MOLECULAR CHARACTERISATION OF MICROBIAL NAD(P)-DEPENDENT ALCOHOL DEHYDROGENASES

Matthew Francis Reid

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Department of Biochemistry
University of Glasgow
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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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Abbreviations

Abbreviations used are those recommended by The Biochemical Journals Instructions to Authors [Biochem. J. (1993) **289**: 1-15] with the following additions.

ADH	Alcohol dehydrogenase
DTT	Dithiothreitol
OD	Optical density
PCR	Polymerase chain reaction
RNase A	Ribonuclease A
r.p.m.	revolutions per minute
SDS	Sodium lauryl sulphate
TEMED	*N, N, N′,N′*-Tetramethylenediamine
Tris	Tris (hydroxymethyl) aminomethane

Summary

1. Alcohol oxidoreductases have been isolated from various sources and classified into five distinct evolutionary categories: Groups I, II and III NAD(P)-dependent alcohol dehydrogenases, NAD(P)-independent alcohol dehydrogenases and alcohol oxidases. Examples of all of these groups are found in microorganisms. The first part of this thesis consists principally of a critical review of information covering the primary, secondary, tertiary and quaternary structures of the microbial NAD(P)-dependent alcohol dehydrogenases and their catalytic mechanisms, in an attempt to clarify the evolutionary relationships amongst them. The second part of the thesis is a description of research into the molecular characterisation of benzyl alcohol dehydrogenase from *Acinetobacter calcoaceticus* NCIB 8250.

2. (a) Primary structure analysis and three-dimensional modelling of the group I alcohol dehydrogenases has shown that the coenzyme specificity is determined by a single residue, Aspartate-223. There does not appear to be a relationship between primary structure, presence of a second structural zinc atom and quaternary structure of the group I alcohol dehydrogenases. There are 14 strictly conserved residues amongst the microbial group I alcohol dehydrogenases, compared to nine amongst the whole of the group I alcohol dehydrogenases. Identity of primary amino acid structure amongst pairs of microbial group I alcohol dehydrogenases ranges from 24% to 94%. In some instances higher sequence identity is seen between prokaryote and eukaryote members than between members of the same cell class.

All microbial group I alcohol dehydrogenases are tetrameric except for the Tol-plasmid (pWW0) encoded benzyl alcohol dehydrogenase from *Pseudomonas putida* and the trimeric nicotinoprotein, 4-nitroso-*N,N* -dimethylaniline-dependent alcohol/aldehyde oxidoreductase from *Amycolatopsis methanolica* . The benzyl alcohol dehydrogenase from *Pseudomonas putida* is dimeric, like plant and mammalian group I alcohol dehydrogenases: the benzyl alcohol dehydrogenase from *Acinetobacter colcoaceticus* NCIB 8250 is tetrameric. Whether the trimeric nitroso-*N,N* -dimethylaniline-dependent aldehyde/alcohol oxidoreductase from *Amycolatopsis methanolica* is a member of a group

I alcohol dehydrogenase sub-group or possibly the first member of a novel group of alcohol dehydrogenase will not be known until its full amino acid sequence is determined.

(b) The three dimensional structure of 3α,20β-hydroxysteroid dehydrogenase (a group II alcohol dehydrogenases) from *Streptomyces hydrogenans* has been solved. The catalytic mechanism is less well understood than is that of the group I alcohol dehydrogenases, although there is no evidence to suggest that coenzyme preference is determined by a single residue as is seen in the group I alcohol dehydrogenases.

Overall, the degree of amino acid identity amongst the group II alcohol dehydrogenases is lower than that seen amongst the group I alcohol dehydrogenases, and ranges from 19% to 59%.

(c) The characteristics of the group III alcohol dehydrogenases are far less conserved than those of either group I or II enzymes. So far, all group III alcohol dehydrogenases have been isolated from micro-organisms, there is no three dimensional structure is available and very little is known about their catalytic mechanism. The degree of identity amongst the group III alcohol dehydrogenases ranges from 18% to 53%. With some members there is a requirement for various metal ions for catalytic activity and in one case an activator protein has been suggested to be involved. Subunit sizes are conserved amongst this group at approximately 40 kDa, but native structure varies considerably from dimers to tetramers and decamers.

Like the group I alcohol dehydrogenases, there are nicotinoproteins which on the basis of partial amino acid sequence analysis have been proposed to be members of the group III alcohol dehydrogenases. Whether these nicotinoproteins form a novel type of alcohol dehydrogenase or constitute a sub-group of the group III alcohol dehydrogenases will only be determined once their full primary sequences are solved.

(d) NAD(P)-independent alcohol dehydrogenases are divided into two functionally distinct groups: the methanol dehydrogenases and the ethanol dehydrogenases. Their exact catalytic mechanism has not yet been defined but it is believed to involve the passing of reducing equivalents to pyrroloquinoline and cytochrome *c*.

(e) Alcohol oxidases differ from alcohol dehydrogenases in that they irreversibly oxidise alcohols to aldehydes and ketones. They have been characterised as flavoproteins

and they are located in the peroxisomes of methylotrophic yeasts and filamentous fungi.

3 (a) Although they differ in quaternary structure, benzyl alcohol dehydrogenases from *Pseudomonas putida* and *Acinetobacter calcoaceticus* are seen to be closely related on the basis of 27% *N*-terminal sequence identity, common substrate specificity, immunolgical cross reactions and subunit size. Determination of the primary structure of benzyl alcohol dehydrogenases from *A. calcoaceticus* would provide an excellent opportunity to examine what its relationship to the *P. putida* enzyme is, why the quaternary structure of these two benzyl alcohol dehydrogenases differ, as well as to examine its relationships with the other alcohol dehydrogenases.

(b) A probe for the *A. calcoaceticus* benzyl alcohol dehydrogenase gene was constructed using polymerase chain reaction. The 109-base sequence that was obtained codes for an amino acid sequence corresponding to the *N*-terminus of benzyl alcohol dehydrogenase and allowed the identification of two previously unidentified residues as Valines.

Preliminary results of probing experiments suggest that the benzyl alcohol dehydrogenase gene is located close to the gene coding for benzaldehyde dehydrogenase II (the next enzyme in the benzyl alcohol dehydrogenase pathway) in the *A. calcoaceticus* genome and therefore may be under the same expression control system.

CONTENTS

Chapter 1: Microbial alcohol oxidoreductases

FIGURES

Chapter 1

Chapter 3

TABLES

Chapter 1

CHAPTER 1

MICROBIAL ALCOHOL OXIDOREDUCTASES

1.1 Introduction

Interconversions of alcohols, aldehydes and ketones are essential processes in both prokaryotes and eukaryotes. The oxidoreductases catalysing these reactions involving the removal and addition of hydrogen atom equivalents use a series of different electron acceptors, and can be divided into three groups: (i) the NAD(P)-dependent alcohol dehydrogenases (ADHs) which are the best characterised of the three groups, (ii) the NAD(P)-independent alcohol dehydrogenases which use quinoproteins and in some cases a haem group in association with the quinoprotein, and (iii) FAD-dependent alcohol oxidases which catalyse an irreversible oxidation of alcohols.

