990). However, alcohol dehydrogenases in potato tubers are induced by a number of other stresses, such as treatment with salicylic acid, arachidonic acid and ultra-violet light (Matton *et al.*, 1990). Such treatments are believed to mimic the effects of pathogenic attack on the plant, causing its defence mechanisms to be induced (Matton *et al.*, 1990). The only prokaryotic enzyme known to respond to stresses other than anaerobiosis is the iron-activated alcohol dehydrogenase 2 of *Z. mobilis*, its synthesis increasing on exposure to ethanol or increased temperature (An *et al.*, 1991). Monoterpene alcohol dehydrogenases in plants and bacteria are involved in terpenoid biosynthesis (e.g. acyclic monoterpene primary alcohol dehydrogenase of *Rauwolfia serpentina*; Ikeda *et al.*, 1991). Long (> C10) chain alcohol dehydrogenases have been implicated in the synthesis and degradation of lipids such as long chain alcohols and wax esters in mammals, plants and micro-organisms (Sargent *et al.*, 1976; Tulloch, 1976; Mahadevan, 1978), alcohol dehydrogenases and aldehyde dehydrogenases are believed to catalyse the interconversion of fatty alcohols and acids in this pathway (for example see Introduction 1.8).

In bacteria, alcohol dehydrogenases have been implicated in other biosynthetic processes such histidine biosynthesis in *Salmonella typhimurium*, histidinol dehydrogenase oxidises histidinol, via histidinal, to histidine (Loper and Adams, 1965). This enzyme is very different from other alcohol dehydrogenases because it catalyses the oxidation of histidinol to histidine, via histidinal (probably bound as a thiohemiacetal), using two molecules of NAD^+ i.e. transferring four electrons (Grubmeyer, 1991).

1.7. Biotransformations of potential industrial significance involving alcohol oxidation enzymes

Many examples exist of the use of biological routes in the production and transformation of aldehydes, ketones and alcohols, using isolated enzymes or whole cell systems from animal, plant or microbial sources. The opportunities available in this area are huge, with many systems which could be profitably exploited to yield products that are often stereospecific.

The advantages of using whole cell or isolated enzyme systems has been discussed in detail (Klibanov, 1983; Hartimer, 1985; Butt and Roberts, 1987) and it is possible to make a few generalisations to summarise the arguments for each system: many step transformations, such as the production of ethanol from cellulose, require different enzymes acting in sequence, and cofactor regeneration is necessary and therefore, the use of whole cells is advantageous for such processes; the use of isolated enzymes is generally superior in one or two step transformations because this avoids problems of sterility, lysis and competing reactions.

(a) Whole cells

Long chain primary alcohols and simple waxes (esters of long chain primary alcohols and acids) are produced in animals, plants and micro-organisms. Such compounds used to be obtained from sperm whales and are currently obtained from plants such as jojoba (Tulloch, 1976). Patents have been filed for the generation of waxes from the bacterium *Acinetobacter sp.* HO1-N, and some mutants of the organism that degrade waxes more slowly than the wild type, grown on ethanol or alkanes (Niedleman and Geigert, 1983; Neidleman and Ervin, 1986).

Production of secondary alcohols from asymmetrical ketones often yields an optically active product and the major enantiomer formed can be predicted from Prelog's rule (Prelog, 1964; Figure 1.4). For example bicycloheptanones are converted to the equivalent alcohols on incubation with yeast, the predominant enantiomer (in > 80 % enantiomeric excess) having the *S* configuration at the newly formed chiral centre (Butt

et al., 1985).

Some diketones can be reduced to yield ketals or diols in a good yield with high optical purity using Baker's yeast (Figure 1.5; Butt and Roberts, 1986; Jones, 1986). For example, ethyl acetoacetate is converted to the S isomer of ethyl-3-hydroxybutyrate in 98 % enantiomeric excess by Baker's yeast (Boccu *et al.*, 1990).

Few examples exist of the use of whole cells in aromatic alcohol biotransformation. Some micro-organisms have been used in detoxification of industrial waste water such as *Rhodotorula muculaginosa* which has been used to produce benzyl alcohol from benzaldehyde (Wisnieski *et al.*, 1983). Some methylotrophs grown on alkanes produce various aromatic alcohols and aldehydes (Higgins *et al.*, 1980).

Both microbial and plant cell cultures have been used in transforming hydroxysteroids. *Mycobacterium fortuitum* has been used to convert steroids to their andosterdione derivatives (Carrera *et al.*, 1984) and a *Digitalis purpurae* cell suspension has been used in conversions involving progesterone, its derivatives and its precursors (Stephen and Dalton, 1987; Minnikin *et al.*, 1982).

Biotransformations can be carried out on many other different, often complex, alcohols such as terpenes e.g. *Cannabis sativa* oxidises geraniol and nerol to their respective citral isomers (Itokawa *et al.*, 1976).

Most research with whole cells has concentrated on the reduction of ketones or aldehydes to stereospecific alcohols, usually using yeast cells, particularly baker's yeast because they are easily available. Often, syntheses involve growing organisms on simple carbon sources so that they can use the "energy" generated to effect a desired transformation, this process is known as co-metabolism.

(b) Isolated enzymes

Horse liver alcohol dehydrogenase is widely used in biotransformations (Jones, 1985). It operates on a wide range of structurally different substrates. Yeast alcohol dehydrogenase has a much narrower substrate specificity, being able to accept only a hydrogen atom or methyl group as the smaller "side chain" to the alcohol/carbonyl group. Both enzymes follow Prelog's Rule (Prelog, 1964; Figure 1.4) in the production of

stereospecific compounds. More recently other alcohol oxidation enzymes have been utilised in biotransformations such as *T. brockii* alcohol dehydrogenase which shows an interesting substrate-size induced reversal of stereospecificity, reducing smaller ketones (methyl ethyl, methyl isopropyl and methyl cyclopropyl ketones) to *R* alcohols but higher ketones to *S* alcohols (Keinan *et al.*, 1986), and *P. pastoris* alcohol oxidase, the enzymes properties in a two-phase system have been examined and differ from the properties in aqueous phase only, the enzyme being able to use longer chain alcohols in a two phase system only (Murray and Duff, 1990).

Many NAD(P)-dependent aromatic alcohol dehydrogenases have been purified from microbial sources (See Introduction 1.3.4 for examples) but their potential for biotransformations has been little-exploited. A number of hydroxysteroid dehydrogenases are commercially available and have been used in a variety of biotransformations (e.g. Klibanov *et al.*, 1982). Glycerol dehydrogenases from bacterial sources produce stereospecific products according to Prelog's Rule (1965), for example stereospecific diols have been obtained using NADP-dependent glycerol dehydrogenase from *Aspergillus niger* (Yamada *et al.*, 1982). Plant-derived enzyme work has revolved around the use of cell-free extracts to elucidate the metabolic pathways for the production of terpenoids, alkaloids and steroids (Anderson *et al.*, 1985).

When using isolated enzymes it is usually necessary to regenerate cofactor by chemical, electrochemical or enzymic means (Bowen and Pugh, 1985). These examples of biotransformations using isolated enzymes illustrate the wide variety of compounds of interest to chemists that can be generated by biological routes using alcohol oxidation enzymes in both oxidative and reductive reactions and serves to demonstrate the future potential in this field.

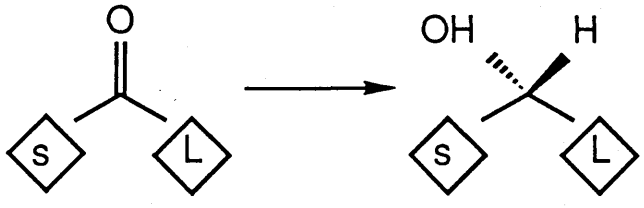


Figure 1.4. Prelog's Rule

Determination of the stereospecificity of the main product of reduction of an asymmetric ketone or aldehyde (Prolog, 1965). "L" represents the larger group; "s" represents the smaller group.

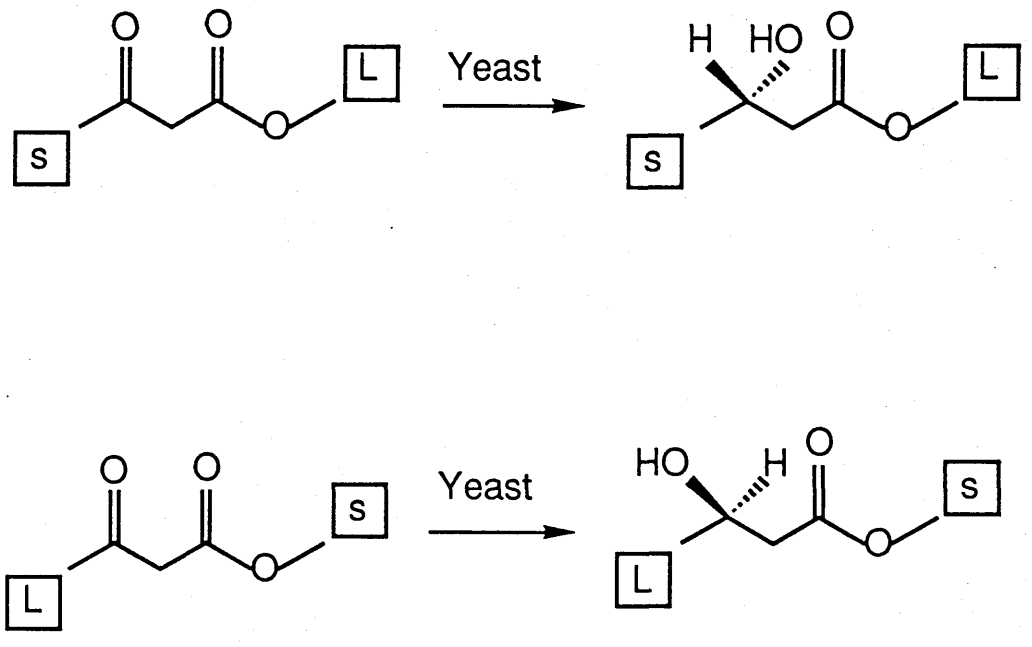


Figure 1.5. Reduction of ketones catalysed by yeasts

Reductions of ketones by yeasts yielding optically active products as shown in this scheme. "L" represents the larger group; "s" represents the smaller group.

1.8. NAD(P)-dependent alcohol dehydrogenases in *Acinetobacter calcoaceticus*

The genus *Acinetobacter* is a group of non-motile, oxidase negative, Gram negative organisms, lacking in pigmentation, that belong to the family *Neisseriaceae* (Towner *et al.*, 1991). A classification of *Acinetobacter* based on 16S rRNA sequencing has been proposed (Bouvet and Grimont, 1986) and results in less ambiguous identification of the genus than older classifications. These organisms are present throughout the environment, are often resistant to antibiotics, can cause nosocomial infections and have been implicated in food spoilage processes (Towner *et al.*, 1991). Of more interest biochemically, is the fact that they can grow on a wide variety of carbon sources, although not generally carbohydrates.

Alcohol dehydrogenases have been identified in different strains of *A. calcoaceticus* (Table 1.8). Constitutive, NADP-dependent, aliphatic alcohol dehydrogenases have been identified in four different strains. The enzyme has been partially purified from *A. calcoaceticus* strain 69V and is reported to have a M_r of 235 000 and to oxidise alcohols with from two to ten carbons (Tauchert *et al.*, 1976). The enzyme from strain NCIB 8250 is reported to have a K_m value of 17 mM for butan-1-ol as substrate in crude extract (Fixter and Nagi, 1984). NADP-dependent alcohol dehydrogenase of *Acinetobacter sp.* HO1-N (*A. calcoaceticus* ATCC 14987) was first identified by Singer and Finnerty (1985) but more recently Fox *et al.*, (1990) have reported that two distinguishable enzyme activities exist in this strain. Ethanol induced NAD-dependent alcohol dehydrogenases have been identified in strain HO1-N and strain NCIB 8250 (Singer and Finnerty, 1985; Fixter and Nagi, 1984). In addition a NAD-dependent butan-2,3-diol dehydrogenase activity, induced by growth on butan-2,3-diol has been identified in strain NCIB 8250 (Fewson, 1966). This enzyme is believed to be involved in the metabolism of butan-2,3-diol via the butan-2,3-diol cycle, where it oxidises butan-2,3-diol to acetyl methyl carbinol (Juni, 1978). The other stages of the cycle convert the acetyl methyl carbinol back to butan-2,3-diol via a thiamin

pyrophosphate derivative of methyl carbinol, diacetyl methyl carbinol and acetyl butanediol (Juni, 1978). Three molecules of NADH are generated per molecule of butan-2,3-diol that undergoes this cycle (Juni, 1978).

A number of acinetobacters grow on alkanes (Asperger and Kleber, 1991). The alkanes are oxidised to alcohols by either a rubredoxin-dependent, or a cytochrome P450-dependent alkane monoxygenase, alcohols being converted via aldehydes to fatty acids which can be further metabolised by β oxidation [Figure 1.6 (a)]. A NAD-dependent hexadecan-1-ol dehydrogenase exists in strain HO1-N which is induced by growth on hexadecan-1-ol has been implicated in this pathway (Singer and Finnerty, 1985).

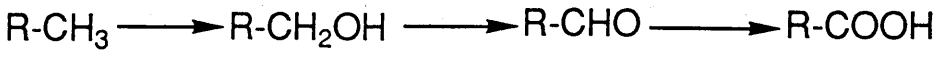
Another metabolic pathway in which alcohol dehydrogenases may be involved is that of wax ester metabolism. Acinetobacters accumulate simple wax esters under certain conditions (Fixter and Sherwani, 1991). This is unusual for two reasons. Firstly, wax metabolism is very uncommon in prokaryotes. Secondly, simple wax esters (monoesters of long chain primary aliphatic alcohols and acids) are rarely seen in nature, complex wax esters (mono- or diesters in which the acid and/or the alcohol moiety have more complex structures) being much more common (Mahadevan, 1978). Simple waxes have been detected only in strains of *Corneybacterium*, *Moraxella*, *Neissera* and *Acinetobacter* (Bryn and Jantzen, 1977). The pathway of wax ester metabolism that is thought to occur in *Acinetobacter*, based on studies in *Acinetobacter* and other organisms, is shown in Figure 1.6 (Fixter and Sherwani, 1991). Under conditions of nitrogen limitation fatty acids, either as their CoA or ACP derivatives, are reduced via aldehydes to long chain (fatty) alcohols. The alcohols are then esterified with more fatty acids yielding the wax esters which are stored in vesicles (Fixter and Sherwani, 1991). Under conditions of carbon limitation wax esters are broken down into their acid and alcohol moieties. The alcohols are oxidised to produce more fatty acids which are then oxidised yielding energy and reducing power (Fixter and Sherwani, 1991). Wax esters are thought to be energy reserve compounds in this organism (Fixter and Sherwani, 1991). Alcohol dehydrogenase(s) has(have) been implicated in the interconversion of fatty alcohols and aldehydes in this pathway and it has been suggested that the constitutive NADP-

dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 is involved at this step, although there was no direct evidence for or against its involvement (Fixter and Nagi, 1984).

The only aromatic alcohol dehydrogenase identified in any strain of *A. calcoaceticus* is benzyl alcohol dehydrogenase of strain NCIB 8250. This enzyme has been purified and partially characterised and on the basis of its *N*-terminal sequence been identified as a member of the zinc-dependent alcohol dehydrogenases (see Introduction 1.3.1). It is believed to play a role in degradation of aromatic compounds.

In addition, membrane-bound NAD(P)-independent alcohol dehydrogenase activities have been identified in crude extracts of two different strains of *A. calcoaceticus*, strain 69-V (Tauchert *et al.*, 1975) and HO1-N (Singer and Finnerty, 1985). Both enzyme activities were not obviously induced and were assayed using ethanol or octan-1-ol as substrates and the dye DCIP as cofactor, although the cofactor(s) used by the enzyme *in vivo* is(are) not known (Tauchert *et al.*, 1975; Singer and Finnerty, 1985).

(a)



(b)

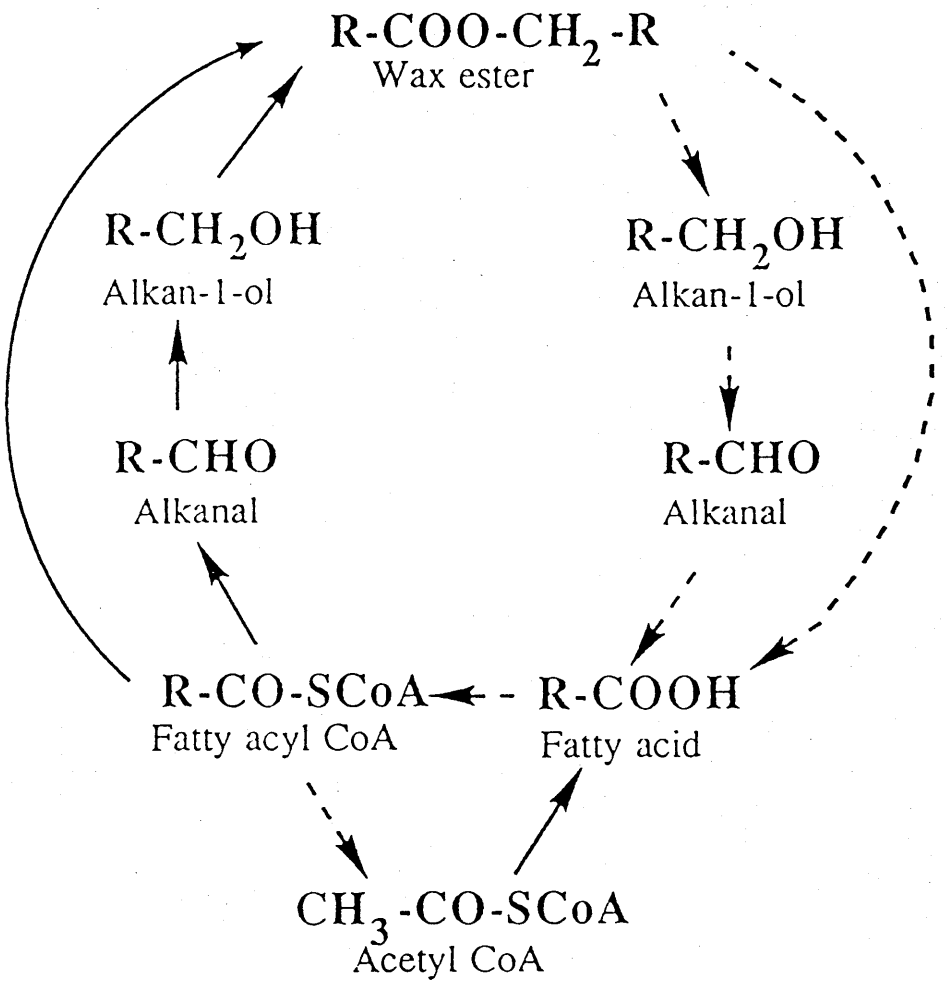


Figure 1.6. Metabolism of alkanes and waxes by *Acinetobacter*

(a) Pathway of alkane metabolism in *Acinetobacter*, taken from Asperger and Kleber (1991); (b) Proposed pathway of wax ester metabolism in *Acinetobacter*, taken from Fixter and Sherwani (1991).

Table 1.8. Soluble NAD(P)-dependent alcohol dehydrogenases in strains of *Acinetobacter calcoaceticus*

References: (1) Fixter & Nagi (1984); (2) Fewson (1966); (3) MacKintosh & Fewson (1988); (4) Tauchert *et al.* (1976); (5) Jirausch *et al.* (1986); (6) Singer & Finnerty (1985).

Strain	Coenzyme	Induction	Activity (substrate) [nmol min^{-1} (mg protein)$^{-1}$]	Reference
NCIB8250	NADP	Constitutive	22 (butan-1-ol)	(1)
	NAD	Ethanol	609 (ethanol)	(1)
	NAD	Butan-2,3-diol	2644 (butan-2,3-diol)	(2)
	NAD	Benzyl alcohol	1500 (benzyl alcohol)	(3)
69V	NADP	Constitutive	7 (octan-1-ol)	(4)
EB104	NADP	Constitutive	24 (octan-1-ol)	(5)
HO1-N	NADP	Constitutive	11 (ethanol)	(6)
	NAD	Ethanol	93 (ethanol)	(6)
	NAD	Hexadecan-1-ol	6 (hexadecan-1-ol)	(6)

1.9. Alcohol dehydrogenases in *Saccharomyces cerevisiae*

The yeast *S. cerevisiae* has been studied in great detail in terms of its biochemistry, genetics and microbiology. Four different alcohol dehydrogenases have been identified in this organism and their genes have all been sequenced. From the deduced primary structures it appears that three of the enzymes belong to the zinc-dependent group of alcohol dehydrogenases (Types 1,2 and 3) whereas the fourth (type 4) is a member of the "iron-activated" group (Williamson and Paquin, 1987).

The classic type 1 yeast alcohol dehydrogenase is soluble and is a fermentative enzyme, reducing acetaldehyde to ethanol (Racker, 1955; Lutsdorf and Megnet, 1968). It is a tetramer of native M_r 140 000 to 150 000 (Table 1.9; Branden *et al.*, 1975) and is thought to contain two zinc atoms per subunit. The type 2 alcohol dehydrogenase is the oxidative isoenzyme of the type 1 enzyme and is repressed by fermentative growth. It is thought to oxidise some of the ethanol that is formed during fermentation (Fowler *et al.*, 1972). The subunits of the type 1 alcohol dehydrogenase can interact with those of the type 2 enzyme forming active, hybrid tetrameric enzymes (Ciriacy, 1975). Type 3 alcohol dehydrogenase is located in mitochondria (Sugar *et al.*, 1970). It appears to be the same native size as the type 1 and 2 enzymes but there is some question as to whether it is a homotetramer or a heterotetramer (Young and Pilgrim, 1985).The function of the enzyme is not known. There is 70 to 80 % sequence amino acid identity among the type 1, 2 and 3 alcohol dehydrogenases.

Type 4 alcohol dehydrogenase shows no sequence homology to any of the other yeast enzymes. On the basis of its primary structure, it appears to belong to the "iron-activated" group of alcohol dehydrogenases (Williamson and Paquin, 1987), although it has one zinc atom per subunit (Drewke and Ciriacy, 1988). Its properties have been described in Introduction 1.3.3.

All four enzymes use NAD as cofactor but have varying substrate specificities (Table 1.9; Williamson and Paquin, 1987) the type 2 enzyme oxidising the broadest range of substrates.

Table 1.9. Alcohol dehydogenases of *Saccharomyces cerevisiae*

The table shows the four alcohol dehydrogenases of *S. cerevisiae*, their subunit number and size and the substrates they are known to oxidise. All information is taken from the following papers: types 1 and 2, Branden *et al.* (1975); type 3, Young and Pilgrim (1985); type 4, Williamson and Paquin (1987).

ADH type	Subunits	Substrates
1	4 x 36 000 to 37 000	ethanol,propan-1-ol,butan-1-ol, allyl alcohol
2	4 x 36 000 to 37 000	ethanol, propan-1-ol, propan-2-ol, allyl alcohol, butan-1-ol
3	4 x 36 000 to 37 000 (?)	ethanol, propan-1-ol, allyl alcohol, butan-1-ol
4	2 x 40 000	ethanol, propan-1-ol, allyl alcohol

1.10. Aims and scope of this thesis

The original aims of this project were:

(1) to purify the NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250;

(2) to characterise the enzyme and to compare it with alcohol dehydrogenases in other organisms;

(3) to determine the physiological role of the enzyme.

Consequently, a method for the purification of the NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 was worked out and the enzyme was partially characterised. Some studies were carried out on the physiological role of the enzyme and these were inconclusive. Also, the enzyme of another strain of *A. calcoaceticus*, strain HO1-N, was purified and some of its properties compared to the enzyme of strain NCIB 8250. The enzyme of strain HO1-N was chosen for three reasons. Firstly, strain HO1-N has been studied in terms of its alcohol and aldehyde dehydrogenases in crude extract (Singer and Finnerty, 1985). Secondly, its metabolism of alkanes/ wax esters had been examined (Neidleman and Geigert, 1984). Thirdly, there were reports that the partially purified enzyme of strain HO1-N was much more kinetically efficient than that of strain NCIB 8250 (Fox *et al.*, 1990). During the course of the study it became apparent that similar NADP-dependent alcohol dehydrogenase activities existed in some quite diverse micro-organisms. Therefore the enzyme of a wild type yeast, *S. cerevisiae* strain D 273-10 B (ATCC 25657; Reid and Schatz, 1982) was purified, partially characterised and compared with the *Acinetobacter* enzyme.

There are five results and discussion chapters in this thesis. The first of these describes the purification of the enzyme of *A. calcoaceticus* NCIB 8250; the other purifications were based on this one and are described in another chapter. Similarly, the enzyme of strain NCIB 8250 was characterised first and to a greater extent than the others. Therefore it is described in a separate results chapter, and the characterisation of the other two enzymes is described in another chapter. The final results chapter considers

the physiological role of the enzyme in the light of its characterisation; the diversity of NADP-dependent alcohol dehydrogenases in micro-organisms is also shown in this chapter. Finally, conclusions are drawn from this work and further studies on NADP-dependent alcohol dehydrogenases in micro-organisms are suggested.

CHAPTER 2
MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

All chemicals used were of the best quality available and most of them were obtained from BDH Chemicals Ltd., Poole, Dorset, UK except for those listed below.

Acetaldehyde was from Aldrich Chemical Co., Gillingham, Dorset, UK.

[1-^{3}H]Glucose was from Amersham International, Little Chalfont, Bucks., UK.

Urea was from Bethesda Research Laboratories, Gaithersburg, MD, USA.

Bistris, DTT, NAD (free acid), NADH (disodium salt), NADP (disodium salt), NADPH (disodium salt) and Tris were from Boehringer Corp. Lewes, Sussex, UK.

Allyl alcohol and ammonium sulphate (specially low in heavy metals) were from Fisons Scientific Equipment, Loughborough, Leics., UK.

TFA and potassium dihydrogen orthophosphate used in determination of stereospecificity of hydride transfer experiments were from Fluka Chemicals Ltd., Glossop, Derbyshire, UK.

Glucose and ammonium sulphate were from Formachem (Research International) Ltd., Strathaven, Midlothian, UK.

Acetic acid, acrylamide, ethanediol, *N,N'*-methylene-*bis*-acrylamide, NaCl, orthophosphoric acid, SDS and sucrose were from FSA Laboratory Supplies, Loughborough, Leics., UK.

Ethanol was from James Burroughs (F.A.D.) Ltd., Witham, Essex, UK.

Silver nitrate was from Johnson Matthey, Royston, Herts., UK.

*cis*Hex-3-en-1-ol and hex-5-en-1-ol were from Lancaster Synthesis, Morcambe, Lancs., UK.

Polybuffer PBE 74 was from Pharmacia Ltd, Milton Keynes, Bucks, UK.

Methanol (h.p.l.c. grade) was from Romil Chemicals, Shepshed, Loughborough, Leics., UK.

Acetyl CoA, ADP (disodium salt), ATP (disodium salt), benzamidine, Bicine, Bistris,

Coomassie Brilliant Blue G250, decan-1-ol, *N*-ethylmaleimide, D,L-isocitrate, Mops, Nitro Blue Tetrazolium, sodium azide and Tween 20 were from Sigma Chemical Co., Poole, Dorset, UK.

2.1.2. Chromatography media

All chromatography media were from Pharmacia Ltd, Milton Keynes, Bucks., UK except Matrex Gel Red A and Matrex Gel Blue A which were from Amicon Ltd., Stonehouse, Glos., UK.

2.1.3. Proteins and enzymes

All proteins and enzymes used were from Sigma Chemical Co., Poole, Dorset, UK except for those listed below.

Pig heart lactate dehydrogenase, rabbit muscle pyruvate dehydrogenase, horse spleen ferritin and horse heart cytochrome *c* were from Boehringer Corp., Lewes, Sussex, UK.

Saccharomyces cerevisiae NAD-dependent alcohol dehydrogenase, bovine pancreas trypsin and bovine pancreas chymotrypsin were from BDH Ltd, Poole, Dorset, UK.

M_r standards for SDS-PAGE were from Pharmacia Ltd., Milton Keynes, Bucks., UK.

Bovine serum albumin powder (fraction V) was from Wilfred Smith Ltd, Edgeware, London, UK.

2.1.4. Miscellaneous materials

Centricon 10 and Centricon 30 microconcentrators and Centricon-Centriprep concentrators were from Amicon, Beverly, MA.

Nutrient broth, bacteriological peptone and yeast extract were from Oxoid, Basingstoke, Hampshire, UK.

Visking tubing (for dialysis) was from The Scientific Instrument Centre, Eastleigh,

Hants, UK.

Chromatography and filter papers were from Whatman International, Maidstone, Kent, UK.

2.2. General methods

2.2.1. pH measurements

The pH values of solutions were measured routinely with a Kent Electronic Instruments Ltd. (Chertsey, Surrey, UK) type 7010 meter. Volumes of < 2 ml (e.g. 1 ml assay mixtures) were measured with a Radiometer (Copenhagen, Denmark) type M26 pH monitor fitted with a GK 2302 micro pH electrode.

2.2.2. Conductivity measurements

The conductivities of solutions were determined with a Radiometer type CDM2e meter.

2.2.3. Glassware

Glassware was washed by immersion in a solution of approximately 1 % Haemo-sol [Alfred Cox (Surgical) Ltd., Coulsdon, Surrey, UK] according to the manufacturers instructions, and then rinsed thoroughly with tap water and then with distilled water.

Glassware for amino acid sequencing was washed in a boiling solution of 10 % (v/v) nitric acid for 30 minutes , then rinsed thoroughly in distilled water.

2.2.4. Dialysis

Dialysis tubing was prepared by boiling for 10 minutes in 1 % (w/v) EDTA, then

rinsed and boiled in distilled water three times for 10 minutes each and stored in 20 % (v/v) ethanol until use.

2.2.5. Protein estimation

Protein concentrations were determined using the method of Bradford (1976) using bovine serum albumin (Fraction V) to construct standard curves.

During enzyme purifications, the A_{280} values of column effluents were determined with an LKB (Milton Keynes, Bucks., UK) Uvicord 2138 monitor. The A_{280} values of effluents from f.p.l.c. (Pharmacia) columns were determined using a Pharmacia UV-1 monitor.

2.2.6. Concentration of protein samples

Unless otherwise stated, protein samples were concentrated using Centricon 10 or Centricon 30 microconcentrators and/or Centricon-Centriprep concentrators according to the manufacturer's instructions.

2.2.7. Lyophilisation

Frozen samples [with their tops covered in Nescofilm (Nippon Shoji Kaisha Ltd., Osaka, Japan) punctured with a needle] were placed in a dessicator attached to an Edwards modulo freeze dryer (Edwards High Vacuum Ltd., Crawley, Sussex, UK) and the dessicator evacuated with an Edwards High Vacuum Pump (Edwards).

2.2.8. Preparation of chromatography media

DEAE Sephacel, Sephacryl S300HR and Phenyl Sepharose were supplied pre-swollen and were poured directly into columns. Matrex Gel Red A, Orange A and Blue A

were washed with 0.1 M-NaOH/ 8 M-urea, following the manufacturers instructions, before use. All columns were equilibrated by washing with at least 10 column volumes of the appropriate buffer. DEAE Sephacel was regenerated after use by washing with 2 M-KCl, then 0.1 M-NaOH. Phenyl Sepharose was regenerated with 6 M-urea, and the Matrex Gel columns were regenerated after unpacking the column with 0.1 M-NaOH/ 8 M-urea. All columns were stored in 0.02 (w/v) % azide at 4 °C, except the f.p.l.c. columns which were stored in 20 % (v/v) ethanol at room temperature.

2.2.9. Buffers and solutions

All buffers were prepared at the temperature that the buffer would be used in by adjusting the pH values of approximately ten ninths strength solutions with the appropriate acid or base and then making to the final volume. All solutions were made using distilled water.

2.3. Sterilisation

2.3.1. Moist heat

Media were sterilised by autoclaving for the appropriate time (C.A. Fewson, unpublished results) and successful sterilisation was confirmed using a Browne's tube (Albert Browne Ltd., Leicester, UK).

2.3.2. Dry heat

Glass pipettes for inoculations were sealed in Kraft paper and heated in an oven at 160 °C for 1.75 hours and again successful sterilisation was confirmed using a Browne's tube. Plastic pipettes were sterilised using ethylene oxide (anprolene; H.W. Anderson Products Limited, Clacton-On-Sea, Essex, UK).

2.3.3. Filtration

Volatile or heat labile compounds, such as alcohols, were filtered through Millex-HV sterile filters (Millipore UK, Watford, UK).

2.4. Maintenance, growth, harvesting and disruption of micro-organisms

2.4.1. Source of micro-organisms

Acinetobacter calcoaceticus NCIB 8250, *Escherichia coli* ML 30, *Pseudomonas putida* 9494, *P. aeruginosa* 10548, *Rhodococcus rhodocrous* and *Bacillus subtilis* NCIMB 3610 were obtained from Professor C.A. Fewson, Department of Biochemistry, Glasgow University. *Acinetobacter sp.* HO1-N and its mutants were donated by Cetus Corporation, Emeryville, CA, USA., *Rhodoturula graminis* KGX 39 which was originally donated by Dr D.R. Durham, Genex Corporation, Gaithersburg USA, *Saccharomyces cerevisiae* D273-10B which was donated by Dr S.M. West, Department of Biochemistry, Glasgow University and *Streptomyces rimosus* 4018 which was donated by Ms. L. Drynan.

2.4.2. Maintenance of bacteria

Stock cultures of *A. calcoaceticus* NCIB 8250, *Acinetobacter sp.* HO1-N and its mutants, and *Escherichia coli* ML30 was maintained on at 4 ^{o}C in a complex medium as described by Allison *et al.* (1985). Stock cultures of *R. graminis* KGX 39 and *Bacillus subtilis* NCIMB 3610 were maintained as lyophilised cultures in a filter-sterilised mixture of three parts heat-inactivated horse serum to one part 30 % (w/v) glucose. Spores of *Streptomyces rimosus* 4018 were stored in sterilised, distilled water at -80 ^{o}C.*Rhodococcus rhodocrous* was maintained at 4 ^{o}C on a nutrient agar slope. *Pseudomanas putida* 9494 and *P. aeruginosa* 10548 were maintained at 4 ^{o}C on agar

slopes containing a defined medium as described by Murray *et al.* (1972). *Saccharomyces cerevisiae* D273-10B was maintained at 4 °C on a PYG agar slope.

2.4.3. Growth media

Minimal media

(a) Z1/succinate medium: KH_2PO_4 (2 g), $(NH_4)_2SO_4$ (1 g) and succinate (1.18 g) were dissolved in distilled water, the pH adjusted to 7.0 with NaOH and the medium made up to 1 litre. After autoclaving and cooling, 20 ml of sterile 2 % (w/v) $MgSO_4.7H_2O$ was added to the medium (Kennedy and Fewson, 1968).

(b) Z1/succinate, carbon-limited medium: medium was made up as in (a) except that only 0.118 g succinate was added per litre instead of 1.18 g. For continuous culture experiments 2 ml of a trace metal supplement [see (g) below] was added to the medium at the same time as the 20 ml of sterile 2 % (w/v) $MgSO_4.7H_2O$.

(c) Z1/succinate, nitrogen-limited medium: medium was made up as in (a) except that only 0.1 g $(NH_4)_2SO_4$ was added per litre instead of 1 g. For continuous culture experiments 2 ml of a trace metal supplement [see (g) below] was added to the medium at the same time as the 20 ml of sterile 2 % (w/v) $MgSO_4.7H_2O$.

(d) Z1/acetate medium: medium was made up as in (a) except that acetate (0.82 g l^{-1}) was added as sole carbon source instead of succinate.

(e) Glycerol/salts medium: KH_2PO_4 (5.4 g) and $(NH_4)_2SO_4$ were dissolved in distilled water, the pH adjusted to 7.0 with NaOH and the volume made up to 600 ml. Separately, glycerol (1.84 g) and $MgSO_4$ (0.13 g) were dissolved in distilled water, the pH adjusted to 7.0 and volume made up to 400 ml. Both solutions were sterilised

(Methods 2.3.1) then mixed and 12 ml of sterile 0.022 % (w/v) $FeSO_4$ was added also (Holms and Bennett, 1971).

(f) Modified Hobb's minimal medium: NaCl (5 g), Na_2SO_4 (5 g), KH_2PO_4 (2 g), Tris base (1.2 g) and MOPS (8.4 g) were dissolved in 300 ml distilled water and sterilised (Methods 2.4.3). Glucose (20 g); $MgSO_4.7H_20$ (2.5 g) and $ZnSO_4$ (0.1 g) were dissolved in 300 ml distilled water and sterilised (Methods 2.4.3). $(NH_4)_2SO_4$ (2.6 g) was dissolved in 300 ml distilled water and sterilised (Methods 2.4.3). All three solutions were mixed, made up to 1 l and 100 µl sterile trace metal supplement [as described in (g)] added aseptically (Hobbs *et al.*, 1989).

(g) Trace metal supplement for continuous culture experiments: Nitrilotriacetic acid (50 g) was added to 625 ml of 1 M-NaOH and the solution titrated to pH 7.0 with HCl. Each of the following were then added in order and allowed to dissolve before the addition of the next component: $FeSO_4.7H_2O$ (1.10 g); $Na_2MoO_4.7H_2O$ (50 mg); $ZnSO_4.7H_2O$ (50 mg); $CuSO_4.5H_2O$ (25 mg); $CoCl_2.6H_2O$. The solution was then made up to a final volume of 1 litre (Beggs and Fewson, 1977).

Complex media

(a) Oxoid nutrient broth: prepared according to the manufacturer's instructions.

(b) MMD medium: Oxoid nutrient broth (26 g), L-glutamic acid (0.9 g), KH_2PO_4 (2 g), $(NH_4)_2SO_4$ (1 g), and $MgSO_4.7H_2O$ (0.4 g) were dissolved in distilled water, the pH adjusted to 7.0 with NaOH and the solution made up to 1 litre. When the medium was used for large scale growth of cells (10 l or more), poly(propylene glycol) 2025 [0.005 % (w/v)] was used as an antifoam agent (after Barrowman, 1982).

(c) MMA medium: The medium was prepared as for MMD medium (a) except that

D,L-lactic acid (1.5 g l^{-1}) was included also.

(d) PYG medium: Oxoid bacteriological peptone (20 g) and Oxoid yeast extract (10 g) were dissolved in 900 ml distilled water and sterilised (Methods 2.3.1). Galactose (20 g) was dissolved in 100ml distilled water, filter-sterilised (Methods 2.3.3) and mixed with the peptone/yeast extract (Reid and Schatz, 1982).

(e) YEME medium: Difco yeast extract (3 g), Difco Bactopeptone (5 g), Oxoid malt extract (3 g), Glucose (10 g) and Sucrose (340 g) were dissolved in 1 l distilled water and sterilised (Methods 2.3.1) (Hopwood *et al.*, 1985).

2.4.4. Growth of micro-organisms

(a) Small scale growth of *A. calcoaceticus* NCIB 8250 *Acinetobacter sp.* HO1-N and its mutants

Stock culture (0.1 ml) was transferred aseptically to 50 ml of the appropriate medium (in a 250 ml flask) and shaken (200 r.p.m.) at 30 °C for 16 h (in complex media) or 24 h (in minimal media). Eight ml of this culture was then transferred aseptically to 400 ml of the same medium (in a 2 l flask) and shaken for 16 h (in complex media) or 24 h (in minimal media) at 30 °C before harvesting.

(b) Large scale growth of *A. calcoaceticus* NCIB 8250

Large scale quantities of *A. calcoaceticus* NCIB 8250 were grown on MMD medium in a 10 l fermenter fitted with a 20 l reservoir, using essentially the method of Allison *et al.* (1985). The inoculum used was a 400 ml culture prepared as described in Methods 2.4.4 (a). The fermenter (Braun Biostat V; F.T. Scientific Instruments, Tewksbury, Glos., UK) was operated at 30 °C with an aeration rate of 4 l of sterile air min^{-1} and stirring at setting 2.5 (approx. 250 r.p.m.). After growth for 20 h, 9.5 l of culture were removed for harvesting and 9.5 l of fresh medium transferred to the fermenter from the reservoir. After a further 20 h growth, 9.5 l of 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.

(c) Growth of A. calcoaceticus NCIB 8250 in continuous culture

A. calcoaceticus NCIB 8250 was grown in continuous culture by Mr M.J. McAvoy, Department of Biochemistry, University of Glasgow. The continuous culture apparatus used was based on that described by Baker (1968). A sterile syringe and needle was used to inoculate 5 ml of the inoculum into the bio-reactor through a septum in one of the ports. The culture was grown initially as a batch and then as a chemostat at various temperatures and growth rates. The medium used was either ammonia-limited, succinate-limited or oxygen limited. At least ten culture volumes were passed through the bio-reactor after changing a growth condition of the culture before harvesting. The bioreactor culture volume was approximately 700 ml.

(d) Large scale growth of *Acinetobacter sp.* HO1-N

Nutrient broth culture [80 ml; prepared as in (a)] was used to inoculate 4 l of nutrient broth (in a 10 l flask). The 4 l culture was grown for 20 h at 30 °C with stirring (using a magnetic stirrer) and aeration (at a rate of 500 ml min^{-1}).

(e) Growth of *E. coli* ML30

Cells were grown by Mrs M.A. Cowan, Department of Biochemistry, Glasgow University. Stock culture (0.1 ml) was transferred aseptically to 50 ml glycerol/salts medium and shaken (200 r.p.m.) for 16 h at 37 °C. One ml of this culture was then transferred aseptically to 250 ml of the same medium and shaken (200 r.p.m.) for 24 h at 37 °C. Four ml of this culture was then transferred aseptically to 1 l of the same medium and stirred (using a magnetic stirrer) with aeration (500 ml min^{-1}) for 24 h at 37 °C.

(f) Growth of *Pseudomonas putida* 9494 and *P. aeruginosa* 10548

Cells were grown by Mr A.J. Scott, Department of Biochemistry, University of Glasgow. A loopful of culture was transferred aseptically to 50 ml MMA (in a 250 ml flask) and shaken (200 r.p.m.) at 30 °C for 16 h. Eight ml of this culture was then transferred aseptically to 400 ml of the same medium (in a 2 l flask) and shaken for 24 h at 30 °C before harvesting.

(g) Growth of *B. subtilis* NCIMB 3610

Cells were grown by Mr A.J. Scott. The lyophilised culture was rehydrated with 10 ml sterile nutrient broth and grown for 16 h at 30 °C. A loopful of culture was plated out on a sterile nutrient agar plate which was then grown for 24 h at 30 °C. One colony was used to inoculate a further 10 ml sterile nutrient broth which was then grown for 24 h at 30 °C. Five ml of this culture was transferred aseptically to 500 ml sterile nutrient broth (in a 2 l flask) and shaken for 16 h at 30 °C

(h) Growth of *Rhodococcus rhodocrous*

A loopful of culture was transferred aseptically to 500 ml sterile nutrient broth and shaken (200 r.p.m.) for 72 h at 30 °C.

(i) Growth of *Streptomyces rimosus* 4018

Cells were grown by Ms L. Drynan, Department of Biochemistry, University of Glasgow. Spores suspended in distilled water (100 µl) were transferred aseptically to 1 l YEME medium in a 2 l flask and shaken (200 r.p.m.) for 72 h at 30 °C. Cells were harvested (Section 2.4.6) and 1 g pellet resuspended in a modified Hobb's minimal medium (100 ml) and shaken (200 r.p.m.) for 24 h at 30 °C.

(j) Small scale growth of *Saccharomyces cerevisiae* D273-10B

A loopful of culture was transferred aseptically to 50 ml PYG (in a 250 ml flask) and shaken (200 r.p.m.) at 30 °C for 20 h. 4 ml of this culture was then transferred aseptically to 400 ml of the same medium (in a 2 l flask) and shaken for 20 h at 30 °C before harvesting.

(k) Large scale growth of *Saccharomyces cerevisiae* D273-10B

Cells were grown by Mr A.J. Scott. 80 ml of nutrient broth culture [prepared as in (a)] was used to inoculate 4 l of nutrient broth (in a 10 l flask). The 4 l culture was grown for 20 h at 30 °C with stirring (using a magnetic stirrer) and aeration (at a rate of 500 ml min^{-1}).

(l) Growth of *Rhodotorula graminis* KGX 39

A lyophilised culture was rehydrated with sterile distilled water and sub-cultured onto Saboraud-dextrose agar and grown at 30 °C for 2 days. A loopful of culture was

aseptically transferred to 50 ml of nutrient broth (in a 250 ml flask) and shaken (250 r.p.m.) at 30 °C for 24 h. Eight ml of this culture was then transferred aseptically to 400 ml nutrient broth (in a 2 l flask) and shaken for 24 h at 30 °C before harvesting.

2.4.5. Optical density measurements

The optical densities of cultures were determined by measuring the OD_{500}, relative to the medium, with a LKB Ultrospec spectrophotometer. Samples were diluted with medium such that the OD_{500} was < 0.8.

2.4.6. Harvesting cells

All harvesting was carried out at 4 °C. Volumes of culture up to 50 ml were harvested by centrifugation at 10 000 r.p.m. for 20 min in a Mistral M.S.E. Highspeed 18 centrifuge (M.S.E. Ltd., London UK). Larger volumes were harvested at 5 000 r.p.m. for 20 minutes in an M.S.E. Mistral 6L centrifuge. Pellets were resuspended in a small volume of cold Z1 buffer [0.2 % (w/v) KH_2PO_4/0.1 % (w/v) $(NH_4)_2SO_4$/NaOH (pH 7.0)], combined and centifuged at 10 000 r.p.m. for 20 min in an M.S.E Highspeed 18 centrifuge. The supernatants were decanted and the pellets either used immediately or stored at -20 °C, unless stated.

2.4.7. Cell disruption

In all cases pellets were suspended in 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0.

(a) Ultrasonic disruption of bacteria

Ultrasonic disruption was carried out using a Lucas-Dawes (London, UK) soniprobe. An ice/water slurry was used to cool the soniprobe and the cell suspension.

Pellets of *A. calcoaceticus* NCIB 8250 were suspended in the appropriate buffer such that the equivalent OD_{500} > 20, and broken by 3 x 30 s periods of exposure to 80

W, alternating with 2 x 30 s cooling periods.

Pellets of *E. coli* ML30, *P. putida* 9494 and *P. aeruginosa* 10548 were suspended in the appropriate buffer such that the equivalent OD_{500} = 50, and broken by 4 x 30 s periods of exposure to 90 W, alternating with 3 x 30 s cooling periods.

Pellets of *Streptomyces rimosus* 4018 (1 g wet weight) were suspended in buffer (6 ml) and broken by 10 x 15 s exposure to 45 W, alternating with 10 x 15 s cooling periods.