1.1.1. Physiological role of alcohol oxidoreductases

The physiological direction of conversion from alcohol to aldehyde and ketone or from ketone and aldehyde to alcohol often depends on the ratio of reduced:oxidised cofactor. Reductive reactions result in the production of alcohol and oxidised cofactor (e.g. NAD^+). This occurs, for instance, in the formation of ethanol during fermentation where the regeneration of NAD^+ is essential for other oxidative metabolic processes to continue. Oxidation of alcohols results in the production of aldehydes or ketones and reduced cofactor (e.g. NADH) and may be involved in feeding alcohols into central metabolism as carbon and energy sources.

The best characterised role of the oxidoreductases is the fermentative role of alcohol dehydrogenases generating alcohol from aldehydes and ketones, with a concurrent regeneration of $NAD(P)^+$. Regeneration of $NAD(P)^+$ is essential for production of ATP and further product generation.

Degradative roles are common among the alcohol oxidoreductases, allowing utilisation of alcohols as carbon sources. This is seen, for instance, in various methylotrophic bacteria where methanol can be used as sole carbon and energy source. Degradation of aromatic alcohols by benzyl alcohol dehydrogenase occurs in species of *Pseudomonas* and in *Acinetobacter calcoaceticus* (Section 1.8). An indirect degradative

role of an alcohol oxidoreductase is carried out by veratryl alcohol oxidase which generates formaldehyde with the concurrent production of hydrogen peroxide, the latter being used by lignin peroxidase in the breakdown of lignin (Section 1.6.2).

1.1.2. The variety of alcohol dehydrogenases

Many different NAD(P)-dependent ADHs, from a wide variety of animals, plants and microorganisms, show certain structural and functional similarities (Jörnvall *et al.*, 1987b). These enzymes catalyse the reaction:

$$\text{ALCOHOL} + \text{NAD(P)}^+ \rightleftharpoons \text{ALDEHYDE} + \text{NAD(P)H} + \text{H}^+$$

The alcohol dehydrogenase superfamily can be divided into three groups (Jörnvall *et al.*, 1987b): zinc-dependent long chain, or group I, ADHs (containing approximately 350 residues per subunit), short chain zinc independent, or group II, ADHs (containing approximately 250 residues per subunit) and the iron activated, or group III, ADHs of subunit size about 380 residues. There is a fourth group of alcohol dehydrogenases which are NAD(P)-independent and pyrroloquinoline quinone (PQQ) linked; most of these are methanol dehydrogenases from various methylotrophic bacteria.

Of the three NAD(P)-dependent groups, the group I ADHs are the most studied and the best characterised (Brändén *et al.*, 1975; Jörnvall *et al.*, 1987a,b; Yokoyama *et al.*, 1990; Sun & Plapp, 1992). Many different group I ADHs have been characterised and they exist in dimeric and tetrameric forms, represented by horse liver alcohol dehydrogenase (HLADH) and *Saccharomyces cerevisiae* yeast alcohol dehydrogenase I (SADHI), respectively. The only tertiary structure to be defined in this group is that of HLADH (Eklund *et al.*, 1974; 1976; 1981). This tertiary structure has since been used for the three dimensional modelling of other group I ADHs (Brändén *et al.*, 1975;Jörnvall *et al.*, 1978). There have been extensive mechanistic studies of alcohol oxidation by modelling the SADHI structure on the tertiary structure of HLADH (Ganzhorn *at al.*, 1987; Jörnvall *et al.*, 1978; Plapp *et al.*, 1990).

Other enzymes have been proposed to be related to the group I ADHs because of their sequence identity and structural/functional similarities. These include *Escherichia coli* threonine dehydrogenase (Aronson *et al.*, 1989), guinea pig lens ζ-crystallin (Borrás *et al.*, 1989), and liver sorbitol dehydrogenase (Jörnvall *et al.*, 1981; Eklund *et al.*, 1985; Jeffery & Jörnvall, 1988; Karlsson *et al.*, 1991).

Group II ADHs, as represented by *Drosophila spp.* alcohol dehydrogenase (Villarroya *et al.*, 1989), have been characterised from both prokaryotes and eukaryotes. Polyol/sugar dehydrogenases have have been suggested to be related to this group on the basis of their primary stucture (Jörnvall *et al.*, 1981).

The group III ADHs are the most recently discovered, represented by the second isoenzyme from *Zymomonas mobilis* (ZMADHII: Conway *et al.*, 1987) and thus far numbers only seven members, all of them microbial; six bacterial and one yeast enzyme.

Comparisons of sequence alignments, as well as structural and functional comparisons help to establish evolutionary relationships that exist amongst enzymes. Using this analysis, one can attempt to determine how the alcohol dehydrogenases have evolved into what appear to be four distinct groups but tackle essentially the same function of alcohol oxidation/reduction.

1.1.3. Evolution of enzyme function

How can the great diversity of microbial catabolic metabolism be accounted for? Early life forms must have been restricted to a limited amount of genetic information encoding a small number of proteins. Bacterial genomes have increased in size and diversity during evolution by several different processes. The increase in size is probably due to duplication and/or fusion of smaller ancestral genomes (Herdman, 1985). The increase in the amount of genetic information appears to have occurred independently in different groups of bacteria which had already diverged phylogenically; however, similarities are still seen in operon organisation and gene sequences between different phylogenic groups. It has been proposed that the

complexity of bacterial genomes was maintained only after the appearance of an aerobic atmosphere, possibly due to the more efficient aerobic metabolism which micoorganisms evolved (Herdman, 1985). Although there is general agreement on the basic processes of genome evolution (replication, translocation, fusion), there are two conflicting hypotheses which attempt to account for the ancestral relationships of existing proteins.

Retrograde evolution (Horowitz, 1965) suggests that enzyme evolution occurred by a stepwise sequential recruitment of enzymes from a single parent enzyme, allowing utilisation of new substrates, in a reverse order through a catabolic pathway. Such retrograde evolution could be achieved by gene duplication and mutation, the mutation accounting for the new-found substrate specificity. The possessor of such a new gene would have a selective advantage, being able to utilise an extra energy source. However, this hypothesis is subject to well-founded criticism. One would expect to find some sequence homology between successive enzymes of the same pathway, but this is rarely observed. The retrograde hypothesis also assumes that each intermediate of an evolving pathway is freely available within the environment, but often this is not the case because some compounds are subject to extreme chemical lability and are stable only when actually bound to an enzyme. Despite these major arguments against retrograde evolution, the hypothesis does attempt to provide some explanation of operonic clustering. Enzymes within an operon are suggested to form a reaction chain, the product of one enzyme being the substrate of the next. Genes within an operon, such as the histidine synthesis operon from *Escherichia coli*, (Ames & Hartman, 1963) or the *trp* operon (Somerville & Yanofsky, 1964) often govern a single biosynthetic process. No enzyme involved in histidine synthesis is found outside the operon and no gene unconcerned with histidine synthesis is in it. The implication of overlapping specificity of enzymes is that there is structural homology, but in well characterised operons such as the *lac* operon no homology has been found between the *lacz,y* and *a* genes (Jacob & Monod, 1961). However, the function of the *lac a* gene product has not yet been defined, indicating there is still a great to learn about operon structure. It has therefore been proposed that operonic genes could have originated at their present sites via

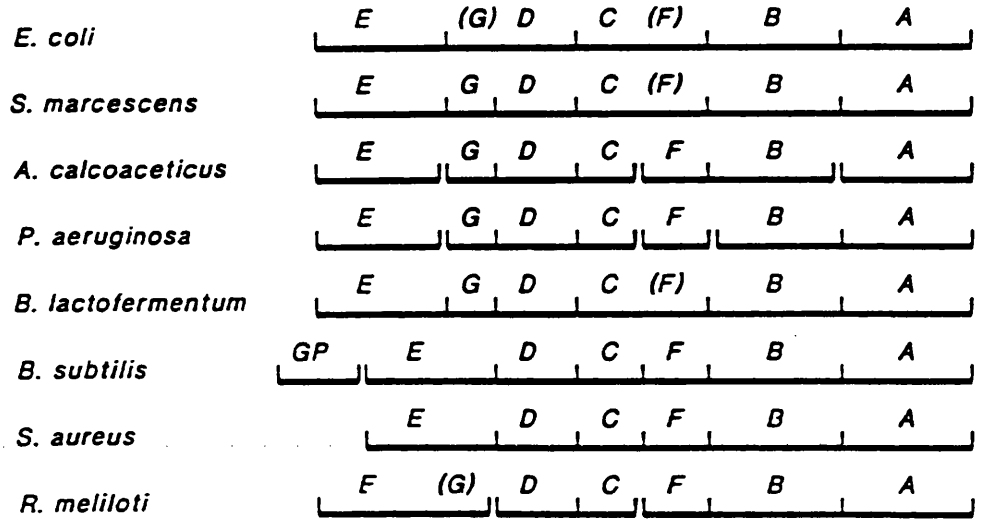
tandem duplication and mutation, thus accounting for related genes being found in the same operonic structures.

The hypothesis of metabolic evolution by gene recruitment (Jensen, 1976) proposes that copies of pre-existing genes are recruited, via gene duplication, mutation and translocation, then integrated into novel pathways performing reactions analogous to that of the original parent enzyme. Pristine life is proposed to have had a limited number of enzymes with broad specificities, allowing metabolism of a wide range of substrates. Gene amplification and mutation would allow increased specialisation of substrate utilisation. This hypothesis is consistent with expectations of early protein evolution, that changes occur to produce a more restrictive state from a less restrictive state.This can account for the homology of enzymes which carry out analogous roles in completely unrelated pathways. Such similarities can be seen for example in a series of reactions in the Krebs cycle (where oxaloacetate is converted to α-ketoglutarate) and in lysine biosynthesis (where α-ketoglutarate is converted to α-ketoadipate); some of the substrates as well the types of reaction, i.e. condensation, oxidation are the same (Jensen, 1976). This hypothesis suggests that not only an individual enzymes can be recruited, but whole operons which perform an analogous series of reactions can also be recruited. Unlike the retrograde hypothesis, gene recruitment provides no explanation for how gene structuring in operons might have occurred and provides no explanation of the adjacent locations of genes coding for enzymes involved in subsequent steps of a pathway. For operon organisation to occur by gene translocation and reshuffling of previously scattered genes might be considered unlikely because both of these processes being extremely rare. However, it is energetically favourable for genes to locate in operonic structures to enable coordinate expression of enzymes within a pathway. If selective pressures are great enough over a long period of time then gene translocation and shuffling could be responsible for arrangement of genes into operons. This hypothesis is partly supported by the observations of different operon structuring, such as in the *trp* operon. from different organisms. The *trp* operon codes for up to seven homologous enzymes required for tryptophan biosynthesis. The gene order within the operon is very highly conserved amongst bacteria, but there are exceptions (Figure 1.1)

Figure 1.1.

The arrangements of the *trp* operon

The genetic organisation of *trp* genes in different prokaryotes. A solid unbroken line indicates a unit of transcription. Gene fusions are indicated by brackets. (Taken from Haspel *et al.*, 1991).



perhaps indicating that the structure of the operon came about as a result of separate gene shuffling within individual organisms to produce a common convergently evolved operon. However, this hypothesis can be criticized in that the arrangement of genes within an established operon could change because of accepted genetic evolutionary events such as duplication and translocation rather than as a result of convergent evolution.

Another way in which genetic material can be acquired is by horizontal gene transfer, which is by no means exclusive of gene recruitment. Conjugative plasmids are used by molecular biologists to transfer genetic material from bacteria to yeast as a standard laboratory technique. This technique is merely the use of a naturally occurring phenomenon which is proposed to play an important role in the evolution of genomes (Smith *et al.*, 1992). The question of horizontal gene transfer is one of the most debated questions in the molecular evolution field because examples of it having occurred are so hard to establish. Indications that gene transfer has occurred are the observations of anomalies within the evolutionary trees, for instance some bacterial proteins appearing to be more closely related to eukaryotic proteins than to other bacterial proteins (Smith *et al.*, 1992). Although there is some strong evidence for horizontal gene transfer occurring with certain enzymes, such as glyceraldehyde-3-phosphate dehydrogenase between *E. coli* and a eukaryotic donor, it is thought to be a rare event, or rarely observed, but it may have an important role to play in the acquisition of genetic diversity (Smith *et al.*, 1992).

If selective pressures result in gene recruitment and organisation it should follow that when these selective pressures are removed the loss of acquired genes should follow. This has been examined by Ornston (1991) who proposed that maintenance of the β-ketoadipate pathway in *Acinetobacter calcoaceticus* is not completely reliant on external selective pressures but is partly due to genetic repair mechanisms of exchange between homologous genes. This repair exchange mechanism is seen with the homologous group of genes *catIJF* and *pcaIJF* coding for enzymes within the β-ketoadipate pathway.

By comparisons of the primary structure of proteins and their genetic arrangement between species and within single organisms a picture of putative evolutionary events can be drawn.

1.2. The group I alcohol dehydrogenase

HLADH is the archetypal group I, or long chain zinc-dependent, alcohol dehydrogenase for which there is a substantial amount of information about the primary (Jörnvall, 1970) and tertiary structure of the apoenzyme (Eklund *et al.*, 1974;1976) and the holoenzyme (Eklund *et al.*, 1981). Because of the large amount of information available on the enzyme, it is used as a paradigm for other members of the group I ADHs.

1.2.1. The three dimensional structure of horse liver alcohol dehydrogenase in relation to catalytic activity

HLADH is a dimer of subunit size 40 kDa, each subunit containing 374 residues arranged in a catalytic domain and a coenzyme binding domain divided by a long cleft. Each catalytic domain binds two zinc atoms, one being structural and the other catalytic, bound at the active site.