Pellets of *Bacillus subtilis* NCIMB 3610 were suspended in the appropriate buffer such that the equivalent OD_{500} = 50, and broken by 5 x 30 s periods of exposure to 90 W, alternating with 4 x 30 s cooling periods.

In all cases the sonicated homogenate was centrifuged in microfuge tubes in a MSE Microcentaur centrifuge at 13 000 r.p.m for 10 minutes at 4 °C and the supernatant saved.

(b) French pressure cell disruption of bacteria and yeasts

All micro-organisms were suspended in 3 volumes of the appropriate buffer and broken by 3 passages through a French pressure cell (FA-073 or FA-003 models; American Instrument company, Silver Spring, MD, USA), which had been pre-cooled in ice, at a pressure of 98 MPa. The homogenate was centrifuged at 100 000 ***g*** for 1 h at 4 °C in a Beckman L5-65 ultracentrifuge and the supernatant saved.

2.5. Enzyme assays

All enzyme assays were carried out in duplicate at 27 °C. One unit of enzyme activity (EU) is defined as 1 μmol of substrate converted per min. The molar absorption coefficient of NAD(P)H was assumed to be 6.3 x 10^3 M^{-1} cm^{-1} at 340 nm (Bergmeyer, 1985). In all cases the reaction was initiated by addition of enzyme.

Initial concentrations of alcohol and aldehyde in the assay buffer were calculated from their M_r, density and purity, the volume calculated being dispensed by Hamilton syringe and checked by its weight. Assay solutions were dispensed using Gilson (Medical Electronics, Villiers-le-Bell, France) Pipetman pipettes, except those containing

> 1 % (w/v) alcohol or aldehyde, which were dispensed using Hamilton syringes.

2.5.1. Assay of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 and *Acinetobacter sp.* HO1-N

(a) Hexan-1-ol oxidation

The oxidation of hexan-1-ol was measured in an assay mixture containing:

800 µl of 100 mM-glycine/NaOH, pH 10.2 (assay concentration 80 mM), containing 25 mM-hexan-1-ol (assay concentration 20 mM);

100 µl of 10 mM-$NADP^+$ (assay concentration 1 mM);

Enzyme;

Glass-distilled water to 1 ml.

The rate of NADP reduction was measured at 340 nm in a 1 ml plastic cuvette with a 1 cm light path. A LKB Ultrospec spectrophotometer was used routinely during enzyme purification. A Pye-Unicam SP8-100 spectrophotometer or a Philips PU8700 series UV/visible spectrophotometer was used for enzyme characterisation and kinetic experiments.

(b) Oxidation of alcohols with > 10 carbons

Alcohols were "solubilised" in buffer using a method adapted from Tassin and Vandecasteele (1971). Alcohol was added to 0.111 M glycine/ 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 altered to 10.2 with NaOH and the buffer made up to volume with 0.7 mg BSA ml^{-1}. The enzyme was then assayed as described in Methods 2.5.1 (a) except the pre-sonicated 0.1 M-glycine/NaOH/ 0.7 mg BSA ml^{-1}/ alcohol, pH 10.2 solution was used instead of a 0.1 M-glycine/NaOH/ hexan-1-ol, pH 10.2 solution. In all cases the final concentration of alcohol in the buffer was 0.5 mM or less

(c) Aldehyde reduction

The reduction of butanal was measured in a mixture containing:

2.4 ml 100 mM-Bistris/ HCl, pH 6.8 (assay concentration 80 mM);

0.3ml 300 mM-butanal (assay concentration 30 mM);

0.2 ml 300 μM-NADPH (assay concentration 20 μM);

Enzyme

Glass-distilled 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 Ltd., Romford, UK) with a 1 cm light path. Reaction velocities were calculated initially in arbitrary fluorescence units/ min/ mg protein, and converted into EU (mg protein)$^{-1}$ using a calibration curve of NADPH against arbitrary fluorescence units prepared each time the fluorimeter was used.

2.5.2. Assay of NADP-dependent alcohol dehydrogenase of *Saccharomyces cerevisiae* D273-10B

(a) Alcohol oxidation

The enzyme was assayed in terms of alcohol oxidation in the same way as described in Methods 2.5.1 (a) except that the pH value of the buffer was 10.7.

(b) Aldehyde reduction

The enzyme was assayed as described in Methods 2.5.1 (b).

2.5.3. Assay of NAD-dependent alcohol dehydrogenase from *S. cerevisiae*

The enzyme was assayed as described for NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 (Methods 2.5.1) except 1 mM-NAD^+ and 250 mM-ethanol were included instead of 1 mM-$NADP^+$ and 25 mM-hexan-1-ol respectively.

2.5.4. Assay of NADP-dependent isocitrate dehydrogenase of *A. calcoaceticus* NCIB 8250

The oxidation of isocitrate was measured using the method of Borthwick *et al.* (1984) in an assay mixture containing:

2.0 ml of 225 mM-Tris/HCl, pH 7.5 (assay concentration 150 mM) containing 0.75 mM-$MnCl_2$ (assay concentration 0.5 mM);

0.1 ml of 12 mM-$NADP^+$ (assay concentration 0.4 mM);

0.1 ml of 75 mM-isocitrate (assay concentration 2.5 mM);

Enzyme;

Glass-distilled water to 3 ml.

The rate of reduction of NADP was measured at 340 nm in a 3 ml plastic cuvette with a 1 cm light path using an LKB Ultrospec spectophotometer.

2.6. Standardisation of substrate concentrations

The exact concentrations of NADP solutions were determined from their A_{260} value using the molar absorption coefficient of 18.0 x 10^3 M^{-1} cm^{-1} (Boehringer Mannheim, 1973). The exact concentrations of NADPH solutions were determined from their A_{340} value using the molar absorption coefficient of 6.3 x 10^3 M^{-1} cm^{-1} (Bergmeyer, 1985). Equilibrium concentrations of hexanal were determined from the calculated equilibrium concentration of NADPH assuming a 1:1 ratio of production of hexanal:NADP (see Section 5.3. also). Equilibrium concentrations of hexan-1-ol were determined by subtracting the calculated equilibrium concentration of hexanal from the initial concentration of hexan-1-ol.

2.7. Analysis of kinetic data

Steady state kinetic data were analysed by the Direct-Linear method (Eisenthal and

Cornish-Bowden, 1974) using the Enzpack computer program (Williams, 1985).

2.8. Averages, standard deviations and linear regression analysis

Averages and standard deviations are given as the mean value +/- standard deviation, with the number of experiments used to obtain these values given in parentheses or brackets. Linear regression analysis was done by the least mean squares method using the Apple Macintosh Cricket Graph Computer program.

2.9. Purification procedures

All steps were carried out at 4 ^{o}C.

2.9.1. Purification of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250

(a) Preparation of cell free extract

Thawed cells (35 g wet weight) of *A. calcoaceticus* NCIB 8250, grown on a rich medium [Methods 2.4.4 (b)] were resuspended in 70 ml 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0 and disrupted by three passages through a French pressure cell and the resulting homogenate was centrifuged (Methods 2.4.7). The supernatant (cell free extract) was decanted and used as the source of enzyme.

(b) Dialysis of cell free extract

Cell free extract was dialysed (Methods 2.2.4) for 2 x 4 h against 2 x 1 l 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0 and 1 x 8 h against 2 l of the same buffer.

(c) Ion exchange chromatography through DEAE Sephacel

An extract was applied at 35 ml h^{-1} to a column of DEAE Sephacel (2.6 x 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 A_{280} of the effluent was < 0.05. Then, elution was carried out with a linear 0 to 0.3 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 the A_{280} of the effluent monitored. Fractions with peak activity were pooled.

(d) Concentration of pooled, active, DEAE Sephacel eluate

The DEAE Sephacel pool was concentrated to < 15 ml in dialysis tubing (Methods 2.2.4) using carboxymethylcellulose. The dialysis tubing was cleaned of carboxymethylcellulose every hour.

(e) 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 x 84 cm). Once loaded, the column was washed with 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0 at 25 ml h^{-1}. Fractions of 6.25 ml were collected and the A_{280} of the effluent was monitored. Fractions with peak activity were pooled.

(f) Hydrophobic interaction chromatography through Phenyl Sepharose CL-4B

$(NH_4)_2SO_4$ was added to the Sephacryl S300HR pool to a final saturation of 5 %, then it was applied at 27 ml h^{-1} to a column of Phenyl Sepharose (2.6 cm x 10 cm). Once loaded and 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) $(NH_4)_2SO_4$, pH 8.0, until the A_{280} of the effluent was < 0.05. The enzyme was eluted with 40 % ethanediol in 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0. Fractions of 6.75 ml were collected and the A_{280} of the eluate was monitored. Fractions with peak activity were pooled.

(g) 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 x 6 cm). Once loaded, and 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 (a 100 ml graduated glass test tube).

(h) Concentration of Matrex Gel Blue A eluate by ion exchange eluate through DEAE Sephacel

As eluate was collected in the reservoir, it was loaded onto a column of DEAE Sephacel (1 cm x 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 the A_{280} of the eluate was monitored. Fractions with peak activity were pooled.

(i) Dialysis of pure protein

The concentrated pool of pure enzyme was dialysed against 6 x 1 l, 3 x 2 l and 2 x 4 l of 50 mM-Tris/HCl/ 2 mM-DTT (pH 8.0) over three days before being stored frozen at -20 ^{o}C in small (< 0.5 ml) aliquots.

2.9.2. Purification of NADP-dependent alcohol dehydrogenase of *Acinetobacter sp.* HO1-N

(a) Preparation of cell free extract

Thawed cells (80 g wet weight) of *Acinetobacter sp.* HO1-N, grown on nutrient broth [Methods 2.4.4 (a)] were resuspended in 100 ml 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0 and disrupted by three passages through a French pressure cell at 98 MPa. and the resulting homogenate was centrifuged (Methods 2.4.7). The supernatant (cell free extract) was decanted and used as the source of enzyme.

(b) Dialysis of cell free extract

The cell free extract was dialysed as described for that of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 [Methods 2.9.1. (b)].

(c) Ion exchange chromatography through DEAE Sephacel

A dialysed extract was applied at 35 ml h^{-1} to a column of DEAE Sephacel (2.6 cm x 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 A_{280} of the effluent was < 0.05. Then, elution was carried out with a linear 0 to 1.0 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 the A_{280} of the effluent monitored. Fractions with peak activity were pooled.

(d) Concentration of pooled, active, DEAE Sephacel eluate

The DEAE Sephacel pool was concentrated as described for that of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 [Methods 2.9.1. (d)].

(e) Gel filtration chromatography through Sephacryl S300HR

The concentrated DEAE Sephacel pool was gel filtered through Sephacryl S300HR as described for that of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 [Methods 2.9.1. (e)].

(f) Hydrophobic interaction chromatography through Phenyl Sepharose CL-4B

$(NH_4)_2SO_4$ was added to the Sephacryl S300HR pool to a final saturation of 20 %. The Sephacryl S300HR pool was applied at 27 ml h^{-1} to a column of Phenyl Sepharose (2.6 cm x 10 cm). Once loaded and after allowing 15 min for the protein to bind, the column was washed, at 27 ml h^{-1}, with at least 5 column volumes of 25 mM-Bistris/HCl/ 2 mM-DTT/ 20 % $(NH_4)_2SO_4$, pH 6.5, until the A_{280} of the effluent was < 0.05. The enzyme was eluted with 25 mM-bisTris/HCl/ 2 mM-DTT, pH 6.5. Fractions of 6.75 ml were collected and the A_{280} of the eluate was monitored. Fractions with peak activity were pooled.

(g) 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 x 6 cm). Once loaded, and after allowing 15 min for the protein to bind, the column was washed, at 25 ml h^{-1}, with at least 5 column volumes of 25 mM-Bistris/HCl/ 2 mM-DTT, pH 6.5 until the A_{280} of the effluent was < 0.02. The enzyme was eluted with 25 mM-Bistris/HCl/ 2 mM-DTT/ 1 mM-NADP/ 5 mM-hexan-1-ol,

pH 6.5 and collected over ice in a reservoir (a 100 ml graduated glass test tube).

(h) Concentration of Matrex Gel Blue A eluate by ion exchange through DEAE Sephacel and dialysis of pure protein

The Matrex Gel Blue A pool was concentrated and dialysed as described for NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 [Methods 2.9.1. (h) and (i) respectively].

2.9.3. Purification of NADP-dependent alcohol dehydrogenase of *S. cerevisiae* D273-10B

(a) Preparation of cell free extract

Thawed cells (80 g wet weight) of *S.cerevisiae* D273-10B, grown on PYG broth [Methods 2.4.4 (d)] were resuspended in 100 ml 50 mM-Tris/HCl/ 2 mM-DTT/ 1 mM-benzamidine/ 1 mM-EDTA, pH 8.0 and disrupted by three passages through a French pressure cell at 98 MPa. and the resulting homogenate was centrifuged (Methods 2.4.7). The supernatant (cell free extract) was decanted and used subsequently.

(b) Dialysis of cell free extract

The cell free extract was dialysed over 2 x 4 hours and 1 x 8 hours against 2 x 1 l and 1 x 2 l 50 mM-Tris/HCl/ 2 mM-DTT/ 1 mM-benzamidine/ 1 mM-EDTA, pH 8.0 respectively.

(c) Ion exchange chromatography through DEAE Sephacel

A dialysed extract was applied at 35 ml h^{-1} to a column of DEAE Sephacel (2.6 cm x 14 cm), pre-equilibrated with 50 mM-Tris/HCl/ 2 mM-DTT/ 1 mM-benzamidine/ 1 mM-EDTA, 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 A_{280} of the effluent was < 0.05. Then, elution was carried out with a linear 0 to 1.0 M-KCl gradient in 50 mM-Tris/HCl/ 2 mM-DTT/ 1 mM-benzamidine/ 1 mM-EDTA, pH 8.0 (500 ml) at a flow rate of 35 ml h^{-1}. Fractions of 5.83 ml were collected and the A_{280} of the effluent monitored. Fractions with peak activity were pooled.

(d) Concentration of pooled, active, DEAE Sephacel eluate

The DEAE Sephacel pool was concentrated by precipitation with $(NH_4)_2SO_4$ to a final saturation of 75 %.

(e) Gel filtration chromatography through Sephacryl S300HR

The concentrated DEAE Sephacel pool was was applied at 25 ml h^{-1} to a column of Sephacryl S300HR (2.6 x 94 cm). Once loaded, the column was washed with 50 mM-Tris/HCl / 2 mM-DTT/ 1 mM-benzamidine/ 1 mM-EDTA, pH 8.0, at 25 ml h^{-1}. Fractions of 6.25 ml were collected and the A_{280} of the effluent was monitored.

(f) Hydrophobic interaction chromatography through Phenyl Sepharose CL-4B

The Sephacryl S300HR pool was applied at 27 ml h^{-1} to a column of Phenyl Sepharose (2.6 x 10 cm). After loading the column was washed with at least 5 column volumes of 50 mM-Tris/HCl/ 2 mM-DTT/ 1 mM-benzamidine/ 1 mM-EDTA. Then, the enzyme was eluted with 40 % (v/v) ethanediol in 50 mM-Tris/HCl/ 2 mM-DTT/ 1 mM-benzamidine/ 1 mM-EDTA, pH 8.0. Fractions of 6.75 ml were collected and the A_{280} of the eluate was monitored. Fractions with peak activity were pooled.

(g) Dye-affinity chromatography through Matrex Gel Red A

$MgCl_2$ (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 x 7.5 cm). Once loaded, the column was washed with at least four column volumes of 50 mM-Tris/HCl/ 2-mM DTT/ 50 mM-$MgCl_2$, pH 8.5. Enzyme was eluted with 50 mM-Tris/HCl/ 2-mM DTT/ 5 mM-$MgCl_2$/ 0.2-mM NADP, pH 8.5. Fractions of 5 ml. were collected and the A_{280} was monitored. Peak active fractions were pooled. Pure enzyme was dialysed as described in Methods 2.9.1 (i) and stored frozen at -20 °C.

2.10. Polyacrylamide gel electrophoresis (PAGE)

2.10.1. SDS-PAGE

SDS-PAGE was carried out according to the method of Laemmli (1970), using the following stock solutions:

solution A, 3.0 M Tris/HCl (pH 8.8)/ 0.23 % (v/v) TEMED;

solution B, 28 % (w/v) acrylamide/ 0.735 % (w/v) *N,N'*-methylene-*bis*-acrylamide (deionised with amberlite);

solution C, 0.1 M-Tris/HCl (pH 6.8)/ 0.8 % (w/v) SDS/ 0.25 % (v/v) TEMED;

solution D, 20 % (w/v) SDS;

solution E (reservoir buffer), 25 mM-Tris/ 192 mM-glycine/ 0.5 % (w/v) SDS.

(a) Resolving gel [10 % (w/v) acrylamide]

Solution A (25 ml), solution B (71.5 ml), solution D (1 ml) and distilled water (99 ml) were mixed and degassed thoroughly. Ammonium persulphate (150 mg) dissolved in distilled water (1 ml) was added and the mixture was degassed. The degassed mixture was poured between thin glass plates, the top of the gel overlaid with a thin layer of propan-1-ol and the gel left to polymerise. After the gel had set, the propan-1-ol was poured off, the top of the gel washed thoroughly with distilled water, and the top of the gel dried with blotting paper.

(b) Stacking gel [5.6 % (w/v) acrylamide]

Solution B (17.5 ml), solution C (10 ml) and distilled water (55 ml) were mixed and degassed thoroughly. Ammonium persulphate (150 mg) dissolved in distilled water (1 ml) was added and the mixture was degassed. The degassed mixture was poured on top of the resolving gel, a 18-track Teflon comb inserted and the gel left to polymerise.

(c) Sample preparation and electrophoresis conditions

Samples (50 µl, diluted with water if necessary) were prepared for electrophoresis by boiling for 2 minutes in a mixture with 5 µl 1 M-DTT and 10 ml of tracker dye mix

[143 mM-Tris/HCl (pH 6.8)/ 2.8 % (w/v) SDS/ 0.029 % (w/v) Bromophenol Blue/ 28.5 % (v/v) glycerol]. Gels were electrophoresed at 90 mA (constant current) and the apparatus was cooled by water circulating from an ice/water bath.

2.10.2. Non-denaturing PAGE

Non-denaturing polyacrylamide gels were prepared exactly as described in Methods 2.10.1 except that SDS was excluded from all solutions and the resolving gel was only 7.5 % (w/v) acrylamide. Samples (50 µl, diluted with distilled water if necessary) were prepared by mixing with 5 µl glycerol and 5 µl 0.01 % (w/v) bromophenol blue. Gels were electrophoresed at 20 mA for 30 minutes prior to sample being loaded, and samples were electrophoresed at 20 mA (constant current) and the apparatus was cooled by water circulating from an ice/water bath.

2.10.3. Staining of gels for protein

(a) Coomassie Blue stain

Gels were stained with Coomassie Brilliant Blue G250 using the method described by MacKintosh and Fewson (1988a).

(b) Silver staining

SDS-PAGE gels were stained by the method of Wray *et al.* (1981).

2.10.4. Staining of gels for activity

Non-denaturing gels were stained for NADP-dependent hexan-1-ol dehydrogenase activity. Gels were incubated in 80 mM-glycine/ NaOH (pH 10.2) for 30 min at 4 °C, then incubated with 80 mM-glycine/ NaOH/ 20 mM-hexan-1-ol (pH 10.2)/ 2 mM-NADP/ 550 µM-PMS/ 5.5 mM-NBT (the staining buffer) in the dark at 30 °C for 5-10 min (i.e. until bands appeared on the gel). The gels were then washed in several volumes of

distilled water and stored in distilled water in the dark at 4 °C.

2.10.5. Gel scanning

Comassie-stained gels [Methods 2.10.3 (a)] were scanned using an LKB 2202 Ultroscan laser densitometer.

2.11. Protein chemistry

2.11.1. Dialysis

Purified NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 was dialysed against 6 x 2 l and 3 x 5 l of 5 mM-ammonium bicarbonate over 4 days and then lyophilised.

2.11.2. Protein Sequencing

The *N*-terminus of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 were sequenced by Dr. J.N. Keen at the SERC Protein Sequencing Unit, Department of Biochemistry and Molecular Biology, University of Leeds on two separate occasions. Run 1 was carried out using an Applied Biosystems 477-A liquid phase sequencer equipped with on-line 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 (Laursen, 1971) with PTH-amino acids being identified off-line by reverse phase (C18) microbore h.p.l.c..

2.11.3. Proteolysis

Proteolysis was carried out according to the methods of Findlay *et al.* (1989).

Protein was suspended in 1 ml 0.1 M-ammonium bicarbonate/HCl, pH 8.1 or 0.1 M-ammonium bicarbonate/HCl / 6 M-urea, pH 8.1. Protease (10 mg ml^{-1}) dissolved in 50 mM Tris/HCl, pH 8.1 (except for trypsin which was dissolved in 0.1 M-HCl) was added to a final amount of 0.1 %, 1 %, 2 % or 5 % (w/w) protein and incubated at 37 °C over 24 h (or 6 h when trypsin was used). Samples were removed at intervals over the timecourse of incubation and immediately frozen in a solid CO_2/ methanol slurry before lyophilisation (Methods 2.2.7). Proteolysis of the protein was examined by SDS-PAGE (Methods 2.10.1) and staining for protein [Methods 2.10.3 (a) or (b)].

2.12. Synthesis of tritiated NADPH

Experiments involving the synthesis and use of tritiated NADPH were carried out in collaboration with Dr. D.P. Baker, Mikrobiologisches Institut, ETH-Zentrum, CH-8092, Zurich, Switzerland.

2.12.1. Synthesis of tritiated pro*S* NADPH

This method is based on the fact that glucose-6-phosphate dehydrogenase of *Leuconstoc mesenteroides* specifically transfers a hydride ion (H^- or $^3H^-$ in the case of tritium) from glucose 6-phosphate to the pro*S* position of NAD^+ or $NADP^+$ (Arnold *et al.*, 1976).

[^{3}H]Glucose 6-phosphate was produced by phosphorylation of [1-^{3}H]glucose by ATP using *S.cerevisiae* hexokinase. The linked assay was carried out in a mixture (500 µl) containing 5 mM-potassium phosphate buffer, 60.5 µM-[1-^{3}H]glucose (specific activity 6 Ci $mmol^{-1}$), 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 ultra-filtration using a Centricon-10 microconcentrator. The tritiated pro*S* NADPH was used

immediately.

2.12.2. Synthesis of tritiated pro*R* NADPH

This method 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 pro*S* tritiated NADPH (Methods 2.12.1) was incubated with 90 mM-dihydrofolate and 0.03 units dihydrofolate reductase. The rate of NADPH oxidation was followed by measuring the change in absorbance at 340 nm and the protein removed as in Methods 2.12.1. Then the tritiated NADP was purified on h.p.l.c. using a Machery & Nagel (Machery & Nagel, Duren, Germany) Nucleoseal 7-C18 reverse-phase h.p.l.c. column (25 cm x 4.6 mm) with pre-column (4 cm x 4.6 mm) run isocratically with 2 % (v/v) methanol/ 0.1 % (v/v) TFA to elute NADP. Fractions containing NADP were collected and lyophilised.

Tritiated pro*R* 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 of glucose-6-phosphate dehydrogenase. The reaction was followed at 340 nm and the protein removed as in Methods 2.12.1. The resulting tritiated pro*R* NADPH was used immediately.

2.12.3. Incubation of tritiated NADPH with NADP-dependent alcohol dehydrogenase

This experiment was carried out in the same way on NADP-dependent alcohol dehydrogenases from both *A. calcoaceticus* NCIB 8250 and *S. cerevisiae* D273-10B. To a reaction mixture (1 ml) containing 10 mM-potassium phosphate buffer (pH 7.0) containing 25 mM-butan-1-ol and NADPH (assay concentration 20 µM) was added either pro*R*-[^{3}H]NADPH (assay concentration 0.7 µM) or pro*S*-[^{3}H]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 as described in Methods 2.12.1. NADP and butan-1-ol were separated by h.p.l.c.. Samples (< 500 μl) were injected onto a Macherey & Nagel Nucleoseal 7-C18 reverse-phase h.p.l.c. column (25 cm x 4.6 mm) with pre-column (4 cm x 4.6 mm) pre-equilibrated with 0.1 % (v/v) TFA. NADP was eluted isocratically in a 2 % (v/v) methanol/ 0.1 % (v/v) TFA run over 15 min, then butan-1-ol was eluted in 100 (v/v) % methanol in 0.1 % (v/v) TFA run over 20 min, in both cases solutions were pumped through the column at 1 ml min^{-1} and fractions were collected at one min intervals and counted for incorporation of ^3H as described in Methods 2.12.4. 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 cold butan-1-ol on a Perkin-Elmer (Perkin-Elmer Limited, Baconsfield, UK) 8700 series gas chromatograph with a Chromosorb (Perkin-Elmer) 104 column at 175 ^oC run by Ms S Strolmeyer, Mikrobiologisches Institut, ETH-Zentrum, Zurich, Switzerland.

2.12.4. Scintillation counting

Radioactivity (^3H) was measured using a LKB model 1209 Rackbeta liquid scintillation counter. Samples of up to 500 μl were added to 5 ml of Ecoscint A (National Diagnostics, Manville, NJ, USA) and mixed thoroughly. Background radiation was measured by determining the counts in an equal volume of solvent in the absence of the radioactive material.

2.13. Safety

Bacterial cultures were killed by autoclaving and all spillages of live bacteria were swabbed with 10 % (v/v) propan-1-ol.

The use and disposal of radiochemicals was carried out at Mikrobiologisches Institut, ETH-Zentrum, Zurich, Switzerland according to Swiss regulations as described

by Dr. G. Braus, Radiation Protection Officer, Mikrobiologisches Institut, ETH-Zentrum, Zurich, Switzerland

All other safety precautions followed, were as described in the University of Glasgow Safety Handbook (1987) and where applicable the University of Glasgow Department of Biochemistry Safety Code and Radiation Rules (1991). From 1990, COSHH assessments were completed for all procedures involving potential hazards.

CHAPTER 3
PURIFICATION OF NADP-DEPENDENT ALCOHOL DEHYDROGENASE OF *ACINETOBACTER CALCOACETICUS* NCIB 8250

3.1. Introduction

Studies on alcohol dehydrogenases in various strains of *Acinetobacter calcoaceticus* revealed the presence of a soluble, constitutive NADP-dependent alcohol dehydrogenase activity (see Introduction 1.8). Some preliminary data on this enzyme had been obtained using crude extracts of strains NCIB 8250 (Fixter & Nagi, 1984) and HO1-N (Singer and Finnerty, 1985), and the enzyme had been partially purified from strain 69V (Tauchert *et al.*, 1976). However, the enzyme had not been purified to homogeneity or adequately characterised. Therefore, the first aim of this work was to purify the enzyme from strain NCIB 8250 so that it could be used for characterisation purposes.

3.2. Preliminary studies on maximising enzyme activity in *A. calcoaceticus* NCIB 8250

No strains of *A. calcoaceticus* which overexpress the NADP-dependent alcohol dehydrogenase had been identified, so it was decided to use *A. calcoaceticus* NCIB 8250 in the first instance because it had been used in the laboratory for many other studies, including work on wax ester metabolism (e.g. Fixter *et al.*, 1986). A variety of complex growth media were tested for any effect on enzyme activity, as were some minimal media containing different alcohols as carbon sources. Also, bacteria were grown in minimal medium under carbon or nitrogen limitation. None of the defined media yielded significantly higher enzyme specific activity than the complex media (see Section 7.3 also). MMD, an enriched nutrient broth medium, gave the highest yield of cells and so it was used in subsequent work. When bacteria were grown in a 10 l fermenter with a 20 l reservoir [see Methods 2.4.4 (b)] up to 180 g (wet weight) of cells were obtained with a specific activity of about 80 nmol min^{-1} (mg protein)$^{-1}$.

The cells, suspended in 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0 were broken by the French pressure cell, optimum activity being obtained by three passages at 98 MPa

[Methods 2.9.1 (a)]. Broken cell extract was centrifuged at 100 000*g* for 1 hour leaving the supernatant containing all of the enzyme activity [Methods 2.9.1 (a)].

DTT was included in the buffer for two reasons: firstly, cell extracts frozen in buffer containing 2 mM-DTT then thawed retained greater activity than those frozen in the absence of DTT; and secondly, enzyme passed down small ion exchange columns of DEAE Sephacel and eluted stepwise with high salt concentrations in buffers containing 2 mM-DTT retained more activity than those treated similarly in buffers containing 1 mM or no DTT.

The enzyme retained 99 % activity in 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0 over 12 h at 4 °C.

3.3. Development of a purification procedure for NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250

This section describes the variety of techniques used in attempts to purify NADP-dependent alcohol dehydrogenase, including some that were not used in the final purification procedure. All techniques were attempted at 4 °C unless stated.

3.3.1. Ammonium sulphate fractionation

NADP-dependent alcohol dehydrogenase was precipitated from an extract in the 40-60 % ammonium sulphate saturated fraction. The resuspended pellet was two-fold purified and 75 % of the initial activity was recovered. This step was not used in the final purification procedure because it gave a low recovery for a first step in a protein purification.

3.3.2. Ion exchange on DEAE Sephacel

Initial experiments using small columns of DEAE Sephacel (0.8 cm x 4.0 cm)

revealed that extracts pre-dialysed into 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0 [Methods 2.9.1 (b)] bound consistently to columns pre-equilibrated with the same buffer, and the enzyme could be eluted with 0.3 M-KCl in 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0.

Dialysed extract was applied to a larger DEAE Sephacel column pre-equilibrated as above. After washing, activity was eluted with a linear gradient of 0 to 0.3 M-KCl in 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0 and active fractions were pooled. This resulted in a 20-fold purification with 100 % recovery. This step was subsequently used as the first step in the purification given its high yield, good purification, low elution volume and the high capacity of DEAE Sephacel [Methods 2.9.1 (c) and Section 3.4.1].

3.3.3. Triazine dye-ligand chromatography on Matrex Gel Red A

In triazine dye-ligand chromatography proteins bind to immobilised synthetic dyes. It has been suggested that the chemical structures of such dyes resemble those of natural substrates and cofactors of the proteins, although in some cases non-specific hydrophobic or ionic interactions have been implicated because some proteins are eluted by a relatively small decrease in polarity, or rise in ionic strength, of the buffer. The binding capacity is highest at low ionic strength, low pH and in the presence of metal ions such as Mg^{2+} (Dean and Watson, 1979).

In an attempt to purify the enzyme in one further step, dye affinity chromatography on the triazine dye Matrex Gel Red A was used. It has been suggested that Matrex Gel Red A resembles the cofactor NADP (Amicon, 1980) so it was hoped that biospecific elution from this dye would result in pure protein.

Initial experiments using small columns of Matrex Gel Red A (0.8 cm x 4.0 cm) pre-equilibrated with 50 mM-Tris/HCl/ 2 mM-DTT/ 0.125 M-KCl, pH 8.0, showed that enzyme in the DEAE Sephacel eluate would bind directly to the column. Furthermore it could be eluted with 50 mM-Tris/HCl/ 2 mM-DTT/ 0.4 M-KCl, pH 8.0, with a 1.5 fold purification and little loss in total activity.

Initial attempts to elute the enzyme biospecifically from small columns were made

with NADP (5 mM) and/or butan-1-ol (200 mM) or butyraldehyde (50 mM), but without success.Further attempts were made with $NADP^+$ and butan-1-ol at various pH values (7 to 9 in Tris buffer, 9.6 in glycine buffer), in the presence or absence of 5 mM-$MgCl_2$, and at various KCl concentrations (0.15 M to 0.3 M). No biospecific elution was seen, although in all cases tested at least some of the initial enzyme activity applied was recovered by washing the column in 50 mM-Tris/HCl/ 2 mM-DTT/ 0.4 M-KCl, pH 8.0. This was not used as a step in the purification because of the absence of any biospecific elution and the poor increase in purity resulting from salt elution.

3.3.4. Gel filtration through Sephacryl S300HR

Prior to gel filtration the DEAE Sephacel pool was concentrated to less than 5 % of the volume of the gel filtration column using carboxymethylcellulose [Methods 2.9.1 (d)]. The concentrated pool was gel filtered through a column of Sephacryl S300HR. The column buffer was 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0. Pooled active eluate was purified a further two to three-fold with 40-60 % recovery of enzyme activity. This step was incorporated in the purification procedure [Methods 2.9.1 (e) and Section 3.4.2].

3.3.5. Hydrophobic interaction chromatography on Phenyl Sepharose CL-4B

In this technique proteins bind to Phenyl Sepharose by hydrophobic interactions. Binding is strongest at high ionic strength and elution can be achieved by decreasing the ionic strength or including reagents that reduce the polarity of the buffer.

In an initial experiment ammonium sulphate was added to the Sephacryl S300HR pool to 30 % saturation. Then the pool was applied to a Phenyl Sepharose column and, after washing, a 30 % to 0 % ammonium sulphate saturation gradient in 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0 was applied to the column. Enzyme activity was eluted slowly at the end of the gradient (i.e. in conditions of no ammonium sulphate). The rate of elution of

enzyme could be increased by addition of ethanediol to the elution buffer and 40 % (v/v) ethanediol in 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0 eluted the enzyme in a reasonably small volume. Further experiments showed that the enzyme would bind to Phenyl Sepharose consistently in 5 % ammonium sulphate saturation in 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0, if the column was pre-equilibrated in the same buffer. From this, a method of hydrophobic interaction chromatography on Phenyl Sepharose was developed which gave a further four-fold increase in purification without any loss of activity. It was incorporated as the third step in the purification procedure [Methods 2.9.1 (f) and Section 3.4.3].

3.3.6. Gel filtration through f.p.l.c. Superose 12

The Phenyl Sepharose pool was concentrated to 200 µl by Centriprep followed by Centricon filtration (Methods 2.2.6). The pool was gel filtered through a f.p.l.c. Superose 12 column (0.5 cm x 30 cm) with 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0 being the column buffer, at 20 °C.This step gave only about 25 % recovery and did not yield pure protein [as judged by SDS-PAGE with silver staining for protein; Methods 2.10.1 and 2.10.3 (b)].

3.3.7. Ion exchange on f.p.l.c. Mono Q

The Superose 12 pool was ion exchanged through a f.p.l.c. Mono Q column (0.5 cm x 5 cm) with a 0 to 0.5 M-KCl gradient in 50 mM-Tris/HCl/ 2mM-DTT, pH 8.0, at 20 °C. About 60 % of the enzyme activity was recovered from the Mono Q column but two proteins still remained. This approach was abandoned at this stage because little activity remained [as judged by SDS-PAGE with silver staining for protein; Methods 2.10.1 and 2.10.3 (b)].

3.3.8. Chromatofocussing on f.p.l.c. Mono P

Ethanediol was removed from Phenyl Sepharose eluate by concentration by Centriprep filtration (Methods 2.2.6) then re-dilution in 25 mM-Bistris/HCl/ 2 mM-DTT, pH 6.3. The eluate was applied to a f.p.l.c. Mono P column (0.5 cm x 20 cm) at 20 °C and after washing, enzyme was eluted in a pH gradient of 6.3 (in bisTris buffer) to 4.0 (in PBE 74/ 2 mM-DTT). SDS-PAGE (Methods 2.10.1) and silver staining [Methods 2.10.3 (b)] of the active fractions for protein revealed that the enzyme was pure. Variable recoveries from 0 to 30 % were obtained at pH 5.7 when the initial activity applied was less than 5 enzyme units. However, any attempts to apply greater amounts of activity led to total loss of activity. This step was therefore abandoned because of its irreproducibility.

3.3.9. Further triazine dye ligand chromatography

Phenyl Sepharose eluate was applied to small columns of the Matrex Gels Blue A and Orange A (0.8 cm x 4 cm). After washing with 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0, protein was eluted with 0.5 M-KCl in the same buffer. Enzyme activity partially eluted from the Orange A in the wash but from the Blue A only in the elution buffer. Therefore it was decided to concentrate on optimising conditions using Matrex Gel Blue A. It was possible to elute the enzyme in a partially biospecific manner with 50 mM-Tris/HCl/ 2 mM-DTT/ 5 mM-NADP, pH 8.0.By addition of 5 mM-hexan-1-ol and 5 mM-$MgCl_2$, decreasing the NADP to 1 mM and altering the pH of the wash and elution buffers to 8.5 it was possible to elute only NADP-dependent alcohol dehydrogenase as judged by SDS-PAGE (Methods 2.10.1) and staining for protein [Methods 2.10.3 (a)]. This technique was successfully scaled up to a larger column [Methods 2.9.1 (g)] and gave a further four-fold increase in purification. It was used in the final purification procedure (Section 3.4.4).

3.3.10. Concentration of pure enzyme by ion exchange on DEAE Sephacel

Pure enzyme was eluted from larger Matrex Gel Blue A columns in a volume of about 300 ml. In this very dilute form the enzyme lost approximately 50 % of its activity in 3 days at 4 °C. Therefore, in order to concentrate it more quickly, the Blue A eluate was collected in a reservoir as it was eluted from the Blue A and pumped onto a small ion exchange column of DEAE Sephacel [(Methods 2.9.1 (h)] at a lower rate. It was eluted from the DEAE Sephacel in high salt concentration [Methods 2.9.1 (h)]. The concentrated enzyme was then dialysed extensively [Methods 2.9.1 (h)] into 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0 to remove salts and residual NADP and hexan-1-ol.

3.4. Purification of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250

A purification procedure based on the preliminary studies (Sections 3.2 and 3.3) was developed to purify NADP-dependent alcohol dehydrogenase from *A. calcoaceticus* NCIB 8250. The purification protocol is described in Methods 2.9.1 and summarised in Table 3.1.

3.4.1. Ion exchange on DEAE Sephacel

A typical elution profile of ion exchange chromatography of dialysed extract of *A. calcoaceticus* NCIB 8250 on DEAE Sephacel is shown in Figure 3.1. NADP-dependent alcohol dehydrogenase was eluted after about 150 ml (i.e. about 0.1 M-KCl) of a 500 ml linear gradient of 0 to 0.3 M-KCl in 50 mM-Tris/HCl + 2 mM-DTT, pH 8.0 . There was a 20-fold purification over the crude extract with a yield of 95-100 %.

3.4.2. Gel filtration through Sephacryl S300HR

The DEAE Sephacel pool was concentrated to less than 5 % of the volume of the Sephacryl S300HR using carboxymethylcellulose [Methods 2.9.1 (c)], and this led to about 10 % loss of activity.A typical elution profile of gel filtration chromatography of the concentrated DEAE Sephacel pool on Sephacryl S300HR is shown in Figure 3.2. This resulted in a further two to three-fold purification with a yield of 50 to 60 %.

3.4.3. Hydrophobic interaction chromatography on Phenyl Sepharose CL-4B

Figure 3.3 shows a typical elution profile of hydrophobic interaction chromatography of a Sephacryl S300HR pool on Phenyl Sepharose CL-4B. This resulted in a four-fold increase in purification with about 100 % yield.

3.4.4. Triazine dye-affinity chromatography on Matrex Gel Blue A

NADP-dependent alcohol dehydrogenase was the only protein eluted from Matrex Gel Blue A under the elution conditions used. It eluted in a volume of 300 ml. No elution profile is shown for this stage because the very low concentration of protein eluted was very difficult to detect by absorbance of light at 280 nm (the only method of detection reasonably possible given the immediate need to concentrate the protein) particularly with the much higher concentrations of NADP and NADPH (both of which absorb light at 280 nm also) present.

3.4.5. Concentration of pure NADP-dependent alcohol dehydrogenase by ion exchange on DEAE Sephacel

As the Matrex Gel Blue A eluate was collected it was pumped onto a small ion

exchange column of DEAE Sephacel (1 cm x 5 cm). A typical elution profile of ion exchange of Matrex Gel Blue A pool on DEAE Sephacel is shown in Figure 3.4. The combination of chromatography on Matrex Gel Blue A then DEAE Sephacel as described gave a further four-fold purification with a yield of 40 % with respect to the Phenyl Sepharose pool.

3.4.6. Stability of the purified protein

The enzyme was stable in 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0 at 4 ^{o}C. Initially the enzyme was stored in 50 % (v/v) ethanediol or 50 % (v/v) glycerol at -20 ^{o}C. However, these storage agents proved to be substrates for the enzyme (Section 5.3.6) and as such were difficult to dialyse out without significant loss of enzyme activity. Therefore the enzyme was stored frozen at -20 ^{0}C in small samples. About 10 % of the total activity was lost on thawing but the length of time the enzyme was frozen had comparatively little effect on this.

3.4.7. Overall purification

A typical purification is summarised in Table 3.1. The specific activity of the pure enzyme was reasonably constant [58 enzyme units (mg protein)$^{-1}$,+/- 5 enzyme units (mg protein)$^{-1}$] among ten independent preparations, if the protein was concentrated immediately as described.The degree of purification varied depending on the specific activity of the enzyme in crude extract which itself varied somewhat amongst preparations.

3.4.8. Purity

The purification of NADP-dependent alcohol dehydrogenase was followed by SDS-PAGE (Methods 2.10.1) and staining for protein (Methods 2.10.3). The enzyme

preparation was also checked for homogeneity using non-denaturing gels (Methods 2.10.2) with both protein staining[Methods 2.10.3 (a)] and activity staining (Methods 2.10.4).

The various stages in the purification are shown in Figure 3.5. Purity was checked by a densitometer scan of a typical SDS-PAGE gel of M_r markers and pure enzyme (Figure 3.6) which showed that the protein was purified to homogeneity.

A non-denaturing gel was stained for both protein and activity (Figure 3.7) and confirms that the major NADP-dependent alcohol dehydrogenase activity in crude extract has been purified to homogeneity.

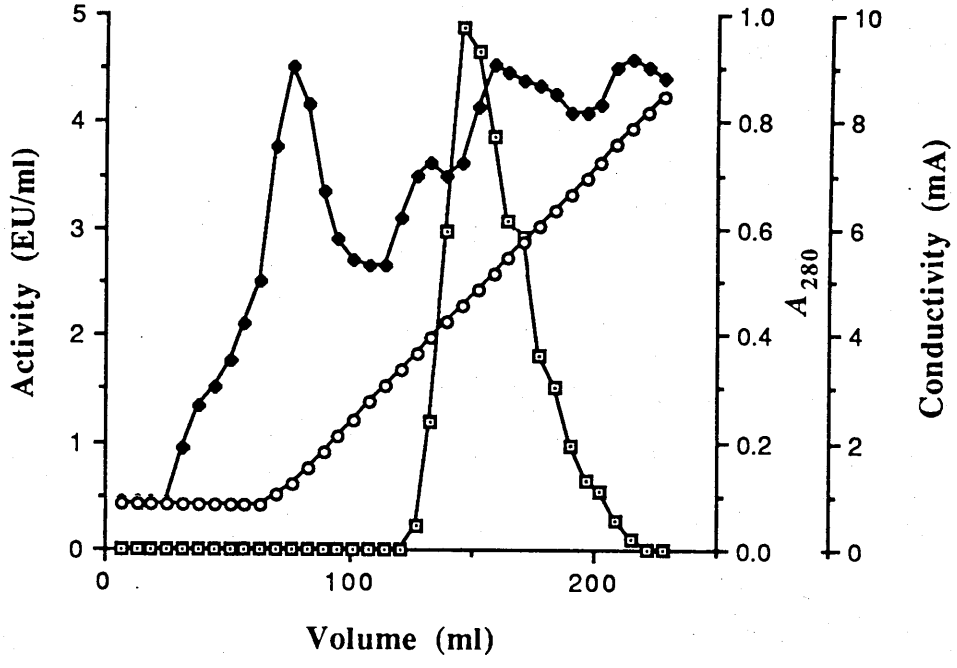


Figure 3.1. Chromatography of an extract of *A.calcoaceticus* NCIB 8250 on DEAE Sephacel

An extract [34.5 ml, Methods 2.9.1 (a) and (b)] was applied at 35 ml h^{-1} to a column of DEAE Sephacel (2.6 cm x 14 cm), pre-equilibrated with 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0. After washing the column with 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0, elution was carried out with a linear 0 to 0.3 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 the A_{280} of the effluent monitored. Enzyme activity was measured as in Methods 2.5.1 (a). Enzyme activity (□), A_{280} (◆) Conductivity (○).

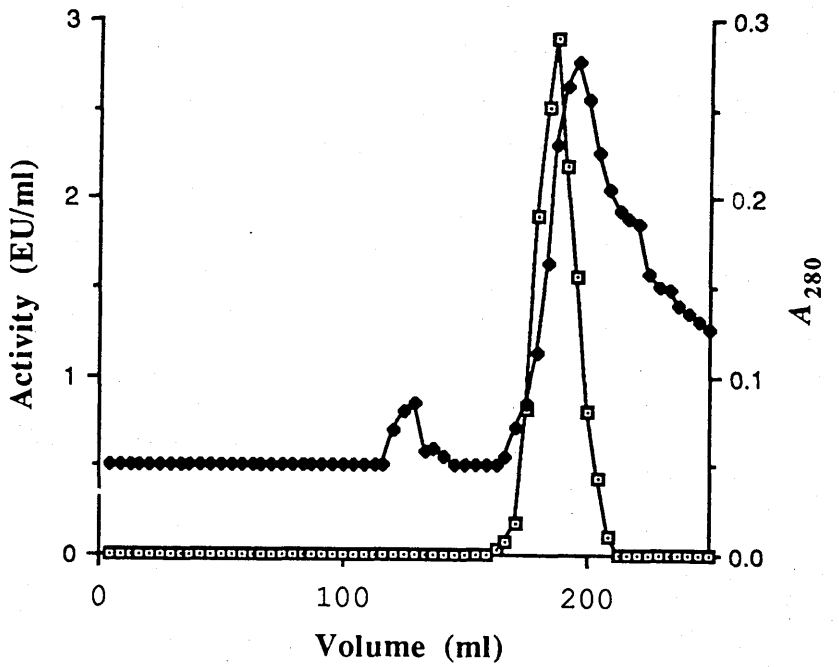


Figure 3.2. Chromatography of a DEAE Sephacel pool on Sephacryl S300HR

A DEAE Sephacel pool [Methods 2.9.1 (c)] was concentrated using carboxymethylcellulose to 11.8 ml [Methods 2.9.1 (d)], then was applied at 25 ml h^{-1} to a column of Sephacryl S300HR (2 cm x 84 cm). Once loaded, the column was washed with 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0 at 25 ml h^{-1}. Fractions of 6.25 ml were collected and the A_{280} of the effluent monitored. Enzyme activity was measured as in Methods 2.5.1 (a). Enzyme activity (□), A_{280} (•).