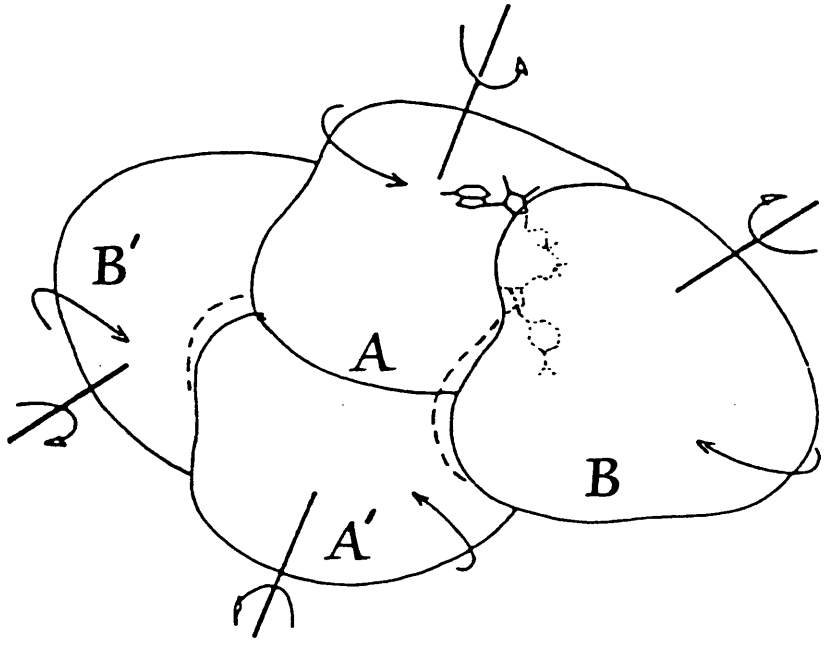
1.2.1.1. Coenzyme binding

The binding of NAD^+ has been described in detail by Eklund (1988). The coenzyme binds in the cleft between the two domains and across the edge of the coenzyme binding domain. The binding of NAD^+ to form the holoenzyme transforms the enzyme from the inactive into the active form by inducing a conformational change which has been well documented (Eklund & Brändén, 1979; Eklund *et al.*, 1981; 1982; Colonna-Cesari *et al.*, 1986; Plapp *et al.*, 1987). This movement consists of the catalytic domains moving towards each other, with respect to the central core of the dimer, causing a narrowing of the active site cleft (Figure 1.2). The loop containing residues 292-298 in the coenzyme domain undergoes a rearrangement to accommodate residues 46-60. Some atoms move as much as 9Å between the two conformations. There is a small movement of the coenzyme binding domain of about 1.5Å, altering the position of

Figure 1.2.

Schematic drawing showing the conformational change of horse liver alcohol dehydrogenase induced by coenzyme binding

A, refers to the catalytic domain; B, refers to the coenzyme binding domain.(Taken from Eklund, 1988).



residues thought to interact directly with the coenzyme, such as Asp-223. The two major effects of this change are to cause a tightening of binding interactions with the coenzyme and a narrowing of the active site substrate binding pocket, creating an anhydrous environment, which is thought to be essential in catalysis (Eklund & Brändén, 1987).

Various residues have been shown to be important in coenzyme binding (Eklund, 1988). Asp-223 forms two hydrogen bonds with the adenosine ribose moiety of NAD^+ (Figure 1.10). Lys-228 hydrogen bonds to the 3'-oxygen atom of the ribose, Arg-47 and Arg-369 form hydrogen bonds to the phosphate oxygen atoms, which are further hydrogen bonded to the amino groups at the end of two helices, one from each domain. Ser-48 and His-51 are hydrogen bonded to the nicotinamide ribose facilitated by the conformational change which increases the number of interactions between enzyme and coenzyme, as well as bringing Ser-48 and His-51 into positions to enable proton translocation (Section 1.2.1.3).

The Gly-Xaa-Gly-Xaa-Xaa-Gly fingerprint pattern of the coenzyme binding domain has been found to be conserved in HLADH as well as other NAD(H)-binding ADHs (Wierenga *et al.*, 1985).

1.2.1.2. Substrate specificity

The substrate binding site is a 20 Å deep cleft which is not seen as a complete pocket in the apoenzyme since one end the pocket is formed by the coenzyme binding. The inner part of the substrate cleft is formed by the catalytic zinc at the bottom, its ligands and the nicotinamide ring of the coenzyme. The main part of the substrate cleft is completely hydrophobic because of the side-chains of Leu-57, -116, -141 and -309, Ser-48, -117 and -310, Phe-93, Pro-296, Met-306, Leu-309 and Ile-318 forming the lining (Figure 1.4; Eklund *et al.*, 1974).

HLADH can oxidise a wide range of primary alcohols in addition to ethanol, but with an increase in alcohol size comes a decrease in activity. It will also oxidise some secondary alcohols (but not propan-2-ol), some aromatic alcohols, primary and

secondary cyclic alcohols, vitamin A and ethanediol (Sund & Theorell, 1963). This is probably due to the size of the substrate cleft being much larger than is needed for a small substrate such as ethanol or benzyl alcohol (Eklund, 1988).

1.2.1.3. Catalytic mechanism

The catalytic mechanism has been extensively studied by various groups (Brändén *et al.*, 1975; Fersht, 1985; Pettersson, 1987). Oxidation of alcohol results in reduction of coenzyme and concurrent release of a proton into solution from the alcohol substrate. The mechanism for ethanol oxidation is ordered, with the NAD^+ binding first. Following oxidation of ethanol to acetaldehyde the NADH is released last (Dalziel, 1975). The release of NADH from the NADH-complex is the rate determining step, although Plapp *et al.* (1987) suggests that the slow isomerization of the ternary complex is partially rate limiting in the turnover with NAD^+ and ethanol. The release of NADH being the rate limiting step is supported by the the observation that free energy of activation for the release of the NADH (17 kcal/mol) is 5 kcal/mol higher than that for the turnover of the ternary complex in ethanol oxidation (Pocker & Page, 1990). The reverse reaction, for acetaldehyde reduction, is random as far as NADH and acetaldehyde binding is concerned, but release of products is ordered with desorption of coenzyme being the last step (Pettersson, 1987).

The pH dependency of NAD^+ binding was identified in early studies where the affinity for NAD^+ was found increase markedly when the pH was raised from 6 to 10, whereas the affinity for the reduced coenzyme remains the same up to pH 9 and then decreases at higher pH values (Brändén *et al.*, 1975). The binding of NADH is reliant upon the ionisation state of a group with a pK_a value of 9.2 in the apoenzyme and the same group has been proposed in the association of NAD^+ with the free enzyme (De Traglia *et al.*, 1977; Andersson *et al.*, 1981). The binding of the NAD^+ to form the holoenzyme perturbs the pK_a of the ionisable group from 9.2 to 7.6.