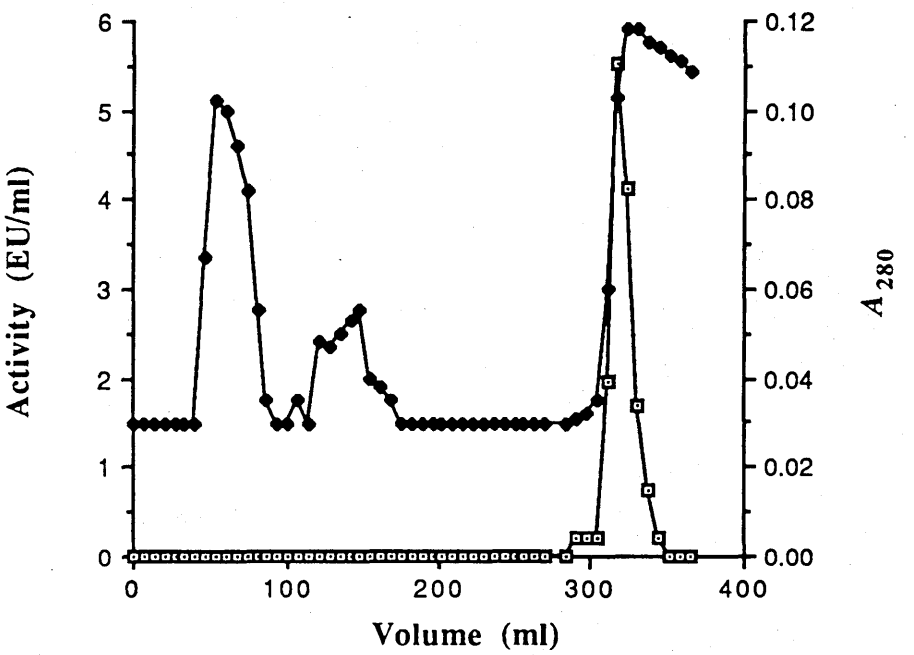


Figure 3.3. Chromatography of a Sephacryl S300HR pool on Phenyl Sepharose

The Sephacryl S300HR pool [63 ml, Methods 2.9.1 (f)], after addition of $(NH_4)_2SO_4$ to a final saturation of 5 %, was applied at 27 ml h^{-1} to a column of Phenyl Sepharose (2.6 cm x 10 cm). After washing the column with 50 mM-Tris/HCl/ 2 mM-DTT/ 5 % $(NH_4)_2SO_4$, pH 8.0, 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 the A_{280} of the effluent monitored. Enzyme activity was measured as in Methods 2.5.1 (a). Enzyme activity (▫), A_{280} (◆).

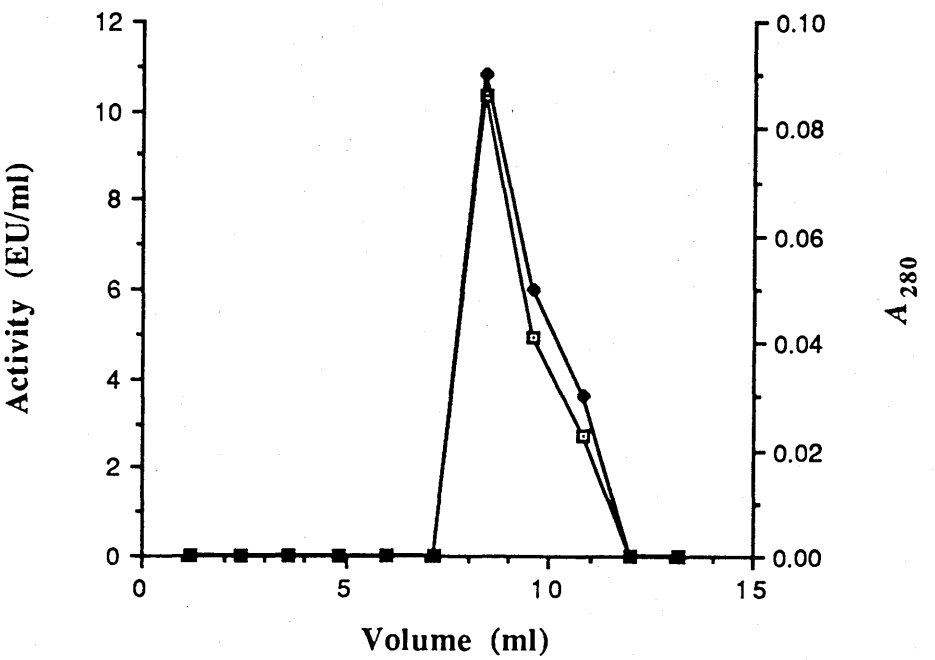


Figure 3.4. Concentration of Matrex Gel Blue A eluate on DEAE Sephacel

Matrex Gel Blue A eluate [Methods 2.9.1 (g)], as it came off the Blue A column, was applied at 10 ml h^{-1} to a column of DEAE Sephacel (1 x 5 cm). After washing the column with 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0, enzyme was eluted with 50 mM-Tris/HCl/ 2 mM-DTT/ 0.3 M-KCl, pH 8.0 at a flow rate of 5 ml h^{-1}. Fractions of 1 ml were collected and A_{280} of the effluent was monitored. Enzyme activity was measured as in Methods 2.5.1 (a). Enzyme activity (⊡), A_{280} (◆).

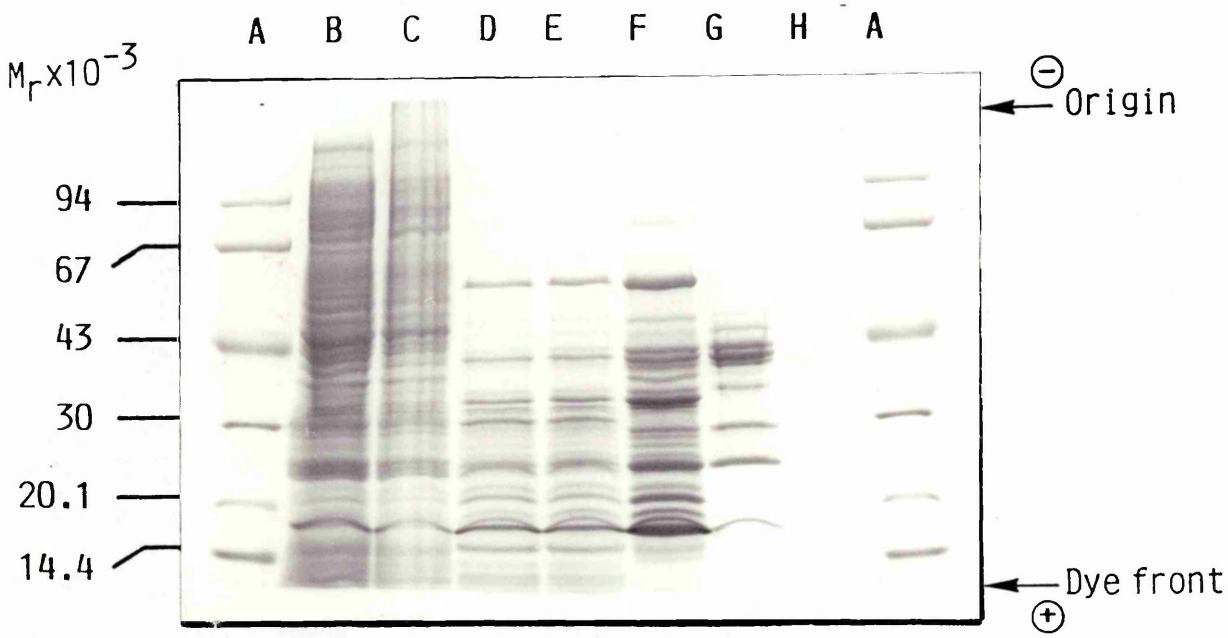


Figure 3.5. Purification of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250

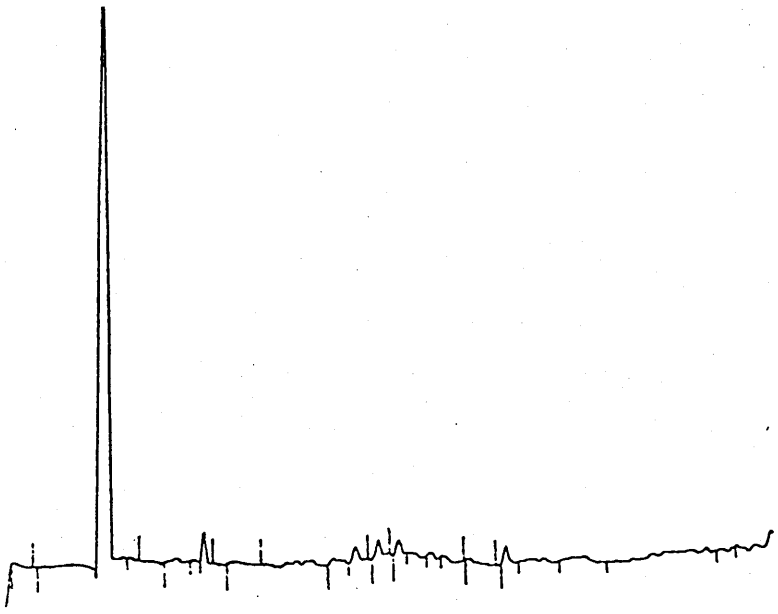
The purification was monitored by SDS-PAGE. A 10 % (w/v) SDS polyacrylamide gel with 5.6 % stacking gel was stained for protein as in Methods 2.10.1 and 2.10 3 (b) respectively. Lane: A, M_r markers; B, 100 µg crude extract; C, 100 µg dialysed crude extract; D, 40 µg DEAE Sephacel pool; E, 40 µg carboxymethylcellulose pool; F, 40 µg Sephacryl S300HR pool; G, 20 µg Phenyl Sepharose pool; H, 1 µg DEAE Sephacel pool.

Figure 3.6. Densitometer scans of purified NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 and M_r markers

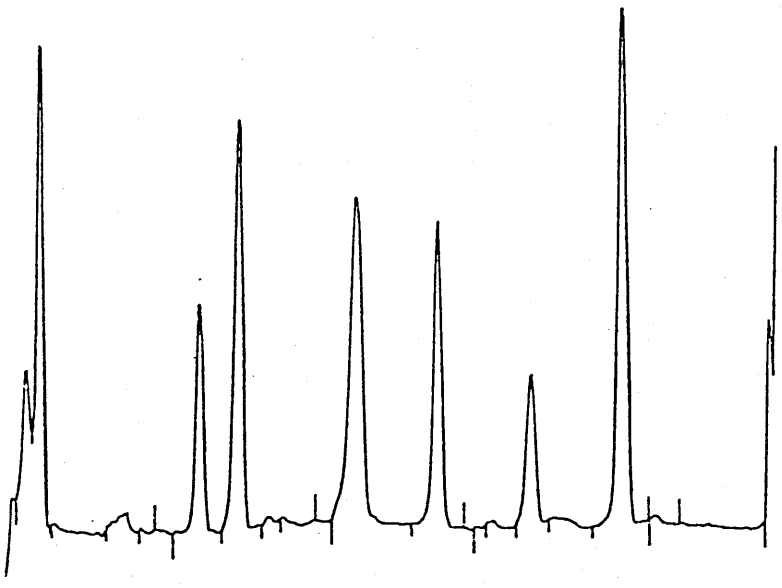
Purified NADP-dependent alcohol dehydrogenase from *A. calcoaceticus* NCIB 8250 and M_r markers were electrophoresed on a 10 % (w/v) SDS polyacrylamide gel with 5.6 % (w/v) stacking gel and stained for protein as in Methods 2.10.1 and 2.10.3 (b) respectively.The gels were scanned with a laser densitometer (Methods 2.10.5). (a) Control (no sample loaded); (b) M_r markers (from left to right) phosphorylase *b* (M_r 94 000), BSA (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soya bean trypsin inhibitor (M_r 20 100) and α-lactalbumin (M_r 14 400); (c) pure NADP-dependent alcohol dehydrogenase (3 μg).

The scans are aligned at the peak marking the start of the resolving gel (far left).

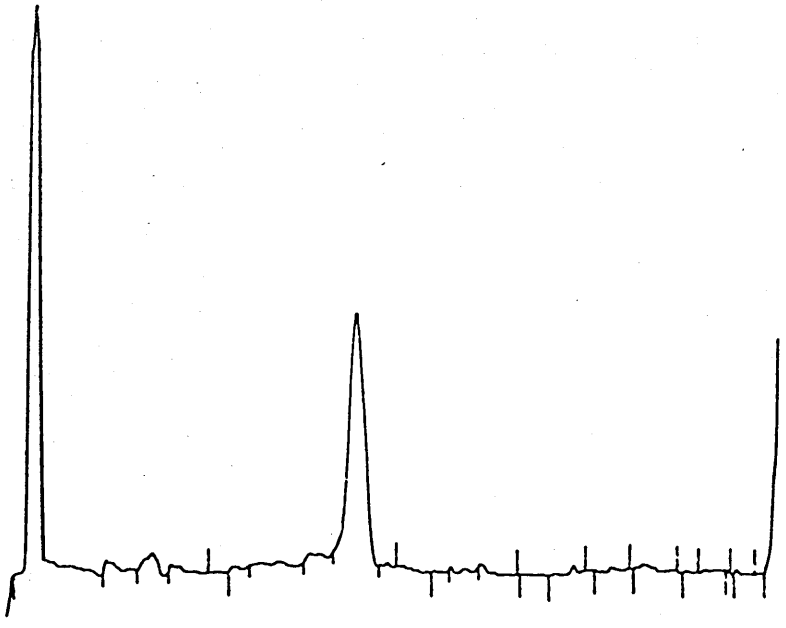
(a)



(b)



(c)



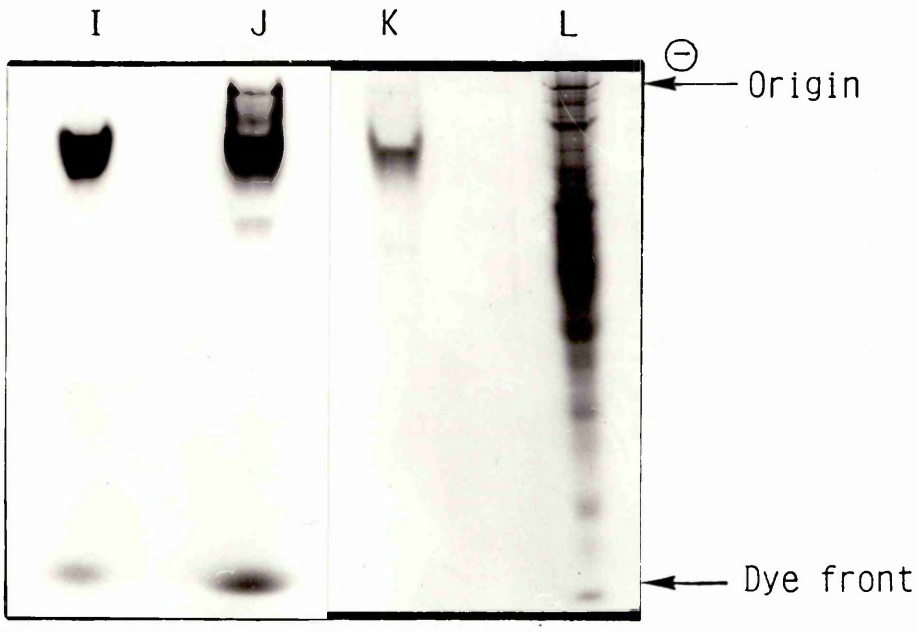


Figure 3.7 Purity of NADP-dependent alcohol dehydrogenase of *A.calcoaceticus* NCIB 8250

The purity of NADP-dependent alcohol dehydrogenase from *A. calcoaceticus* NCIB 8250 was checked using non-denaturing PAGE. A 7.5 % (w/v) polyacrylamide gel with 5.6 % (w/v) stacking gel was run as in Methods 2.10.2. It was then cut in half with one half was stained for protein and the other half was stained for activity as in Methods 2.10.3 (a) and 2.10.4 respectively.Lanes: I and K, 12 µg pure alcohol dehydrogenase; J and L, 100 µg of crude extract.

Table 3.1. Purification of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250

EU: μmol min^{-1}; CMC: carboxymethylcellulose.

	Volume (ml)	Total activity (EU)	Total protein (mg)	Specific activity [EU (mg protein)$^{-1}$]	Yield (%)	Purification (fold)
Crude extract	34.5	139	1725	0.08	100	1
Dialysis	45.5	141	1442	0.10	100	1
DEAE Sephacel	56.0	139	86.8	1.61	99	20
CMC	11.8	129	90.7	1.53	92	19
Sephacryl S300HR	63.0	70	19.5	3.61	50	45
Phenyl Sepharose	20.0	68	4.8	14.2	48	175
Blue A/DEAE Sephacel	4.7	28	0.47	61.7	20	762
Dialysis	6.0	24	0.42	60.9	17	752

3.5 Discussion

NADP-dependent alcohol dehydrogenase from *A. calcoaceticus* NCIB 8250 has been purified to homogeneity by ion exchange chromatography, gel filtration chromatography, hydrophobic interaction chromatography and triazine dye-ligand chromatography. The purification protocol takes 7 days to complete and gives a 750-fold purification over crude extract with 17 % recovery. The enzyme is stable at 4 °C in the presence of DTT (Section 3.2). The loss of enzyme during the purification results predominantly from incomplete recovery at certain stages (e.g. only 50 % recovery from gel filtration), not from instability of the enzyme. However, the enzyme is unstable in very dilute solution so it is important in terms of maximising yield to concentrate it as quickly as possible when it is eluted from Matrex Gel Blue A (Section 3.3.10).

Although the enzyme is soluble and precipitates at 40-60 % ammonium sulphate saturation, it binds to Phenyl Sepharose in only 5 % ammonium sulphate. Most soluble enzymes bind at a much higher ammonium sulphate saturation. This may be due either to the enzyme having a high proportion of hydrophobic residues on its surface, or to specific surface interactions occurring between the enzyme and the matrix phenyl groups (Scopes, 1987).

Much effort was devoted to try to develop a triazine dye-ligand chromatography step in the purification, particularly on Matrex Gel Red A which, it has been suggested, binds NADP (Amicon, 1980). No biospecific elution could be obtained from Red A with NADP and/or butan-1-ol or butyraldehyde. NADPH was not tested in the elution buffer. This would have proved very expensive given the cost of NADPH, and seemed unlikely to succeed anyway in the light of the other attempts. However, successful biospecific elution was achieved from Matrex Gel Blue A (Section 3.4.4). This triazine dye-ligand is thought to resemble NAD but presumably weaker non-specific interactions are part of the reason for the success of Blue A over Red A. The fact that addition of hexan-1-ol decreases the amount of NADP necessary to elute the enzyme specifically may have something to do with the order in which the substrates bind to the enzyme.

Purification of the enzyme was necessary for its successful characterisation. However it may prove difficult and expensive to scale up this purification to any great extent (i.e. to yield greater than low mg amounts of enzyme) because of potential problems in concentrating the large volume of protein of low concentration that would be eluted from the dye affinity column if much larger amounts of protein were applied to it and the large amount of NADP necessary for the dye affinity step respectively.

CHAPTER 4
PURIFICATION OF THE NADP-DEPENDENT ALCOHOL DEHYDROGENASES OF *ACINETOBACTER SP.* HO1-N AND *SACCHAROMYCES CEREVISIAE* D273-10B

4.1. Introduction.

Acinetobacter sp. HO1-N has been much studied with respect to its enzymology, and studies on partially purified NADP-dependent alcohol dehydrogenase from *Acinetobacter sp.* HO1-N suggested that it was much more kinetically efficient than the enzyme in *A. calcoaceticus* NCIB 8250 (Fox *et al.*, 1990). Therefore, it was decided to purify it to homogeneity and partially characterise it so that it could be studied in more detail and compared with the enzyme from strain NCIB 8250.

During this project NADP-dependent alcohol dehydrogenases have been identified in a variety of other micro-organisms grown aerobically (Table 7.3), so a purification of the enzyme from the yeast *Saccharomyces cerevisiae* D273-10B (ATCC 25657) was attempted. In both cases purification was tried using methods based on the procedure used to purify the enzyme from *A. calcoaceticus* NCIB 8250. All steps were carried out at 4 ^{o}C.

4.2. Purification of the NADP-dependent alcohol dehydrogenase of *Acinetobacter sp.* HO1-N.

Bacteria were grown on nutrient broth [Methods 2.4.4 (d)], a commonly used growth medium for this organism (e.g. Singer and Finnerty, 1985). Four 10 l flasks, each containing 3 l of medium yielded 120 g of cells. The cells, suspended in 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0, were broken by three passages at 98 MPa. through a French pressure cell, and broken cell extract was centrifuged at 100 000*g* for 1 h leaving all of the enzyme activity [30 nmol min^{-1} (mg protein)$^{-1}$] in the supernatant [Methods 2.9.2 (a)], i.e. as in strain NCIB 8250 the enzyme is soluble. The purification protocol used finally is described in Methods 2.9.2 and the results are summarised in Table 4.1.

4.2.1. Ion exchange on DEAE Sephacel.

This step was carried out as for the enzyme from strain NCIB 8250 except that the linear elution gradient was 1 l of 0 to 0.5 M-KCl. A typical elution profile of ion exchange chromatography of extract from *Acinetobacter sp.* HO1-N on DEAE Sephacel is shown in Figure 4.1. NADP-dependent alcohol dehydrogenase activity eluted after about 130 ml (approx. 0.06 M-KCl) of the linear elution gradient. The enzyme was purified two to three-fold over crude extract, with over 100 % recovery.

4.2.2. Gel filtration through Sephacryl S300HR.

The DEAE Sephacel pool was concentrated to less than 5 % of the volume of the Sephacryl S300HR column using carboxymethylcellulose [Methods 2.9.2 (d)] and this led to a 4-10 % loss of activity. A typical elution profile of gel filtration chromatography of a concentrated DEAE Sephacel pool on Sephacryl S300HR is shown in Figure 4.2. This step led to a further two-fold increase in purification with a 60-80 % yield.

4.2.3. Hydrophobic interaction chromatography on Phenyl Sepharose.

Unlike the enzyme from *A. calcoaceticus* NCIB 8250, the NADP-dependent alcohol dehydrogenase activity from *Acinetobacter sp.* HO1-N did not bind to Phenyl Sepharose in 5 % ammonium sulphate saturation in 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0. Further experiments revealed that the enzyme did bind in 20 % ammonium sulphate saturation in the same buffer and could be eluted in either 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0, or 25 mM-Bistris/HCl/ 2 mM-DTT, pH 6.5 in a reasonably small volume. The latter elution buffer was used [Methods 2.9.2 (f)] to facilitate the next stage in the purification (Section 4.2.4).

A typical elution profile of hydrophobic interaction chromatography of a Sephacryl S300HR pool on Phenyl Sepharose is shown in Figure 4.3. This step gave a further

four-fold purification with over 100 % yield.

4.2.4. Triazine dye-ligand chromatography on Matrex Gel Blue A.

Unlike the enzyme from strain NCIB 8250, the Phenyl Sepharose pool containing the NADP-dependent alcohol dehydrogenase activity from *Acinetobacter sp.* HO1-N did not bind to Matrex Gel Blue A in 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0. Further experiments showed that it would bind in 25 mM-Bistris/ 2 mM-DTT, pH 6.5. Therefore it was eluted from the previous, Phenyl Sepharose, column in 25 mM-Bistris/ 2 mM-DTT, pH 6.5 [Methods 2.9.2 (f)] and applied to the Blue A column in that buffer. Elution in 25 mM-bisTris/ 2 mM-DTT/ 1 mM-NADP/ 5 mM-$MgCl_2$/ 5 mM-hexan-1-ol, pH 6.5 i.e. the same biospecific components as in the elution buffer for the enzyme from strain NCIB 8250 [Methods 2.9.1 (g)] proved to be biospecific for the NADP-dependent alcohol dehydrogenase. It was the only protein eluted from Matrex Gel Blue A and was eluted in a volume of 250 ml. No elution profile is shown for this stage for the same reasons given in Section 3.4.4 for the NADP-dependent alcohol dehydrogenase from *A. calcoaceticus* NCIB 8250.

4.2.5. Concentration of pure NADP-dependent alcohol dehydrogenase by ion exchange chromatography on DEAE Sephacel.

As the Matrex Gel Blue A eluate was collected it was pumped onto a small ion exchange column of DEAE Sephacel [Methods 2.9.1 (g)]. A typical elution profile of ion exchange of Matrex Gel Blue A on DEAE Sephacel is shown in Figure 4.4.

Chromatography on Matrex Gel Blue A then on DEAE Sephacel as described led to a further 50-fold purification over the Phenyl Sepharose pool with a 50 % yield. The pure enzyme was dialysed to remove salt and residual NADP and hexan-1-ol.

4.2.6. Stability of the purified protein.

The enzyme was assumed to have similar stability to the enzyme purified from strain NCIB 8250 and was stored in the same way (Section 3.4.6).

4.2.7 Overall purification.

A typical purification is summarised in table 4.1. The specific activity of the pure enzyme was reasonably constant [53.7 and 56.0 enzyme units (mg protein)$^{-1}$] in two independent preparations, if the protein was concentrated immediately as described. The degree of purification varied depending on the specific activity of the enzyme in crude extract which itself varied between preparations.

4.2.8. Purity.

The purification of NADP-dependent alcohol dehydrogenase was followed by SDS-PAGE (Methods 2.10.1) and staining for protein [Methods 2.10.3 (a); Figure 4.5]. A typical SDS polyacrylamide gel of M_r markers and pure enzyme stained for protein [Methods 2.10.1 and 2.10.3 (a) respectively] was densitometer scanned [Methods 2.10.5; Figure 4.6] The enzyme preparation was also checked for homogeneity using non-denaturing PAGE (Methods 2.10.2) with both protein staining and activity staining [Methods 2.10.3 (a) and 2.10.4; Figure 4.7]. The above methods show that the major NADP-dependent alcohol dehydrogenase activity in crude extract has been purified to homogeneity.

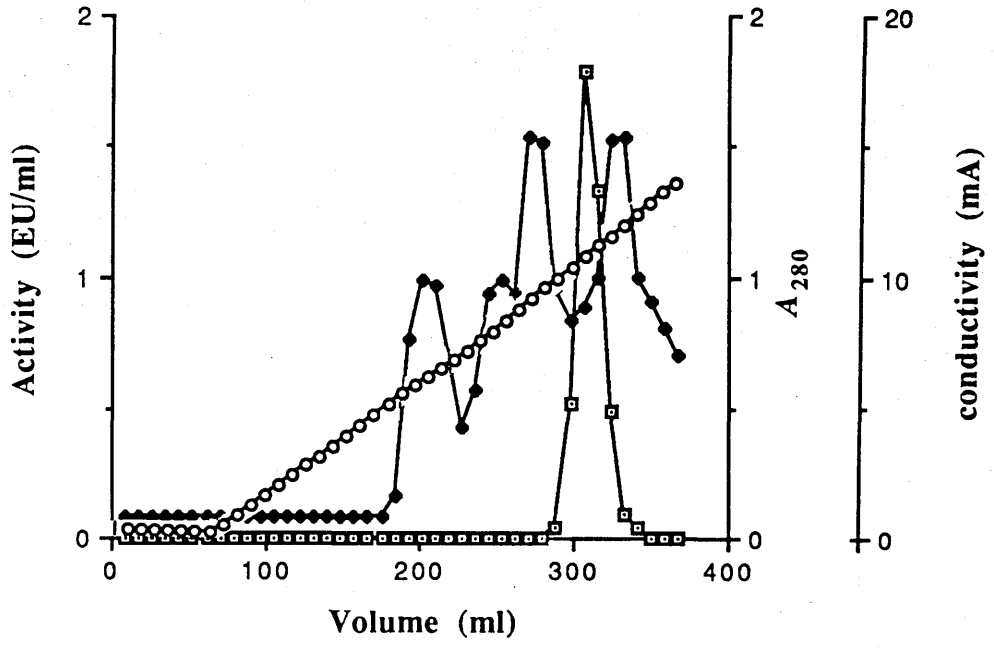


Figure 4.1. Chromatography of an extract of *Acinetobacter sp.* HO1-N on DEAE Sephacel

An extract [34.5 ml, Methods 2.9.2 (a) and 2.9.2 (b)] was applied at 35 ml h^{-1} to a column of DEAE Sephacel (2.6 cm x 14 cm), pre-equilibrated with 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0. After washing the column with 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0, elution was carried out with a linear 0 to 0.5 M-KCl gradient in 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0 (1 l) at a flow rate of 35 ml h^{-1}. Fractions of 5.83 ml were collected and the A_{280} of the effluent monitored. Enzyme activity was measured as in Methods 2.5.1 (a). Enzyme activity (▫), A_{280} (•), Conductivity (o).

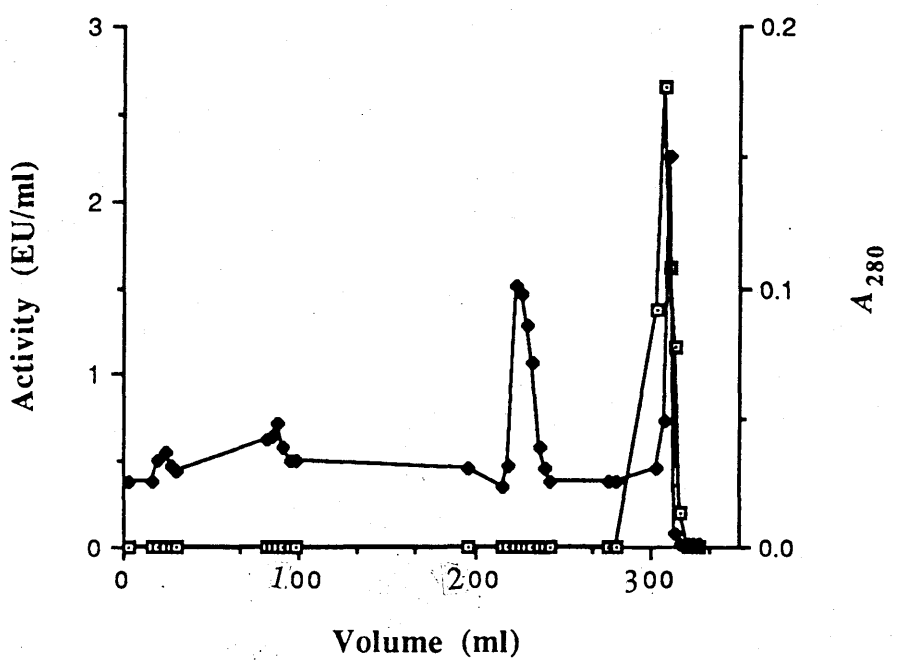


Figure 4.2. Chromatography of a DEAE Sephacel pool on Sephacryl S300HR

A DEAE Sephacel pool [Methods 2.9.2 (c)], concentrated using carboxymethylcellulose to 12.0 ml [Methods 2.9.2 (d)], was applied at 25 ml h^{-1} to a column of Sephacryl S300HR (2 cm x 84 cm). Once loaded, the column was washed with 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0, at 25 ml h^{-1}. Fractions of 6.25 ml were collected and the A_{280} of the effluent monitored. Enzyme activity was measured as in Methods 2.5.1 (a). Enzyme activity (▣), A_{280} (◆).

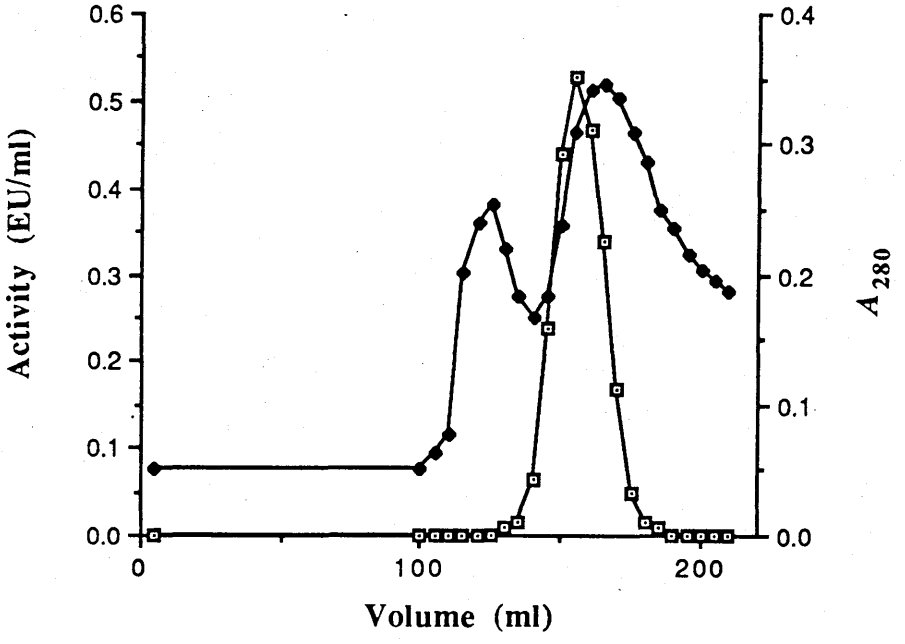


Figure 4.3. Chromatography of a Sephacryl S300HR pool on Phenyl Sepharose

The Sephacryl S300HR pool [71 ml, Methods 2.9.2 (e)], after addition of $(NH_4)_2SO_4$ to a final saturation of 20 %, was applied at 27 ml h^{-1} to a column of Phenyl Sepharose (2.6 cm x 10 cm). After washing the column with 25 mM-Bistris/HCl/ 2 mM-DTT/ 20 % $(NH_4)_2SO_4$, pH 6.5, the enzyme was eluted with 25 mM-bisTris/HCl/ 2 mM-DTT, pH 6.5. Fractions of 6.75 ml were collected and the A_{280} of the effluent monitored. Enzyme activity was measured as in Methods 2.5.1 (a). Enzyme activity (▫), A_{280} (◆).

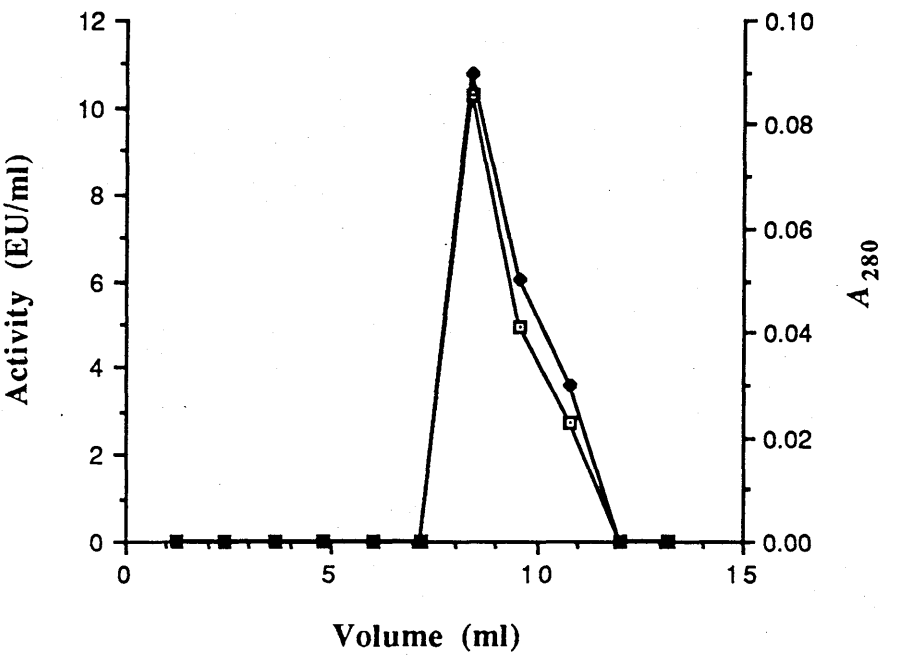


Figure 4.4. Concentration of Matrex Gel Blue A eluate on DEAE Sephacel

Matrex Gel Blue A eluate [Methods 2.9.2 (g)], as it came off the Blue A column, was applied at 10 ml h^{-1} to a column of DEAE Sephacel (1 cm x 5 cm). Enzyme was eluted with 50 mM-Tris/HCl/ 2 mM-DTT/0.3 M-KCl, pH 8.0 at a flow rate of 5 ml h^{-1}. Fractions of 1 ml were collected and A_{280} was monitored. Enzyme activity was measured as in Methods 2.5.1 (a). Enzyme activity (▣), A_{280} (●).

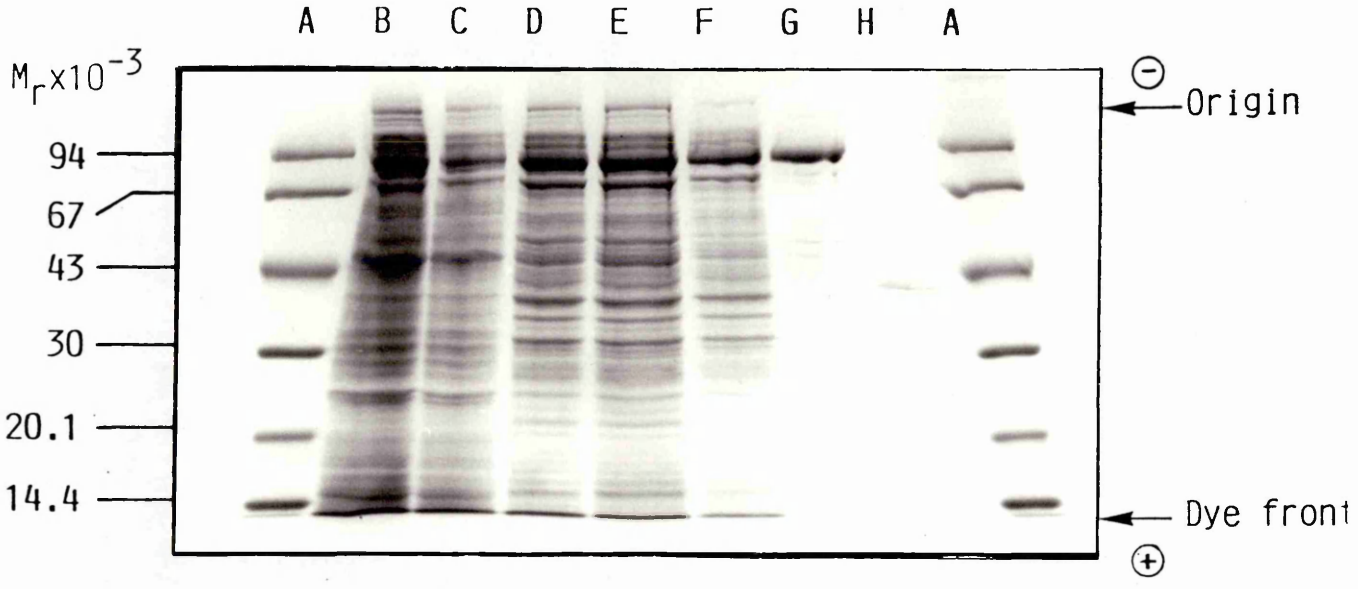


Figure 4.5. Purification of NADP-dependent alcohol dehydrogenase of *Acinetobacter sp.* HO1-N

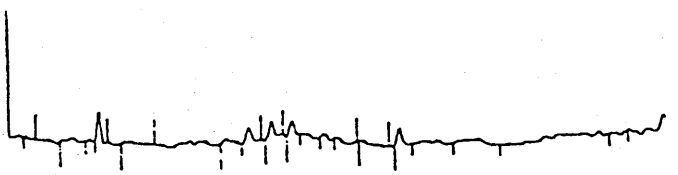
The purification was monitored by SDS-PAGE. A 10 % (w/v) SDS polyacrylamide gel with 5.6 % (w/v) stacking gel was stained for protein as in Methods 2.10.1 and 2.10.3 (a) respectively. Lane: A, M_r markers; B, 100 µg crude extract; C, 100 µg dialysed crude extract; D, 50 µg DEAE Sephacel pool; E, 50 µg carboxymethylcellulose pool; F, 40 µg Sephacryl S300HR pool; G, 40 µg Phenyl Sepharose pool; H, 1.5 µg DEAE Sephacel pool.

Figure 4.6 Densitometer scans of purified NADP-dependent alcohol dehydrogenase of *Acinetobacter sp.* HO1-N and M_r markers

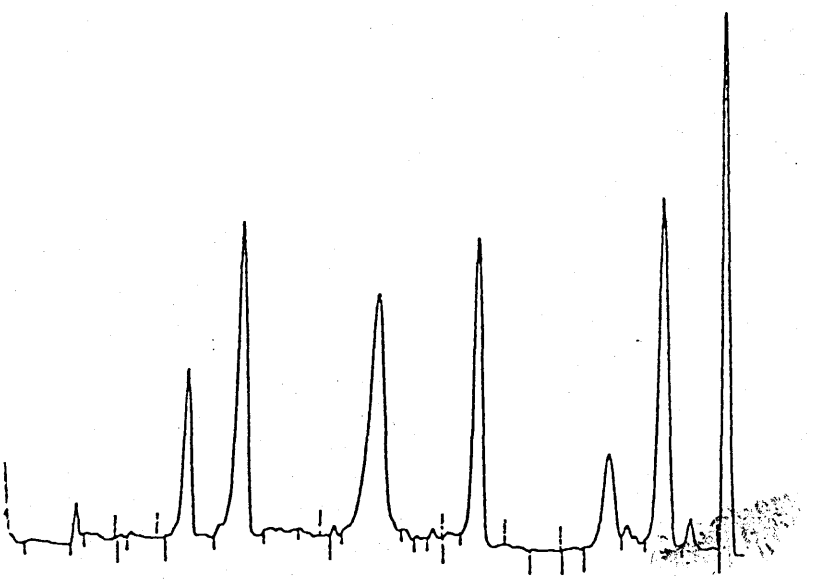
Purified NADP-dependent alcohol dehydrogenase from *Acinetobacter sp.* H01-N and M_r markerswere electrophoresed on a 10 % (w/v) SDS polyacrylamide gel with 5.6 % (w/v) stacking gel and stained for protein as in Methods 2.10.2 and 2.10.4 respectively.Then they were scanned with a laser densitometer (Methods 2.10.5). (a) Control (no sample loaded); (b) M_r markers (from left to right) phosphorylase *b* (M_r 94 000), BSA (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soya bean trypsin inhibitor (M_r 20 100) and α-lactalbumin (M_r 14 400); (c) pure NADP-dependent alcohol dehydrogenase (3 μg).

The scans are aligned at the peak marking the start of the resolving gel (not shown).

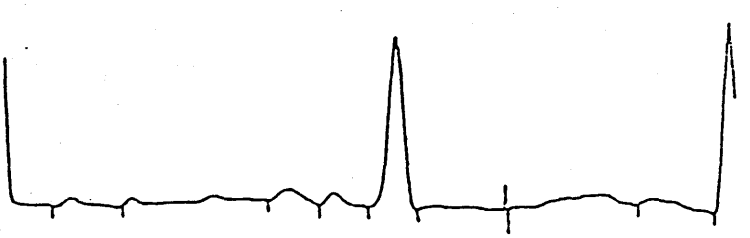
(a)



(b)



(c)



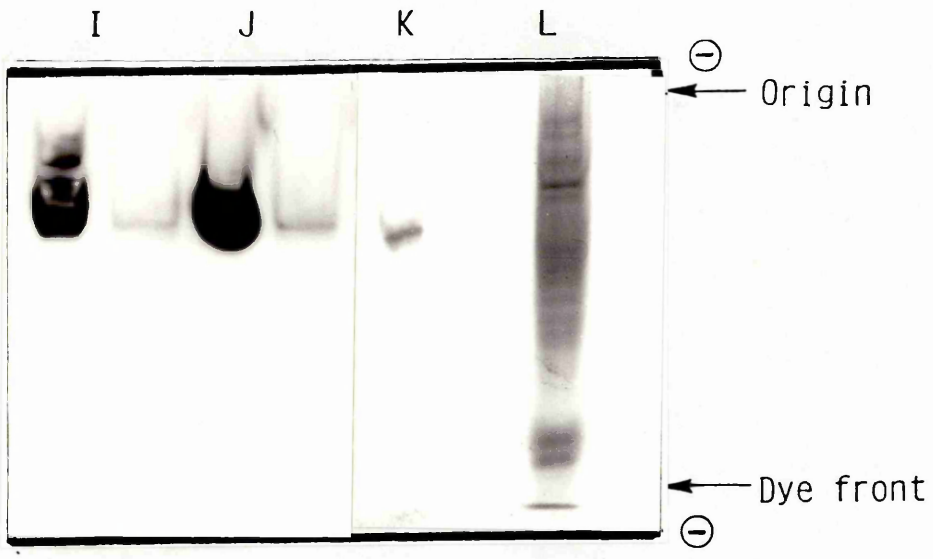


Figure 4.7. Purity of NADP-dependent alcohol dehydrogenase from *Acinetobacter sp.* HO1-N

The purity of NADP-dependent alcohol dehydrogenase from *Acinetobacter sp.* HO1-N was checked using non-denaturing PAGE. A 7.5 % (w/v) polyacrylamide gel with 5.6 % (w/v) stacking gel was run as in Methods 2.10.2. It was then cut in half and one half was stained for protein and the other half was stained for activity as in Methods 2.10.3 (a) and 2.10.4 respectively. Lanes: J and K, 12 μg pure alcohol dehydrogenase; I and L, 100 μg of crude extract.

Table 4.1. Purification of NADP-dependent alcohol dehydrogenase of *Acinetobacter sp.* HO1-N

EU mg^{-1} = μmol min^{-1} (mg protein)$^{-1}$; CMC = carboxymethylcellulose.

	Volume (ml)	Total activity (EU)	Total protein (mg)	Specific activity (EU mg^{-1})	Yield (%)	Purification (fold)
Crude extract	24.0	34.2	1061	0.03	100	1
Dialysis	33.0	40.4	561	0.07	118	2
DEAE Sephacel	36.0	40.7	259	0.16	120	5
CMC	12.0	39.7	270	0.15	116	5
Sephacryl S300HR	71.0	20.8	81.6	0.26	61	8
Phenyl Sepharose	20.7	27.4	26.9	1.03	80	32
Blue A/DEAE Sephacel	8.0	16.2	0.29	55.9	47	1745
Dialysis	13.0	13.2	0.24	53.7	39	1680

4.3. Purification of the NADP-dependent alcohol dehydrogenase of *S. cerevisiae* D273-10B.

Cells were grown on the complex medium YPG under aerobic conditions [Methods 2.4.4 (k)],with four 10 l flasks each containing 3 l medium yielding 247 g cells. Cells were broken by three passages through a French pressure cell at 98 MPa. with broken cell extract being centrifuged at 100 000*g* for 1 hour leaving all the enzyme activity [10 nmol min^{-1} (mg protein)$^{-1}$] in the supernatant (as for *A. calcoaceticus* NCIB 8250). The purification used for the same enzyme in *A. calcoaceticus* NCIB 8250 was followed in the first instance, except 1 mM-benzamidine and 1 mM-EDTA were included in buffers as protease inhibitors during dialysis and in the first three purification steps because of an apparent 20-30 % loss of activity during dialysis. Further modifications were made to the buffers where necessary for binding to, or elution from, columns. The triazine dye ligands Blue A and Red A were tested for the final purification step. The purification protocol finally used is described in Methods 2.9.3 and the results are summarised in Table 4.2.