The catalytic zinc atom is essential for stabilisation and orientation of the alcohol during oxidation. In aldehyde reduction the zinc atom polarises the substrate in such a

way that the first carbon atom increases in its electrophilicity making the transfer of a proton from the coenzyme favourable. Crystallographic evidence has shown that in the apoenzyme a water molecule binds in the substrate cleft acting as a fourth ligand to the catalytic zinc (Eklund *et al.*, 1976; Figure 1.3.).

Proton release is associated with binding NAD^+at pH 7.6 when concentrations of NAD^+ are saturating.(Shore *et al.*, 1974). It was originally thought that the alcohol substrate would interact with the unprotonated form of the ionising group, suggested to be the zinc-bound water molecule. However, it has been shown that for ethanol association, protonation of the group with a pK_a of 7.6 is required (Kvassman & Pettersson, 1980) and the requirement for ionisation of the zinc-bound water has been shown to be a non-essential in the main catalytic pathway (Schmidt *et al.*, 1979). Hence the alcohol displaces the zinc-bound water from the active site (Figure 1.4).

A major point of contention in interpreting the catalytic mechanism has been the form in which the alcohol is bound in the ternary complex, whether it is bound as the native alcohol or as the alcoholate ion. This contention arises because crystallographic evidence cannot determine the position of a proton. Hydride transfer from ethanol to NAD^+ is dependent on the ionisation of a group with a pK_a 6.4 or lower within the productive ternary complex (Brooks *et al.*, 1972) A similar pK_a dependence was observed for hydride transfer from benzyl alcohol to NAD^+, although no similar pH dependence was seen for the reverse reaction in which benzaldehyde and NADH were substrates (Kvassman & Pettersson, 1978). The pK_a value of 6.4 is a dramatically lower pK_a value than that of the alcohol in solution (Ethanol pK_a =15.9), hence perturbation of the pK_a of the ternary complex to 6.4 would ensure that the alcohol is bound predominantly as the alcoholate at physiological pH. It was concluded that ternary complex deprotonation is an essential and distinct step in activating the substrate prior to hydride transfer (Kvassman & Pettersson, 1980; Kvassman *et al.*, 1981).

The characterisation of ionisation states of alcohols in the ternary complex was investigated by Pocker & Page (1990) using small inhibitory alcohols. Their work, coupled with crystallographic evidence of the LADH-NAD^+-*p* -bromobenzyl alcohol ternary complex (Eklund *et al.*, 1982), indicated that inhibition was caused by the

Figure 1.3.

The active site of horse liver alcohol dehydrogenase

Drawing showing the catalytic zinc ligands and water molecule bound at the active site in the apoenzyme. (Taken from Fersht, 1985).

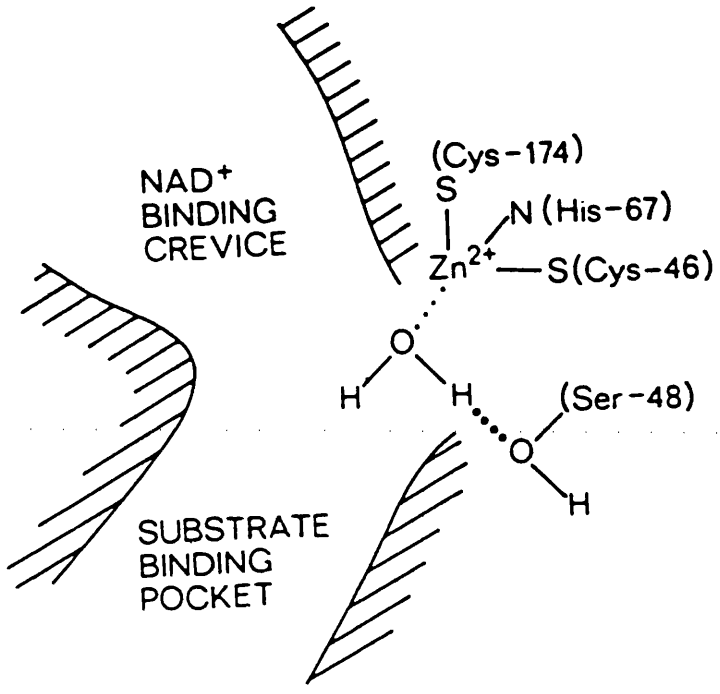
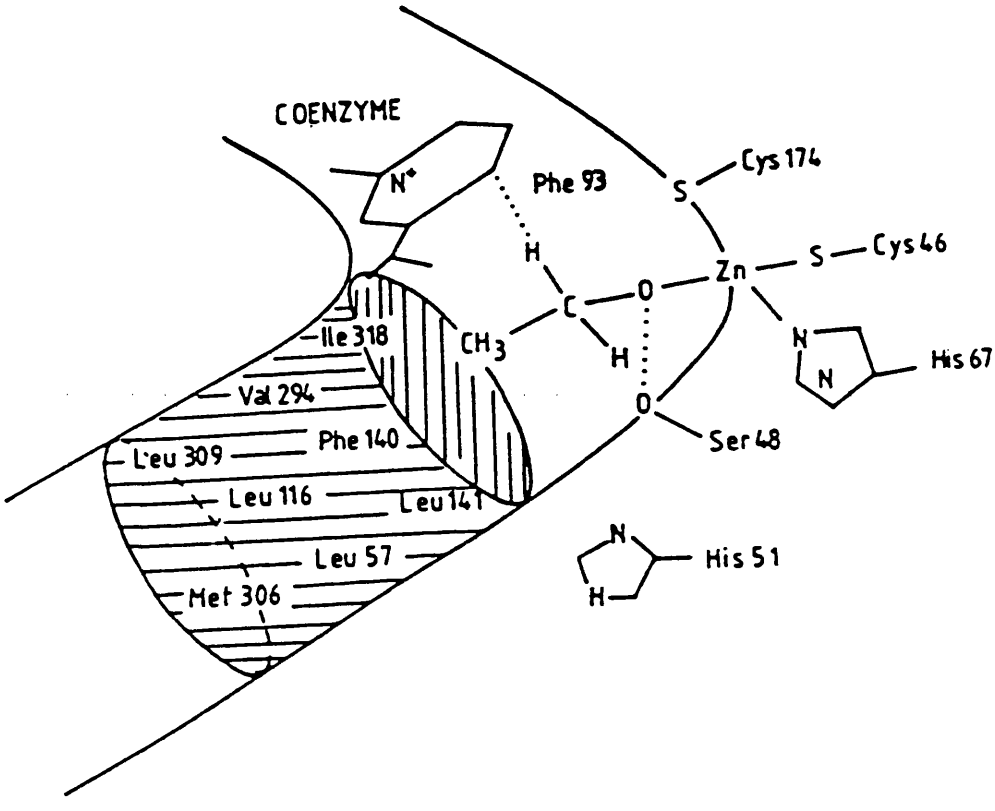


Figure 1.4.