4.3.1. Ion exchange on DEAE Sephacel.

This step was carried out as for the enzyme in *A. calcoaceticus* NCIB 8250 except: the linear elution gradient was 500 ml of 0 to 0.2 M-KCl. A typical elution profile of ion exchange chromatography of dialysed extract from *S. cerevisiae* on DEAE Sephacel is shown in Figure 4.8. NADP-dependent alcohol dehydrogenase activity was eluted after about 190 ml (approx. 0.2 M-KCl) of the linear elution gradient. The enzyme was 9 fold purified compared with crude extract, with 70-80 % recovery.

4.3.2. Gel filtration through Sephacryl S300 HR.

The DEAE Sephacel pool was concentrated to less than 5 % of the volume of the

Sephacryl S300HR by ammonium sulphate precipitation [Methods 2.9.3 (d)], which led to a 10-20 % loss in activity. A typical profile of elution of enzyme activity resulting from gel filtration chromatography of concentrated DEAE Sephacel pool on Sephacryl S300HR is shown in Figure 4.9. This step led to a further five-fold increase in purification with a 70-80 % yield.

4.3.3. Hydrophobic interaction chromatography on Phenyl Sepharose.

Unlike the enzyme from *A. calcoaceticus* NCIB 8250, the Sephacryl S300HR pool containing the NADP-dependent alcohol dehydrogenase from *S. cerevisiae* D273-10B bound in the absence of ammonium sulphate. The enzyme was eluted in 40 % (v/v) ethanediol in 50 mM-Tris/HCl/ 2 mM-DTT/ 1 mM-benzamidine/ 1 mM-EDTA, pH 8.0. A typical elution profile of hydrophobic interaction chromatography of Sephacryl S300HR pool on Phenyl Sepharose is shown in Figure 4.10. This step led to a further four-five fold increase in purification with a 50 % yield.

4.3.4. Triazine dye-ligand chromatography.

Initial experiments were carried out on small columns of Matrex Gel Blue A (0.8 cm x 4 cm). It was shown that the enzyme activity would bind directly to a Blue A column and could be eluted in in 50 mM-Tris/HCl/ 2 mM-DTT/ 0.1 mM-NADP, pH 8.0. Further experiments tested the effects of varying pH (6.5, 7.5, 8.0, 8.5), varying the concentration of NADP (0.01, 0.02, 0.05, 0.1, 0.2, 1 mM), including 5 mM-$MgCl_2$ and including 1 mM-hexan-1-ol on purity as judged by SDS-PAGE and staining for protein [Methods 2.10.1 and 2.10.3 (a)]. Three bands around M_r 40 000 appeared consistently, as well as some lower M_r weight bands in some cases.

Similar experiments were carried out on small columns of Matrex Gel Red A (0.8 cm x 4 cm). These revealed that the enzyme could be purified to homogeneity by including 5 mM-$MgCl_2$ in the Phenyl Sepharose pool and in the wash and elution buffers

and altering the pH of the wash and elution buffers to pH 8.5. The elution buffer was 50 mM-Tris/HCl/ 2 mM-DTT/ 0.2 mM-NADP/ 5-mM $MgCl_2$, pH 8.5. This method was scaled up to a 40 ml column and was used in the final purification [Methods 2.9.3 (g)] resulting in a further 15-fold increase in purification with 100 % recovery compared to the Phenyl Sepharose pool. A typical elution profile of triazine dye ligand chromatography of a Phenyl Sepharose pool on Matrex Gel Red A is shown in Figure 4.11. The much lower NADP concentration used compared to that used in purifying NADP-dependent alcohol dehydrogenase from *A. calcoaceticus* NCIB 8250 and the enzyme's more "classical" behaviour in eluting from the dye over a smaller volume (40 ml compared to 250-300 ml) makes it possible to show an elution profile for this stage. The pure enzyme was dialysed in 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0 to remove $NADP^+$ [Methods 2.9.3 (h)].

4.3.5. Overall purification.

A typical purification is summarised in Table 4.2. The specific activity of the enzyme was reasonably consistent [34.0 and 30.8 enzyme units $(mg\ protein)^{-1}$] in two independent preparations. The degree of purification varied depending upon the specific activity of the enzyme in crude extract, which itself varied between the preparations.

4.3.6. Purity.

The purification of NADP-dependent alcohol dehydrogenase from *S. cerevisiae* D273-10B was monitored by SDS-PAGE (Methods 2.10.1) and staining for protein [Methods 2.10.3 (a); Figure 4.12]. A typical SDS polyacrylamide gel of M_r markers and pure enzyme stained for protein [Methods 2.10.1 and 2.10.3 (a)] was densitometer scanned (Methods 2.10.5; Figure 4.13).

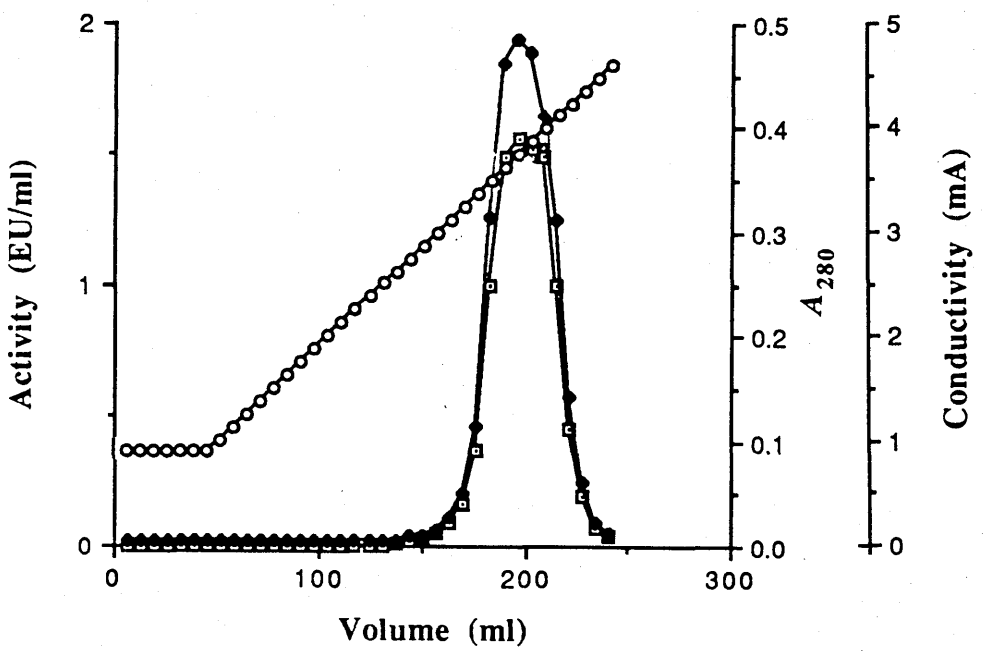


Figure 4.8. Chromatography of an extract of *S. cerevisiae* D273-10B on DEAE Sephacel

An extract [34.5 ml, Methods 2.9.3 (a) and 2.9.3 (b)] was applied at 35 ml h^{-1} to a column of DEAE Sephacel (2.6 cm x 14 cm), pre-equilibrated with 50 mM-Tris/HCl/ 2 mM-DTT/ 1 mM-benzamidine/ 1 mM-EDTA, pH 8.0. After washing the column with 50 mM-Tris/HCl/ 2 mM-DTT/ 1 mM-benzamidine/ 1 mM-EDTA, pH 8.0, elution was carried out with a linear 0 to 0.2 M-KCl gradient in 50 mM-Tris/HCl/ 2 mM-DTT/ 1 mM-benzamidine/ 1 mM-EDTA, pH 8.0 (500 ml) at a flow rate of 35 ml h^{-1}. Fractions of 5.83 ml were collected and the A_{280} of the effluent monitored. Enzyme activity was measured as in Methods 2.5.1 (a). Enzyme activity (□), A_{280} (●), Conductivity (○).

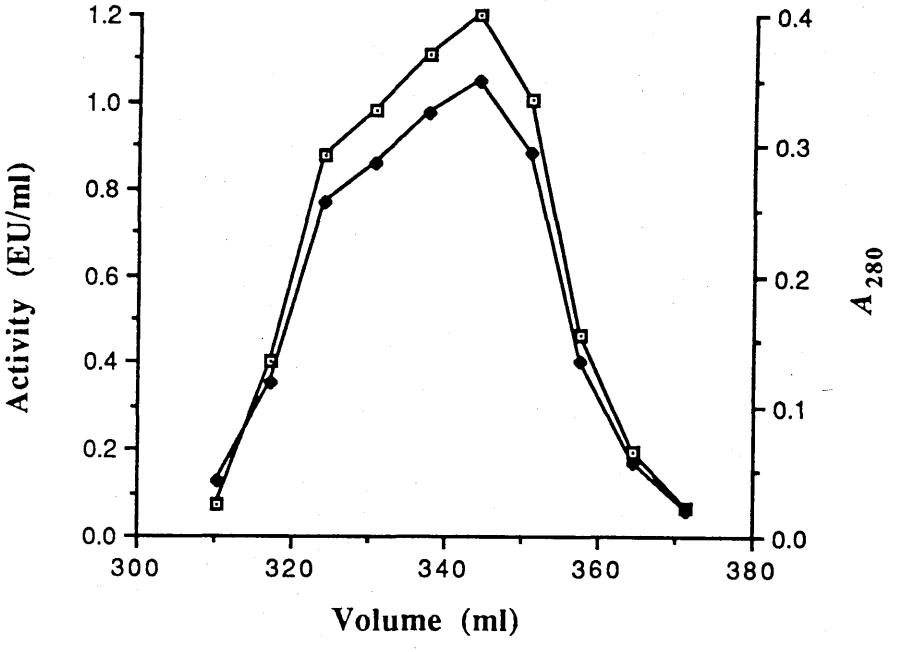


Figure 4.9. Chromatography of a DEAE Sephacel pool on Sephacryl S300HR

A DEAE Sephacel pool [Methods 2.9.3 (c)], concentrated by $(NH_4)_2SO_4$ precipitation to 11.5 ml [Methods 2.9.3 (d)], was applied at 25 ml h^{-1} to a column of Sephacryl S300HR (2.6 cm x 94 cm). Once loaded, the column was washed with 50 mM-Tris/HCl/ 2 mM-DTT/ 1 mM-benzamidine/ 1 mM-EDTA, pH 8.0, at 25 ml h^{-1}. Fractions of 6.25 ml were collected and the A_{280} of the active effluent monitored. Enzyme activity was measured as in Methods 2.5.1 (a). Enzyme activity (▫), A_{280} (•).

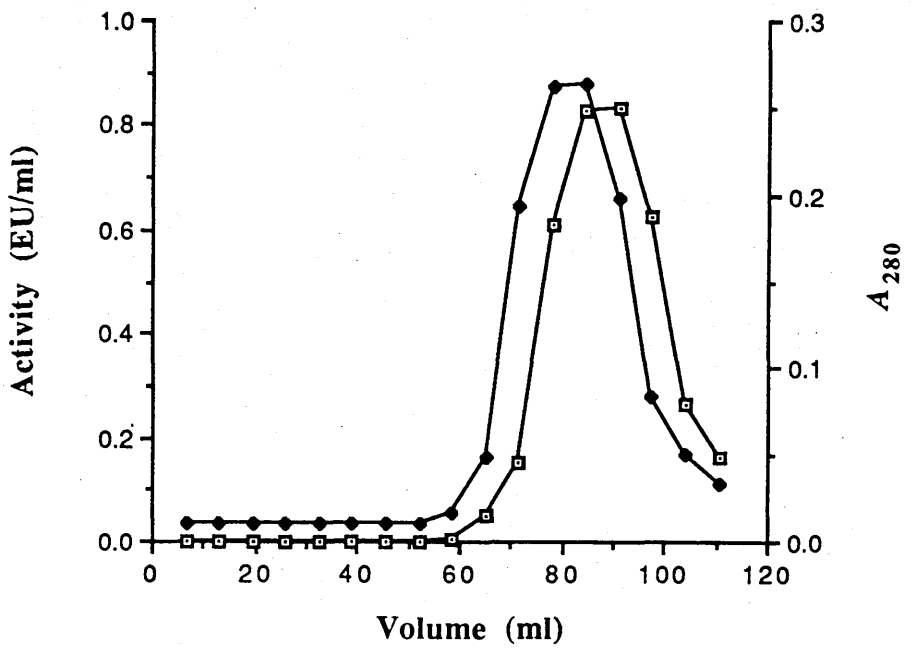


Figure 4.10. Chromatography of a Sephacryl S300HR pool on Phenyl Sepharose

The Sephacryl S300HR pool [46 ml, Methods 2.9.3 (e)],was applied at 26 ml h^{-1} to a column of Phenyl Sepharose (2.6 cm x 10 cm). After washing the column with 50 mM-Tris/HCl/ 2 mM-DTT/ 1 mM-benzamidine/ 1 mM-EDTA, pH 8.0, the enzyme was eluted with 40 % (v/v) ethanediol in 50 mM-Tris/HCl/ 2 mM-DTT/ 1 mM-benzamidine/ 1 mM-EDTA, pH 8.0. Fractions of 6.5 ml were collected and the A_{280} of the eluate monitored. Enzyme activity was measured as in Methods 2.5.1 (a). Enzyme activity (□), A_{280} (♦).

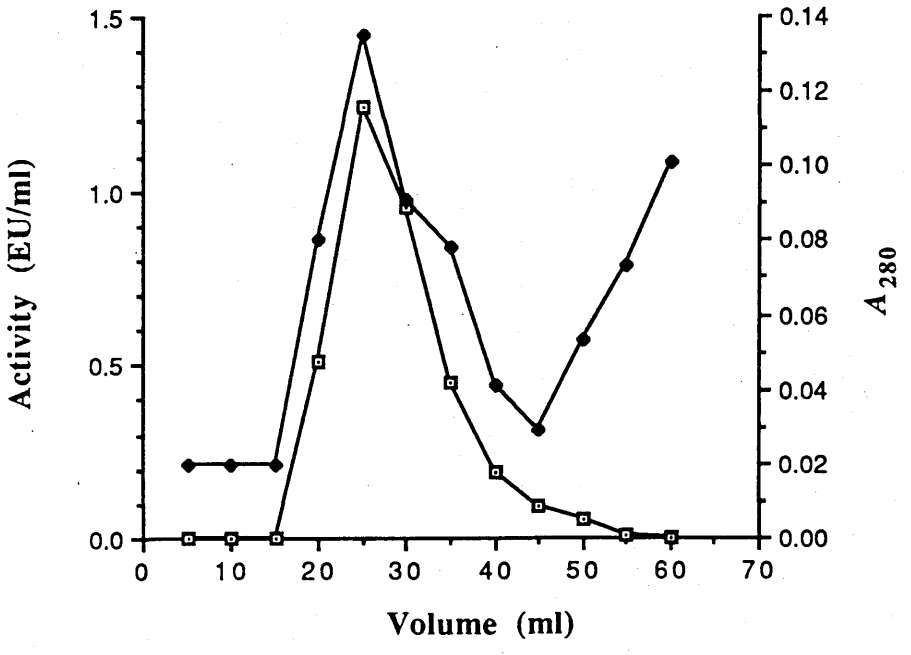
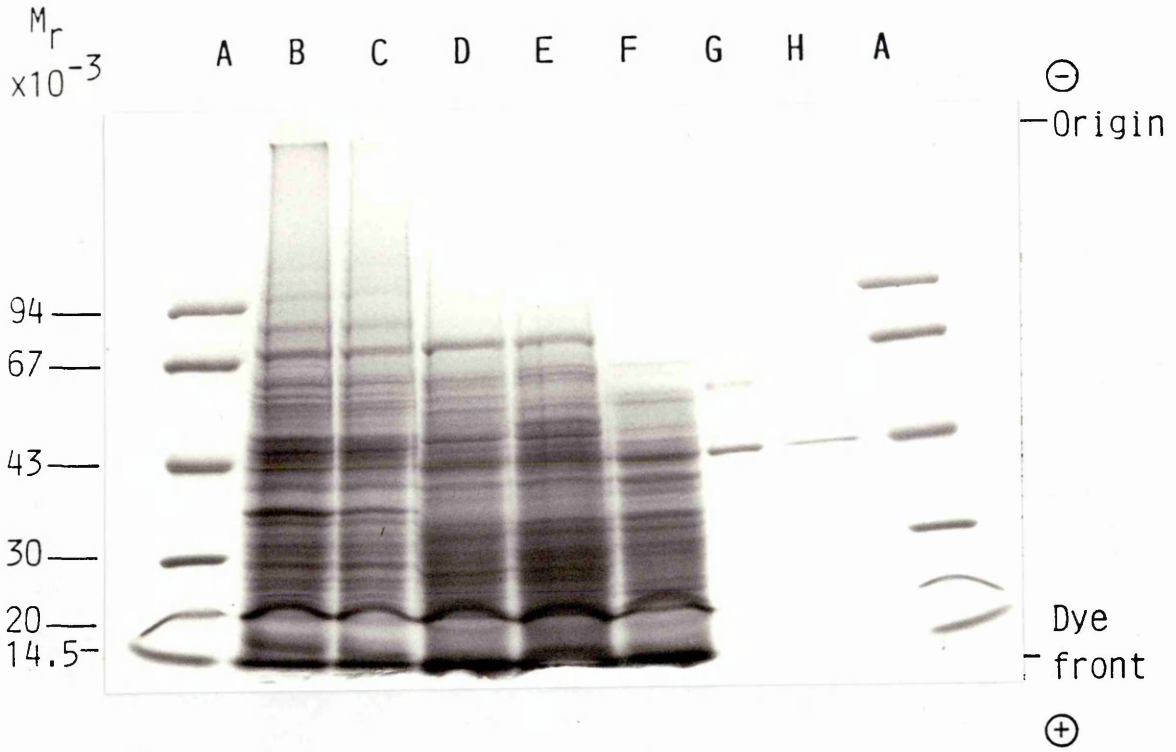


Figure 4.11. Chromatography of a Phenyl Sepharose pool on Matrex Gel Red A

$MgCl_2$ (100 mM) was added to a Phenyl Sepharose pool [39 ml, Methods 2.9.3 (f)] to 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 cm x 7.5 cm). Once loaded, the column was washed with 50 mM-Tris/HCl/ 2 mM-DTT/ 5 mM-$MgCl_2$, pH 8.5. Enzyme was eluted with 50 mM-Tris/HCl/ 2 mM-DTT/ 5 mM-$MgCl_2$/ 0.2 mM-NADP, pH 8.5. Fractions of 5 ml were collected and the A_{280} of the effluent monitored. Enzyme activity was measured as in Methods 2.5.1 (a). Enzyme activity (⊡), A_{280} (●).



A, M_r markers;
B, Crude extract;
C, Dialysis;
D, DEAE Sephacel;
E, $(NH_4)_2SO_4$ ppte;
F, Sephacryl S300 HR;
G, Phenyl Sepharose;
H, Matrex Gel Red A;

Figure 4.12. Purification of NADP-dependent alcohol dehydrogenase of S. *cerevisiae* D273-10B

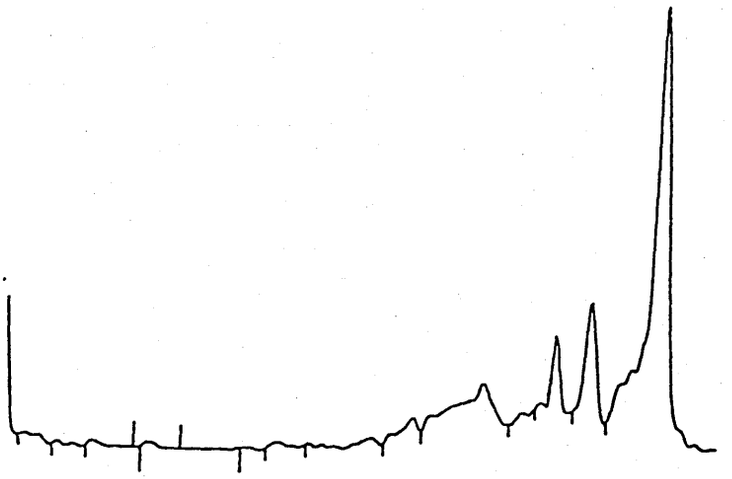
The purification was monitored by SDS-PAGE. A 10 % (w/v) SDS polyacrylamide gel with 5.6 % (w/v) stacking gel was stained for protein as in Methods 2.10.1 and 2.10.3 (a). Lane: A, M_r markers; B, 100 µg crude extract; C, 100 µg dialysed crude extract; D, 50 µg DEAE Sephacel pool; E, 50 µg $(NH_4)_2SO_4$ pool; F, 40 µg Sephacryl S300HR pool; G, 40 µg Phenyl Sepharose pool; H, 2 µg Matrex Gel Red A pool.

Figure 4.13. Densitometer scans of purified NADP-dependent alcohol dehydrogenase of *S. cerevisiae* D273-10B and M_r markers

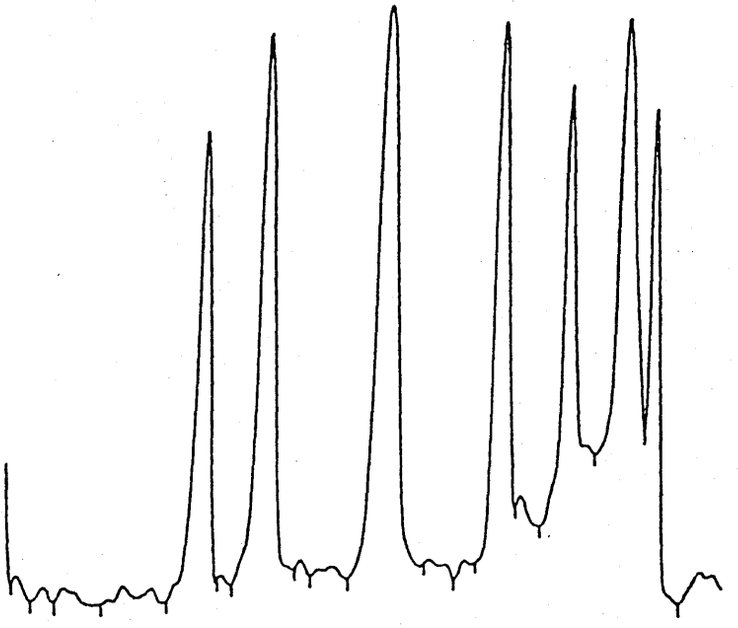
Purified NADP-dependent alcohol dehydrogenase from *S. cerevisiae* D273-10B and M_r markers were electrophoresed on a 10 % (w/v) SDS polyacrylamide gel with 5.6 % (w/v) stacking gel and stained for protein as in Methods 2.10.1 and 2.10.3 (a).They were then scanned with a laser densitometer (Methods 2.10.5). (a) Control (no sample loaded); (b) M_r markers (from left to right) phosphorylase *b* (M_r 94 000), BSA (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soya bean trypsin inhibitor (M_r 20100) and α-lactalbumin (M_r 14 400); (c) pure NADP-dependent alcohol dehydrogenase (2 μg).

The scans are aligned at the peak marking the start of the resolving gel (not shown).

(a)



(b)



(c)

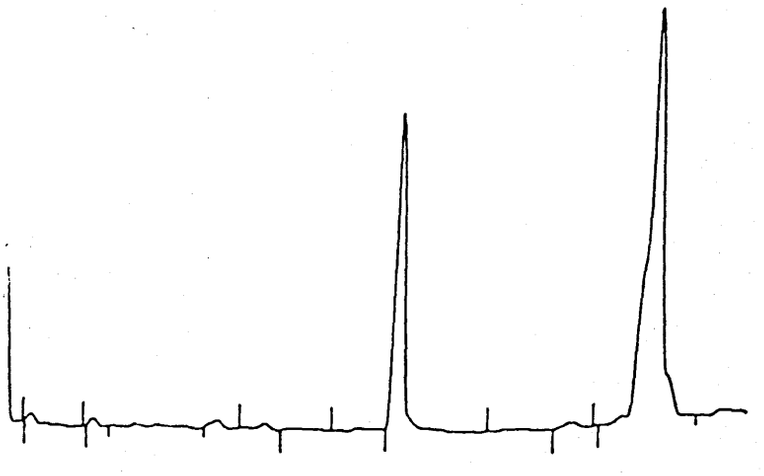


Table 4.2. Purification of NADP-dependent alcohol dehydrogenase of *S. cerevisiae* D273-10B

EU mg^{-1} = $\mu mol.min^{-1}$(mg protein)$^{-1}$

	Volume (ml)	Total activity (EU)	Total protein (mg)	Specific activity (EU.mg^{-1})	Yield (%)	Purification (fold)
Crude extract	110	73.0	6050	0.012	100	1.0
Dialysis	124	50.6	6200	0.008	70	0.7
DEAE Sephacel	76	49.9	449	0.111	68	9.0
$(NH_4)_2SO_4$ precipitation.	11.5	38.5	381	0.101	53	8.4
Sephacryl S300	46	31.8	59.8	0.532	44	44
Phenyl Sepharose	39	16.6	6.63	2.50	23	208
Red A	34	18.9	0.44	42.8	26	3020
Dialysis	40	13.1	0.34	34.0	15	2833

4.4. Discussion.

The method used to purify NADP-dependent alcohol dehydrogenase from *A. calcoaceticus* NCIB 8250 was basically applicable to purifying the same enzyme activity from *Acinetobacter sp.* HO1-N and from *S. cerevisiae* D273-10B.

For the enzyme purified from *Acinetobacter sp.* HO1-N, the purification protocol takes 7 days to complete and gives a 1680 fold increase in purification over crude extract with 39 % recovery. Elution from DEAE Sephacel and from Sephacryl S300HR occurred similarly to that of the enzyme from *A. calcoaceticus* NCIB 8250. The enzyme from *Acinetobacter sp.* HO1-N bound to Phenyl Sepharose in a higher saturation (20 %) of ammonium sulphate than the enzyme from *A. calcoaceticus* NCIB 8250 (5 %) perhaps suggesting that it is less hydrophobic. The enzyme purified from *Acinetobacter sp.* HO1-N bound to Matrex Gel Blue A at lower pH than that from *A. calcoaceticus* NCIB 8250. Lower pH increases non-specific binding of proteins to triazine dye-ligands so it may be that a decrease in hydrophobic interactions of the enzyme purified from *Acinetobacter. sp.* HO1-N compared to that from *A. calcoaceticus* NCIB 8250 makes the lower pH necessary for binding. There is a very large increase in purification fold at this stage, which results from removal of a high M_r contaminant (see Figure 4.5). This contaminating protein does not bind to the triazine dye ligand. It appears green/yellow in colour and has absorbance maxima at 280 nm and 405 nm.

Purification of NADP-dependent alcohol dehydrogenase from *S. cerevisiae* D273-10B as described (Methods 2.9.3) takes 6 days to complete and gives about a 3000 fold increase in activity with 15-20 % recovery. Elution from DEAE Sephacel occurred similarly to that of the enzyme from *A. calcoaceticus* NCIB 8250. It was concentrated prior to gel filtration by ammonium sulphate precipitation for reasons of speed and convenience. Less activity is lost in ammonium sulphate precipitation with the yeast enzyme compared to that of *A. calcoaceticus* NCIB 8250. A larger gel filtration column was used in this purification so direct comparisons with the elution profile of the enzyme of *A. calcoaceticus* NCIB 8250 cannot be made. The enzyme from yeast binds to Phenyl

Sepharose in the absence of ammonium sulphate whereas that from *A. calcoaceticus* NCIB 8250 needs 5 % ammonium sulphate to bind. This suggests the enzyme from yeast is more hydrophobic than that from *A. calcoaceticus* NCIB 8250. The enzyme from yeast bound to Matrex Gel Blue A like the others but could not be purified to homogeneity on this triazine dye ligand. Unlike the enzyme from *A. calcoaceticus* NCIB 8250 the yeast enzyme could be biospecifically eluted from Matrex Gel Red A and it behaved in a different manner in its elution, eluting in a much smaller volume with an obvious peak of activity. This suggests that it either lacks the features that lead to both *Acinetobacter* enzymes binding strongly and non-specifically to triazine dyes or that some much weaker interactions occur. Mg^{2+} ions are necessary throughout this stage in the purification, presumably to ensure some potential contaminating proteins stay bound to the column.

NADP-dependent alcohol dehydrogenase can be purified from all three organisms using the similar procedures. Small changes in surface amino acid residues can have significant effects on the behaviour of proteins during ion exchange and hydrophobic interaction chromatography. However, the fact that they do behave relatively similarly in these chromatographic steps suggests there is some degree of similarity amongst the three proteins. This may be expected of the enzyme from the two different strains of *Acinetobacter* but it is perhaps more surprising that NADP-dependent alcohol dehydrogenase of *S. cerevisiae* D273-10B is purified in a similar manner to the *Acinetobacter* enzymes. The major difference between the *Acinetobacter* enzymes and the yeast enzyme is that the latter can be biospecifically eluted from Matrex Gel Red A whereas this is not true for the former enzymes. This change could be due to relatively small changes in surface amino acid residues and surface amino acids are more likely to vary amongst homologous proteins than internal residues. In summary, the similarities in the purification procedures of these enzymes suggest that they have similar structures.

CHAPTER 5
CHARACTERISATION OF NADP-DEPENDENT ALCOHOL DEHYDROGENASE OF *ACINETOBACTER CALCOACETICUS* NCIB 8250

5.1. Introduction

Having achieved the first part of this work, purifying the NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250, the chief aim of the work described in this chapter was to characterise the enzyme in order to compare it with other alcohol dehydrogenases and to provide some information that might give clues as to its physiological function.

5.2. Physical and chemical characterisation

5.2.1. Relative molecular mass

The native M_r of the enzyme, estimated by gel filtration chromatography through a f.p.l.c. Superose 6 column (Figure 5.1), was 158 500 (151 500, 165 500).

The subunit M_r of the enzyme was estimated using calibrated SDS-PAGE gels. A representative standard plot of mobility against $\log_{10} M_r$ is shown in Figure 5.2. The subunit M_r value was determined to be 40 300 (+/- 2000, n = 10 samples of enzyme).

From comparison of the subunit and native M_r values, the enzyme appears to be a tetramer.

5.2.2. Absorption spectrum

The absorption spectrum of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 had a maximum at 280 nm (Figure 5.3) but no absorbance above 340 nm indicating that the enzyme is not associated with bound cytochrome, haem, PQQ or flavin as prosthetic group.

5.2.3. Isoelectric point

The isoelectric point of the enzyme was at pH 5.7 as judged by chromatofocussing on a f.p.l.c. Mono P column (see Section 3.3.8).

5.2.4. *N*-Terminal amino acid sequence

The sequence of the first 38 amino acids of NADP-dependent alcohol dehydrogenase were determined from two separate samples of the protein (Table 5.1).

In October 1991 the sequence was compared with other protein sequences using two algorithms, Wordsearch and Fasta, from the University of Wisconsin Genetic Computer Group (Deveraux *et al.*, 1984). Wordsearch, a Wilbur and Lipman type search (Wilbur and Lipman, 1983), compared the sequence to protein sequences in the Genbank and EMBL databases [Release 60.0 (6/89) and Release 26.0 (2/91) respectively]. Fasta, a Pearson and Lipman type search (Pearson and Lipman, 1988), compared the sequence to protein sequences in the NBRF-protein database [Release 26.0 (9/90)]. Also, the sequence was compared by eye to the *N*-terminal amino acid sequences (to the 80th amino acid) of those bacterial alcohol dehydrogenases that have been sequenced. The only other alcohol dehydrogenase, or related enzyme, to which any significant homology was observed with the NADP-dependent alcohol dehydrogenase sequence, by eye or by computer search, was with a fermentative alcohol dehydrogenase from *E. coli* (see Section 5.5.1).

Attempts to generate some internal peptides for *N*-terminal amino acid sequencing by enzymic methods, using V8 protease, trypsin, chymotrypsin or proteinase K, in the presence and absence of urea as denaturant (Methods 2.11.3), were unsuccessful and so it was not possible to obtain any information about internal sequences. In the absence of denaturant there was very little proteolysis whereas in its presence proteolysis did proceed but without the generation of discrete, large peptides. Control experiments with pig heart lactate dehydrogenase and chymotrypsin were successful. It therefore appears that it is

difficult to generate internal peptides of NADP-dependent alcohol dehydrogenase by limited proteolysis, perhaps because of the way in which the protein is folded.

Table 5.1. *N*-Terminal amino acid sequence of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250

The *N*-terminal amino acid sequence of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 was determined for two separate samples of protein (Methods 2.11.2). Approximately 12 picomoles and 35 picomoles of enzyme were sequenced in two separate runs (Run 1 and Run 2 respectively) and the PTH amino acid derivative identified and the amount recovered in picomoles at each cycle are given for each run. The deduced amino acid sequence is given also. Abbreviations: ?, identification tentative; X, not identified.

	Run 1		Run 2		Sequence
Cycle	PTH amino acid	pmoles	PTH amino acid	pmoles	
1	X	-	X	-	X
2	N	8	N	34	N
3	N	7	N	34	N
4	T	1	T	18	T
5	F	6	F	23	F
6	H	5	H	20	H
7	A	8	A	21	A
8	Y	12	Y	23	Y
9	A	3	A	16	A
10	A	6	A	11	A
11	L	9	L	14	L
12	E	5	K?	1	E?
13	A	5	A	9	A
14	G	4	G	16	G
15	A	6	A	9	A
16	A	8	A	10	A
17	L	5	L	14	L
18	V	5	V	13	V
19	P	1	P	2	P
20	Y	6	Y	17	Y
21	Q	3	Q	13	Q
22	F	4	F	14	F
23	D	3	D	10	D
24	A	2	A	3	A
25	G	2	G	9	G

Continued over page

	Run 1		Run 2		Sequence
Cycle	PTH amino acid	pmoles	PTH amino acid	pmoles	
26	E	3	E	6	E
27	L	2	L	7	L
28	Q	3	Q	3	Q
29	X	-	X	-	X
30	H	3	H	3	H
31	Q	0	Q	5	Q
32	V	2	V	9	V
33	E	1	X	-	E?
34	V	1	E?	3	V?
35	X	-	X	-	X
36	V	0	X	-	V?
37	E?	0	X	-	E?
38	Y	1	Y	5	Y

Figure 5.1. Determination of the native M_r of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 by gel filtration

The native M_r value of NADP-dependent alcohol dehydrogenase from *A. calcoaceticus* NCIB 8250 was estimated under non-denaturing conditions using a Superose 6 gel filtration column (1 cm x 30 cm) pre-equilibrated with 50 mM-Tris/HCl/ 2 mM-DTT/ 0.1 M-NaCl, pH 8.0. Proteins were eluted at 0.3 ml h^{-1} and the A_{280} of the effluent was monitored (Methods 2.2.5). When enzyme was eluted fractions were collected and assayed for enzyme activity [Methods 2.5.1 (a)]. The elution volumes of the proteins were plotted against log M_r. Samples were run in duplicate and all data points are shown. Where values were identical only one point is shown. The standard proteins used, all shown as black circles, were:

1. cytochrome *c* (M_r 12 500, 25 µg);
2. ovalbumin (M_r 45 000, 100 µg);
3. lactate dehydrogenase (M_r 140 000, 50 µg);
4. aldolase (M_r 158 000, 200 µg);
5. pyruvate kinase (M_r 237 000, 100 µg);
6. ferritin (M_r 450 000, 50 µg).

The elution volume of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 (20 µg) is indicated by an arrow.

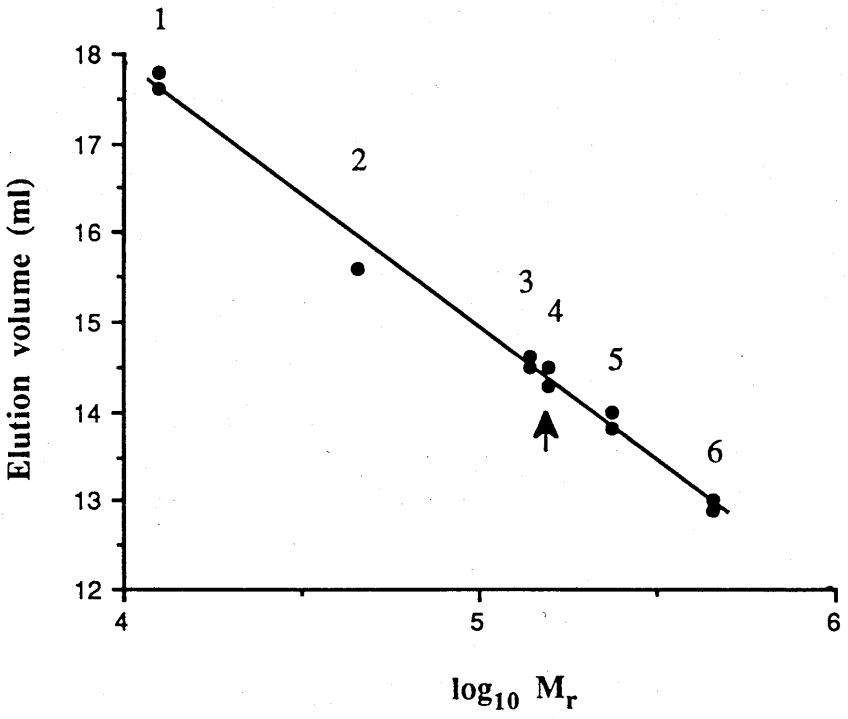
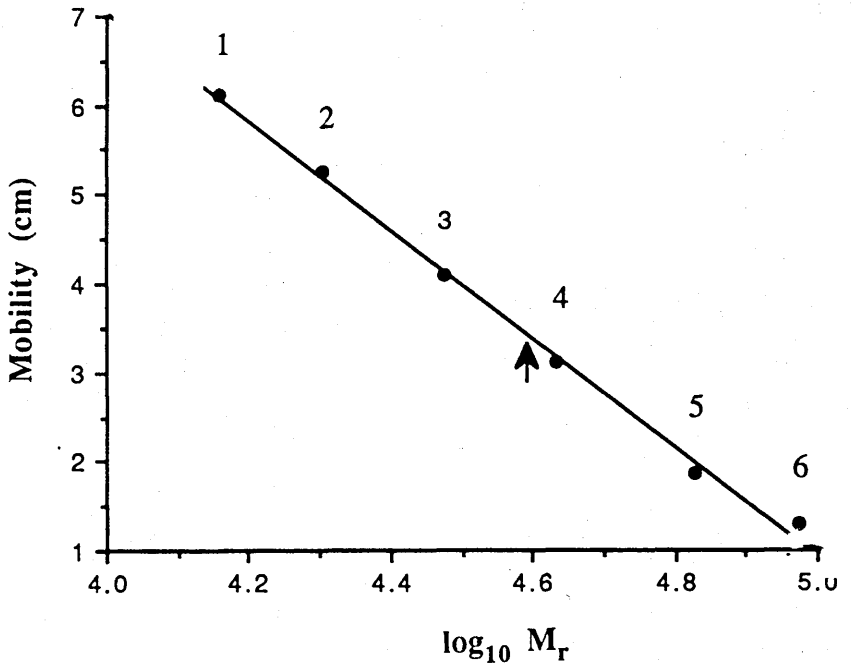

1
2
3
4
5
6
18
17
16
15
14
13
12
Elution volume (ml)
4
5
6
log10 Mr

Figure 5.2. Determination of the subunit M_r of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 by SDS-polyacrylamide gel electrophoresis

The subunit M_r value of NADP-dependent alcohol dehydrogenase from *A. calcoaceticus* NCIB 8250 was estimated under denaturing conditions using 10 % (w/v) SDS-polyacrylamide gels calibrated with proteins of known M_r. Gels were run and stained for proteins as in Methods 2.10.1 and 2.10.3 (a) respectively. The distance migrated by the calibration proteins down the gel was plotted against $\log_{10} M_r$ in each case. A typical standard curve is shown, as is the position of the NADP-dependent alcohol dehydrogenase. The standard proteins used, all shown as black circles, were:

1. α-lactalbumin (M_r 14 400);
2. soya bean trypsin inhibitor (M_r 20 100);
3. carbonic anhydrase (M_r 30 000);
4. ovalbumin (M_r 43 000);
5. bovine serum albumin (M_r 67 000);
6. phosphorylase *b* (M_r 94 000).

The mobility of NADP-dependent alcohol dehydrogenase from *A. calcoaceticus* NCIB 8250 is shown as an arrow.


Mobility (cm)
7
6
5
4
3
2
1
1
2
3
4
5
6
4.0
4.2
4.4
4.6
4.8
5.0
log10 Mr

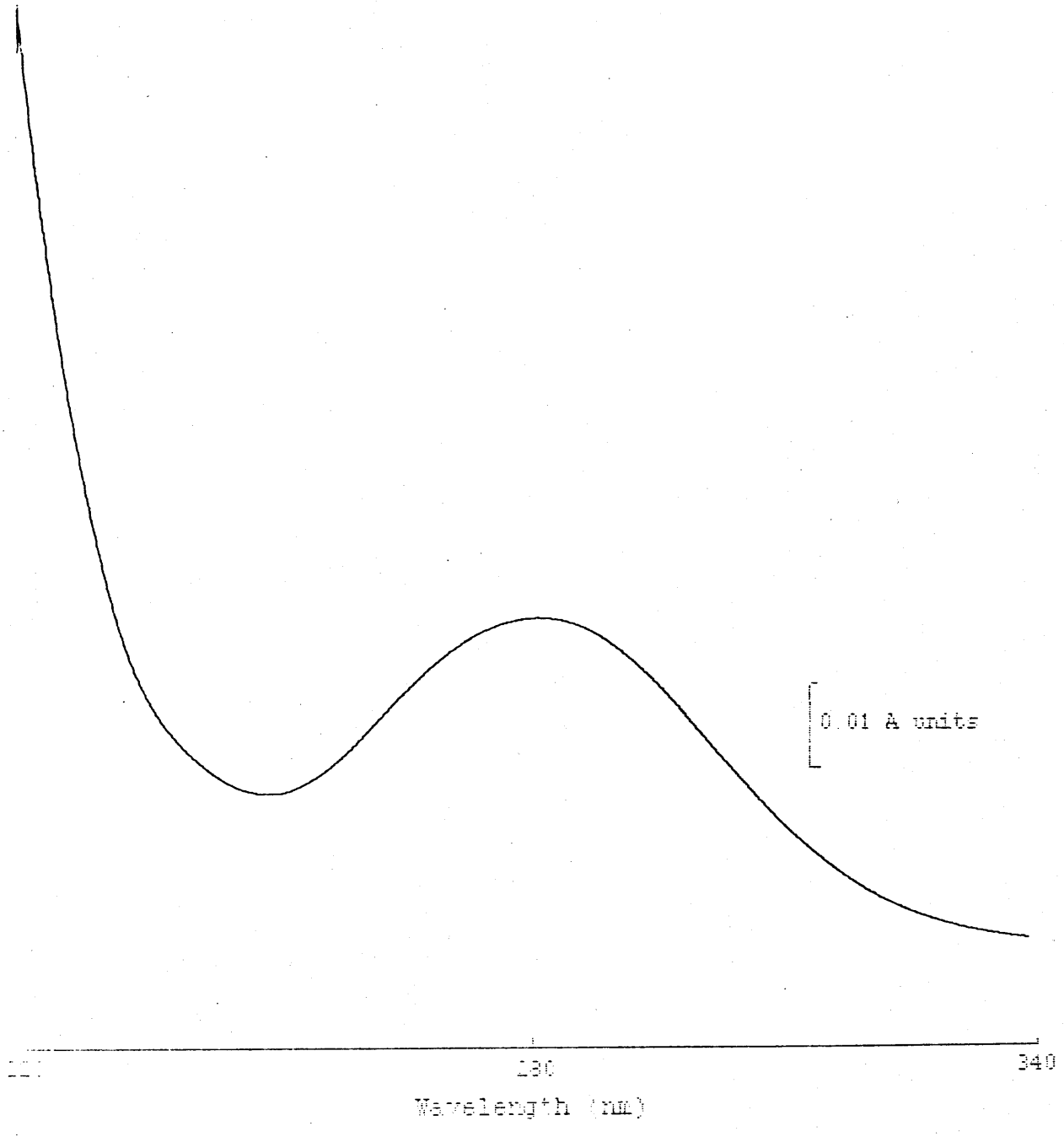


Figure 5.3. Absorption spectrum of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250

The absorption spectrum of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 in 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0 was recorded in a Pye Unicam SP8-100 spectrophotometer. The light path was 1 cm and buffer without protein was placed in the reference beam. The protein concentration of the sample was 0.06 mg ml^{-1}.

5.3. Preliminary kinetic characterisation

5.3.1. Assay development

The alcohol oxidation assay used initially to measure the enzyme in crude extracts was that described by Fixter and Nagi (1984), the assay mixture (volume 1 ml) containing 80 mM-glycine/NaOH, pH 9.5, 2 mM-$NADP^+$ and 0.3 to 0.5 mg bacterial extract protein, the reaction being initiated by addition of 5 µl butan-1-ol to give a final concentration of 53.7 mM. This assay was not very reproducible and did not give a linear reaction rate for longer than two minutes. The assay could be made reproducible and give a linear rate over a longer time by increasing the pH of the assay mixture to 10.2 and either increasing the butan-1-ol concentration to 100 mM or using 20 mM-hexan-1-ol as substrate. This can be explained by considering the equation for the reaction:

$$\text{Alcohol} + NADP^+ \rightleftharpoons \text{Aldehyde} + NADPH + H^+$$

Presumably the reaction was tending towards equilibrium. By increasing the substrate concentration relative to its K_m value and by increasing the pH, thereby decreasing the H^+ ion concentration, the reaction is driven more towards alcohol oxidation and not tending towards equilibrium significantly for at least five minutes with the usual enzyme concentrations. The concentration of $NADP^+$ could be decreased from 2 mM to 1 mM without significantly altering the initial reaction rate, presumably because it is effectively saturating the enzyme at either concentration. The reaction mixture described in Methods 2.5.1 (a) was used for all later experiments with pure enzyme.

5.3.2. pH optimum

(a) Alcohol oxidation

The alcohol oxidation reaction was assayed at a range of pH values between 6.4 and 11.0 [Figure 5.4 (a)]. As no single buffer covers this pH range it was necessary to use a number of different buffers. The optimal pH for alcohol oxidation was pH 10.2. A higher reaction velocity resulted when the buffer used in the assay mixture was glycine rather

than CAPS. Above pH 9.8 the pH curve showed a sharp upswing when glycine was used as the assay buffer compared to the pH curve when CAPS was the assay buffer. A possible explanation for this is that the pK value of the amino group of glycine is 9.75, i.e. above pH 9.75 the amino group of glycine exists predominantly in the uncharged NH_2 form. It may be that this uncharged amino group is acting as a Schiff's base reacting with the aldehyde product of the alcohol oxidation reaction thus trapping it and lowering the extent of the back reaction whereas with CAPS buffer where trapping of this type cannot occur, the back reaction lowers the observed, apparent rate. Attempts to obtain a similar effect by including hydrazine in the assay mixture, which had been used successfully to maximise reaction rates of benzyl alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 (MacKintosh and Fewson, 1988a), failed because it completely inhibited the enzyme.

(b) Aldehyde reduction

The aldehyde reduction reaction was assayed at a range of pH values between 5.4 and 7.45 in Bistris buffer [Figure 5.4 (b)]. The pH optimum of the aldehyde reduction assay lies at around pH 6.8, although the reaction rate is high (> 95 % of maximal rate) over a broader range of pH (pH 6.4 to pH 7.15) than the alcohol oxidation reaction (pH 10.15 to pH 10.25). Other buffers tested were Tris and potassium phosphate but they gave consistently lower reaction rates at pH 7.0.

5.3.3. Reproducibility and linearity of reaction

(a) Alcohol oxidation

The initial rate of reaction was linear over six minutes and proportional to the amount of enzyme added for protein concentrations of up to 500 ng ml^{-1}. Measurements of the initial reaction rate were reproducible [55.4 +/- 0.35 EU $(mg\ protein)^{-1}$] when assayed ten times as described in Methods 2.5.1 (a) with a protein concentration of 300 ng ml^{-1}.

(b) Aldehyde reduction

The initial rate of reaction was linear over six minutes and proportional to the amount of enzyme added for protein concentrations of up to 50 ng ml^{-1}. Measurements of the initial reaction rate were reproducible [39.15 +/- 0.17 EU $(mg\ protein)^{-1}$] when assayed five times as described in Methods 2.5.1 (c) with a protein concentration of 25 ng ml^{-1}.

5.3.4. Apparent K_{eq} and ΔG_0 values

The apparent K_{eq} value was determined at five different initial substrate concentrations with glycine as assay buffer and two different initial substrate concentrations with CAPS as assay buffer [Figures 5.5 (a) and 5.5 (b) respectively], initial substrate concentrations being determined as described in Methods 2.5. The progress curve in each case was followed until there was no further change in A_{340}. From this value the NADPH concentration at equilibrium was determined. It was assumed that the equation of the reaction was:

$$\text{Hexan-1-ol} + NADP^+ \rightleftharpoons \text{Hexanal} + NADPH + H^+$$

and the equilibrium concentrations of the other substrates and products were calculated from the equilibrium concentration of NADPH and the initial concentrations of hexan-1-ol and NADP; the pH was measured directly (Methods 2.2.1).

Therefore

$$K_{eq} = \frac{[\text{Hexanal}][NADPH][H^+]}{[\text{Hexan-1-ol}][NADP^+]}$$

From the values of apparent K_{eq}, the apparent ΔG_0 could be calculated from the equation:

$$\Delta G_0 = -RT \ln K_{eq}$$

where R = 8.303 J mol^{-1} and T = 300 K.

The final (equilibrium) concentrations of substrates and products, apparent K_{eq} and apparent ΔG_0 values determined are shown in Table 5.2.

Regardless of the assay buffer used in the reaction, aldehyde reduction appears strongly favoured. However, in glycine buffer the aldehyde reduction reaction is

significantly less favoured than in CAPS buffer, which agrees with the observation that the apparent maximal velocity is larger in glycine buffer than in CAPS buffer and adds credence to the proposal that glycine is acting as a trapping agent for the aldehyde hence driving the equilibrium more towards alcohol oxidation (see Section 5.3.3).

Figure 5.4. Effects of pH on the oxidation and reduction reactions of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250

(a) The alcohol oxidation reaction was measured in a reaction mixture (1 ml) containing 80 mM-buffer, 1 mM-$NADP^+$, 20 mM-hexan-1-ol and NADP-dependent alcohol dehydrogenase (300 ng). The pH values of the reaction mixtures were determined after assay (Methods 2.2.1). The maximum (100%) velocity was 57 EU $(mg\ protein)^{-1}$.

(◈) Potassium phosphate buffer, pH 6.4 to 7.5

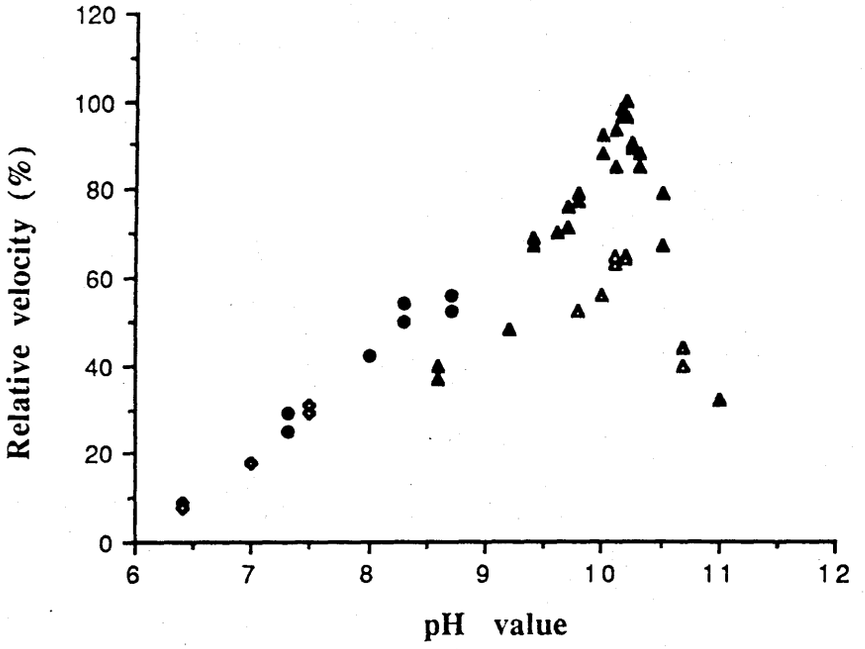
(●) Tris buffer, pH 7.3 to 8.7

(▲) Glycine buffer, pH 8.6 to 11

(▲) CAPS buffer, pH 9.8 to 10.7

(b) The aldehyde reduction reaction was measured in a reaction mixture (3 ml) containing 80 mM-bisTris buffer, 10 μM-NADPH, 20 mM butan-1-ol and NADP-dependent alcohol dehydrogenase (20 ng). The pH values of the reaction mixtures were determined after assay (Methods 2.2.1). The maximum (100%) velocity was 38 EU $(mg\ protein)^{-1}$.

(a)


Relative velocity (%)
120
100
80
60
40
20
0
6
7
8
9
10
11
12
pH value

b)

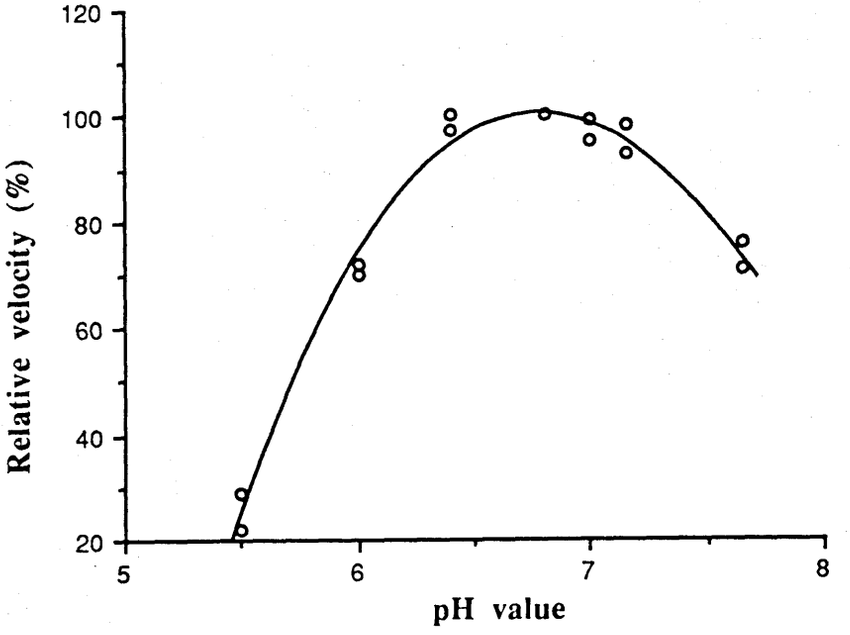

Relative velocity (%)
120
100
80
60
40
20
5
6
7
8
pH value

Figure 5.5. Progress curves of the alcohol oxidation reaction of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250

The alcohol oxidation of the reaction was assayed in either (a) 80 mM-glycine buffer, pH 10.2 or (b) 80 mM-CAPS buffer, pH 10.2, with enzyme (850 ng) and various limiting concentrations of hexan-1-ol and NADP, using a Pye-Unicam SP8-100 spectrophotometer. The A_{340} was followed until it showed no further change. Initial substrate concentrations:

(a) (1) 1000 mM-hexan-1-ol, 80 mM-NADP

(2) 1000 mM-hexan-1-ol, 40 mM-NADP

(3) 500 mM-hexan-1-ol, 80 mM-NADP

(4) 500 mM-hexan-1-ol, 40 mM-NADP

(5) 100 mM-hexan-1-ol, 40 mM-NADP

(b) (6) 1000 mM-hexan-1-ol, 80 mM-NADP

(7) 500 mM-hexan-1-ol, 80 mM-NADP

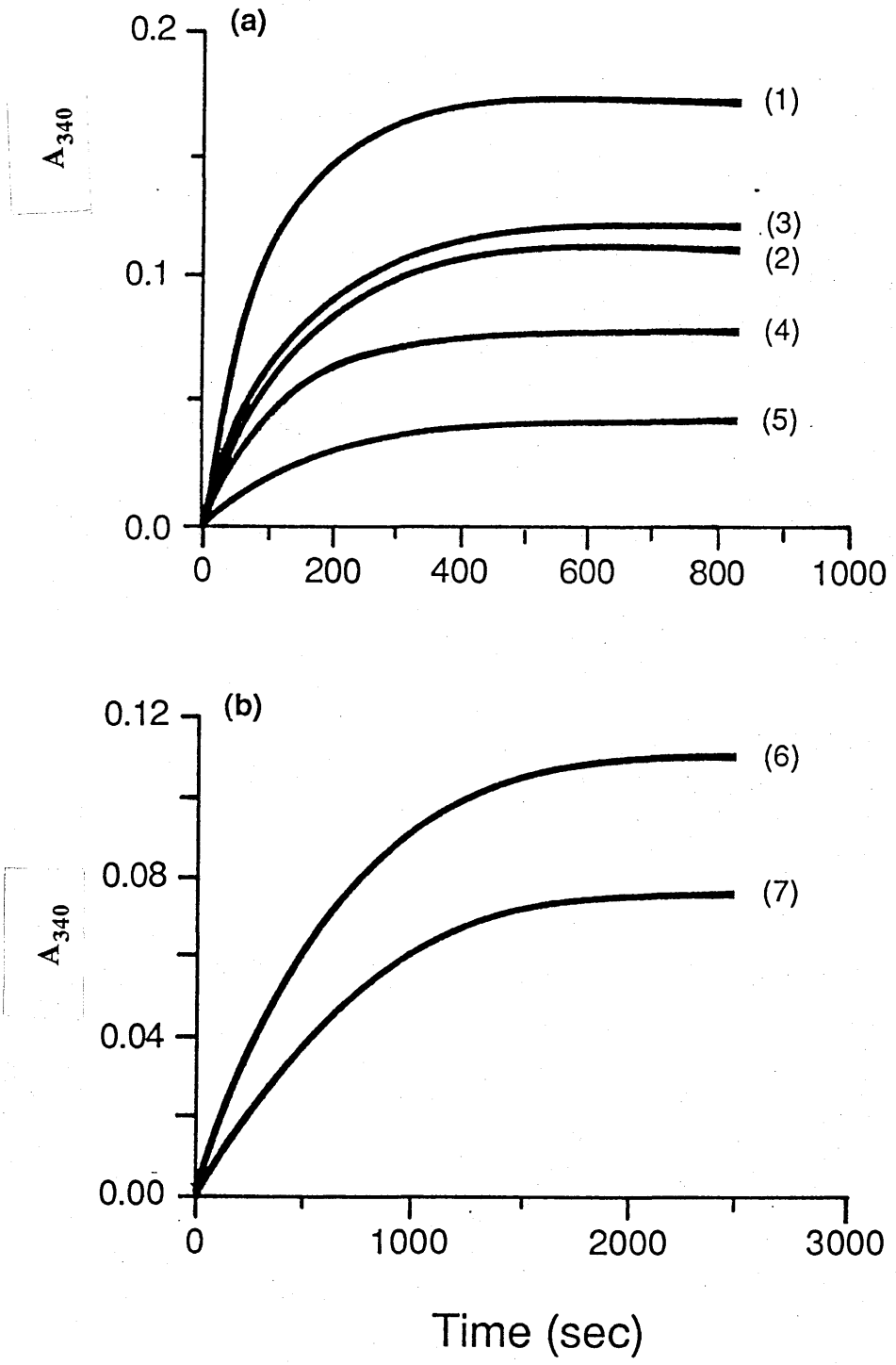

(a)
0.2
0.1
0.0
A_{340}
(1)
(3)
(2)
(4)
(5)
0
200
400
600
800
1000
(b)
0.12
0.08
0.04
0.00
A_{340}
(6)
(7)
0
1000
2000
3000
Time (sec)

Table 5.2. Apparent K_{eq} and ΔG_o values for NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250

K_{eq} values were calculated from the final A_{340} values of reactions shown in Figure 5.5. From the average K_{eq} values (standard deviations are given in parentheses, where appropriate), ΔG_o values were calculated using the equation $\Delta G_o = -RT \ln K_{eq}$ where $R = 8.303$ kJ mol^{-1} and $T = 300$ K.

(a) In glycine buffer

Progress curve:	Equilibrium concentration (μM) of:				K_{eq}(M)
	Hexan-1-ol	NADP	Hexanal	NADPH	(x 10^{13})
(1)	972.1	52.1	27.9	27.9	9.7
(2)	986.1	21.6	18.4	18.4	10.1
(3)	480.3	60.3	19.7	19.7	8.5
(4)	487.3	27.3	12.7	12.7	7.7
(5)	93.0	33.0	7.0	7.0	10.0

Average $K_{eq} = 9.2 \times 10^{-13}$ (+/- 1.1×10^{-13})

$\Delta G_o = +51.9$ kJ mol^{-1}

(b) In CAPS buffer

Progress curve:	Equilibrium concentration (μM) of:				K_{eq}(M)
	Hexan-1-ol	NADP	Hexanal	NADPH	(x 10^{13})
(6)	982.9	62.9	17.1	17.1	3.0
(7)	487.9	67.9	12.1	12.1	2.8

Average $K_{eq} = 2.9 \times 10^{-13}$

$\Delta G_o = +71.9$ kJ mol^{-1}

5.3.5. Cofactor specificity

(a) Electron acceptor specificity

Alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 did not detectably (< 1 %) reduce NAD^+ when assayed as described in Methods 2.5.1 (a) but with 1 mM-NAD^+ instead of 1 mM-$NADP^+$ in the reaction mixture. Also, the enzyme was unable to use the dye DCIP (200 mM) or DCIP and PMS (1 mM) as electron acceptors when assayed as described in Methods 2.5.1 (a) except that the dye replaced $NADP^+$ and A_{600} was followed.

(b) Stereospecificity of hydride transfer

The stereospecificity of hydride transfer was determined by assaying the enzyme in the presence of pro*S*-[^{3}H]NADPH or pro*R*-[^{3}H]NADPH and butanal (Methods 2.12). The criteria used to determine the stereospecificity were as follows:

If incubating the enzyme with pro*S*-[^{3}H]NADPH and butanal resulted in most of the radioactivity being identified in butan-1-ol the enzyme had transferred the ^{3}H labelled pro*S* hydride. However , if the radioactivity was found in $NADP^+$ the enzyme had transferred the "cold" pro*R* hydride. Similarly, if incubating the enzyme with pro *R* [^{3}H]NADPH and butanal resulted in most of the radioactivity being identified in butan-1-ol the enzyme had transferred the ^{3}H labelled pro*R* hydride. However, if the radioactivity was identified in NADP then the enzyme had transferred the "cold" pro*S* hydride.

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

5.3.6. Substrate specificity

NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 is specific for primary aliphatic alcohols (Table 5.3). Primary aliphatic alcohols with more than about ten carbons are insoluble in water (Hodgman *et al.*, 1959) and were made accessible as substrates to the enzyme as described in Methods 2.5.1 (b). When hexan-1-ol was pretreated by this method [Methods 2.5.1 (b)] the activity measured was a third to a quarter of that measured when the enzyme was assayed normally (Table 5.4). A decrease in activity was observed with increasing chain length of the primary alcohol substrate at the concentrations assayed (Table 5.4). The activity of the enzyme with tetradecan-1-ol as substrate is very low, and no activity is observed with hexadecan-1-ol.

No detectable enzyme activity was observed with the secondary alcohols propan-2-ol, pentan-2-ol and octan-2-ol (Table 5.5) and neither pentan-2-ol (50 mM) nor octan-2-ol (10 mM) inhibited enzyme activity when the enzyme was assayed as described in Methods 2.5.1 (a) but with pentan-1-ol (at 10 mM or 50 mM assay concentration) or octan-1-ol (at 1 mM or 10 mM assay concentration) respectively as substrate.

The enzyme could use the 1,x diols ethanediol and butan-1,4-diol as substrates (Table 5.3.) but not the 2,x diol butan-2,3-diol (Table 5.5). The activities obtained with 1,x diols were similar to those of their equivalent primary alcohols (Table 5.3). The enzyme used the mono-unsaturated alcohols allyl alcohol and *cis*-hex-3-en-1-ol (Table 5.3) as substrates but not hex-5-en-1-ol (a 50:50 *cis*:*trans* mixture; Table 5.5). Much lower activity was observed towards *cis* 3 hexan-1-ol compared with hexan-1-ol, its equivalent primary alcohol. Furthermore the enzyme used the polyols ribitol and mannitol as substrates with low rates (Table 5.3) but not erythritol (Table 5.5). Other potential substrates that were not oxidised by the enzyme include methanol, cyclohexanol, glyceraldehyde, the sugars glucose and fructose, the amino acid alcohols serine, threonine and histidinol, or hexahydrobenzyl alcohol or phenylethanol (Table 5.5) although it could use benzyl alcohol (Table 5.6). Formaldehyde (50 mM) and acetaldehyde (50 mM) were not oxidised by the enzyme 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).

Table 5.3. Relative activities of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 with various alcohol substrates

The enzyme was assayed as described in Methods 2.5.1 (a) except that the substrates listed below were used in place of hexan-1-ol at the concentration stated. The relative activity of the enzyme towards each substrate is given with respect to the activity with hexan-1-ol and to the activity of the enzyme towards the primary aliphatic alcohol with the same number of carbons as that substrate (the equivalent primary alcohol) e.g. ethanol is the equivalent primary alcohol of ethanediol. The activity with hexan-1-ol as substrate was 57.3 EU (mg protein)$^{-1}$.

Substrate	Concentration. (mM)	% Hexan-1-ol Activity	% Equivalent Primary Alcohol Activity
Ethanol	50	8.5	100
Ethanediol	50	8.5	100
	10	8.5	100
Propan-1-ol	50	28.0	100
Allyl alcohol	50	28.0	100
Butan-1-ol	50	36.3	100
Butan-1,4-diol	50	29.0	80
Pentan-1-ol	50	133	100
Ribitol	50	4.0	3.0
Hexan-1-ol	50	100	100
Mannitol	50	6.5	6.5
cis-Hex3-en-1-ol	10	18.7	18.7

Table 5.4. Ability of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 to use alcohols with chain lengths > 10 as substrates

All of the alcohols used were pre-sonicated in BSA (0.7 mg ml^{-1}) and assayed as described in [Methods 2.5.1 (b)] except that the alcohols listed in the table replaced hexan-1-ol. The apparent concentration is based on the concentration of the alcohol that was sonicated. Values in parentheses refer to activities with no pre-sonication with BSA at these alcohol concentrations [enzyme assayed as in Methods 2.5.1 (a)]. For all alcohols tested in this way the initial A_{340} of the pre-sonicated alcohol/BSA/buffer solution was < 0.1 compared to buffer/BSA solution alone; n.d. = not determined because the above criterion was not met.

Alcohol	Apparent Concentration (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	n.d.
	250	0.00
	100	0.00

Table 5.5. Potential substrates that were not oxidised by NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250

The enzyme was assayed as described in Methods 2.5.1 (a) except that 50 mM of the potential substrate was included in place of 20 mM-hexan-1-ol (unless stated).

(a) Aliphatic alcohols:

methanol; propan-2-ol; butan-2,3-diol; pentan-2-ol; hex-5-en-1-ol (10 mM); octan-2-ol (10 mM).

(b) Cylic alcohols:

hexahydrobenzyl alcohol; phenyl ethanol; cyclohexanol.

(c) Amino acid alcohols:

serine; threonine; histidinol.

(d) Sugars and polyols:

glyceraldehyde; erythritol; glucose; fructose; galactose.

5.3.7. Effects of salts, metal ions, metal binding agents and common metabolites

No significant effect (i.e. less than 10 % variation in enzyme activity) was observed when the enzyme (5 μg ml^{-1}) was pre-incubated at 27 °C over five minutes with 1 mM concentrations each of the following salts and common metabolites and then assayed for activity as described in Methods 2.5.1 (a), except that the salt/metabolite was included in the assay mixture at the same concentration that was used in the pre-incubation.:
NaCl; KCl; $(NH_4)_2SO_4$; $ZnCl_2$; $CuCl_2$; $MgSO_4$; $MgCl_2$; $MnCl_2$; $FeSO_4$; $FeCl_3$; ATP-Mg^{2+}; ADP-Mg^{2+}; acetyl CoA; succinate.

Pre-incubation of the enzyme (5 μg ml^{-1}) with the metal binding agents 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 minutes had no significant effect on enzyme activity when assayed for activity as described in Methods 2.5.1 (a), except that the potential inhibitor was included in the assay mixture at the same concentration that was used in the pre-incubation.. Incubation of yeast alcohol dehydrogenase 1 with 50 mM-pyrazole at 27 °C completely inhibited the enzyme after 5 minutes, as expected (Branden *et al.*, 1975).

5.3.8. Effects of thiol-acting agents

Pre-incubation of the enzyme (5 μg ml^{-1}) at 27 °C for 20 minutes with the thiol-acting agents iodoacetate (10 mM), iodoacetamide (10 mM) or *N*'-ethylmaleimide (10 mM) had no effect on enzyme activity when assayed as described in Methods 2.5.1 (a), except that the potential inhibitor was included in the assay mixture at the same concentration that was used in the pre-incubation. However, incubation of the enzyme with *p*-chloromercuribenzoate did inhibit the enzyme (Figure 5.6). No protection from this inhibition was observed in the presence of hexan-1-ol (10 mM) and/or $NADP^+$ (1 mM).

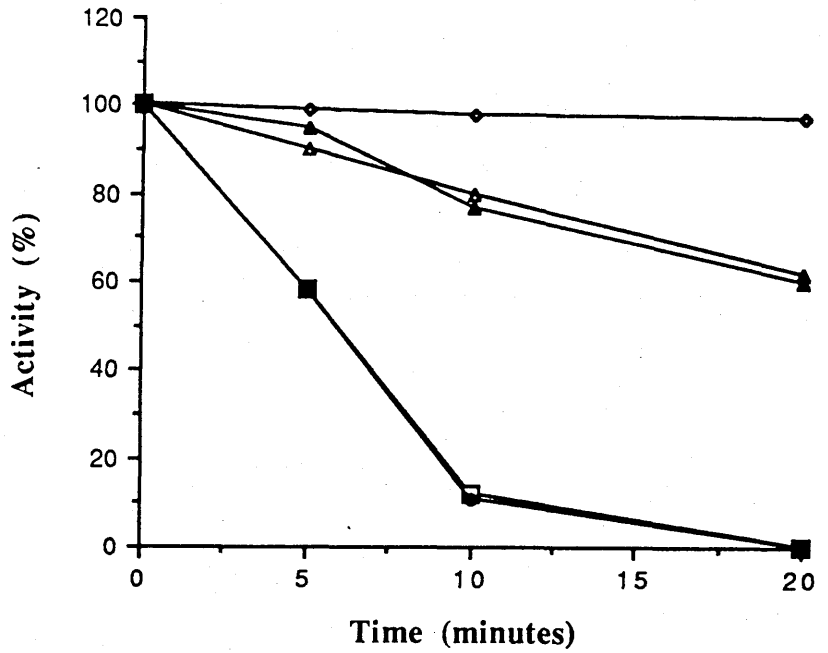


Figure 5.6. Effect of *p*-chloromercuribenzoate on NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250

The enzyme was incubated at 27 °C with either 100 μM or 10 μM *p*-chloromercuribenzoate both in the presence and in the absence of 10 mM-hexan-1-ol and 1 mM-NADP for 20 minutes. Samples were taken at 5, 10 and 20 minutes and assayed for enzyme activity [Methods 2.5.1(a)]. Each incubation contained 5 μg ml^{-1} enzyme and each assay contained 250 ng enzyme and the appropriate concentration of *p*-chloromercuribenzoate. Maximum (100 %) activity i.e at time = 0 in the absence of *p*-chloromercuribenzoate was 56 EU (mg protein)$^{-1}$. The results shown are the average of two experiments which, in all cases, agreed to each other to within 10 %.

(◆) control;

(□) 100 μM *p*-chloromercuribenzoate;

(●) 100 μM *p*-chloromercuribenzoate, 10 mM-hexan-1-ol, and 1 mM-NADP;

(▲) 10 μM *p*-chloromercuribenzoate;

(▲) 10 μM *p*-chloromercuribenzoate, 10 mM-hexan-1-ol, and 1 mM-NADP.

5.4. Steady state kinetics

5.4.1. K_m and V_{max} values for alcohol oxidation and aldehyde reduction

K'_m and V'_{max} values were determined for primary alcohols from ethanol to decan-1-ol (at pH 10.2), the aldehyde butanal (at pH 6.8), and the cofactor(s) NADP(H) 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. The kinetic constants calculated are shown in Table 5.6.

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 kinetic coefficients for decan-1-ol were obtained from decan-1-ol concentrations below its K'_m value because of its low solubility (Hodgman *et al.*,1959).

The K'_m values for primary alcohols decrease as chain length of the primary alcohol increases from ethanol to hexan-1-ol/octan-1-ol with decan-1-ol having a slightly higher K'_m than octan-1-ol. The V'_{max} values increase as chain length increases from ethanol to pentan-1-ol, then decrease with increasing chain length to decan-1-ol. The V'_{max} is slightly lower for butanal than for butan-1-ol [39.2 EU (mg protein)$^{-1}$ and 57.1 EU (mg protein)$^{-1}$ respectively], however, the K'_m value for butanal is much lower than for butan-1-ol (3.97 mM and 52.7 mM respectively). In addition the K'_m value for NADPH is much lower than that for $NADP^+$ (4.3 μM and 38 μM respectively) although the K'_m of both cofactors is much lower than that of either alcohol or aldehyde.

K'_m and V'_{max} 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 Methods 2.5.1 (a)]. This pH value was chosen because it was as close to the pH optimum of aldehyde reduction (pH 6.8) as the enzyme could be reasonably assayed at. The K'_m and V'_{max} values were 12.1 mM (68 % confidence limits, 11.4-17.2) and 41.0 EU (mg protein)$^{-1}$ (68 % confidence limits, 38.1-56.4) respectively. The K'_m value is ten fold higher than, and the V'_{max} value 70 % of, that obtained at pH 10.2 which suggests that these

variations are because of ionisations in the free enzyme as opposed to the enzyme-substrate complex (Fersht, 1985).

5.4.3. Turnover numbers and specificity constants

The turnover number (k'_{cat}) and the specificity constant (k'_{cat}/K'_m) of the enzyme were determined for primary alcohols and for butanal from the data in Table 5.7, assuming that there was one active site/subunit enzyme, that the subunit M_r of the enzyme was 40 300 (Section 5.2.1) and that $V'_{max} = k'_{cat}[E_t]$ where $[E]_t$ = the concentration of active sites.

The highest turnover number of the enzyme is for pentan-1-ol as substrate. The highest specificity constants of the enzyme are for hexan-1-ol and heptan-1-ol as substrates.

Using V'_{max} values for butan-1-ol and butanal, specificity constants can be calculated for $NADP^+$ and NADPH respectively. The specificity constant calculated for NADPH is 6-7 fold higher than that calculated for $NADP^+$ (6.6×10^6 and 1.0×10^6 respectively). For both cofactors the calculated specificity constants are 10^3-10^4 times greater than those for butan-1-ol and butanal.

Table 5.6. Kinetic constants of selected substrates of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250

The K'_m and V'_{max} 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 concentration of the other substrate (1 mM-NADP, 20 μM NADPH, 20 mM hexan-1-ol or 20 mM-butanal as appropriate) at pH 10.2. The standard assay mixtures (Methods 2.5.1) were used except that the concentration of one of the substrates was varied. The aldehyde reduction reaction was assayed fluorimetrically [Methods 2.5.1 (c)]. 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 (Methods 2.7). Where only one value was determined the 68 % confidence limits (Methods 2.7) 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 average is given with the standard deviation and the sample size in parentheses.

n.d. = not determined.

Substrate	K_m (mM)	V_{max} [EU (mg protein)$^{-1}$]
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)
Decan-1-ol	2.56 (2.32,2.80)	12.1 (11.0,13.1)
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 (hexan-1-ol)	38.0×10^{-3} (39.7×10^{-3}, 36.2×10^{-3})	n.d.
NADPH (butan-1-ol)	4.33×10^{-3} (3.77×10^{-3}, 4.88×10^{-3})	n.d.

Table 5.7. Apparent turnover numbers and specificity constants for selected substrates of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250

Apparent turnover numbers (k'_{cat}) and specificity constants (k'_{cat}/K'_m) were determined using the average values for kinetic coefficients given in Table 5.6.

Substrate	k'_{cat} (s^{-1})	k'_{cat}/K'_m (s^{-1} M^{-1})
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
Decan-1-ol	8.1	3.17×10^3
Butanal	26.3	6.62×10^3

5.5. Discussion

5.5.1. Protein structure

The NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 appears to be a tetramer (native M_r 158 500) of identical subunits (M_r 40 300; Section 5.2.1). It is somewhat smaller than the partially purified enzyme from *A. calcoaceticus* 69V which is a tetramer of native M_r value 235 000 (Introduction 1.8). The only other alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 to be purified and characterised is benzyl alcohol dehydrogenase which is also a tetramer of similar subunit M_r (39 700; MacIntosh and Fewson, 1988).

Many other bacterial alcohol dehydrogenases have been purified and their subunit and native M_r values estimated (see Introduction 1.3, 1.4 and 1.5). It is most useful and informative to make comparisons between NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 and those bacterial enzymes for which amino acid sequences (complete or partial) are known and the group of alcohol dehydrogenases to which they belong can be identified (see Introduction 1.3).

All seven bacterial alcohol dehydrogenases of the zinc-dependent group, including benzyl alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250, are tetramers of subunit M_r about 40 000 (Introduction 1.3.1). Those bacterial enzymes identified as belonging to the short chain, non-metallo-enzyme group are much smaller, with subunit M_r values of about 28 000, although their tertiary structures are not known (Introduction 1.3.2). Of the iron-activated alcohol dehydrogenases identified, all except a fermentative alcohol dehydrogenase from *E. coli* have subunit M_r values of about 40 000 (Introduction 1.3.3). However, only alcohol dehydrogenase 2 of *Z. mobilis*, a fermentative enzyme, is known to be a tetramer. Of the others, methanol dehydrogenase of *B. methanolicus* is a decamer and the quaternary structures of the other enzymes in this group are not known (Table 1.5). The *E. coli* enzyme was found to be a dimer of subunit M_r 96 000 encoding both alcohol and CoA-linked aldehyde dehydrogenase activities (Clark, 1989). More

recently, it has been proposed that the enzyme may exist *in vivo* as a homopolymer of 40 subunits and that this homopolymer has pyruvate-formate-lyase deactivase activity associated with it as well as the alcohol and aldehyde dehydrogenase activities (Kessler *et al.*,1991). NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 could belong to either the zinc-dependent or the iron-activated groups of alcohol dehydrogenases as judged by its subunit M_r and quaternary structure but not to the smaller non-metallo-enzyme group.

Comparison of the *N*-terminal amino acid sequence of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 with other sequences (partial and complete) in the GenBank, EMBL and NBRF-Protein databases showed no significant homologies between the enzyme and any other alcohol dehydrogenases (Section 5.2.4). The maximum homologies identified using the Wordsearch program were with fruit fly homeotic proteins, whereas those identified with the Fasta program were with internal sequences of glucosyl transferase of *Streptococcus mutans* and chicken acetyl CoA carboxylase. In all of these cases the maximal percentage identity between NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 and these proteins was < 30 %.

Comparison by eye of the amino acid sequence with the *N*-terminal amino acid sequences of bacterial alcohol dehydrogenases as well as with that of horse liver alcohol dehydrogenase showed no homologies except with the fermentative alcohol dehydrogenase of *E. coli* (Section 5.2.4; Figure 5.7.) There is 34 % identity between the *N*-terminal amino acid sequence of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 and residues 31-68 of the *E. coli* enzyme if one gap is introduced into each sequence. The significance of this is questionable, but it is higher than any of the homologies identified by computer searches, and many alcohol dehydrogenases accepted as belonging to the same family show less homology (Introduction 1.3).

As stated earlier, the *E. coli* enzyme is a 96 kDa protein which codes for both alcohol and CoA-linked aldehyde dehydrogenase activities. Removal of the third of the

gene which codes for the *C*-terminal third of the protein results in a smaller protein which possesses alcohol dehydrogenase activity but lacks acetaldehyde dehydrogenase activity (Clark, 1992). However, curiously it is the *C*-terminal half of the protein which shows strong homology with the iron-activated alcohol dehydrogenases (Clark, 1992). Figure 5.8 shows the regions of the *E. coli* enzyme which have homologies with other alcohol dehydrogenases and the enzyme activity identified with various regions of the enzyme.This makes it very difficult to judge the significance of the homology between NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 and the *E. coli* protein. It should be noted that the NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 has no aldehyde dehydrogenase activity in the presence, or the absence, of coenzyme A (Section 5.3.6).

The *N*-terminal amino acid sequence of the enzyme lacks the GxGxxG/A "fingerprint" motif for NAD(P) binding which is present at the *N*-terminus of some NAD(P)-dependent dehydrogenases (Wierenga *et al.*, 1986). However, the *N*-terminal amino acid sequence of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 constitutes only about 10 % of the total protein sequence and it will be necessary to obtain the entire sequence before the question as to which group of alcohol dehydrogenases the enzyme belongs to can be answered. Also, the absence of much homology between the enzyme and other alcohol dehydrogenases does not rule out the existence of homology at other structural levels, e.g. the main chain of pig heart mitochondrial and cytoplasmic malate dehydrogenases are folded in a similar fashion resulting in similar tertiary structures despite the fact that they have only 20 % amino acid sequence identity (Birkoft *et al.*, 1982; Roderick and Banaszak, 1986). Only determination of the crystal structures of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 and other alcohol dehydrogenases (horse liver alcohol dehydrogenase is the only crystal structure determined so far, Branden *et al.*, 1975) will permit a full comparison of alcohol dehydrogenases.

The *N*-terminal amino acid sequence was analysed to determine any runs of 10-11 amino acids with minimal degeneracy which could act as probes for cloning the gene. One

low-degeneracy sequence was identified that ran from position 2 to position 12:

Position	2	3	4	5	6	7	8	9	10	11	12
Amino acid	N	N	T	F	H	A	Y	A	A	L	E
Degeneracy	2	2	4	2	2	4	2	4	2	6	2

Ideally, a probe of the non-coding strand of DNA would be synthesised which would allow probing of mRNA for expression studies.

An alternative method of generating a larger probe would be to synthesise two smaller probes, one of the coding strand and one of the non-coding strand of DNA, based on 4-5 amino acid sequences, with minimal degeneracy, far apart in the *N*-terminal amino acid sequence and using the polymerase chain reaction to generate a longer, identical probe. Two suitable amino acid sequences lie from position 2 to position 6 and from position 30 to position 34 in the *N*-terminal sequence:

Position	2	3	4	5	6		30	31	32	33	34
Amino acid	N	N	T	F	H	and	H	Q	U	E	U
Degeneracy	2	2	4	2	2		2	2	4	2	4

Degeneracies could be minimised further by taking into account codon usage in those acinetobacter structural genes which have already been sequenced (White *et al.*, 1991). A + T constitute about 58 % of those genes sequenced with over 66 % of bases in the third codon position and nearly 62 % of bases in the second codon position being A + T. Furthermore, G is preferred in the first codon position (34.3 %) but is uncommon in the second codon position (16.2 %). Additionally, some specific codons are preferred for certain amino acids, e.g. the codon for glutamine is GAA in 78 % of cases whereas some codons are rarely used, e.g. ATA is used less than 5 % of the time for isoleucine (White *et al.*, 1991). Such considerations should make cloning the gene for NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 relatively straightforward.

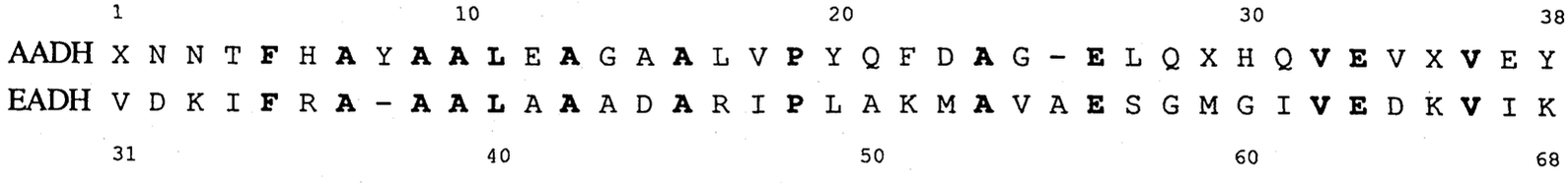


Figure 5.7. Comparison of N-terminal amino acid sequences of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 and fermentative alcohol dehydrogenase of *E. coli*

The *N*-terminal amino acid sequence of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 (AADH) was compared to that of fermentative alcohol dehydrogenase of *E. coli* (EADH) by eye. 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.

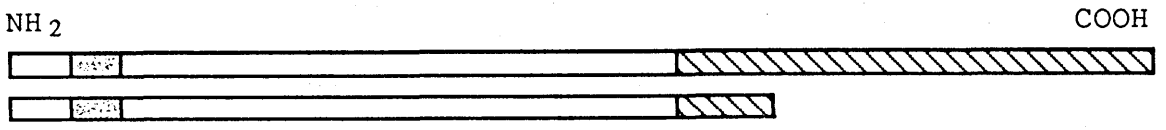


Figure 5.8. Diagrammatic representation of the functional domains of fermentative alcohol dehydrogenase of *E. coli* and its amino acid sequence homologies with other alcohol dehydrogenases

The information in this figure is taken from Goodlove *et al.* (1990), Clark (1992) and this thesis. The fermentative alcohol dehydrogenase of *E. coli* is represented by the top bar. The size and position of the truncated protein with alcohol dehydrogenase activity but no aldehyde dehydrogenase activity is indicated by the bottom bar. The sizes and positions of amino acid sequence homologies with other proteins are indicated by shaded areas on the protein:

(▒) 34 % identity with the *N*-terminal amino acid sequence of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250;

(▨) 32 to 35 % identity with iron-activated alcohol dehydrogenases (Table 1.4).

5.5.2 Cofactor specificity

The NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 is specific for NADP(H) as cofactor. There is no activity of the enzyme with NAD^+ or DCIP or DCIP and PMS as cofactor [Section 5.3.5 (a)]. The lack of activity with the latter suggests the enzyme does not use flavin or PQQ or one of its derivatives as electron acceptors.

The enzyme transfers the proR hydrogen to/from NADP(H) [Section 5.3.5 (b)]. This was shown using pro*S*-[^{3}H]NADPH and confirmed using pro*R*[^{3}H]NADPH. The results are unambiguous but the ratio of percentages of ^{3}H incorporated into $NADP^+$ and butan-1-ol is less than 100 % in both cases. One reason for this is that transfer of ^{3}H from stereospecifically-tritiated NADPH to NADP in a random manner occurs in solution where both forms of the cofactor are present in similar proportions (Glasfeld *et al.*, 1990). The enzyme is much slower than many other enzymes for which the stereospecificity has been determined in this way, as judged by k'_{cat}/K'_m values [e.g benzyl alcohol dehydrogenase, see Appendix and MacKintosh and Fewson (1988a)]. In order to drive the reaction to completion in seconds rather than minutes, hence minimising the random exchange of ^{3}H between cofactors, it would be necessary to add hundreds of μg of enzyme which was not possible in this instance. The ratio of $NADP^+$:butan-1-ol resulting from incubation with pro*R*-[^{3}H]NADPH is further from 100 %: 0 % than that of incubation with pro*S*-[^{3}H]NADPH. This may be because pro*R*-[^{3}H]NADPH is generated from [^{3}H]$NADP^+$ and it is possible that not all of the [^{3}H]$NADP^+$ was converted to pro*R*-[^{3}H]NADPH and this may have increased the pool of [^{3}H]$NADP^+$ after reaction, therefore affecting the result.

The stereospecificities of about 200 dehydrogenases have been determined. Half transfer the pro*S* hydrogen whereas the other half transfer the pro*R* hydrogen (Benner *et al.*, 1987, 1989). It has been shown from crystallographic studies that those enzymes which transfer the pro*S* hydrogen, e.g. lobster glyceraldehyde-3-phosphate dehydrogenase, have the dihydronicotinamide ring oriented in the *syn* position whereas

those that transfer the pro*R* hydrogen, e.g. horse liver alcohol dehydrogenase, have the ring oriented in the *anti* position (Rossman *et al.*, 1975). Therefore, it is likely that NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250, which transfers the pro*R* hydrogen, binds NADPH in an *anti* conformation orienting the *re* face of the dihydronicotinamide ring towards the substrate.

The first popular generalisation about stereospecificity of dehydrogenases, Bentley's first rule, states that "enzymes accepting the same substrates generally have the same stereospecificity" (Glasfeld *et al.*, 1990, after Bentley, 1970). This assumption does not prove correct for alcohol dehydrogenases. The non-metallo-enzymes transfer the pro*S* hydrogen, whereas both the groups of metallo-enzymes (zinc-dependent and iron-activated) transfer the pro*R* hydrogen.

Various historical and functional models have been suggested to explain the non-randomness of stereospecificity in dehydrogenases. A complete historical model had not been constructed until recently when stereospecificities of seven dehydrogenases of *Acholeplasma ladlawii*, a mycoplasma, were determined and compared with existing data (Glasfeld *et al.*, 1990). From these data a complex model was constructed incorporating many *ad hoc* assumptions, and the authors themselves implicitly questioned the validity of such a model.

A number of functional hypotheses have been proposed to explain stereospecificities in dehydrogenases (Donkersloot and Buck, 1981; Benner, 1982; Sviarastara and Bernhard, 1985). The only hypothesis that attempts to explain the different stereospecificities in alcohol dehydrogenases is that of Benner and colleagues (Benner 1982, Benner *et al.*, 1985; Alleman *et al.*, 1988). This hypothesis proposes that stereospecificity corresponds with the redox potential of the substrate, i.e. dehydrogenases which reduce unstable carbonyls transfer the pro*R* hydrogen, those which reduce stable carbonyls transfer the pro*S* hydrogen and neither stereospecificity is strongly selected in the enzymes which reduce carbonyls that are not obviously stable or unstable. This intermediate stability corresponds to reactions with a K_{eq} of about 10^{-11} M. The zinc-dependent alcohol dehydrogenases and the non-metallo-enzymes both have K_{eq}

values near this value. More recently, Glasfeld and Benner (1989) have proposed that the metal ion in the active site of the zinc-dependent and the iron-activated enzymes destabilises the carbonyl group and hence they transfer the pro*R* hydrogen whereas the non-metalloenzymes transfer the pro*S* hydrogen to/from the more stable carbonyl. The NADP-dependent alcohol dehydrogenase from *A. calcoaceticus* NCIB 8250 has a K'_{eq} of > 10^{-12} M (in glycine or in CAPS buffer), therefore (assuming hexan-1-ol is, or is at least similar to, the physiological substrate) it would be expected that it would transfer the pro*R* hydrogen, as it does. The carbonyl group of hexan-1-ol is less stable than that of ethanol, the CH_2 group of hexan-1-ol next to the carbonyl group being slightly more electropositive than the CH_3 group of ethanol therefore destabilising the carbonyl group to a greater extent. However, this makes it difficult to deduce, from Glasfeld and Benner's argument (1989), whether the enzyme has a metal ion or not.

5.5.3. Substrate specificity

NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 is specific for compounds with a primary alcohol group. Activity is maximal for aliphatic, saturated, primary alcohols with chain lengths from five to eight carbons (Table 5.6). The minimum chain length of primary alcohol used is two, i.e.ethanol (Table 5.3). Like most bacterial alcohol dehydrogenases it will not use methanol as substrate (Table 5.5). The maximal chain length of primary alcohol used appears to be 14 (Table 5.4). However, the enzyme activity detected with tetradecan-1-ol (14 carbons) as substrate is so low as to make it questionable whether this results from a genuine inability of the enzyme to utilise cetyl alcohol (16 carbons) or simply that the assay used is not sensitive enough to detect a possible very low rate of cetyl alcohol conversion to aldehyde. This range of primary alcohol specificity is very similar to NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* 69V which oxidises alcohols with from two to ten carbons (Introduction 1.8).

The enzyme does not use secondary alcohols (Table 5.5), and they have no

apparent inhibitory effect on the enzyme (Section 5.3.6), therefore it seems that secondary alcohols are not bound by the enzyme at all.

The enzyme uses 1,x diols but not other diols (Tables 5.3 and 5.5). Even when the -OH groups are at either end of the carbon skeleton, i.e. both form part of primary alcohol groups (i.e. $-CH_2OH$), the enzyme activity is not any higher than for the equivalent "mono"-primary alcohol. Therefore it can be assumed that the enzyme converts only one of the primary alcohol groups to aldehyde. Presumably the aldehyde/alcohol product of reaction is not a substrate for the enzyme which may in part explain why glyceraldehyde is not a substrate (Table 5.5).