The active site in the horse liver alcohol dehydrogenase ternary complex

Schematic representation of the active site region in horse liver alcohol dehydrogenase, showing catalytic zinc with its ligands, nicotinamide-binding, and the residues constituting hydrophobic substrate-binding pocket. (Taken from Eklund & Brändén, 1987).



formation of a zinc-bound inhibitory alkoxide. The inhibition constants and the pK_a value of inhibiting alcohol indicated that the pK_a value of an alcohol in the ternary complex is dependent on its pK_a in solution.

Thus, it seems that major roles of the coenzyme binding in the catalytic mechanism are to induce a conformational change and to shift the pK_a of an ionisable group involved in alcohol binding, so that the alcoholate ion is formed for the oxidation reaction.

Since the substrate binding pocket becomes anhydrous when NAD^+ binds (Section 1.2.1.1) the proton cannot be released directly into the cleft. His-51 is proposed to act as a base catalyst for alcohol oxidation, accepting a proton from the substrate and via a framework of hydrogen bonds formed between residues Ser-48, the 2'-hydroxyl of the coenzyme ribose ring allows shuttling of the proton to the surface of the enzyme. (Andersson *et al.*, 1981; Eklund *et al.*, 1982; Figure 1.5).

A scheme for the reactive mechanism has been proposed by Kvassman and Pettersson (1980) which accounts for the change in pK_a and for the activation of the alcohol substrate before hydride transfer (Figure 1.6). It is thought that the formation of the alcoholate is also essential in order to induce the closed conformation of the enzyme before hydride transfer can occur. Proton release, as a consequence of alcohol oxidation, results in activation of the substrate to the alcoholate form. The hydride transfer is then a nucleophilic attack that transfers hydride ion from the polarised substrate to the 4-position on the nicotinamide ring of the coenzyme (Figure 1.7).

Hydride transfer by dehydrogenases is always stereospecific in that some dehydrogenases transfer the hydride ion to one side of the nicotinamide ring and others transfer to the opposite side. On this basis enzymes are classified as "Class-A" or "Class-B", transferring the hydride ion to the pro-*R* and pro-*S* positions, respectively. Structural analysis has shown that class-A enzymes bind the coenzyme with the nicotinamide ring in the *anti* conformation and class-B enzymes bind the coenzyme with the nicotinamide ring in the *syn* conformation. The differences between the two conformation is a 180° rotation of the nicotinamide ring around the glycosidic bond with respect to the adenosine ribose moiety and so different sides of the ring are

Figure 1.5.

The proton translocation mechanism in horse liver alcohol dehydrogenase

Representation of the proton shuttle in horse liver alcohol dehydrogenase proposed by Andersson *et al.* (1981) and Eklund *et al.* (1982). ET corresponds to ethanol, the 2' hydroxyl group is that of the coenzyme and :B corresponds to the proton acceptor at the enzyme surface. (Taken from Fersht, 1985).

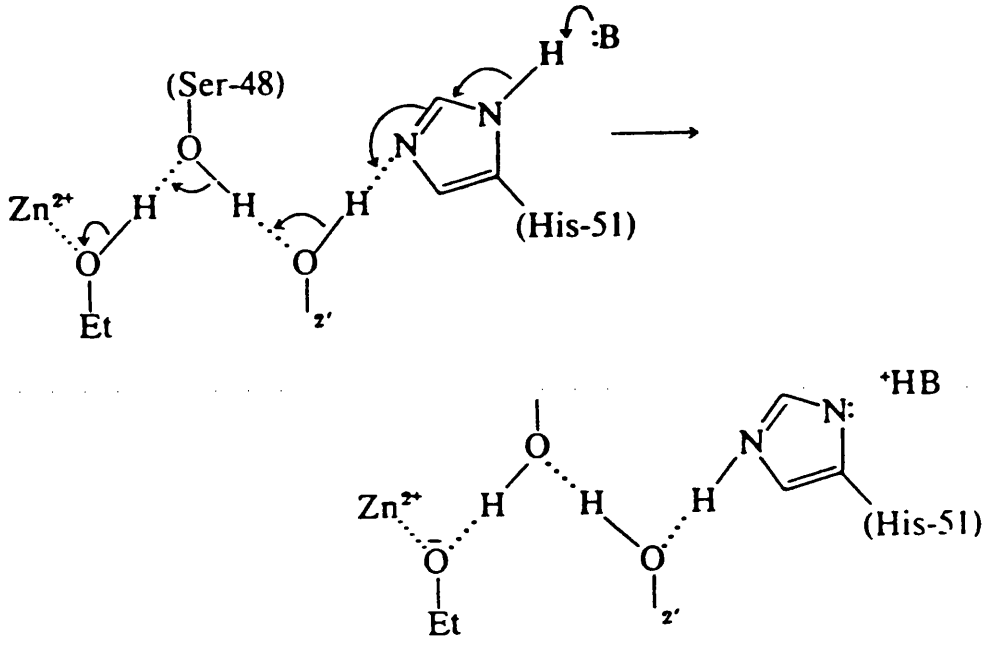


Figure 1.6.

The reaction scheme of horse liver alcohol dehydrogenase

Proposed reaction mechanism of horse liver alcohol dehydrogenase, in which the catalytic step of proton transfer is located to the ternary enzyme-NAD^+-alcohol complex and attributed to the ionisation of the bound alcohol. Numbers in brackets refer to pK_a of the ionisable group. (Adapted from Kvassman & Pettersson, 1980).