The enzyme can use some unsaturated alcohols as substrates but not others (Tables 5.3 and 5.5 respectively), probably due to steric constraints. A similar explanation may account for the fact that the enzyme uses benzyl alcohol (Table 5.6) but not hexahydrobenzyl alcohol, phenyl ethanol or cyclohexanol (Table 5.5). The inability of the enzyme to use amino acid alcohols (Table 5.5) probably results from a combination of their size, shape and charge.

Sugars and polyols were tested as substrates because some enzymes of the different groups of alcohol dehydrogenases use sugars or polyols as substrates, e.g. sheep liver sorbitol dehydrogenase (Section 1.3.1), glucose dehydrogenase of *B. megaterium* (Section 1.3.2). Although the enzyme can use ribitol and mannitol (Table 5.4) it does so with very low activity relative to primary alcohol substrates and it seems unlikely that such substrates are utilised *in vivo*. The enzyme could not use the sugars tested as substrates (Table 5.5).

Microbial alcohol and aldehyde dehydrogenases have been classified according to their substrate specificities (Introduction 1.3.4). However, such classifications involve broad groupings, which stem in part from the lack of enzyme specificity, and result in overlapping groupings which do not, for the most part, reflect genuine homologies or evolutionary relationships (MacKintosh and Fewson, 1987). The zinc-dependent alcohol dehydrogenases consist mostly of enzymes which have a preference for primary aliphatic alcohols from two-eight carbons but will use some secondary alcohols (Introduction

1.3.1). However, some eukaryotic sorbitol dehydrogenases and steroid dehydrogenases are known to belong to this group, as do some prokaryotic aromatic alcohol dehydrogenases (Introduction 1.3.1). Fewer iron-activated alcohol dehydrogenases have been identified and they have been less well characterised but they mostly prefer primary aliphatic alcohols with from two to eight carbons also (Introduction 1.3.3). The only prokaryotic enzymes belonging to the non-metallo-enzyme group of alcohol dehydrogenases are dihydrodiol, hydroxysteroid, sugar or polyol dehydrogenases (Introduction 1.3.2). Other alcohol dehydrogenases that have been purified and partially characterised show a wide range of substrate specificities (Introduction 1.3.4). However, it is quite unusual to find an enzyme which uses primary alcohols only (Section 1.3.4; MacKintosh and Fewson, 1987), so this relatively narrow substrate specificity makes NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 unusual.

5.5.4. Kinetics

The pH optimum of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 for alcohol oxidation is 10.2 [Section 5.3.2 (a)]. Most NAD(P)-dependent dehydrogenases have pH optima above 8.0 for oxidation simply because protons are generated in the enzymic oxidation reaction as the cofactor is reduced (NAD(P) NAD(P)H + H^+), therefore the higher pH favours the oxidation reaction (i.e. fewer protons in solution therefore reaction driven more in favour of oxidation, see Section 5.3.1 also). Presumably, in this case, the ionisation state of the active site residues in the protein is altered above pH 10.2. This is supported by the fact that pH appears to have a marked effect on K'_m at lower pH, the K'_m value for hexan-1-ol is 10 times higher at pH 7.2 than at pH 10.2 (Section 5.4.1) and the same effect may occur at pH values > 10.2 also. The pH optimum for the aldehyde reduction reaction is pH 6.8 [Section 5.3.2 (b)], i.e. much closer to neutral pH, protons being utilised, not generated, in the reaction.

The K'_m value can be regarded as an apparent dissociation constant (Fersht, 1985).

Hexan-1-ol, heptan-1-ol and octan-1-ol have the lowest K'_m values of those primary alcohols tested (Table 5.6). The very high K'_m value for ethanol as substrate (Table 5.6) suggests that it is not a physiological substrate for the enzyme, unlike most of the alcohol dehydrogenases for which amino acid sequence is available.

The turnover number of the enzyme, k_{cat}, represents "the maximum number of substrate molecules converted to product per unit time" (Fersht, 1985). The highest apparent k_{cat} value for primary alcohol substrates of the enzyme is with pentan-1-ol as substrate, hexan-1-ol and heptan-1-ol having slightly lower values (Table 5.7). These values are much lower than the largest known values (e.g. carbonic anhydrase $k_{cat} = 6 \times 10^6\ s^{-1}$) but well within the range of most enzymes for their physiological substrates (1 to $10^4\ s^{-1}$; Stryer,1988). The specificity constant, k_{cat}/K'_m, is an apparent second order rate constant relating the reaction rate to the concentration of free enzyme (Fersht, 1985). It cannot be higher than the rate at which the enzyme-substrate (ES) complex forms. The upper limit of ES formation is set by diffusion. As a result k_{cat}/K_m cannot be higher than 10^8-$10^9\ M^{-1}\ s^{-1}$ (Stryer, 1988). The highest k'_{cat}/K'_m values of the enzyme for primary alcohol, aldehyde or cofactor are well below this limit.

Comparison of the kinetic coefficients of butan-1-ol and butanal reveals that the K'_m value is much lower, and the k'_{cat}/K'_m value much higher, for the aldehyde, i.e. the aldehyde is the "best" substrate for NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250, although this does not indicate that it is necessarily the physiological substrate.

5.5.5. Presence of a metal ion in the active site of the enzyme

The absence of obvious inhibition of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 by metal binding agents implies that no metal ion is present at the active site of the enzyme. However, the pro*R* stereospecificity of hydrogen transfer to/from NADP(H) of the enzyme and its subunit M_r suggest that it might belong to the zinc-dependent or the iron-activated group of alcohol dehydrogenases. It may be that the

conditions used were not harsh enough to remove a metal ion from the enzyme and heating the enzyme in the presence of a chelating agent may be necessary to inhibit the enzyme (DeBruyn *et al.*, 1980). Extensive dialysis of the enzyme against a metal chelator, purification of the enzyme in the absence of trace metals and/or some form of metal analysis may be necessary to determine whether any metal ion is present and functional. Whether NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 is a member of the iron-activated alcohol dehydrogenases, the zinc-dependent alcohol dehydrogenases or the first protein to be identified in a new group of alcohol dehydrogenases which may lack a metal ion at the active site cannot be deduced from its *N*-terminal amino acid sequence. However, determination of the complete amino acid sequence of the enzyme may reveal the presence of possible metal ligands.

CHAPTER 6
CHARACTERISATION OF NADP-DEPENDENT ALCOHOL DEHYDROGENASES OF *ACINETOBACTER SP.* HO1-N AND *SACCHAROMYCES CEREVISIAE* D273-10B

6.1. Introduction

NADP-dependent alcohol dehydrogenase from a second strain of *Acinetobacter*, *Acinetobacter sp.* HO1-N, was characterised in order to compare it to the enzyme from strain NCIB 8250 and to discover whether the latter enzyme was a typical example of NADP-dependent alcohol dehydrogenase in *A. calcoaceticus*, in so far as can be judged from comparison of two enzymes. Strain HO1-N was used because it has been much studied in terms of its growth on hydrocarbons and because partially purified NADP-dependent alcohol dehydrogenase of *Acinetobacter sp.* HO1-N was reported to have significantly different kinetic coefficients when compared with the enzyme of *A. calcoaceticus* NCIB 8250 (Introduction 1.8).

NADP-dependent alcohol dehydrogenases were identified in a number of other bacteria and yeasts (Section 7.6) and the enzyme of *Saccharomyces cerevisiae* D273-10B was characterised in order to compare it to the enzyme of *A. calcoaceticus*.

6.2. Characterisation of NADP-dependent alcohol dehydrogenase of *Acinetobacter sp.* HO1-N

6.2.1. Relative molecular mass

The native M_r of the enzyme, estimated by gel filtration chromatography through a f.p.l.c. Superose 6 column (Figure 6.1), was 172 800 (177 800, 167 900).

The subunit M_r value was estimated using calibrated SDS-PAGE gels. A representative standard plot of mobility against $\log_{10} M_r$ is shown in Figure 6.2. The subunit M_r was determined to be 40 600 (+/- 2000, n = 5 samples of enzyme).

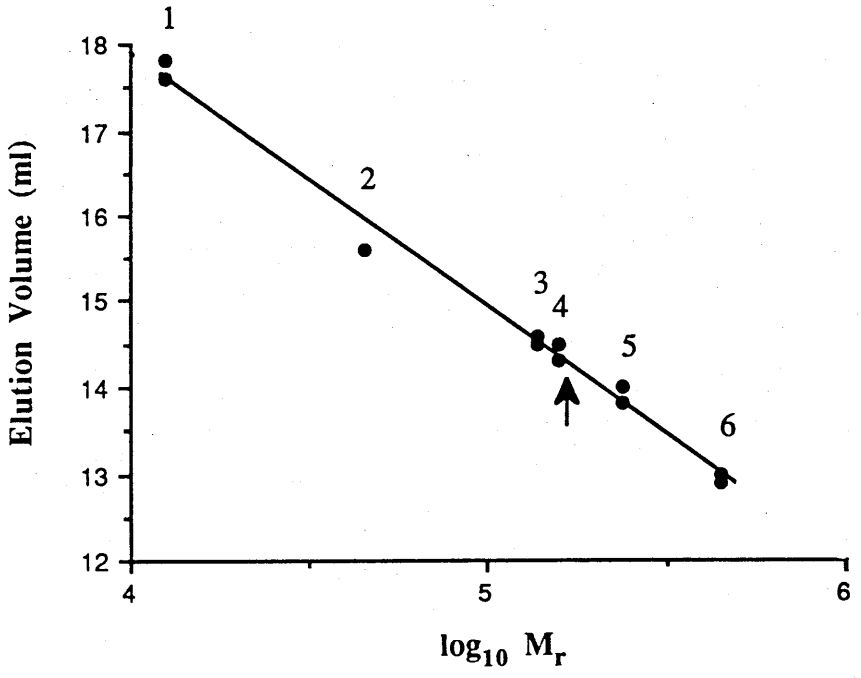
From comparison of subunit and total M_r values, the enzyme appears to be a tetramer.

Figure 6.1. Determination of the native M_r of NADP-dependent alcohol dehydrogenase of *Acinetobacter sp.* HO1-N by gel filtration

The native M_r value of NADP-dependent alcohol dehydrogenase from *Acinetobacter sp.* HO1-N was estimated under non-denaturing conditions using a Superose 6 gel filtration column (1 cm x 30 cm) pre-equilibrated with 50 mM-Tris/HCl/ 2 mM-DTT/ 0.1 M-NaCl, pH 8.0. Proteins were eluted at 0.3 ml h^{-1} and the A_{280} of the effluent was monitored (Methods 2.2.5). When enzyme was eluted fractions were collected and assayed for enzyme activity [Methods 2.5.1 (a)]. The elution volumes of the proteins were plotted against log M_r. Samples were run in duplicate and all data points are shown. Where values were identical only one point is shown. The standard proteins used, all shown as black circles, were:

1. cytochrome *c* (M_r 12 500, 25 μg);
2. ovalbumin (M_r 45 000, 100 μg);
3. lactate dehydrogenase (M_r 140 000, 50 μg);
4. aldolase (M_r 158 000, 200 μg);
5. pyruvate kinase (M_r 237 000, 100 μg);
6. ferritin (M_r 450 000, 50 μg);

The elution volume of NADP-dependent alcohol dehydrogenase from *Acinetobacter sp.* HO1-N (20 μg) is shown by an arrow.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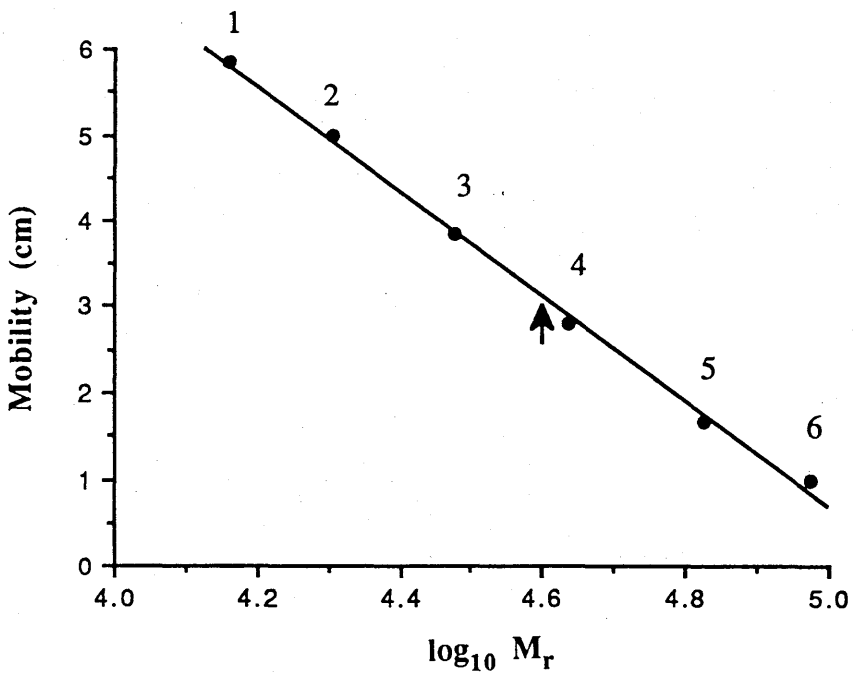
15
14
13
12
4
5
6
Elution Volume (ml)
log10 Mr

Figure 6.2. Determination of the subunit M_r of NADP-dependent alcohol dehydrogenase of *Acinetobacter sp.* HO1-N by SDS-polyacrylamide gel electrophoresis

The subunit M_r value of NADP-dependent alcohol dehydrogenase from *Acinetobacter sp.* HO1-N was estimated under denaturing conditions using 10 % (w/v) SDS-polyacrylamide gels calibrated with proteins of known M_r. Gels were run and stained for proteins as in Methods 2.10.1 and 2.10.3 (a) respectively. The distance migrated by the calibration proteins down the gel was plotted against $\log_{10} M_r$. A typical standard curve is shown, as is the position of the NADP-dependent alcohol dehydrogenase. The standard proteins used, all shown as black circles, were:

1. α-lactalbumin (M_r 14 400);
2. soya bean trypsin inhibitor (M_r 20 100);
3. carbonic anhydrase (M_r 30 000);
4. ovalbumin (M_r 43 000);
5. bovine serum albumin (M_r 67 000);
6. phosphorylase b (M_r 94 000);

The elution volume of NADP-dependent alcohol dehydrogenase from *Acinetobacter sp.* HO1-N is shown by an arrow.


1
2
3
4
5
6
Mobility (cm)
0
1
2
3
4
5
6
4.0
4.2
4.4
4.6
4.8
5.0
log10 Mr

6.2.2. Assay development

It was assumed that conditions for assay of the enzyme would be very similar to NADP-dependent alcohol dehydrogenase of strain NCIB 8250. This proved to be the case and the assay was linear and reproducible, as for the assay described for the enzyme from strain NCIB 8250 [Methods 2.5.1 (a)].

6.2.3. Substrate specificity

NADP-dependent alcohol dehydrogenase of *Acinetobacter sp.* HO1-N oxidised primary alcohols (Table 6.1). Primary alcohols with more than ten carbons were made accessible as substrates to the enzyme as described in Methods 2.5.1(b). Enzyme activity decreased with increasing chain length of the primary alcohol and no activity was observed with hexadecan-1-ol as substrate (Table 6.2).

6.2.4. Kinetic coefficients

The K'_m and V'_{max} values were determined for primary alcohols from butan-1-ol (four carbons) to decan-1-ol (ten carbons), by measuring the initial reaction rates at various non-saturating concentrations of the substrate in the presence of a fixed, high concentration of $NADP^+$ (Table 6.1). The K'_m values decreased from butan-1-ol to hexan-1-ol then increased slightly to decan-1-ol. The V'_{max} values increased from butan-1-ol to pentan-1-ol then decreased to decan-1-ol. Also, determinations of the K'_m at pH values 9.5, 10.0, 10.2 and 10.5 showed the lowest K'_m value at pH 10.2.

The turnover number (k'_{cat}) and the specificity constant (k'_{cat}/K'_m) of the enzyme were determined for primary alcohols using the data in Table 6.1 and assuming that there was one active site per subunit, that the subunit M_r of the enzyme was 40600 (Section 6.1.) and making the other assumptions stated in Section 5.4.2. The highest turnover number of the enzyme was for pentan-1-ol as substrate. The highest specificity constant

of the enzyme was with hexan-1-ol as substrate.

Table 6.1. Kinetic constants of selected substrates of NADP-dependent alcohol dehydrogenase of *Acinetobacter sp.* HO1-N

The K'_m and V'_{max} 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 concentration of $NADP^+$ (1 mM). The standard assay mixtures [Methods 2.5.1 (a)] were used except that the concentration of one of the substrates was varied. The kinetic constants were calculated by the Direct Linear method (Methods 2.7). Where one value is given the 68 % confidence limits (Methods 2.7) are quoted in parentheses. Where two values were determined for a kinetic constant the average is given with the actual values in parentheses.

n.d. = not determined.

Substrate	K'_m (mM)	V'_{max} [EU (mg protein)$^{-1}$]
Butan-1-ol	15.3 (12.8-19.1)	55.1 (47.6-62.0)
Pentan-1-ol	2.42 (1.70-3.10)	88.5 (72.5-110.1)
Hexan-1-ol	0.26 (0.30,0.22)	70.0 (71.0,69.0)
Octan-1-ol	0.34 (0.36,0.31)	47.5 (55.5,39.5)
Decan-1-ol	0.43 (0.46,0.39)	26.8 (25.5,28.0)

Table 6.2. Ability of NADP-dependent alcohol dehydrogenase of *Acinetobacter sp.* HO1-N to use alcohols with chain lengths > 10 as substrates

All of the alcohols used were pre-sonicated in BSA (0.7 mg ml^{-1}) and assayed as described in [Methods 2.5.1(b)] except that the alcohols listed in the table replaced hexan-1-ol. The apparent concentration is based on the concentration of the alcohol that was sonicated. Values in parentheses refer to activities with no pre-sonication with BSA at these alcohol concentrations [enzyme assayed as in Methods 2.5.1(a)]. For all alcohols tested in this way the initial A_{340} of the pre-sonicated alcohol/ BSA/ buffer solution was < 0.1 compared to buffer/BSA solution alone; n.d. = not determined because the above criterion was not met. Conc.; concentration

Alcohol	Apparent Conc. (μM)	Activity [nmol min (mg protein)$^{-1}$]
Hexan-1-ol	500	27.7 (70.0)
	250	18.4 (21.4)
	100	11.3 (10.1)
Octan-1-ol	500	21.0
	250	17.5
	100	10.0
Decan-1-ol	500	30.7
	250	19.0
	100	14.0
Dodecan-1-ol	500	17.4
	250	19.4
	100	13.3
Tetradecan-1-ol	500	10.5
	250	4.60
	100	3.15
Hexadecan-1-ol	500	n.d.
	250	0.00
	100	0.00

Table 6.3. Turnover numbers and specificity constants for selected substrates of NADP-dependent alcohol dehydrogenase of *Acinetobacter sp.* HO1-N

Turnover numbers (k'_{cat}) and specificity constants (k'_{cat}/K'_m) values were determined using the average values for kinetic coefficients given in Table 6.1.

Substrate	k'_{cat} (s^{-1})	k'_{cat}/K'_m (s^{-1} M^{-1})
Butan-1-ol	37.3	2.43×10^3
Pentan-1-ol	59.9	2.47×10^4
Hexan-1-ol	47.4	1.81×10^5
Octan-1-ol	32.1	9.59×10^4
Decan-1-ol	18.1	4.27×10^4

6.3. Characterisation of NADP-dependent alcohol dehydrogenase of *S. cerevisiae* D273-10B

6.3.1. Relative molecular mass

The native M_r of the enzyme, estimated by gel filtration chromatography through a f.p.l.c. Superose 6 column (Figure 6.3), was 46 200 (47 800, 45 700). NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 was included as a control in this experiment, and its estimated native M_r was the same value as determined previously (Section 5.2.1).

The subunit M_r of the enzyme was estimated using calibrated SDS-PAGE gels. A representative standard plot of mobility against $\log_{10} M_r$ is shown in Figure 6.4. The subunit M_r was determined to be 43 500 (+/- 700, n = 5 samples of enzyme).

From comparison of the subunit and native M_r values, the enzyme appeared to be a monomer.

6.3.2. *N*-Terminal amino acid sequence

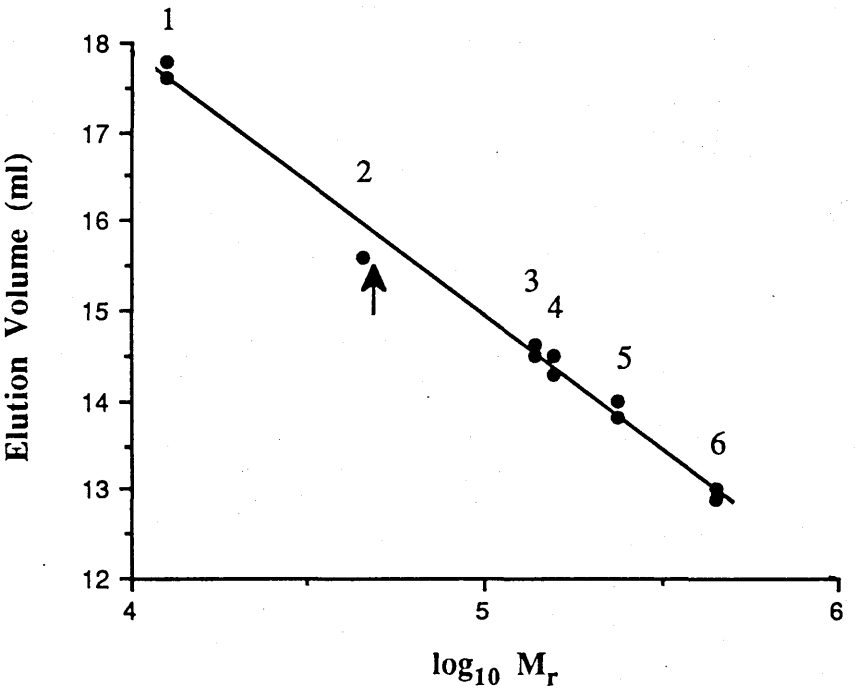
Attempts at determining the *N*-terminal amino acid sequence of the enzyme at the SERC Protein Sequencing Unit at Leeds University were unsuccessful. It is thought that the *N*-terminus is blocked (J.N. Keen, personal communication).

Figure 6.3. Determination of the native M_r of NADP-dependent alcohol dehydrogenase of *S. cerevisiae* D273-10B by gel filtration

The native M_r value of NADP-dependent alcohol dehydrogenase from *S. cerevisiae* D273-10B was estimated under non-denaturing conditions using a Superose 6 gel filtration column (1 cm x 30 cm) pre-equilibrated with 50 mM-Tris/HCl/ 2 mM-DTT/ 0.1 M-NaCl, pH 8.0. Proteins were eluted at 0.3 ml h^{-1} and the A_{280} of the effluent was monitored (Methods 2.2.5). When enzyme was eluted fractions were collected and assayed for enzyme activity [Methods 2.5.2 (a)]. The elution volumes of the proteins were plotted against $\log_{10} M_r$. Samples were run in duplicate and all data points are shown. Where values were identical only one point is shown. The standard proteins used, all shown as black circles, were:

1. cytochrome *c* (M_r 12 500, 25 μg);
2. ovalbumin (M_r 45 000, 100 μg);
3. lactate dehydrogenase (M_r 140 000, 50 μg);
4. aldolase (M_r 158 000, 200 μg);
5. pyruvate kinase (M_r 237 000, 100 μg);
6. ferritin (M_r 450 000, 50 μg).

The elution volume of NADP-dependent alcohol dehydrogenase from *S. cerevisiae* D273-10B (20 μg) is shown as an arrow.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13
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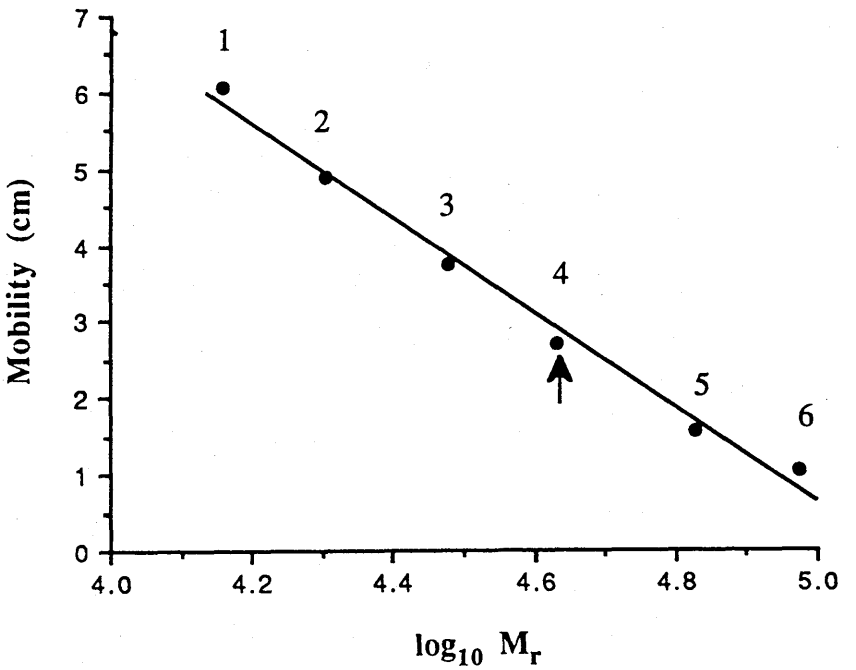
4
5
6
Elution Volume (ml)
log10 Mr

Figure 6.4. Determination of the subunit M_r of NADP-dependent alcohol dehydrogenase of *S. cerevisiae* D273-10B by SDS-polyacrylamide gel electrophoresis

The subunit M_r value of NADP-dependent alcohol dehydrogenase from *S. cerevisiae* D273-10B was estimated under denaturing conditions using 10% (w/v) SDS-polyacrylamide gels calibrated with proteins of known M_r. Gels were run and stained for proteins as in Methods 2.10.1 and 2.10.3 (a) respectively. The distance migrated by the calibration proteins down the gel was plotted against $\log_{10} M_r$. A typical standard curve is shown. The standard proteins used, all shown as black circles, were:

1. α-lactalbumin (M_r 14 400);
2. soya bean trypsin inhibitor (M_r 20 100);
3. carbonic anhydrase (M_r 30 000);
4. ovalbumin (M_r 43 000);
5. bovine serum albumin (M_r 67 000);
6. phosphorylase b (M_r 94 000);

The elution volume of NADP-dependent alcohol dehydrogenase from *S. cerevisiae* D273-10B is shown by an arrow.


Mobility (cm)
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6
5
4
3
2
1
0
1
2
3
4
5
6
4.0
4.2
4.4
4.6
4.8
5.0
log10 Mr

6.3.3. pH optimum

(a) Alcohol oxidation

The alcohol oxidation was assayed at a range of pH values from 8.9 to 11.2 in glycine buffer [Figure 6.5. (a)]. The optimum pH value for alcohol oxidation was pH 10.7. The activity was high (> 95 % maximal value) between pH 10.6 and pH 10.85. Above pH 9.8 the enzyme activity dramatically increased. This resembles the situation observed with NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 and presumably occurred for the same reasons (Section 5.3.2).

(b) Aldehyde reduction

The aldehyde reduction reaction was assayed at a range of pH values between 5.5 and 7.5 in Bistris buffer [Figure 6.5 (b)]. The pH optimum of aldehyde reduction lay at around 6.8, although the reaction rate was high (> 90 %) between pH 6.4 and pH 7.0, a broader pH range than that for alcohol oxidation.

6.3.4. Reproducibility and linearity of reaction

(a) Alcohol oxidation

The initial rate of reaction was linear over 5 minutes and proportional to the amount of protein added for protein concentrations of up to 600 ng ml^{-1}. The initial rate of reaction was the same [53.0 +/- 0.1 EU $(mg\ protein)^{-1}$] when assayed six times consecutively. In both cases the enzyme was assayed as described in Methods 2.5.2 (a).

(b) Aldehyde reduction

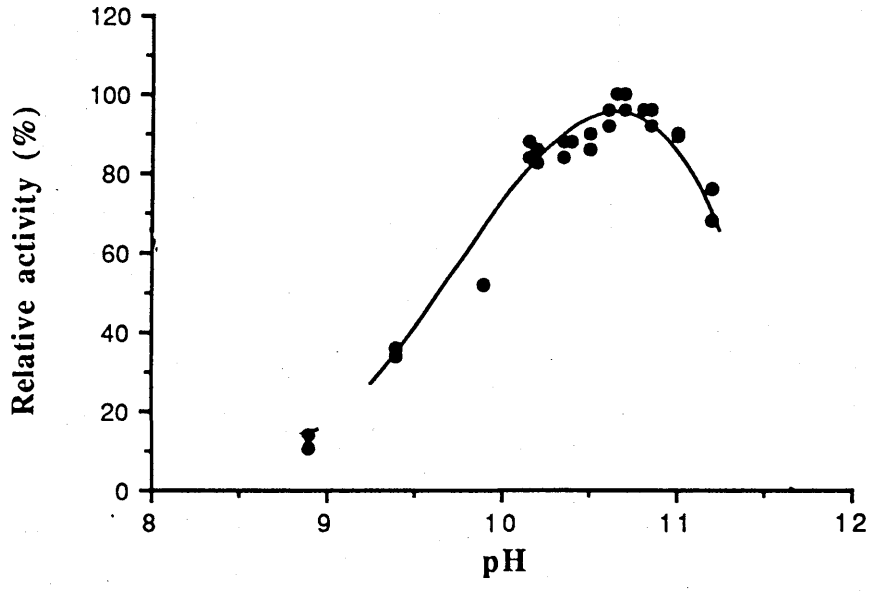
The initial rate of reaction was linear over 5 minutes and proportional to the amount of protein added for protein concentrations of up to 65 ng ml^{-1}. The initial rate of reaction was the same [27.0 +/- 0.4 EU $(mg.protein)^{-1}$] when assayed five times consecutively. In both cases the enzyme was assayed as described in Methods 2.5.2 (b).

Figure 6.5. Effects of pH on the oxidation and reduction reactions of NADP-dependent alcohol dehydrogenase of *S. cerevisiae* D273-10B

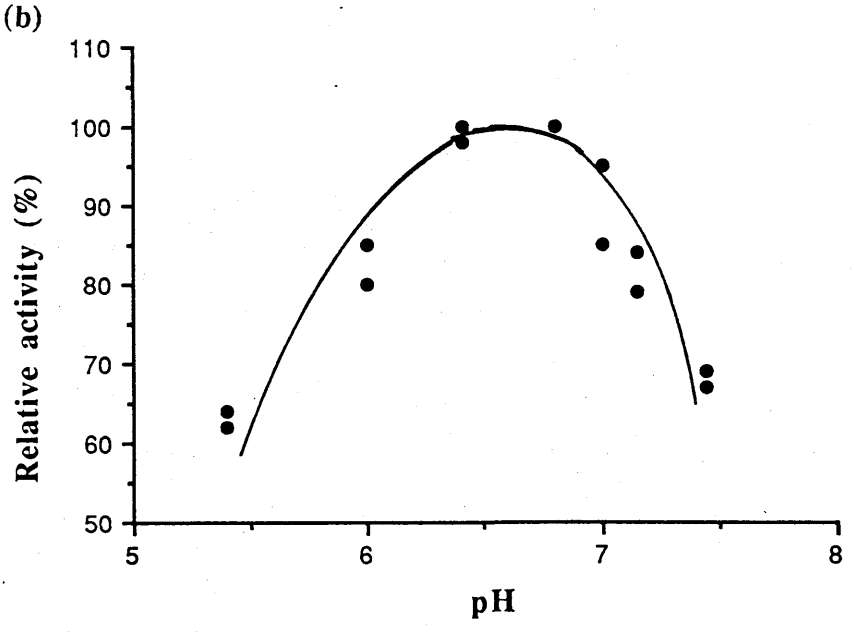
(a) The alcohol oxidation reaction was measured in a reaction mixture (1 ml) containing 80 mM-glycine buffer, 1 mM-$NADP^+$, 20 mM-hexan-1-ol and NADP-dependent alcohol dehydrogenase (250 ng). The pH values of the mixtures were determined after assay (Methods 2.2.1). The maximum (100%) velocity was 55 EU $(mg\ protein)^{-1}$.

(b) The aldehyde reduction reaction was measured in a reaction mixture (3 ml) containing 80 mM-Bistris buffer, 10 μM-NADPH, 20 mM butan-1-ol and NADP-dependent alcohol dehydrogenase (25 ng). The pH values of the mixtures were determined after assay (Methods 2.2.1). The maximum (100%) velocity was 26 EU $(mg\ protein)^{-1}$.

(a)


Relative activity (%)
120
100
80
60
40
20
0
8
9
10
11
12
pH

(b)


Relative activity (%)
110
100
90
80
70
60
50
5
6
7
8
pH

6.3.5. Cofactor specificity

(a) Electron acceptor specificity

NADP-dependent alcohol dehydrogenase of *S. cerevisiae* D273-10B did not reduce NAD^+ when assayed as described in Methods 2.5.2 (a) except with 1 mM-NAD^+ instead of 1 mM-$NADP^+$.

(b) Stereospecificity of hydride transfer

The stereospecificity of hydride transfer was determined by assaying the enzyme in the presence of pro*S*-[^{3}H]NADPH or pro*R*-[^{3}H]NADPH and butanal (Methods 2.12). The criteria used to determine the stereospecificity were as described in Section 5.3.5.

When the enzyme was incubated with butanal and proS [^{3}H]NADPH the ratio of counts incorporated into the resulting $NADP^+$ and butanal was 92.5 %: 7.5 % respectively, i.e. the enzyme was stereospecific for the pro*R* hydride of NADPH. This was confirmed by incubation of the enzyme with butanal and pro*R*-[^{3}H]NADPH, the ratio of counts incorporated into the resulting $NADP^+$ and butan-1-ol being , 30.0 %: 70.0 % respectively.

6.3.6. Substrate specificity and kinetic coefficients

The enzyme utilised primary alcohols with from two to eight carbons (Table 6.4). No other primary alcohols were tested. No activity was observed with the secondary alcohol pentan-2-ol as substrate.

The K'_m and V'_{max} values were determined for primary alcohols from ethanol to octan-1-ol, the aldehyde butanal and the cofactor(s) NADP(H) by measuring the initial reaction rates at various non-saturating concentrations of the substrate in the presence of a fixed, high concentration of the other substrate (Table 6.4). The K'_m values for primary alcohols decreased from ethanol to hexan-1-ol and octan-1-ol. The V'_{max} values increased as chain length increased from ethanol to pentan-1-ol, then decreased slightly. The V'_{max}

value determined for the aldehyde butanal was only half that of its equivalent alcohol butan-1-ol. However, the K'_m value for butanal was much lower than that of butan-1-ol. Similarly the K'_m for NADPH was much lower than for $NADP^+$, although the K'_m of both cofactors was much lower than that of the alcohol or the aldehyde.

The turnover number (k'_{cat}) and the specificity constant (k'_{cat}/K'_m) of the enzyme were determined for primary alcohols and for butanal using the data in Table 6.4 and assuming that there was one active site per monomer, that the molecular weight of the enzyme was 43 500 (Section 6.3.1) and making the other assumptions stated in Section 5.4.2. The highest turnover number of the enzyme was for pentan-1-ol as substrate (Table 6.5). 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 (Table 6.5).

Using V'_{max} values for butan-1-ol and butanal, specificity constants could be calculated for $NADP^+$ and NADPH respectively, and compared. The specificity constant calculated for NADPH was 10-fold higher than that of $NADP^+$ (2.5×10^7 and 2.5×10^6 respectively). For both cofactors the calculated specificity constants were 10^2 to 10^3 times greater than those of butanal and butan-1-ol.

6.3.7. Effect of metal binding agents

Incubation of the enzyme (5 $\mu g\ ml^{-1}$) at 27 °C over 20 minutes with the metal binding agents pyrazole (50 mM) or 2'2'bipyridyl (20 mM) had no significant effect on enzyme activity. However, incubation of the enzyme with the metal binding agents EDTA (100 mM or 10 mM) or sodium azide (100 mM, 10 mM or 1 mM) did partially inhibit the enzyme (Table 6.6).

Table 6.4. Kinetic constants of selected substrates of NADP-dependent alcohol dehydrogenase of *S. cerevisiae* D273-10B

The K'_m and V'_{max} 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 concentration of the other substrate [$NADP^+$ (1 mM), NADPH (20 mM), hexan-1-ol (20 mM) or butanal (20 mM) as appropriate]. The standard assay mixtures (Methods 2.5.2) were used except that the concentration of one of the substrates was varied. 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 (Methods 2.7). Where only one value was determined for a kinetic constant the 68 % confidence limits (Methods 2.7) are given in parentheses. Where two values were determined for a kinetic constant the average is given with the actual values in parentheses. n.d. = not determined.

Substrate	K'_m (mM)	V'_{max} [EU (mg protein)$^{-1}$]
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(hexan-1-ol)	20.2×10^{-3} (15.8×10^{-3}, 24.6×10^{-3})	n.d.
NADPH(butanal)	7.9×10^{-4} (6.7×10^{-4}, 9.0×10^{-4})	n.d.

Table 6.5. Turnover numbers and specificity constants values for selected substrates of NADP-dependent alcohol dehydrogenase of *S. cerevisiae* D273-10B

Turnover numbers (k'_{cat}) and specificity constants (k'_{cat}/K'_m) values were determined using the average values for kinetic coefficients given in Table 6.4.

Substrate	k'_{cat} (s^{-1})	k'_{cat}/K'_m (s^{-1} M^{-1})
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	2.6×10^5

Table 6.6. Effect of the metal binding agents, EDTA and sodium azide, on the activity of NADP-dependent alcohol dehydrogenase of *S. cerevisiae* D273-10B

The enzyme (5 μg ml^{-1}) was incubated at 27 °C with either EDTA or sodium azide at various concentrations for 20 minutes. The enzyme was assayed as described in Methods 2.5.2 (a), except that the metal chelating agent was included in the assay mixture at the incubation concentration. The maximum velocity i.e. in the absence of metal binding agent was 42 EU (mg protein)$^{-1}$.n.d. = not determined.

	Relative activity (%) in:	
Concentration of chelating agent (mM)	EDTA	Sodium azide
100	60	20
10	80	60
1	100	93
0.1	n.d.	97

6.3.8. Effect of thiol-acting agents

Incubation of the enzyme (5 μg ml^{-1}) at 27 °C with the thiol-acting agent iodoacetete (10 mM) had no effect on enzyme activity. However, incubation of the enzyme similarly with iodoacetamide (to 1 mM), *N*'ethylmaleimide (to 1 mM) or *p*-chloromercuribenzoate (to 1 μM) did partially or completely inhibit the enzyme (Figure 6.6.). 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, partial protection of the inhibition resulting from 1 mM-*p*-chloromercuribenzoate occurred in the presence of 1 mM-$NADP^+$, and almost complete protection occurred in the presence of 10 mM-hexan-1-ol (Figure 6.6).

Figure 6.6. Effect of thiol-acting reagents on the activity of NADP-dependent alcohol dehydrogenase of *S. cerevisiae* D273-10B

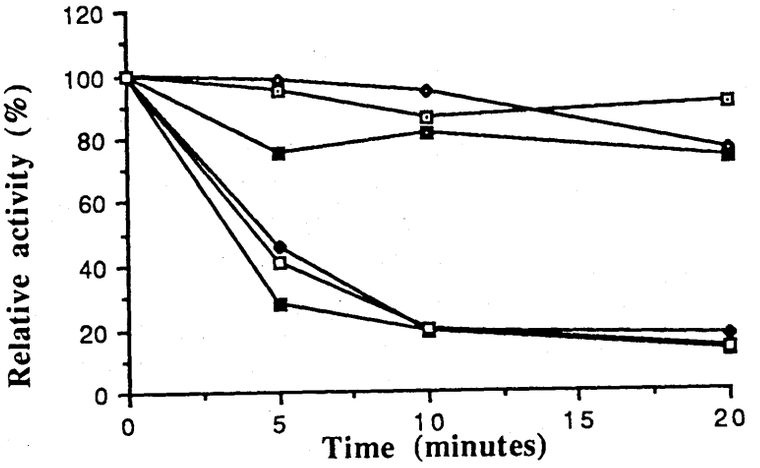
Enzyme was incubated with various concentrations of each sulphydryl reagent at 27 °C and samples were taken at 5, 10 and 20 minutes. In some incubations 1 mM-$NADP^+$ or 5 mM-hexan-1-ol was included. The enzyme was assayed as described in Methods 2.5.2 (a) except that the appropriate thiol-acting reagent was included in the assay mixture at the incubation concentration. The concentration of enzyme was 5 μg ml^{-1} in each incubation and 250 ng ml^{-1} in each assay. 100 % enzyme activity was 68 EU (mg protein)$^{-1}$.The results shown are the average of two experiments which, in all cases, agreed with each other to within 10 %.

(a) Iodoacetamide: (□) control; (◆)10 mM; (■) 1 mM;(◇) 0.1 mM; (▬) 10 mM and 1 mM-NADP; (▫) 10 mM and 10 mM-hexan-1-ol.

(b) NEM: (○) control; (★) 10 mM; (▲) 1 mM; (■) 0.1 mM; (+) 10 mM and 1 mM-NADP; (▬) 10 mM and 10 mM-hexan-1-ol.

(c) *p*–Chloromercuribenzoate: (○) control; (✱) 10 μM; (▬) 1 μM; (▾) 1 μM and 1 mM-NADP; (▾) 1 μM and 10 mM hexan-1-ol.

(a)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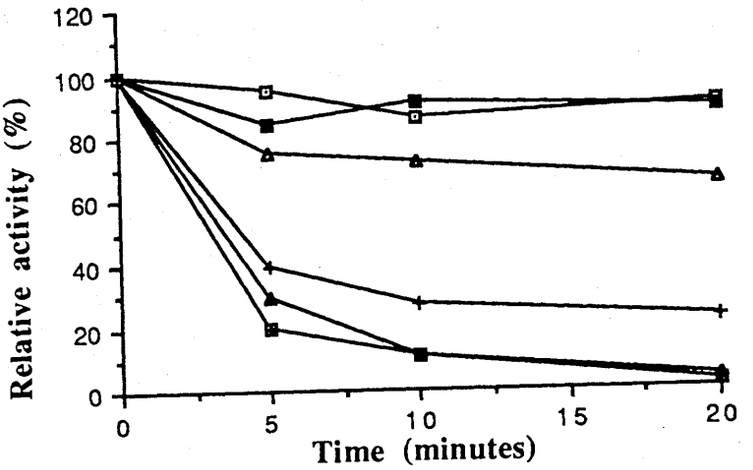
100
80
60
40
20
0
0
5
10
15
20
Time (minutes)

(b)


Relative activity (%)
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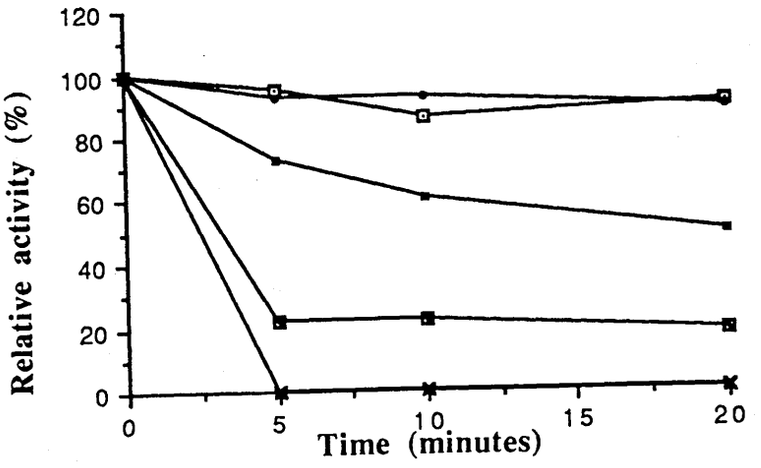
80
60
40
20
0
0
5
10
15
20
Time (minutes)

(c)


Relative activity (%)
120
100
80
60
40
20
0
0
5
10
15
20
Time (minutes)

6.4. Discussion

6.4.1. NADP-dependent alcohol dehydrogenase of *Acinetobacter sp.* HO1-N

NADP-dependent alcohol dehydrogenase of *Acinetobacter sp.* HO1-N is purified in a similar fashion to the enzyme of *A. calcoaceticus* NCIB 8250 (Section 4.5). The former enzyme appears to be a tetramer (native M_r 172 800) of subunit M_r 40 600 (Section 6.2.1) and is therefore very similar to the latter enzyme which is also a tetramer of subunit M_r 40 300. These enzymes resemble other bacterial alcohol dehydrogenases of both the iron-activated, and the zinc-dependent, groups in being tetramers of subunit M_r about 40 000 (Introduction 1.3).

The enzyme of strain HO1-N is kinetically very similar to that of strain NCIB 8250, being assayed in the same way with the same optimum pH (pH 10.2). The kinetic coefficients of the enzyme from both strains for primary alcohols can be compared (Table 6.7) and similar patterns of variation of kinetic constants with varying chain length of primary alcohol are observed. Hexan-1-ol and octan-1-ol have the lowest K'_m values for the enzyme from both strains, although for the enzyme of strain HO1-N the K'_m value for hexan-1-ol is slightly lower than that of octan-1-ol whereas for the enzyme of strain NCIB 8250 the converse is true (Table 6.7). The V'_{max} and k'_{cat} values of the enzyme from both strains are highest with pentan-1-ol as substrate. However, hexan-1-ol and octan-1-ol have the highest specificity constants for the enzyme from both strains (Table 6.7).

The major differences in kinetic coefficients of the enzymes of the two strains are that the K'_m values for the enzyme of strain HO1-N are about five-fold lower than those for the enzyme of strain NCIB 8250. Also, the V'_{max} values, and therefore the k'_{cat} values, for the enzyme of strain HO1-N are, in general, slightly higher than those for the enzyme of strain NCIB 8250. As a consequence of these variations, the specificity constants for the enzyme of strain HO1-N are 2-6 fold higher than those of the enzyme of

strain NCIB 8250. These differences in the enzymes of the two strains are much less significant than expected from the results reported for partially pure enzyme of strain HO1-N. (Fox *et al.*, 1990). The variations in kinetic coefficients observed between the enzymes from the two strains could be due to very few differences in the primary structure of the enzymes and similar, or larger, differences in kinetic coefficients are often observed for the same enzyme from two different strains of the same species, e.g. the benzaldehyde dehydrogenases of *Pseudomonas putida* strains MT53 and PaW1 have K'_m values for benzaldehyde of 0.79 μM and 457.5 μM respectively (Chalmers *et al.*, 1990 and Shaw and Harayama, 1990 respectively).

NADP-dependent alcohol dehydrogenase of *Acinetobacter sp.* HO1-N uses primary alcohols up to a maximal chain length of 14 carbons, like the enzyme of strain NCIB 8250 (Table 6.2). The activity of the enzyme of strain HO1-N detected with tetradecan-1-ol as substrate is much higher than that of the enzyme of strain NCIB 8250 (Tables 6.2 and 5.3), yet no activity can be detected for the enzyme of either strain with hexadecan-1-ol as substrate. Given the strong similarities between the two enzymes it seems probable that both enzymes use primary alcohols up to a maximal chain length of 14 carbons but not those with 16 carbons. Furthermore, the results obtained for the enzyme from strain HO1-N are so alike those for the enzyme from strain NCIB 8250 that no further characterisation of the former enzyme was attempted.

Table 6.7. Comparison of kinetic coefficients of NADP-dependent alcohol dehydrogenases of *A. calcoaceticus* NCIB 8250 and *Acinetobacter sp.* HO1-N

Average kinetic coefficients were taken from Tables 5.6. and 5.7. for *A. calcoaceticus* NCIB 8250 and Tables 6.1. and 6.2. for *Acinetobacter sp.* HO1-N. 8250 = *A. calcoaceticus* NCIB 8250, HO1-N. = *Acinetobacter sp.* HO1-N.

Substrate	K'_m (mM)		V'_{max} [EU (mg protein)$^{-1}$]		k'_{cat} (s^{-1})		k'_{cat}/K'_m (M^{-1} s^{-1})	
	8250	HO1-N	8250	HO1-N	8250	HO1-N	8250	HO1-N
Butan-1-ol	52.7	15.3	57.1	55.1	38.3	37.3	7.3×10^2	2.4×10^3
Pentan-1-ol	5.86	2.42	66.7	88.5	44.7	59.9	7.7×10^3	2.5×10^4
Hexan-1-ol	1.22	0.26	58.8	70.0	39.4	47.4	3.2×10^4	1.8×10^5
Octan-1-ol	1.10	0.34	37.9	47.5	25.4	32.1	2.3×10^4	9.6×10^4
Decan-1-ol	2.56	0.43	12.1	26.8	8.1	18.1	3.2×10^3	4.3×10^4

6.4.2. NADP-dependent alcohol dehydrogenase of the yeast *S. cerevisiae* D273-10B

(a) Protein structure

NADP-dependent alcohol dehydrogenase of *S. cerevisiae* D273-10B appears to be a monomer of M_r between 43 500, as judged by SDS gel electrophoresis, and 46 200, as judged by gel filtration chromatography, (Section 6.3.1). This M_r value is slightly larger than the subunit M_r value for NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* (about 40 000; Section 6.4.1). However, the *A. calcoaceticus* enzyme is a tetramer, whereas the yeast enzyme is a monomer which is somewhat unusual in one sense, most alcohol dehydrogenases being multimers, but the variations observed in quaternary structure of different enzymes belonging to the same group of alcohol dehydrogenases such as the "iron-activated" alcohol dehydrogenases (Section 1.3.3) suggest that differences in quaternary structures are not important in terms of the yeast and the bacterial enzymes having homologous primary structures.

The M_r value of NADP-dependent alcohol dehydrogenase of *S. cerevisiae* is larger than the subunit M_r values (36 000-37 000) of the three NAD-dependent alcohol dehydrogenases, which belong to the zinc-dependent group of alcohol dehydrogenases, and that of the "iron-activated" alcohol dehydrogenase (M_r = 40 000), identified in the same organism (Section 1.9). Unlike the NADP-dependent alcohol dehydrogenase, the zinc-dependent, NAD-dependent alcohol dehydrogenases are all tetramers (Section 1.9), whereas the "iron-activated" alcohol dehydrogenase is a dimer (Introduction 1.3.3).

The subunit M_r value of NADP-dependent alcohol dehydrogenase of *S. cerevisiae* D273-10B is perhaps consistent with it being a member of the zinc-dependent, or the iron-activated alcohol dehydrogenases but not the non-metallo-enzyme group which have subunit M_r values of 28 000.

(b) Cofactor specificity

NADP-dependent alcohol dehydrogenase of *S. cerevisiae* D273-10B is specific for NADP(H) as cofactor. The enzyme transfers the pro*R* hydrogen to/from NADP(H) as shown using pro*S*-[^{3}H]NADPH and confirmed using pro*R*-[^{3}H]NADPH [Section

6.3.5 (b)]. The ratio of percentages of ^3H incorporated into $NADP^+$ and butan-1-ol is less than 100 % in both cases probably for similar reasons to those stated in Section 5.5.2. The yeast enzyme is the same as the enzyme of *A. calcoaceticus* NCIB 8250 in its pro*R* stereospecificity of hydride transfer to/from NADP(H) [Section 5.3.5 (b)].

(c) Substrate specificity and kinetics

The pH optimum of NADP-dependent alcohol dehydrogenase of *S. cerevisiae* D273-10B with respect to alcohol oxidation is 10.7 [Section 6.3.3(a)]. As stated in Section 5.5.4, higher pH values favour oxidation reactions of NAD(P)-dependent dehydrogenases, but very few such enzymes have pH optima as high as 10.7. Presumably this is a reflection of the ionic state(s) in which the enzyme is active. This pH optimum is a little higher than that of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 (pH optimum 10.2; Section 5.3.2 (a).

Like the enzyme of *A. calcoaceticus* NCIB 8250 and few other bacterial alcohol dehydrogenases (Introduction 1.3.4), NADP-dependent alcohol dehydrogenase of *S. cerevisiae* D273-10B oxidises primary alcohols but not secondary alcohols (Section 6.3.6).

The kinetic coefficients of the NADP-dependent alcohol dehydrogenases of *S. cerevisiae* D273-10B, and of *A. calcoaceticus* NCIB 8250 for primary alcohols, the aldehyde butanal and the cofactor are compared in Table 6.8. The K'_m values for primary alcohols of the yeast enzyme and the bacterial enzyme show a similar pattern with respect to chain length of the primary alcohol, the lowest values occurring with octan-1-ol as substrate, with hexan-1-ol having similar values (Table 6.8). However, the K'_m values for primary alcohols of the yeast enzyme are between two and eight-fold higher than those of the bacterial enzyme (Table 6.8). Conversely, the K'_m values for butanal and the cofactor(s) are between two and eight-fold lower for the yeast enzyme compared with the bacterial enzyme (Table 6.8).

Comparison of the V'_{max} values and apparent turnover numbers (k'_{cat}) of the enzyme in both organisms reveals that the yeast enzyme has slightly higher values for primary alcohols but lower values for the aldehyde butanal (Table 6.8). Also, the V'_{max}

and k'_{cat} values of the yeast enzyme show less variation than those of the bacterial enzyme, the V'_{max} value for ethanol being 56 % of that for pentan-1-ol for the yeast enzyme but 31 % for the bacterial enzyme (Table 6.8). For both enzymes the highest V'_{max} and k'_{cat} values were obtained for pentan-1-ol as substrate (Table 6.8).

Comparison of the apparent specificity constants (k'_{cat}/K'_m) for the yeast enzyme and for the bacterial enzyme reveals that the values for primary alcohols obtained for the yeast enzyme are between one and five-fold lower than those of the bacterial enzyme (Table 6.8). However, the specificity constant for the aldehyde, butanal, obtained for the yeast enzyme is forty-fold higher than that for the bacterial enzyme (Table 6.8). The calculated specificity constants for the cofactor are two to four-fold greater for the yeast enzyme compared to the bacterial enzyme (Table 6.8)

For the yeast enzyme, the specificity for butanal over butan-1-ol is greater than that of the bacterial enzyme which itself "prefers" the aldehyde over the alcohol. Similarly, the difference in K'_m, the apparent dissociation constant, for butanal compared to butan-1-ol, is greater for the yeast enzyme than the bacterial enzyme which itself binds the aldehyde in preference to the alcohol.

(d) Possible presence of a metal ion in the active site of the enzyme

Partial inhibition of the yeast enzyme results from incubation with comparatively high concentrations (100 mM or 10 mM) of the metal binding agents EDTA and sodium azide (Table 6.6). However, no inhibition resulted from incubation of the enzyme with high concentrations of pyrazole or 2'2'bipyridyl (Section 6.3.7). It may be that EDTA and sodium azide are inhibiting the enzyme by some means other than a metal binding effect, i.e. the effect could be artefactual, which may explain why the inhibition is only partial and the concentrations of the chelating agents necessary to cause the partial inhibition are so high. These results are similar to those obtained for NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250, which showed no inhibition by metal chelating agents. However, the subunit M_r and the pro*R* stereospecificity of the yeast enzyme, like the bacterial enzyme suggest that it may belong to one of the metal dependent group of alcohol dehydrogenases. More sophisticated techniques may be

necessary to identify any metal dependency of the enzyme. Also, determination of the amino acid sequence of the enzyme may enable identification of possible metal ligands.

(e) Possible presence of an active site thiol group in the enzyme

Iodoacetamide and *N*'-ethylmaleimide inhibit the enzyme almost completely at 10 mM concentrations after 5 minutes incubation (Figure 6.6). No substrate protection of this inhibition is observed by either $NADP^+$ or hexan-1-ol. Therefore, it may be that these thiol acting agents are reacting with a thiol group that is not in, or near, the active site of the enzyme. *p*-Chloromercuribenzoate completely inhibits the enzyme after five minutes incubation at a concentration of 10 μM (Figure 6.6), and almost completely inhibits it at 1 mM concentration. Partial protection of this inhibition (at 1 μM - *p*-chloromercuribenzoate) can be obtained by including $NADP^+$ (1 mM) in the incubation, and almost complete protection can be obtained with hexan-1-ol (10 mM) (Figure 6.6). It may be that *p*-chloromercuribenzoate reacts with a thiol group at, or near, the active site of the enzyme which is exposed in the absence of substrate or cofactor or that the enzyme is very sensitive to mercuric ions. Another thiol acting agent, iodoacetate, has no effect on enzyme activity, possibly because its negative charge prevents it getting close enough to any thiol group to react with it. However, the negatively charged *p*-chloromercuribenzoate does inhibit the enzyme, so there may be some other reason for the lack of inhibition by iodoacetate.

The enzyme of *S. cerevisiae* D273-10B differs somewhat from that of *A. calcoaceticus* NCIB 8250 in the effects of thiol acting agents, the enzyme from the latter organism being sensitive only to *p*-chloromercuribenzoate and there is no substrate protection of this inhibition. This difference in effect of thiol acting agents on the enzymes of the two organisms could be due to anything from minor structural variations between the enzymes to major differences. It could be argued (perhaps naively) that the apparent tetrameric structure of the enzyme of *A. calcoaceticus* NCIB 8250 protects thiol groups that are exposed in the monomeric *S. cerevisiae* D273-10B enzyme and this may also explain the sensitivity of the yeast enzyme to high concentrations of EDTA and sodium

azide. However, it is impossible to draw any significant conclusions on the differences in the enzymes of the two organisms from these observations alone.

Table 6.8. Comparison of kinetic coefficients of NADP-dependent alcohol dehydrogenases of *A. calcoaceticus* NCIB 8250 and *S. cerevisiae* D273-10B

Average kinetic coefficients were taken from Tables 5.6. and 5.7. for *A. calcoaceticus* NCIB 8250 and Tables 6.4. and 6.5. for *S. cerevisiae* D273-10B. The k'_{cat}/K'_m values quoted for NADP and NADPH were calculated using k'_{cat} values for butan-1-ol and butanal as described in Sections 5.4.3 and 6.3.6 for *A. calcoaceticus* NCIB 8250 and *S. cerevisiae* D273-10B respectively.A. = *A. calcoaceticus* NCIB 8250, S. = *S. cerevisiae* D273-10B; n.d. = not determined.

Substrate	K'_m (mM)		V'_{max} [EU (mg protein)$^{-1}$]		k'_{cat} (s^{-1})		k'_{cat}/K'_m (M^{-1} s^{-1})	
	A.	S.	A.	S.	A.	S.	A.	S.
Ethanol	727	4790	20.8	48.8	13.9	35.1	1.9×10^1	7.3
Butan-1-ol	52.7	78.6	57.1	69.8	38.3	50.6	7.3×10^2	6.4×10^2
Pentan-1-ol	5.86	14.1	66.7	86.7	44.7	62.9	7.7×10^3	4.5×10^3
Hexan-1-ol	1.22	6.80	58.8	55.6	39.4	40.3	3.2×10^4	5.8×10^3
Octan-1-ol	1.10	6.20	37.9	54.9	25.4	39.8	2.3×10^4	6.4×10^3
Butanal	3.97	0.75	39.2	27.1	26.3	19.6	6.6×10^3	2.6×10^5
NADP	3.8×10^{-2}	2.0×10^{-2}	n.d.	n.d.	n.d.	n.d.	(1.0×10^6)	(2.5×10^6)
NADPH	4.3×10^{-3}	7.9×10^{-4}	n.d.	n.d.	n.d.	n.d.	(6.6×10^6)	(2.5×10^7)

CHAPTER 7
STUDIES ON THE PHYSIOLOGICAL ROLE AND LOCATION OF NADP-DEPENDENT ALCOHOL DEHYDROGENASE OF *ACINETOBACTER CALCOACETICUS* AND OCCURRENCE OF THE ENZYME IN OTHER ORGANISMS

7.1. Introduction

Several approaches were used to investigate the physiological role of NADP-dependent alcohol dehydrogenase of *Acinetobacter calcoaceticus* NCIB 8250.

Fixter and Nagi (1984) thought that the enzyme was soluble although only a superficial study had been made of its location. Therefore, more detailed experiments were carried out to determine whether the enzyme is cytoplasmic, periplasmic or membrane-bound.

The inducibility of the NADP-dependent alcohol dehydrogenase and other soluble NAD(P)-dependent alcohol dehydrogenases in *A. calcoaceticus* NCIB 8250 was tested by growing on different carbon sources.

The only suggestion that has been made as to the physiological role of the enzyme is that it may be involved in an unusual bacterial metabolic pathway, that of wax ester metabolism (Introduction 1.7; Fixter and Nagi, 1984). Attempts to verify this revolved mainly around continuous culture studies on *A. calcoaceticus* NCIB 8250. In addition, NADP-dependent alcohol dehydrogenase activities were examined in mutants of *Acinetobacter sp.* HO1-N which accumulate wax esters to a greater extent than the wild type.

Very few NADP-dependent alcohol dehydrogenases have been identified in micro-organisms that are not induced by either growth on alcohols as carbon sources or during growth under fermentative conditions. Therefore, prokaryotic and eukaryotic organisms were screened for this enzyme activity.

7.2. Location of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250

In order to determine whether any membrane-bound NADP-dependent alcohol dehydrogenase activity exists in *A. calcoaceticus* NCIB 8250, bacteria were grown in nutrient broth (Methods 2.4.4), harvested (Methods 2.4.6) and immediately broken by three passages through the French pressure cell at 98 MPa. The broken cell homogenate was assayed for enzyme activity, then centrifuged at 100 000 ***g*** for one hour, and the resulting supernatant and pellet separated, the pellet resuspended in 50 mM-Tris/HCl/ 2 mM-DTT, pH 8.0 and both pellet and supernatant assayed for enzyme activity [as in Methods 2.5.1 (a)]. All the NADP-dependent alcohol dehydrogenase activity detected in the broken cell extract was recovered in the supernatant after centrifugation, no activity being detected in the pellet. This method had been used successfully to distinguish both soluble and membrane-bound enzymes previously (e.g. Hoey, 1986).

In order to determine whether any periplasmic NADP-dependent alcohol dehydrogenase activity exists in *A. calcoaceticus* NCIB 8250 the wall of harvested cells was disrupted by lysozyme treatment using the method of Hoey (1986), thereby generating spheroplasts. NADP-dependent alcohol dehydrogenase activity [Methods 2.5.1(a)] and NADP-dependent isocitrate dehydrogenase activity (Methods 2.5.4; as a control for a cytoplasmic enzyme) were determined in samples of the intact spheroplast preparation and in samples of the spheroplasts lysed osmotically, over a 150 minute time course [Figure 7.1 (a) and (b)]. In addition, results obtained by Hoey (1986) for isocitrate dehydrogenase in a similar experiment are shown [Figure 7.1 (c)].

There is no evidence to suggest that NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 is membrane-bound or periplasmic.

Figure 7.1. NADP-dependent alcohol dehydrogenase activity and isocitrate dehydrogenase activity in lysozyme-treated cells of *A. calcoaceticus* NCIB 8250

Cells were incubated in 50 mM-Tris/HCl, pH 8.0 containing lysozyme (Section 7.2.2). Samples were taken at 30 minute intervals and diluted ten-fold in buffer or buffer containing 0.6 M-sucrose, then assayed for NADP-dependent alcohol dehydrogenase activity [Methods 2.5.1 (a)] or isocitrate dehydrogenase activity (Methods 2.5.4). Also, the results of the same experiment, carried out by Hoey (1986) for isocitrate dehydrogenase only are shown.

(◆) = samples diluted in buffer only; (◘) = samples diluted in buffer with 0.6 M-sucrose.

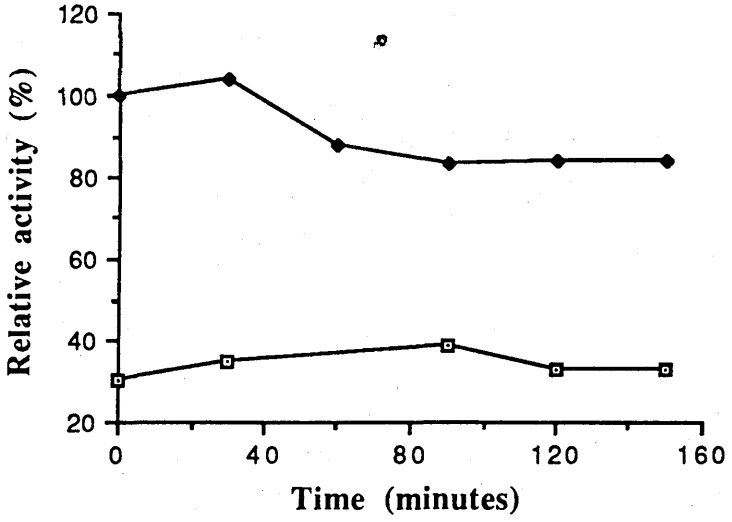
Graphs:

(a) NADP-dependent alcohol dehydrogenase, 100 % activity (for sucrose treated cells at time = 0 minutes) = 100 nmol min^{-1} (mg protein)$^{-1}$;

(b) isocitrate dehydrogenase, 100 % activity (for sucrose treated cells at time = 0 minutes) = 142 nmol min^{-1} (mg protein)$^{-1}$;

(c) isocitrate dehydrogenase (after Hoey, 1986), 100 % activity (for sucrose treated cells at time = 0 minutes) = 150 nmol min^{-1} (mg protein)$^{-1}$.

(a)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40
20
Relative activity (%)
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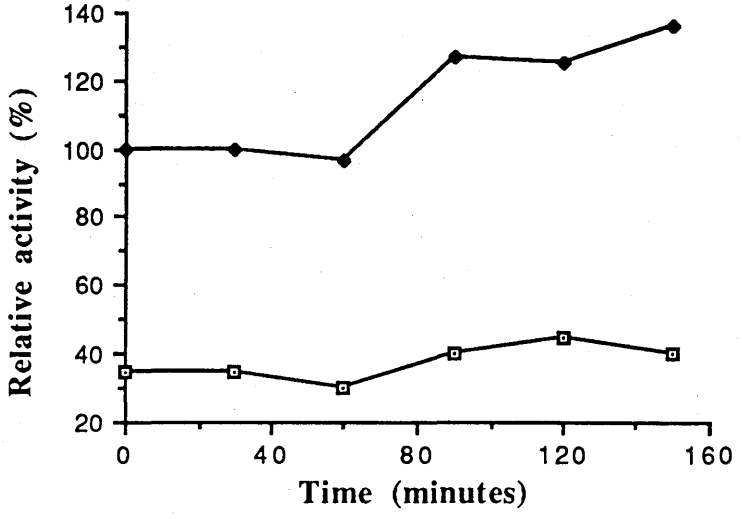
40
80
120
160
Time (minutes)

(b)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100
80
60
40
20
Relative activity (%)
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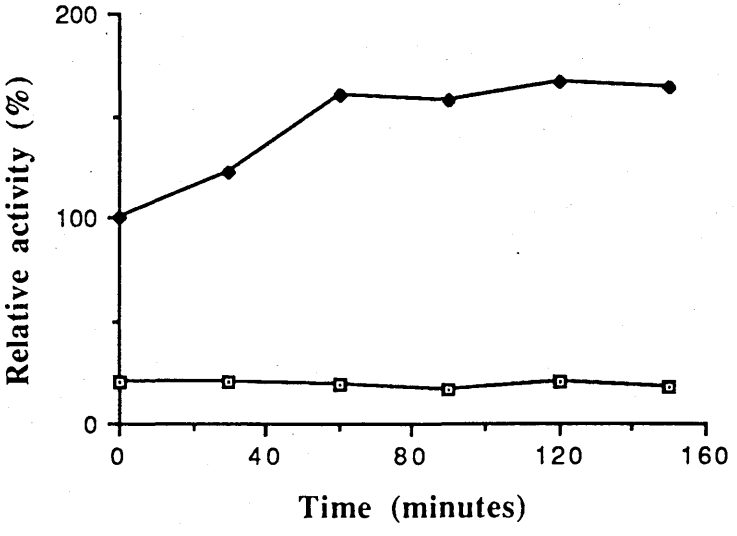
40
80
120
160
Time (minutes)

(c)


200
100
0
Relative activity (%)
0
40
80
120
160
Time (minutes)

7.3. Variations in soluble NAD(P)H-dependent aliphatic alcohol dehydrogenase activities in *A. calcoaceticus* NCIB 8250 grown on various different carbon sources

A. calcoaceticus NCIB 8250 was grown in batch culture on a variety of different carbon sources, under carbon limitation and under nitrogen limitation and the effects of the different growth media on NADP-dependent alcohol dehydrogenase, NAD-dependent ethanol dehydrogenase and NAD-dependent butan-2,3-diol dehydrogenase activity in soluble extracts were determined (Table 7.1). NADP-dependent alcohol dehydrogenase activity was present regardless of carbon source. Levels of enzyme activity did vary somewhat, the maximum activity being found under nitrogen limitation. Both of the other enzyme activities were induced to varying extents by growth on different alcohols as sole carbon source.

NAD-dependent hexan-1-ol dehydrogenase activity was always three to four-fold lower than NAD-dependent ethanol dehydrogenase activity (whereas NADP-dependent hexan-1-ol dehydrogenase activity was always four to five-fold higher than NADP-dependent alcohol dehydrogenase activity) and from this it could be deduced that there was no detectable NAD-dependent alcohol dehydrogenase activity on hexan-1-ol other than the activity of NAD-dependent ethanol dehydrogenase on hexan-1-ol, when assayed in 80 mM-glycine/ 20 mM-hexan-1-ol/ 1 mM-NAD, pH 9.5.

Table 7.1. Alcohol dehydrogenase activities of extracts of *A. calcoaceticus* NCIB 8250 grown on various carbon sources

A. calcoaceticus NCIB 8250 was grown on a variety of different carbon sources (see Methods 2.4.3 for details of different media used). Cells were harvested (Methods 2.4.6), broken by sonication [Methods 2.4.7 (a)], and assayed for: NADP-dependent alcohol dehydrogenase activity [Methods 2.5.1 (a)]; NAD-dependent ethanol dehydrogenase activity [as described in Methods 2.5.1 (a), except 1 mM-NAD replaced 1 mM-NADP and 50 mM-ethanol replaced 20 mM-hexan-1-ol]; NAD-dependent butan-2,3-diol dehydrogenase activity [as described in Methods 2.5.1 (a), except 1 mM-NAD replaced 1 mM-NADP and 50 mM-butan-2,3-diol replaced 20 mM-hexan-1-ol]. n.d. = not determined.

Growth substrate	**Enzyme activity [nmol min^{-1} (mg protein)$^{-1}$]**		
	NADP-dependent alcohol dehydrogenase	**NAD-dependent ethanol dehydrogenase**	**NAD-dependent butan-2,3-diol dehydrogenase**
Nutrient broth	150	9	n.d.
Succinate (nitrogen limited)	300	4	n.d.
Succinate (carbon limited)	184	4	n.d.
Ethanol	142	733	910
Butan-1-ol	162	231	730
Hexan-1-ol	178	80	530
Butan-2,3-diol	213	227	2644

7.4. NADP-dependent alcohol dehydrogenase activity of *A. calcoaceticus* NCIB 8250 when the organism was grown in continuous culture under various limitations

A. calcoaceticus NCIB 8250 was grown in continuous culture under various limiting conditions [Methods 2.4.4 (c)]. Samples of cells were collected from the effluent line of the continuous culture vessel over ice for up to 15 minute periods then immediately harvested (Methods 2.4.6). After harvesting, samples collected at any one time (i.e. any one growth rate and limitation) were pooled and frozen at -80 °C. As a control experiment, *A. calcoaceticus* NCIB 8250 was grown in batch culture under nitrogen and under carbon limitation. Cells were harvested and some of the cells from each growth limitation were broken by sonication (Methods 2.7.1) and the resulting cell free homogenate assayed for enzyme and protein [Methods 2.5.1(a) and 2.7.5 respectively]. The rest of the cells from each batch culture were frozen at -80 °C.

When samples had been taken from the continuous culture vessels under a variety of different growth rates and conditions (over a 6-8 week period), all samples (including the batch culture controls) were thawed, broken by sonication [Methods 2.4.7 (a)] and assayed for NADP-dependent alcohol dehydrogenase activity [Methods 2.5.1(a)] and for protein (Methods 2.2.5) on the same day. The average enzyme activity of the control samples for each growth condition taken after freezing varied by < 7 % compared to those control samples assayed before freezing [before freezing: nitrogen limitation, 209 nmol min^{-1} (mg protein)$^{-1}$, (211, 207); carbon limitation 145 nmol min^{-1} (mg protein)$^{-1}$, (141, 148). After freezing: nitrogen limitation, 198 +/- 12 nmol min^{-1} (mg protein)$^{-1}$, n = 4 samples; carbon limitation 138 +/- 4 nmol min^{-1} (mg protein)$^{-1}$, n = 4 samples].

Details of the actual limitation of each sample were given by Mr M.J. MacAvoy, Department of Biochemistry, University of Glasgow. "Nitrogen limitation" or "low nitrogen concentration" refer to the nitrogen source supplied in the medium, i.e. ammonium sulphate. Similarly, "carbon limitation" or "low carbon concentration" refer to the carbon source supplied in the medium, i.e. succinate. Where the phrase "low ....

concentration" is used, < 5 % of the initial amount of that nutrient was left in the medium but oxygen rather than the nitrogen or carbon source was probably limiting because the yield increased slightly when the rate of aeration was increased.

For each growth condition, at 30 °C, enzyme activity was plotted against growth rate [Figures 7.2 (a) to (c)]. In all cases there was a decrease in enzyme activity with increasing growth rate.

The effect on enzyme activity of temperature under nitrogen limitation and under oxygen limitation with low nitrogen concentration are shown in Figure 7.3 [(a) and (b) respectively]. The enzyme activity was lower at 15 °C or 20 °C compared to 30 °C, regardless of growth rate.

The variation of enzyme activity with total wax content of the cells is shown for conditions of nitrogen limitation and oxygen limitation with low nitrogen concentrations at 30 °C, carbon limitation and oxygen limitation at low carbon concentrations at 30 °C, and for conditions of oxygen limitation with low nitrogen concentrations at 15 °C in Figure 7.4 [(a), (b) and (c) respectively].

Statistical analyses of some of the data was carried out in order to determine if there was any correlation between enzyme activity and:

1. total wax content;
2. growth rate;
3. growth temperature.

ANOVA analysis was used to determine significant relationships and was carried out by Mr D. Buchanan, Paisley College, Paisley, Scotland using the suite of programs at the Department of Statistics, Glasgow University. Where appropriate, simple linear regression analysis (Methods 2.8) was used to show relationships (Figures 7.5, 7.6 and 7.7). Only those relationships with a probability of random occurrence of < 0.05 (i.e. less than one in twenty) were deemed to be significant. It should be noted that in order to obtain enough data for significant statistical analysis, some of the data obtained for slightly different growth conditions had to be grouped together.

1. In order to examine the relationship between enzyme activity and total wax content, the

data were grouped as follows: (i) data obtained from cells grown at 30 °C in the presence of low or limiting amounts of carbon, regardless of any oxygen limitation; (ii) data obtained from cells grown at 30 °C in the presence of low or limiting amounts of nitrogen, regardless of any oxygen limitation; and (iii) data obtained from cells grown at 15 °C in the presence of low or limiting amounts of nitrogen, regardless of any oxygen limitation.

For group (i), i.e 30 °C/ low carbon, there was no significant relationship between enzyme activity and wax content, as judged by the statistical analysis used, although it should be noted that wax values for this group are much smaller than for the other groups [Figure 7.5, (▫)]. There did seem to be an apparent correlation when the Figure (7.5) was examined by eye but the correlation coefficient (r) for these data was only 0.764. For group (ii), i.e. 30 °C/ low nitrogen, the sample with the highest wax content [41 mg (g dry weight)$^{-1}$ in Figure 7.4 (a)] did not appear to conform to the pattern of the rest of the data and had a disproportionate amount of influence on any models fitted to these data. If this sample was ignored, and it was omitted from Figure 7.4, there was a strongly linear relationship between enzyme activity and wax content, enzyme activity increasing with increasing wax content [Figure 7.5 (▪)]. For group (iii), i.e. 15 °C/ low nitrogen, there was a different significant linear relationship between enzyme activity and wax content, enzyme activity increasing with wax content but not as steeply as for group (i) [Figure 7.5 (▬)].

2. In order to examine the relationship between growth rate and enzyme activity the data obtained at 30 °C only were split into two groups: (i) low amounts of carbon, regardless of oxygen concentration; and (ii) low amounts of nitrogen, regardless of oxygen concentration. A linear relationship existed between enzyme activity and growth rate, enzyme activity decreasing with increasing growth rate in both groups but the degree of decrease was greater for cells grown in low concentrations of nitrogen than for those grown in low concentrations of carbon (Figure 7.6). Furthermore, the cells grown in low concentrations of carbon had generally lower enzyme activities than those grown in low concentrations of nitrogen.

3. In order to examine the relationship between growth temperature and enzyme activity the data obtained at 15 °C and 20 °C were grouped together forming a single group, 20⁻, because there appeared to be little variation in enzyme activities between these two temperatures (Figure 7.4). Since growth rate is known to affect enzyme activity (see above) it was included as a covariant to increase the precision of the analysis. The two groups did not decrease to the same extent with growth rate and there was a low correlation of enzyme activity and growth temperature for the group 20⁻ (Figure 7.7) but there was a significant temperature effect on enzyme activity.

Figure 7.2. Effect of growth rate on NADP-dependent alcohol dehydrogenase activity for cells grown under various limitations

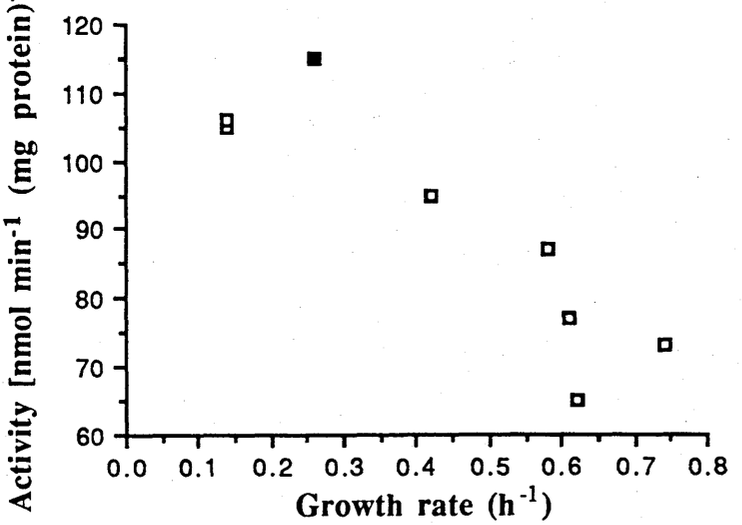
Acinetobacter calcoaceticus NCIB 8250 was grown in continuous culture at 30 °C [Methods 2.4.4 (c)] and the growth limitation determined by Mr M.J.MacAvoy (Section 7.4). Cells were harvested as described in Section 7.4, broken as described in Methods 2.4.7 (a) and enzyme activity assayed as described in Methods 2.5.1 (a).

(a) (■), carbon limitation; (□), oxygen limitation, low carbon concentration,

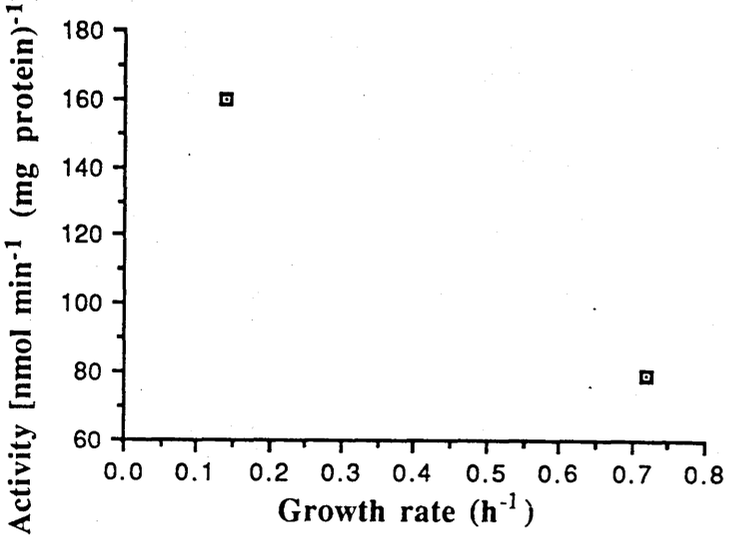
(b) nitrogen limitation,

(c) oxygen limitation, low nitrogen concentration.

(a)


Activity [nmol min-1 (mg protein)-1]
120
110
100
90
80
70
60
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8
Growth rate (h-1)

(b)


Activity [nmol min-1 (mg protein)-1]
180
160
140
120
100
80
60
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8
Growth rate (h-1)

(c)

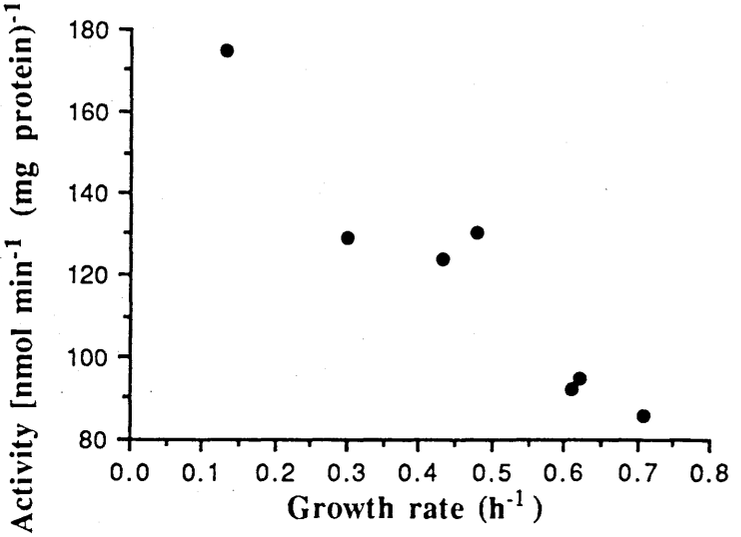

Activity [nmol min-1 (mg protein)-1]
180
160
140
120
100
80
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8
Growth rate (h-1)

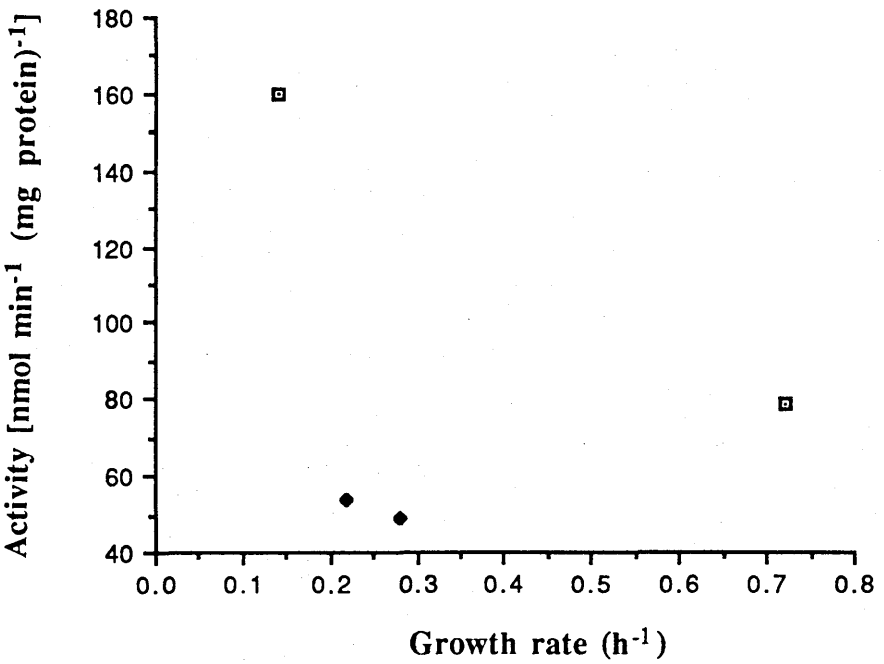
Figure 7.3. Effect of growth rate at different growth temperatures on NADP-dependent alcohol dehydrogenase activity

Acinetobacter calcoaceticus NCIB 8250 was grown in continuous culture at 15 °C, 20 °C and 30 °C [Methods 2.4.4 (c)] and the growth limitation determined by Mr M.J. MacAvoy (Section 7.4), cells were harvested as described in Section 7.4, broken as described in Methods 2.4.7 (a) and enzyme activity assayed as described in Methods 2.5.1 (a).

(a) nitrogen limitation, (▫) 30 °C, (•) 15 °C,

(b) oxygen limitation, low nitrogen concentration: (▫) 30 °C, (◆) 20 °C, (■) 15 °C.

(a)


Activity [nmol min-1 (mg protein)-1]
180
160
140
120
100
80
60
40
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Growth rate (h-1)

(b)

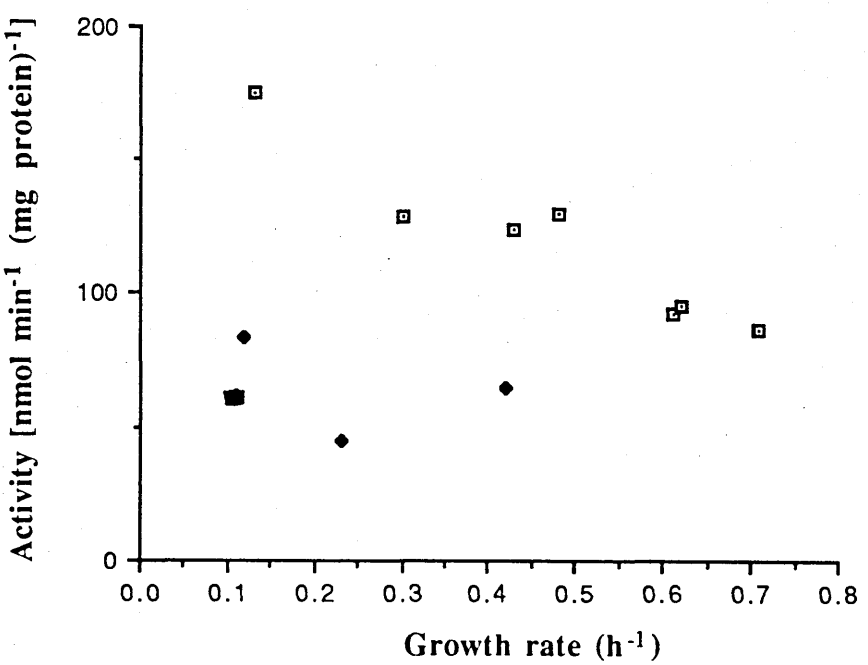

Activity [nmol min-1 (mg protein)-1]
200
100
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Growth rate (h-1)

Figure 7.4. Possible relationship of wax content and NADP-dependent alcohol dehydrogenase activity for cells grown under various conditions

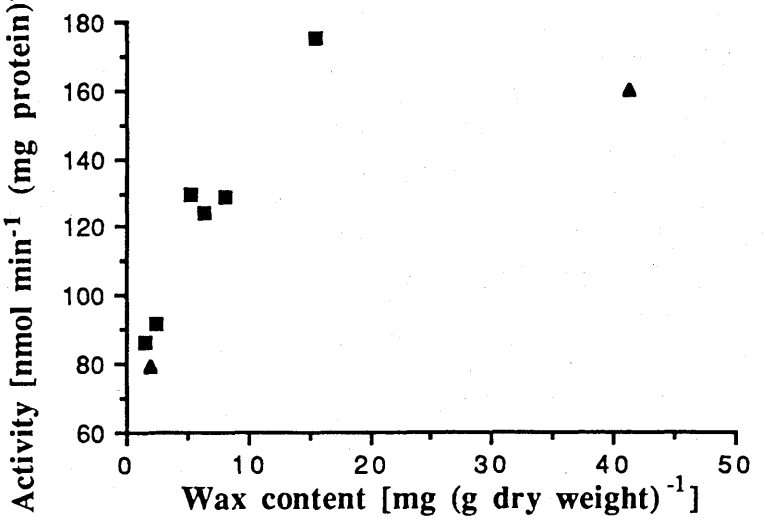
Acinetobacter calcoaceticus NCIB 8250 was grown in continuous culture at 15 °C and 30 °C [Methods 2.4.4 (c)] and the growth limitation determined by Mr M.J. MacAvoy (Section 7.4), cells were harvested as described in Section 7.4, broken as described in Methods 2.4.7 (a) and enzyme activity assayed as described in Methods 2.5.1 (a). Wax contents were determined using g.l.c., by Mr M.J. MacAvoy.

(a) All cells grown at 30 °C: (▲) nitrogen limitation; (■) oxygen limitation, low nitrogen concentration.

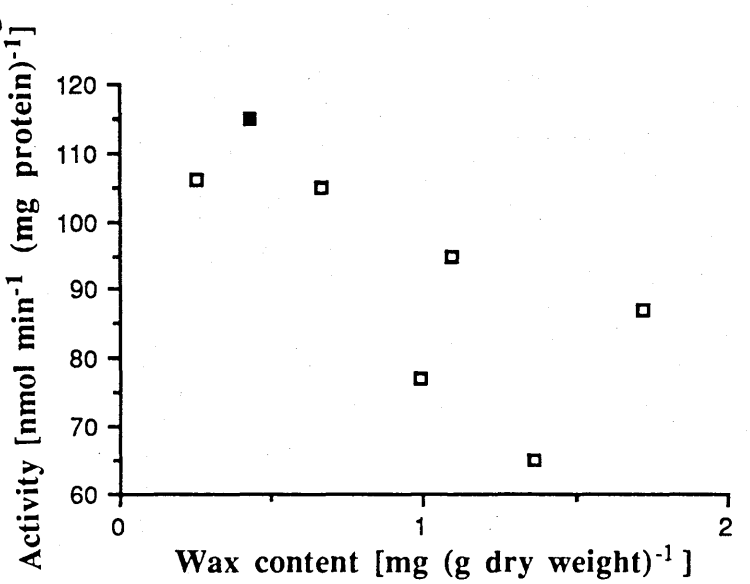
(b) All cells grown at 30 °C: (■) carbon limitation; (□) oxygen limitation, low carbon concentration.

(c) All cells grown at 15 °C: (⧈) nitrogen limitation; (◆) oxygen limitation, low nitrogen concentration.

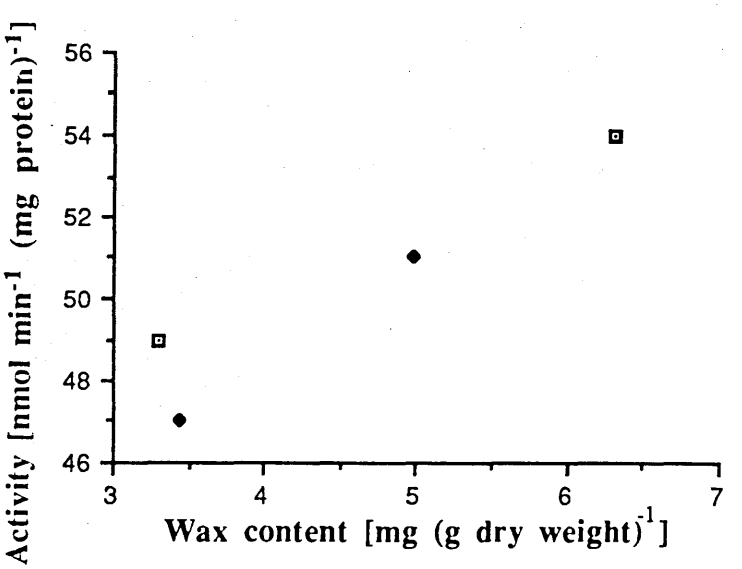
(a)


Activity [nmol min-1 (mg protein)-1]
180
160
140
120
100
80
60
0
10
20
30
40
50
Wax content [mg (g dry weight)-1]

(b)


Activity [nmol min-1 (mg protein)-1]
120
110
100
90
80
70
60
0
1
2
Wax content [mg (g dry weight)-1]

(c)


Activity [nmol min-1 (mg protein)-1]
56
54
52
50
48
46
3
4
5
6
7
Wax content [mg (g dry weight)-1]

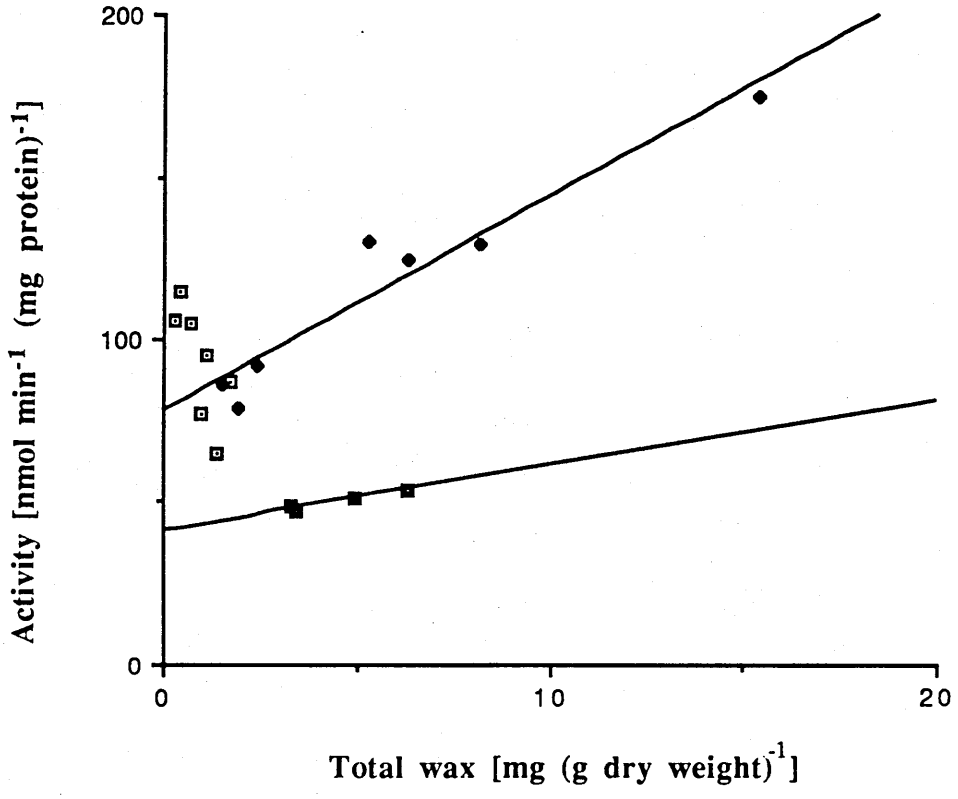


Figure 7.5. Correlation of enzyme activity with wax content in cells grown under various growth conditions

The data (Figure 7.4) were grouped and analysed as described in Section 7.4. Simple linear regression analysis (Methods 2.8) was used to plot the correlations shown. Where a line has been extrapolated, a dashed line is shown. The regression equations of the lines and their correlation coefficients (r) are given below.