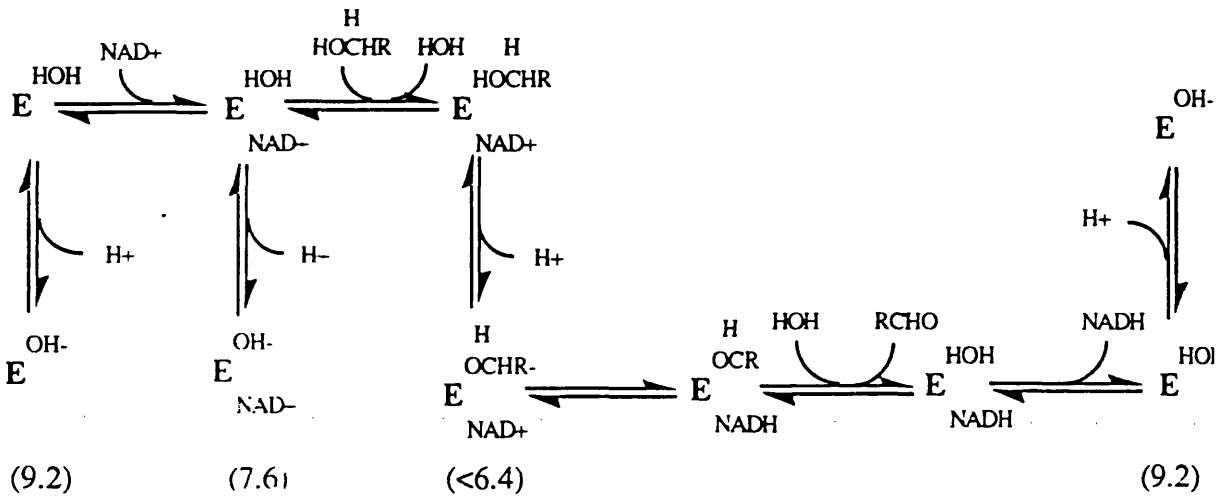
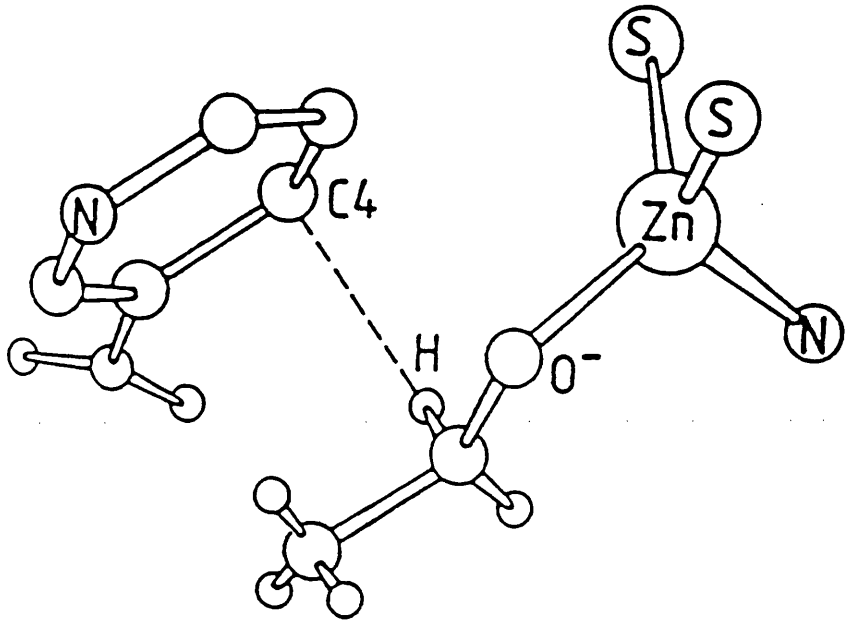


Figure 1.7.

The geometry of hydride transfer

Schematic drawing of the geometry for hydride transfer between ethanol and NAD^+. (Taken from Eklund, 1988).



presented to the active site in enzymes of different classes. HLADH is a class A enzyme (Figure 1.8).

1.2.2. Comparisons of the microbial group I alcohol dehydrogenases

This section compares both eukaryotic and prokaryotic group I ADHs in terms of primary and quaternary structure, substrate specificity and cofactor requirement to establish trends in evolutionary origin. All sequence comparisons with HLADH refer to the ethanol-active (E) isoenzyme (Section 1.2.4).

1.2.2.1. Primary sequences

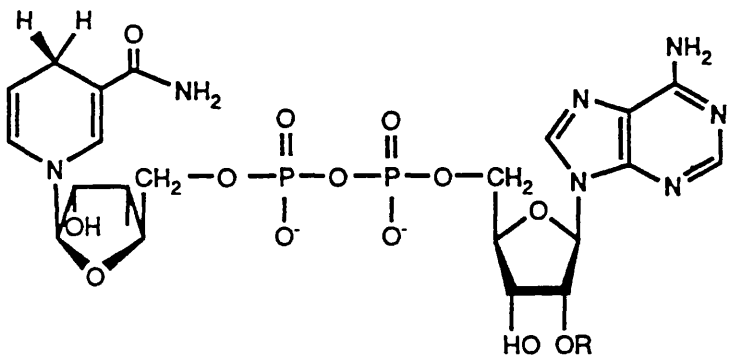
Evolutionary relationships among enzymes can be examined by comprehensive analysis of both the nucleotide and amino acid sequence. Primary amino acid structures available for group I microbial ADHs are listed in Table 1.1. These include the fermentative NAD(H)-dependent ADH from *Alcaligenes eutrophus* (AEADH: Jendrossek *et al.*, 1988) type I NAD(H)-dependent ADH from *Zymomonas mobilis* (ZMADHI: Keshav *et al.*, 1990), NAD-dependent ADH from *Bacillus stearothermophilus* (BSADH1503: Sakoda & Imanaka, 1992), NAD(H)-dependent benzyl alcohol dehydrogenase from *Pseudomonas putida* (TOL-BADH: Shaw *et al.*, 1993) and NADP(H)-dependent ADHs from *Thermoanaerobium brockii* (TBADH: Peretz & Burstein, 1989), *Entamoeba histolytica* (EADH: Kumar *et al.*, 1992) and *Clostridium beijerinckii* (CBADH: Rifaat & Chen, 1992). The primary structure of NAD(H)-dependendent L-threonine dehydrogenase from *Escherishia coli* has indicated that it is also a member of the group I alcohol/polyol dehydrogenases (Aronson *et al.*, 1989). Sequences available of eukaryotic microorganism ADHs include the NAD(H)-dependent isoenzymes I, II and III from *Sacchromyces cerevisiae* (SADHI: Bennetzen & Hall, 1982;SADHII Russell *et al.*, 1983;SADHIII: Young & Pilgrim, 1985) and *Schizosaccharomyces pombe* (SPADH: Russell & Hall, 1983). Other eukaryotic group I ADH sequences available are four isoenzymes from the yeast *Kluyveromyces lactis*

Figure 1.8.

Structure of NAD(P)H and stereospecificity of hydride transfer

1, shows the structure of NAD(P)H; 2, shows the stereospecificity of hydride transfer.

1



R=H :NADH

R=PO_3^- :NADPH

2

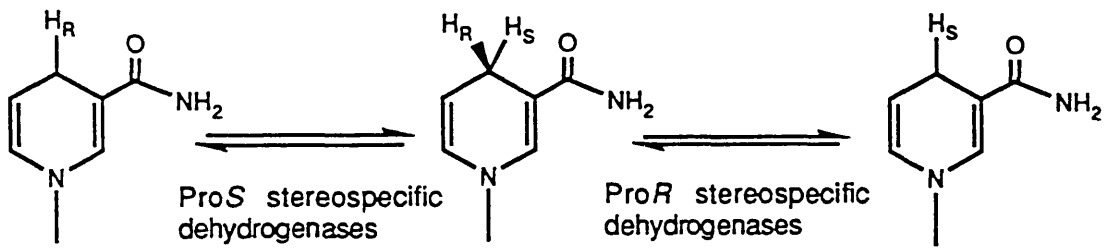


Table 1.1.