(▫) group (i), 30 °C, low carbon;

(•) group (ii), 30 °C, low nitrogen, $y = 6.614x + 77.691$, $r = 0.964$;

(▪) group (iii), 15 °C, low nitrogen, $y = 1.996x + 41.262$, $r = 0.950$.

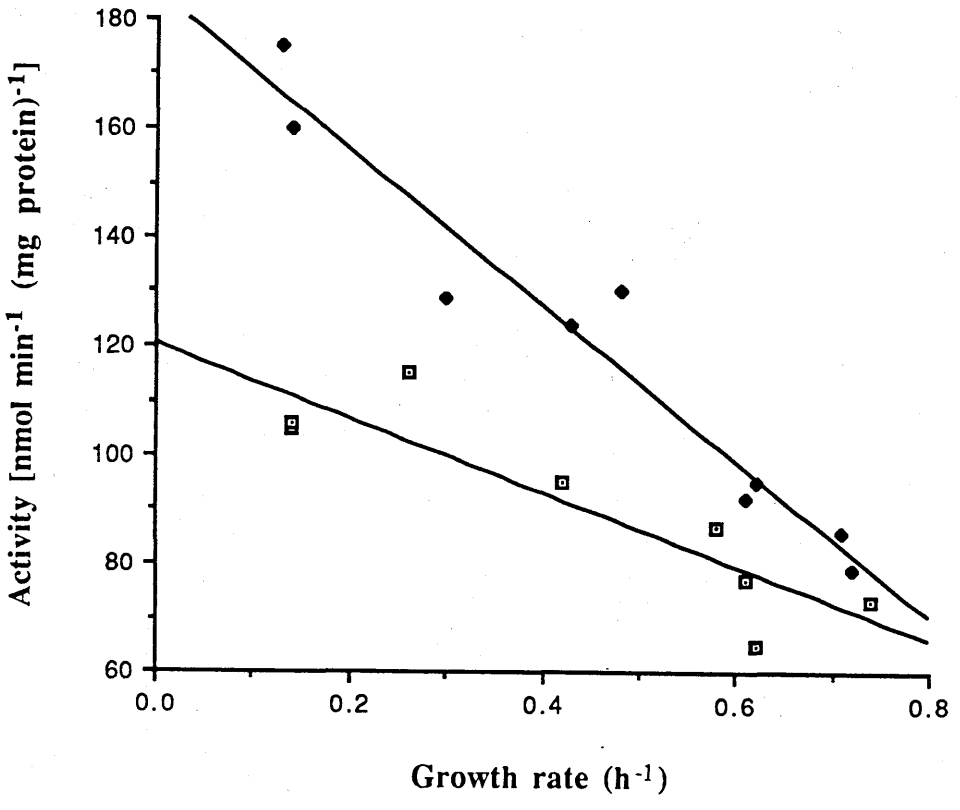


Figure 7.6. Correlation of enzyme activity with growth rate in cells grown under various growth conditions

The data (Figure 7.2) were grouped and analysed as described in Section 7.4. Simple linear regression analysis (Methods 2.8) was used to plot the correlations shown. The regression equations of the lines and their correlation coefficients (r) are given below.

(▫) group (i), low carbon, $y = -68.093x + 120.25$, $r = 0.895$;

(◆) group (ii), low nitrogen, $y = -143.3x + 184.81$, $r = 0.972$.

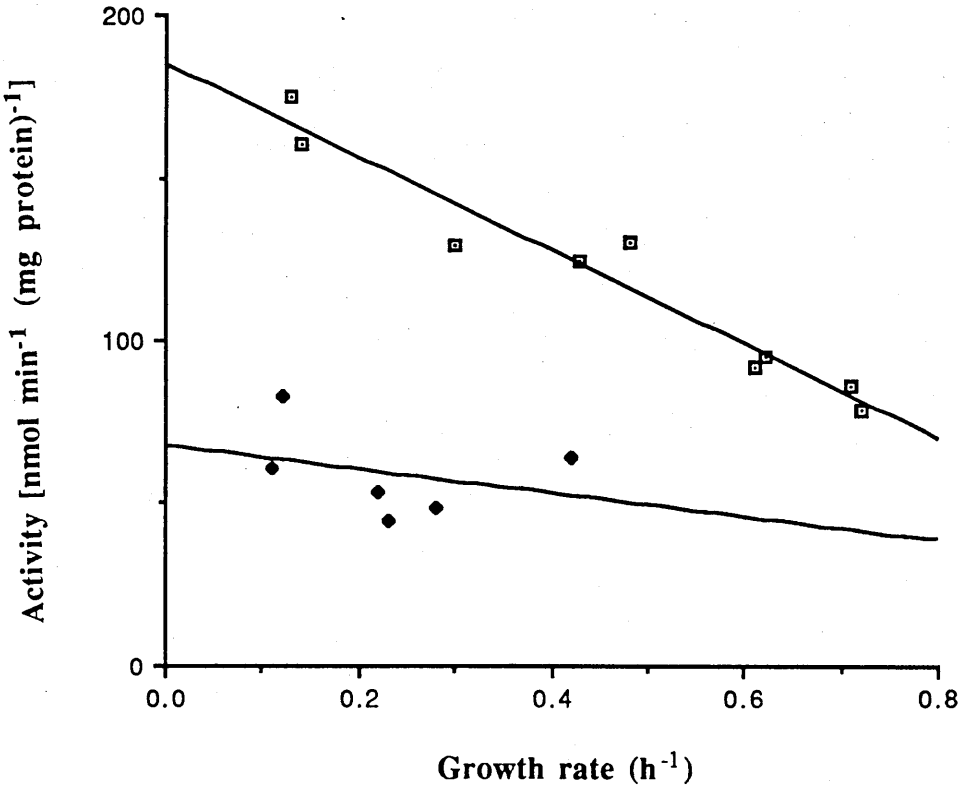


Figure 7.7. Correlation of enzyme activity at various growth temperatures with growth rate in cells grown under low nitrogen concentrations

The data (Figure 7.3) were grouped and analysed as described in Section 7.4. Simple linear regression analysis (Methods 2.8) was used to plot the correlations shown. Where a line has been extrapolated, a dashed line is shown. The regression equations of the lines and their correlation coefficients (r) are given below.

(▫) group (i), 30 °C, y = -143.3x + 184.81, r = 0.972;

(♦) group (ii), 20°, y = -36.503x + 67.729, r = 0.307.

7.5. NADP-dependent alcohol dehydrogenase activities in wax ester catabolic mutants of *Acinetobacter sp.* HO1-N

Five mutants of *Acinetobacter sp.* HO1-N, all of which are believed to break down wax esters more slowly than the wild type, thus accumulating wax esters to at least a ten-fold greater extent than the wild type when grown on acetate, propionate or hexadecane, (Geigert *et al.*, 1984; Niedleman *et al.*, 1986) were donated by Cetus Corporation, Emeryville, CA, USA. These mutants and the wild type were grown on an acetate medium [Methods 2.4.3 (d)], harvested (Methods 2.4.6), broken by sonication [Methods 2.4.7 (a)] and assayed for NADP-dependent alcohol dehydrogenase activity and for isocitrate dehydrogenase activity (Table 7.2).

Levels of isocitrate dehydrogenase activity were similar in all five mutants and the wild type organism [136.5 +/- 18.7 nmol min^{-1} (mg protein)$^{-1}$]. The actual rates of alcohol dehydrogenase activity and the ratio of rates of activity of isocitrate dehydrogenase activity: alcohol dehydrogenase activity of the mutants can be compared to those of the wild type. For all the mutants, the actual rates of alcohol dehydrogenase activity were slightly higher (up to two-fold) than the wild type. Mutant cp 148-7 had very similar alcohol dehydrogenase and isocitrate dehydrogenase activities. Mutant cp 3-14 had a very similar ratio of isocitrate dehydrogenase: alcohol dehydrogenase activity although both activities were higher than for the wild type. Mutant cp 1-16 had a lower ratio of isocitrate dehydrogenase: alcohol dehydrogenase activity than the wild type mainly due to an increase in alcohol dehydrogenase activity of the mutant. Mutants cp 4-5 and cp 3-24 had ratios of isocitrate dehydrogenase: alcohol dehydrogenase activity that were about half that of the wild type, mainly due to their alcohol dehydrogenases being twice as high as the wild type.

Table 7.2. NADP-dependent alcohol dehydrogenase activity and isocitrate dehydrogenase activity in *Acinetobacter sp.* HO1-N and mutants of *Acinetobacter sp.* HO1-N

Extracts of *Acinetobacter sp.* HO1-N and five mutants of *Acinetobacter sp.* HO1-N that accumulate wax esters to a greater extent than the wild type, grown on Z1/acetate medium [Methods 2.4.1 (d)], were assayed for NADP-dependent alcohol dehydrogenase activity [Methods 2.5.1 (a)] and isocitrate dehydrogenase activity (Methods 2.5.4).

Strain	Enzyme activity [nmol min^{-1} (mg protein)$^{-1}$]	
	alcohol dehydrogenase	isocitrate dehydrogenase
HO1-N	59	139
cp 4-5	119	130
cp 3-24	120	109
cp 1-16	82	146
cp 148-7	60	130
cp 3-14	86	165

7.6. Occurrence of NADP-dependent alcohol dehydrogenase activities in other micro-organisms

In an attempt to identify any non-fermentative, constitutive NADP-dependent alcohol dehydrogenases, selected micro-organisms were grown aerobically in batch culture into stationary phase on growth media with non-alcohol carbon sources (Methods 2.4). Organisms grown in this way were assayed for soluble, NADP-dependent hexan-1-ol dehydrogenase activity [Methods 2.5.1 (a)] and the approximate K'_m value of any such activity found was determined (Table 7.3). 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 in Methods 2.5.1 (a) except that 0.5 M ethanol was included instead of hexan-1-ol, was less than 20 % of the NADP-dependent hexan-1-ol dehydrogenase activity. However, no such activity was identified in the halophilic archaebacterium, *Haloferax mediterranei*.

Table 7.3. Identification of NADP-dependent hexanol dehydrogenase activities in micro-organisms

Organisms were grown into stationary phase under aerobic conditions on the media listed (see Methods 2.4.3 and 2.4.4 for details of the different media and growth conditions used). Cells were harvested (Methods 2.4.6), broken by sonication (Methods 2.4.7) and assayed for NADP-dependent alcohol dehydrogenase activity [Methods 2.5.1 (a)].

Organism	**Medium:**	**Specific activity** [nmol min^{-1} (mg protein)$^{-1}$]	**K'_m (hexan-1-ol)** (mM)
Acinetobacter. calcoaceticus NCIB 8250	MMD	104	1.16
Escherichia coli ML30	Glycerol/salts	11.0	0.88
Pseudomonas putida 9494	MMA	5.4	0.46
Pseudomonas aeruginosa 10548	MMA	7.7	1.11
Bacillus subtilis NCIMB 3610	Nutrient broth	2.8	7.50
Rhodococcus rhodocrous	Nutrient broth	6.6	2.86
Streptomyces rimosus 4018	Hobb's medium	3.6	12.1
Saccharomyces cerevisiae D273-10B	PYG	12.7	3.35
Rhodotorula graminis KGX 39	MMB	74.0	2.16

7.7. Discussion

7.7.1. Location of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250

The enzyme is located in the cytoplasm and is not membrane-associated or periplasmic (Section 7.2). Although some enzyme could be detected after removal of the cell wall by lysozyme treatment, the activity, as a percentage of that detected when the spheroplasts were lysed, was no greater than that of isocitrate dehydrogenase, a known cytoplasmic enzyme, even though this apparent leakage of cytoplasmic enzymes was greater than that of isocitrate dehydrogenase shown by Hoey [1986; Figure 7.1(c)].

7.7.2. Soluble aliphatic NAD(P)-dependent alcohol dehydrogenase activities in *A. calcoaceticus* NCIB 8250

Only those soluble, aliphatic NAD(P)-dependent alcohol dehydrogenase activities that have been previously identified (Introduction 1.8) were identified in *A. calcoaceticus* NCIB 8250.

7.7.3. Is NADP-dependent alcohol dehydrogenase involved in wax ester metabolism in *Acinetobacter calcoaceticus* ?

Statistical analysis of the data obtained from growth of *A. calcoaceticus* NCIB 8250 in continuous culture shows different significant linear relationships between enzyme activity and wax content, for low amounts of wax, for cells grown at 30 ^{o}C with low nitrogen concentration and for cells grown at 15 ^{o}C with low nitrogen concentration (Section 7.4). In both cases there was a positive correlation between wax content and enzyme activity although the numerical values of the relationships are different (Figure 7.5). However, no such statistically significant relationship was apparent for cells grown

with low carbon concentrations (Figure 7.5). It may well be that those relationships between enzyme activity and wax content identified are simply reflections of the correlation between enzyme activity and growth rate because it has been established that under nitrogen limitation wax content increases with decreasing growth rate (Figure 7.8; MacAvoy, M.J., Fixter, L.M. and Fewson C.A., unpublished results), and given that enzyme activity increases with decreasing growth rate (Figure 7.6) it is hardly surprising that enzyme activity increases with increasing wax content. Conversely, wax content increases, although only slightly, with increasing growth rate under carbon limitation (Figure 7.8) and this fact, combined with the small variation in wax content observed with variation in enzyme activity (Figure 7.5), accounts for the lack of apparent correlation between wax content and enzyme activity under conditions of low or limiting amounts of carbon. Overall, therefore, it is possible that any correlations between wax content and enzyme activity are not causal but are reflections of these two parameters' independent correlations with growth rate.

The mutants of *Acinetobacter sp.* HO1-N all accumulate wax esters, in theory, because of slow breakdown of wax esters (Neidleman and Geigert, 1983; Neidleman and Geigert, 1984; Neidleman & Ervin, 1986). Therefore, if NADP-dependent alcohol dehydrogenase is involved in the breakdown of wax esters, at least one of the mutants assayed might have been expected to have a much lower enzyme activity compared to the wild type. This proved not to be the case because none of these mutants had a lowered enzyme activity (Table 7.2). On the other hand if the previous works' (Neidleman and Geigert, 1983; Neidleman and Geigert, 1984; Neidleman & Ervin, 1986) conclusions were incorrect and one, or more, of the mutants could synthesise waxes at a higher rate than the wild type (rather than breaking them down more slowly), it might have been that enzyme activity in one or more of the mutants would be significantly higher than in the wild type. Two mutants have enzyme activities that are twice that of the wild type (Table 7.2) but whether this increase in activity would be sufficient to account for the > ten-fold increase in waxes accumulated is questionable. This experiment does not preclude the possibility of the NADP-dependent alcohol dehydrogenase being involved in wax ester

catabolism or synthesis but they do not provide evidence for its involvement.

Further evidence against the involvement of NADP-dependent alcohol dehydrogenase comes from studies on the ability of the enzyme from strain NCIB 8250 and strain HO1-N to use long chain alcohols (Table 5.4 and 6.2 respectively). The enzyme was unable to use primary alcohols with a chain length > 14 whereas wax esters in *Acinetobacter* consist predominantly of alcohol and acid moieties of 16 or more carbons (Fixter *et al.*, 1986).

Considering these studies together it seems unlikely that the enzyme is involved directly in wax degradation, although it is possible that it is involved in wax synthesis. Given the lipophilic (hydrophobic) nature of the physiological substrates and products of a long chain alcohol dehydrogenase involved in wax ester metabolism it is possible that any enzyme involved, is somehow associated with a membrane.

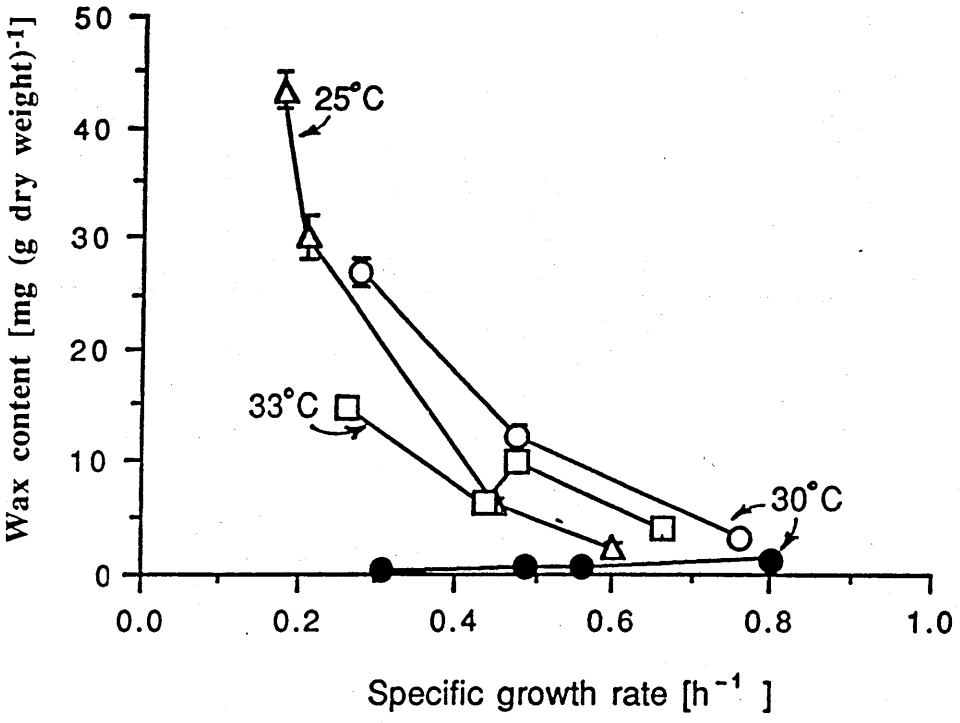


Figure 7.8. Effect of growth rate, growth temperature and specific nutrient limitation on total wax content

Open symbols represent nitrogen limited cells, closed symbols represent carbon limited cells. From M.J. MacAvoy, L.M. Fixter and C.A. Fewson, unpublished results.

7.7.4. Regulation of activity of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250

NADP-dependent alcohol dehydrogenase activity did not increase significantly (< two-fold; Table 7.1) after growth in defined media containing alcohols or under nutrient limitation compared with that in nutrient broth grown cells regardless of carbon source, suggesting that it is a constitutive enzyme. However, when the organism was grown in either batch culture or in continuous culture, nitrogen limited cells had a higher enzyme activity than carbon limited cells (Sections 7.3 and 7.4 respectively) so there is some sort of control of enzyme activity.

It is difficult to draw any conclusions from the fact that enzyme activity decreases, to different degrees depending on the nutrient limitation, with increasing growth rate (Figure 7.6). A decrease in enzyme activity with increasing growth rate has been observed for a number of different enzymes such as histidase of *Klebsiella aerogenes*, isocitrate dehydrogenase of *Candida utilis* and glutamate-oxalate transaminase of *Escherichia coli* and no obvious rules have been deduced for the growth rate dependence of enzyme activity, the situation being more complex in some examples that in others (Dean, 1972). However, the observed decrease in enzyme activity with increasing growth rate would be consistent with a global regulatory mechanism, such as catabolite repression (Clarke, 1981). Alternatively, such changes could result from regulation at the level of the enzyme activity, e.g. by some form of covalent modification but this has never been observed for any alcohol dehydrogenase although it might be worth checking in this case.

7.7.5. Apparently constitutive NADP-dependent aliphatic alcohol dehydrogenases seem to be common in micro-organisms

Table 7.4 shows the data given in Table 7.3 together with other examples of soluble NADP-dependent aliphatic alcohol dehydrogenases described in the literature that were

produced by growing organisms in aerobic conditions on non-alcohol carbon sources, i.e. conditions unlikely to induce fermentative alcohol dehydrogenase activity.

The inability to detect this enzyme activity in the halophilic archaebacterium *H. mediterranei* may simply be because the enzyme requires a high salt concentration for enzyme activity. Alternatively, such an enzyme activity may have evolved after archaebacteria diverged evolutionarily from eubacteria. It would be worth testing other archaebacteria for the presence of this activity.

Alcohol dehydrogenase 1 from *P. aeruginosa* 196 Aa has been partially purified and shown to use primary alcohols with from 2 to 16 carbons, with a minimum K'_m value (80 μM) quoted for decan-1-ol and a highest V'_{max} value for ethanol [136 nmol min^{-1} (mg protein)$^{-1}$; Tassin and Vandecasteele, 1972]. Alcohol dehydrogenase 2 from the same organism uses primary alcohols with from 2 to 12 carbons, with minimum K'_m values for octan-1-ol and decan-1-ol (200 μM) and a highest V'_{max} value for ethanol [44 nmol min^{-1} (mg protein)$^{-1}$; Tassin and Vandecasteele, 1972]. Both of these enzymes are apparently different from the enzymes identified in *P. putida* 9494 and *P. aeruginosa* 10548 in that they have highest V'_{max} values for ethanol whereas the latter enzymes have much lower velocities for ethanol than hexan-1-ol under the conditions tested. However, this may be explained by the fact that the *P. putida* 9494 and *P. aeruginosa* 10548 enzymes were assayed at an ethanol concentration well below the K'_m value of the *P. aeruginosa* 196Aa enzymes (over 1 M). Both *P. aeruginosa* 196Aa enzymes are kinetically similar to the enzymes from *Acinetobacter* and from yeast in that they have very high K'_m values for ethanol, but they differ somewhat in that their highest V'_{max} values are with ethanol as substrate and their lowest K'_m values are with longer primary alcohols than the *Acinetobacter* and yeast enzymes (Tassin and Vandecasteele, 1972). Also, alcohol dehydrogenase 1 of *P. aeruginosa* can use hexadecan-1-ol as substrate (Tassin and Vandecasteele, 1972) unlike the enzymes from either strain of *Acinetobacter* (Tables 5.4 and 6.2).

The *Mycobacterium* enzyme has been purified and partially characterised also (DeBruyn *et al.*, 1981). Kinetically it is similar to the *Acinetobacter* enzyme and the

yeast enzyme in that its K'_m value for butanal (0.125 mM) is much lower than that of butan-1-ol (220 mM) (DeBruyn *et al.*, 1981). The substrate specificities of the enzyme from these organisms are similar, the *Mycobacterium* enzyme using primary alcohols and 1,x diols but not secondary alcohols (DeBruyn *et al.*, 1981) like the enzymes from *Acinetobacter* and yeast. However, the enzymes differ in terms of their structure, the *Mycobacterium* enzyme being a dimer of subunit M_r 37 500 (DeBruyn *et al.*, 1981) whereas the *Acinetobacter* enzyme is a tetramer of subunit M_r 40 000 (Sections 5.2.1 and 6.2.1) and the yeast enzyme a monomer of subunit M_r 43 500 (Section 6.3.1). Also, the *Mycobacterium* enzyme is inhibited by heating to 55 ^{o}C in the presence of EDTA, and this inhibition can be reversed by addition of zinc, suggesting that the enzyme may be zinc-dependent (DeBruyn *et al.*, 1981), although the *Acinetobacter* and yeast enzymes were not tested in this way.

Overall, these enzymes are all similar. The variations in quarternary structure may be trivial given the dimeric/tetrameric nature of the zinc-dependent alcohol dehydrogenases (Introduction 1.3.1) and the variations in quarternary structure of the "iron-activated" enzymes (Introduction 1.3.3). It is interesting that the *Mycobacterium* enzyme , like the *Acinetobacter* and yeast enzymes, is kinetically biased towards aldehyde reduction. It may be that these NADP-dependent aliphatic alcohol dehydrogenases identified in bacteria and yeasts perform similar physiological roles and/or may be structurally related.

Table 7.4. NADP-dependent aliphatic alcohol dehydrogenases identified in micro-organisms

References: (1) Table 7.3 of this thesis; (2) Tassin & Vandecasteele (1972); (3) DeBruyn *et al.* (1981). n.d. = not determined.

Organism	Medium	Specific activity [nmol min^{-1} (mg protein)$^{-1}$]	K'_m (hexan-1-ol) (mM)	Reference
Acinetobacter calcoaceticus NCIB 8250	MMD	104	1.16	(1)
Escherichia coli ML30	Glycerol/salts	11.0	0.88	(1)
Pseudomonas putida 9494	MMA	5.4	0.46	(1)
Pseudomonas aeruginosa 10548	MMA	7.7	1.11	(1)
Pseudomonas aeruginosa 196Aa	glucose	57.8 (ADH1)	0.79	(2)
		10.2 (ADH2)	1.20	(2)
Bacillus subtilis NCIMB 3610	Nutrient broth	2.8	7.50	(1)
Rhodococcus rhodocrous	Nutrient broth	6.6	2.86	(1)
Streptomyces rimosus 4018	Hobb's medium	3.6	12.1	(1)
Mycobacterium tuberculosis var. *bovis*	Sauton	43.0	n.d.	(3)
S. cerevisiae D273-10B	PYG	12.7	3.35	(1)
Rhodotorula graminis KGX 39	MMB	74.0	2.16	(1)

CHAPTER 8
CONCLUSIONS AND FUTURE WORK

8.1 Conclusions

The constitutive NADP-dependent alcohol dehydrogenase of *Acinetobacter calcoaceticus* NCIB 8250 (Section 7.3; Introduction 1.8) appears to be a member of either the zinc-dependent alcohol dehydrogenases or the "iron-activated" alcohol dehydrogenases (Introduction 1.3) on the basis of its subunit size (M_r value 40 000; Section 5.2.1) and its pro*R* stereospecificity of hydride transfer to/from NADP/H (Section 5.3.5), both characteristics of either of these groups but not of the non-metallo-enzyme alcohol dehydrogenases which have smaller subunits (M_r value 28 000; Introduction 1.3.2) and transfer the pro*S* hydride to/from NAD(P)/H. Alcohol dehydrogenases are grouped on the basis of their primary structures (Introduction 1.2) but the *N*-terminal amino acid sequence data available for the enzyme (Section 5.2.4) sheds little light on which group it belongs to, not resembling any recognised alcohol dehydrogenase directly (Section 5.5.1), although there is some confusion as to whether it resembles the alcohol dehydrogenase portion of a fermentative alcohol/aldehyde dehydrogenase in *Escherichia coli*, believed to be a member of the "iron-activated" group of alcohol dehydrogenases (Section 5.5.1). It may be that the *Acinetobacter* enzyme is not, in fact, a member of either group but is the first member to be identified of a new fourth group of alcohol dehydrogenases.

NADP-dependent alcohol dehydrogenase of *Acinetobacter calcoaceticus* NCIB 8250 has no obvious metal ion dependency for catalysis, as judged by the lack of inhibition of the enzyme by metal binding agents (Section 5.3.7). However, it is possible that any active site metal is tightly bound and further experiments may be needed to remove it and/or identify it (see Section 8.2). Its similarities with "iron-activated" alcohol dehydrogenases and with zinc-dependent alcohol dehydrogenases outlined above implies the existence of a metal ion at the active site but it is possible that no such metal exists and confirmation of this would provide further evidence in favour of the enzyme being in a new group of alcohol dehydrogenases.

The specificity of the enzyme for alcohols with primary hydroxyls, its preference

for aliphatic alcohols and its inability to use alcohols with a chain length longer than 14 carbons and some smaller unsaturated alcohols (Section 5.3.6) suggests that the substrate specificity pocket of the enzyme is hydrophobic in nature, unable to accept charged groups and binds alcohols only on one side of the hydroxylated carbon such that the hydroxyl group is oriented at one end of the pocket. These studies combined with the kinetic studies on the enzyme (Section 5.4), showing that it oxidises hexan-1-ol, heptan-1-ol and octan-1-ol optimally i.e these alcohols have the lowest K'_m values and highest specificity constants, give indications as to the size and shape of the pocket. The enzyme is also highly specific for its cofactor, NADP(H), being unable to use even NAD^+ (Sections 5.2.2 and 5.3.5). However, it should be noted that the enzyme shows a significant "preference" for catalysing aldehyde reduction over alcohol oxidation as judged by comparison of its kinetic coefficients for butanal (and NADPH) and butanol (and NADP) respectively (Section 5.4) and its apparent K_{eq} value (Section 5.3.4).

Purification and some characterisation of NADP-dependent alcohol dehydrogenase of another strain of *A. calcoaceticus*, *Acinetobacter sp.* HO1-N, showed that the enzymes are similar, both structurally and in terms of their kinetics (Section 6.4.1). The enzymes are purified in a very similar fashion suggesting structural likenesses (Section 4.5) and are both tetramers of subunit M_r value about 40 000 (Sections 5.2.1 and 6.2.1). Kinetically, the enzymes show virtually the same pattern with respect to primary alcohols. The enzyme from strain HO1-N has K'_m values about five-fold lower, V'_{max} values for the most part slightly higher and, consequently, specificity constants are two to six-fold higher than those of the enzyme from strain NCIB 8250 (Section 6.2.4). Furthermore, both enzymes oxidise primary alcohols with from two to fourteen carbons (Section 6.2.3). Such variations probably result from small differences in amino acid structure and, in as far as can be judged from only two enzymes, the enzyme from strain NCIB 8250 is likely to be a typical example of NADP-dependent alcohol dehydrogenase in *A. calcoaceticus*.

Purification and partial characterisation of an apparently constitutive NADP-dependent alcohol dehydrogenase identified in the yeast *Saccharomyces cerevisiae*

D273-10B (Sections 4.3 and 6.3) reveals some similarities and some differences between it and the NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250. The purification of the yeast enzyme is similar to that of the bacterial enzyme, differing most significantly in the dye affinity columns used in the final step of the protocol i.e. Matrex Gel Red A being used to purify the yeast enzyme, whereas the Blue A was used to purify the bacterial enzyme (Sections 4.3.4 and 3.4.5 respectively). Structurally the yeast enzyme has a slightly larger subunit M_r value (43 500; Section 6.3.1) than the bacterial enzyme (M_r value 40 200; Section 5.2.1) but their quaternary structures are very different the yeast enzyme being a monomer (Section 6.3.1), which is unusual for an alcohol dehydrogenase, whereas the bacterial enzyme is a tetramer (Section 5.2.1). However, given the variation in quaternary structures of the different zinc-dependent alcohol dehydrogenases and of the different "iron-activated" enzymes (Introduction 1.3), the variation in structures between the yeast and bacterial enzymes may not be important in considering whether the enzymes are homologous.

The substrate specificities of the two enzymes are very similar, the yeast enzyme oxidising primary alcohols (Section 6.3.6) with $NADP^+$ (not NAD^+; Section 6.3.5) as cofactor and transferring the pro*R* hydrogen to/from NADP(H) (Section 6.3.5), like the bacterial enzyme. The pH optimum for alcohol oxidation by the yeast enzyme is slightly higher than that of the bacterial enzyme [pH 10.7 *cf* pH 10.2; Sections 6.3.3 (a) and 5.3.2 (a) respectively] whereas the pH optimum for aldehyde reduction is the same for both enzymes [pH 6.8; Sections 5.3.2 (b) and 6.3.3 (b)]. In terms of its kinetic coefficients for alcohol oxidation the yeast enzyme is less efficient than the bacterial enzyme, K'_m values being two to eight-fold higher, V'_{max} values being slightly higher and consequently specificity constants being up to five-fold lower (Section 6.3.6). However, aldehyde reduction is kinetically favoured over alcohol oxidation to an even greater extent than in the bacterial enzyme, the yeast enzyme having much lower K'_m values for butanal and NADPH than for butanol and $NADP^+$ (Section 6.3.6). Further similarities between the two enzymes come in the lack of inhibition of enzyme activity by metal binding agents, although the yeast enzyme is partially inhibited by high

concentrations of EDTA and sodium azide (Section 6.3.7), unlike the bacterial enzyme. Like the bacterial enzyme it remains an open question as to the presence of a metal ion at the active site of the yeast enzyme. There are differences in the effect of thiol acting agents on the enzymes, the yeast enzyme being susceptible to a wider variety of such agents (Section 6.3.8). Overall there are enough structural and kinetic similarities between the yeast and bacterial enzymes to suggest that the enzymes may be homologous and that they may have the same physiological role (Section 6.4).

Studies on crude extracts of NADP-dependent alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 revealed that the enzyme is located only in the cytoplasm (Section 7.2). It is difficult to see a role for the bacterial enzyme in wax ester metabolism, as had been previously suggested (Fixter and Nagi, 1984) in the light of its inability to metabolise alcohols with > 14 carbons (Sections 5.3.6 and 6.2.3). Further evidence against this, particularly against any involvement in wax degradation, comes from: (a) the lack of correlation of wax concentration with enzyme activity for cells grown in continuous culture under low carbon concentrations (Section 7.4), i.e conditions that promote wax breakdown; (b) the poor correlation of wax concentration with enzyme activity for cells grown in continuous culture under low nitrogen concentration (Section 7.4), i.e. conditions that promote wax synthesis; (c) the lack of variation of enzyme activity in mutants of *Acinetobacter sp.* HO1-N that breakdown wax esters more slowly compared to the wild type (Section 7.5); and (d) the presence of apparently similar enzymes in micro-organisms that are not known to produce wax esters e.g. *Bacillus subtilis* (Section 7.6).

The only variations in enzyme activity observed are: (a) that enzyme activity decreases, to varying degrees depending on growth condition, with increasing growth rate in continuous culture (Section 7.4); (b) enzyme activity increases with increasing growth temperature in continuous culture (Section 7.4); and (c) that enzyme activity is always higher in cells grown either in batch culture (Section 7.3) or in continuous culture under nitrogen limitation when grown at the same growth rate (Section 7.4). The enzyme is not induced by growth of cells on alcohols (Section 7.3) and the enzyme activity does

not vary > 2-3 fold whether grown in batch culture (Section 7.3) or in continuous culture (Section 7.4). Therefore it appears that the enzyme is constitutive. The variations in enzyme activity observed may result from regulation of the enzyme itself e.g. covalent modification, or from control of enzyme synthesis, possibly by some catabolite repression-type mechanism (Section 7.7.4).

The fact that NADP-dependent alcohol dehydrogenases of both *A. calcoaceticus* NCIB 8250 and *S. cerevisiae* D273-10B are strongly kinetically biased towards the aldehyde reduction reaction suggests that it may be this reaction that is important *in vivo* although kinetic favourability of reversible reactions cannot be taken as sole judge of an enzyme's physiological direction of catalysis.

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 such enzymes may be ubiquitous in the microbial world, carrying out some common function as yet undefined (Sections 7.5 and 7.7.5).

8.2 Future work

In this section a number of experiments are outlined that would allow extension of this work to enable further confirmation of the existence a new group of structurally and/or functionally similar enzymes and to identify their physiological role(s).

It should be possible to clone and sequence the gene for NADP-dependent alcohol dehydrogenase of *Acinetobacter calcoaceticus* NCIB 8250 using a probe based on the *N*-terminal amino acid sequence of the enzyme (Section 5.5.1). Complete sequence data should enable determination of the group of alcohol dehydrogenases to which the enzyme belongs and would also allow identification of possible metal ligands. Site directed mutagenesis experiments, combined with further structural and mechanistic studies should enable determination of the enzyme mechanism. Overexpression of the protein would be helpful, enabling protein chemical experiments, such as affinity labelling, which are expensive of protein, to be carried out. Furthermore, it should be possible to generate mutants completely lacking in enzyme activity or reduced in activity, either by site directed mutagenesis, insertion of DNA into the alcohol dehydrogenase gene or deletion of part or all of the gene. "Marker rescue" type techniques (Smithies *et al.*, 1985) could be used to insert the altered gene into the chromosome and such mutants could be used in investigations into the physiological role of the enzyme e.g. by examining the effects of a reduction or complete loss of enzyme activity on the organism when grown under various different conditions. Such a method of generating mutants would be easier than random mutagenesis because of the problems in selecting for mutants of an enzyme whose physiological substrate and function is not known. Expression studies, and studies on the promotor of the gene would enable investigations into a possible global regulatory mechanism of the enzyme by catabolite repression.

In order to compare the structures of the bacterial and yeast enzymes, cloning and sequencing of the yeast enzyme is desirable. However, in the absence of *N*-terminal amino acid sequence for the yeast enzyme (Section 6.3.2) it would be necessary to generate some internal sequence in order to identify possible probes and attempts to do so

are in progress (J.N. Keen, personal communication). The internal sequence itself could be used to deduce the group of alcohol dehydrogenases to which the enzyme belongs and, if the sequence of the *Acinetobacter* enzyme was known, would allow comparison with it. Cross-linking experiments (Coggins, 1978) on the yeast enzyme would also be desirable in order to confirm its apparent monomeric structure (Section 6.3.1). Further substrate specificity experiments on the yeast enzyme, to the same degree as those on the bacterial enzyme (Section 5.3.6), would also be helpful in comparison of the enzymes.

The pro*R* stereospecificity of hydrogen transfer to/from NADP(H) of both enzymes may imply the existence of a metal ion at the active site of the enzymes (Section 5.5.2). Although complete sequence data for the NADP-dependent alcohol dehydrogenases of *A. calcoaceticus* NCIB 8250 and *S. cerevisiae* D273-10B would enable identification of metal ligands, further studies on the enzymes would be necessary to identify what metal, if any, is involved. Heating the enzyme to 55 ^{o}C when incubated in the presence of a metal chelating agent, then, after removal of the chelating agent, attempting to regain any enzyme activity inhibited by incubating the enzyme with various metal ions may give some indication of its metal dependency (DeBruyn *et al.*, 1981). However, purification of the enzyme in the absence of trace metals then metal analysis of the enzyme by atomic absorption chromatography is necessary to give an unambiguous answer as to what active site metal(s) is(are) present in the enzyme.

Purification and characterisation of one or more of the other enzyme activities identified in different micro-organisms would enable comparisons with the *Acinetobacter* and *Saccharomyces* enzymes and would provide more evidence as to whether a group of functionally and/or structurally NADP-dependent aliphatic alcohol dehydrogenases do exist. The apparent lack of such an activity in the archaebacterium *Haloferax mediterranei* requires further investigation i.e. does such an activity exist in cells assayed in the presence of high salt concentrations i.e resembling those intracellularly. Also, it would be interesting to determine whether such activities exist in plant and animal tissues and, if so, to purify and characterise such an activity and compare it with the yeast and bacterial enzymes.

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APPENDIX

Appendix: Stereospecificity of hydrogen transfer to/from NAD(H) catalysed by benzyl alcohol dehydrogenase of *Acinetobacter calcoaceticus* NCIB 8250

This work was carried out in collaboration with Dr. D.P. Baker, Mikrobiologisches Institut, ETH, Zurich, Switzerland.

Introduction

Benzyl alcohol dehydrogenase from *Acinetobacter calcoaceticus* NCIB 8250 is thought to be a zinc-dependent alcohol dehydrogenase on the basis of its *N*-terminal amino acid sequence (Chalmers *et al.*, 1991) even though it is not inhibited by metal-chelating agents (MacKintosh and Fewson, 1988). Determining the stereospecificity of hydrogen transfer to/from NAD(H) of this enzyme was of interest for two main reasons. Firstly, other zinc-dependent alcohol dehydrogenases transfer the pro*R* hydrogen to/from NAD(H) (Benner *et al.*, 1985). Secondly, the TOL-plasmid encoded benzyl alcohol dehydrogenase of *Pseudomonas putida*, which has also been identified as a zinc-dependent alcohol dehydrogenase (Chalmers *et al.*, 1991) transfers the pro*R* hydrogen to/from NAD(H) (Shaw and Harayama, 1990) and this enzyme is homologous with the *Acinetobacter* enzyme in terms of its *N*-terminal amino acid sequence (Chalmers *et al.*, 1991). Therefore it was important to determine the stereospecificity of the *Acinetobacter* enzyme to see if it did, in fact, transfer the pro*R* hydrogen to/from the cofactor, which would provide further indirect evidence for the existence of a metal ion at the active site of the enzyme and for the notion that the chromosomally-encoded *Acinetobacter* enzyme and the plasmid-encoded *Pseudomonas* enzyme are homologous.

Materials

As described in Materials 2.1.

Methods

Source of benzyl alcohol dehydrogenase

The enzyme was purified from *A. calcoaceticus* NCIB 8250 using the procedure of MacKintosh and Fewson (1988) by Mr A.J. Scott, Department of Biochemistry, University of Glasgow.

Synthesis of tritiated pro*S* NADH

As described in Methods 2.12.1 except 60.5 μM-NAD^+ was included in the assay instead of 60.5 μM-$NADP^+$.

Synthesis of tritiated pro*R* NADH

This method is based on the fact that yeast alcohol dehydrogenase 1 specifically removes the pro*R* hydrogen from NADH (Benner *et al.*, 1985). The filtered mixture containing the pro*S* tritiated NADH was incubated with 23.6 mM-acetaldehyde and 23.5 units yeast alcohol dehydrogenase 1. The rate of NADH oxidation was followed by measuring the change in absorbance at 340 nm and the protein removed as in Methods 2.12.1. Then the tritiated NAD^+ was purified on h.p.l.c. using a Machery & Nagel (Duren, Germany) Nucleoseal 7-C18 reverse-phase h.p.l.c. column (25 cm x 4.6 cm) with pre-column (4 cm x 2.6 cm) run isocratically with 2.4 % (v/v) methanol/ 0.1 % (v/v) TFA to elute NAD^+. Fractions containing NAD^+ were collected and lyophilised.

Tritiated pro*R* NADH was synthesised from tritiated pro*R* NAD^+ as described in Methods 2.12.2 except that 15.8 μM-[^{3}H]NAD was added instead of 8.8 μM-[^{3}H]NADP.

Incubation of tritiated NADH with benzyl alcohol dehydrogenase

To a reaction mixture containing 10 mM-potassium phosphate buffer, pH 7.0, 125 μM -benzyl alcohol and 100 μM-NADH (assay concentration) was added either pro*R*-[^{3}H]NADH (assay concentration 0.5 μM) or pro*S*-[^{3}H]NADH (assay

concentration 0.15 μM). The reaction was initiated by the addition of benzyl alcohol dehydrogenase (3 μg) and followed by measuring the the change in A_{340}. When the reaction had reached equilibrium, protein was removed as described in Methods 2.12.1. NAD^+ and benzyl alcohol were separated by h.p.l.c. Samples (< 500 μl) were injected onto a Machery & Nagel Nucleoseal 7-C18 reverse-phase h.p.l.c. column (25 cm x 4.6 cm) with pre-column (4 cm x 2.6 cm) pre-equilibrated with 0.1 % (v/v) TFA. NAD^+ and benzyl alcohol were eluted isocratically, the former in 2.4 % (v/v) methanol/ 0.1 % (v/v) TFA and the latter in 80 % (v/v) methanol/ 0.1 % (v/v) TFA, solutions being pumped through the column at 1 ml min^{-1} and fractions collected at 1 minute intervals and counted for the incorporation of ^3H as described in Methods 2.12.4. The absorbance of the effluent was monitored between 190 nm and 370 nm, NAD^+ and benzyl alcohol being identified by their distinctive absorbance patterns.

Results

The stereospecificity of hydride transfer was determined by assaying the enzyme in the presence of pro*S*-[^{3}H]NADH or pro*R*-[^{3}H]NADH and benzaldehyde (Methods 2.12). The criteria used to determine the stereospecificity were as follows:
If incubating the enzyme with pro*S*-[^{3}H]NADH and benzaldehyde resulted in most of the radioactivity being identified in benzyl alcohol the enzyme had transferred the ^3H labelled pro*S* hydride. However , if the radioactivity was determined in NAD the enzyme had transferred the "cold" pro*R* hydride. Similarly, if incubating the enzyme with pro*R*-[^{3}H]NADH and benzaldehyde resulted in most of the radioactivity being identified in benzyl alcohol the enzyme had transferred the ^3H labelled pro*R* hydride. However, if the radioactivity was identified in NAD then the enzyme had transferred the "cold" pro*S* hydride.

When the enzyme was incubated with benzaldehyde and pro*S* [^{3}H]-NADH the ratio of counts incorporated into the resultant NAD^+ and benzyl alcohol was 98.2 % : 1.8 % respectively, i.e. the enzyme was stereospecific for the pro*R* hydride of NADH.

This was confirmed by incubation of the enzyme with benzaldehyde and pro*R*-[^{3}H]NADH, the ratio of counts incorporated into the resultant NAD^+ and benzyl alcohol being 4.8 % : 95.2 % respectively.

Conclusions

Benzyl alcohol dehydrogenase of *A. calcoaceticus* NCIB 8250 transfers the pro*R* hydrogen to/from NAD(H). This result is what would be expected given that the enzyme has been identified as a member of the group of zinc-dependent alcohol dehydrogenases on the basis of its *N*-terminal amino acid sequence (Chalmers *et al.*, 1991) and these enzymes transfer the pro*R* hydrogen (Schneider-Bernlohr *et al.*, 1988; Glasfield and Benner, 1989). This result is the same as that obtained for the TOL-plasmid encoded benzyl alcohol dehydrogenase of *P. putida* (Shaw and Harayama, 1990) and provides further evidence as to the similarity of the chromosomally encoded *Acinetobacter* enzyme and the plasmid encoded *Pseudomonas* enzyme.

No metal ion dependency has been shown for the *Acinetobacter* enzyme, the absence of enzyme inhibition when incubated with chelating agents implies that the enzyme lacks any metal ion dependency (MacKintosh and Fewson, 1988). However, it has been shown recently that heating the enzyme to 55 ^{o}C in the presence of EDTA does inhibit the enzyme (M.F. Reid and C.A. Fewson, unpublished result) suggesting that a metal ion is necessary for activity, which fits in with both the sequence homology to zinc-dependent alcohol dehydrogenases and the pro*R* stereospecificity of hydrogen transfer to/from NAD(H) of the enzyme.

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