Microbial group I alcohol dehydrogenases

Enzyme	Organism	Abbreviation
Fermentative ADH	*Alcaligenes eutrophus*	AEADH
Type I ADH	*Aspergillus nidulans*	ANADHI
Type III ADH	*A. nidulans*	ANADHIII
Non-methanol using ADH	*Bacillus stearothermophilus*	BSADH
ADH	*Clostridium beijerinicki*	CBADH
ADH	*Entoamoeba histolytica*	EADH
L-Threonine dehydrogenase	*Escherichia coli*	ECTH
Isoenzyme I	*Kluveromyces lactis*	KADHI
Isoenzyme II	*K. lactis*	KADHII
Isoenzyme III	*K. lactis*	KADHIII
Isoenzyme IV	*K. lactis*	KADHIV
Isoenzyme I	*Sacchromyces cerevisiae*	SADHI
Isoenzyme II	*S. cerevisiae*	SADHII
Isoenzyme III	*S. cerevisiae*	SADHIII
fermentative ADH	*Schizosaccharomyces pombe*	SPADH
Secondary ADH	*Thermoanaerobium brockii*	TBADH
Benzyl ADH	*Pseudomonas putida*	TOL-BADH
Type I ADH	*Zymomonas mobilis*	ZMADH

(KADHI, KADHII, KADHIV: Saliola *et al.*, 1990; 1991) (KADHIII: Shain *et al.*, 1992) and two from *Aspergillus nidulans* (ANADHI and ANADHII: Gwynne *et al.*, 1987; Mcknight *et al.*, 1985).

Comparisons of amino acid primary structures may not always provide an accurate representation of evolutionary distances when isoenzymes are concerned because of complications arising from non-reciprocal gene exchanges. Isoenzymes are genetically-encoded multiple forms of enzymes. Such an exchange has been proposed to have occurred between the genes coding for SADHI and SADHII (Russell *et al.*, 1983; Figure 1.17; Section 1.2.5). Nucleotide sequence is not the best way to determine evolutionary relationships of similar enzymes from different organisms because of codon bias (especially in highly expressed genes) and the unlikelihood of a non-reciprocal gene crosses occurring between species (Smith *et al.*, 1992). Therefore, in this discussion interspecies comparisons will be restricted to amino acid sequence whereas isoenzyme comparisons will use both nucleotide and amino acid sequences.

1.2.2.2. Conserved residues

The identity among the microbial ADHs is much greater than seen with other members of the group (Table 1.2). HLADH has low values of identity when compared to microbial group I ADH sequences (32% maximum identity) in contrast to the microbial comparisons which have much higher levels of identity amongst them such as CBADH and TBADH which have 75% identity. Identity of 75% or above is often seen between isoenzymes, such as the yeast ADHs (KADHs, SADHs and ANADHs) but the level of identity between CBADH and TBADH is exceptionally high for ADHs isolated from two different organisms. There is a definite pattern of high sequence conservation amongst the NADP(H)-dependent ADHs: CBADH, TBADH and EADH (Table 1.2). The identity of TOL-BADH with the other microbial group I ADHs is comparable with that of HLADH (31% maximum identity) and is discussed in section 1.2.5.

Alignment of the amino acid sequences of 47 members of the group I ADHs showed that there are only nine residues conserved throughout all members (Sun &

Table 1.2. Percentage amino acid sequence identity amongst the group I microbial alcohol dehydrogenases

Identities were calculated using the "Bestfit" program from the VMS3 network system. Gap weight=3, gap weight length=0.1. Abbreviations used are those quoted in table 1.1. All sequences were obtained as stated in figure 1.9.

% IDENTITY

	CBADH	TBADH	EADH	AEADH	BSADH	ZMADHI	SADHI	SADHII	KADHI	KADHII	KADHIII	SADHIII	KADHIV	ANADHIII	ANADHI	SPADH	HLADH	TOL-BADH	ECTH
CBADH	100	75	62	37	24	21	21	22	22	21	21	24	24	24	23	24	27	24	25
TBADH		100	66	37	27	25	21	22	21	22	25	26	23	25	23	22	29	27	27
EADH			100	36	25	24	18	17	22	19	21	24	21	23	21	23	26	25	28
AEADH				100	32	25	26	26	23	23	26	22	23	26	24	26	25	25	30
BSADH					100	54	41	43	41	43	43	42	41	45	42	47	32	32	29
ZMADHI						100	36	38	37	37	37	37	38	41	36	38	31	26	27
SADHI							100	94	85	81	80	79	77	55	56	52	26	28	30
SADHII								100	84	81	80	78	77	56	55	52	26	25	29
KADHI									100	90	83	81	77	57	56	50	26	27	28
KADHII										100	82	78	76	55	55	49	27	26	28
KADHIII											100	82	81	56	55	49	26	26	30
SADHIII												100	81	56	55	49	26	27	29
KADHIV													100	58	57	48	26	23	27
ANADHIII														100	78	54	24	27	29
ANADHI															100	49	26	25	29
SPADH																100	29	29	24
HLADH																	100	31	27
TOL-BADH																		100	27
ECTH																			100

Plapp 1992). None of the nine conserved residues, rather surprisingly, are involved in catalysis directly but they all have structural roles; there are eight Gly and one Val. Among the microbial enzymes there are a further five strictly conserved residues: Cys-46, Asp-49, His-67, Glu-68 and Ala-211 (all residue numbering is based on alignment to HLADH except where otherwise stated; Figure 1.9) These residues are both structural and catalytic, indicating that there are greater evolutionary links among these enzymes, compared with the non-microbial enzymes. As well as conserved residues, there are some residues which are conservatively replaced. There is a conserved sequence of four residues, from 46 to 49, of Cys , His, Thr, Asp in all microbial ADHs except for TBADH, CBADH and EADH which have Thr-47 and Ser-48, and AEADH and ECTH which have Gly-47. All group I ADHs maintain a Thr or Ser at position 48 (Sun & Plapp, 1992).

The exchanges at positions 48 and 51 are particularly interesting because of their proposed involvement in substrate and coenzyme binding and, more importantly, their roles in the proton-relay system (Eklund *et al.*, 1982; Andersson *et al.*, 1981; Section 1.2.1.3). Site-directed mutagenesis of SADHI have shown that both Ser and Thr at position 48 might be able to play a similar role in the proton-relay system, although they have been shown to have different effects on substrate specificity (Section 1.2.3.3). His-51 is conserved all group I ADHs apart from TOL-BADH, human class II π subunit, human ADH6 and human class III χ subunit in which position 51 is replaced replaced by Val, Ser, Lys and Tyr respectively (Shaw *et al.*, 1993; Höög *et al.*, 1987; Yasunami *et al.*, 1991; Julia *et al.*, 1988). The His/Ser/Lys/Tyr -51 exchanges have been suggested to correlate with the effects of pH on activity (Parés & Jörnvall, 1988). Tyr-51, like His-51, is able form a direct hydrogen bond to the 2' ribose oxygen of the coenzyme (Eklund *et al.*, 1990). However, Sun & Plapp (1992) suggest that Tyr-51 does not play a role in base catalysis under normal physiological conditions. It is also interesting to note that the only non-catalytic group I ADH, guinea pig eye lens ζ-crystallin, also conserves a Tyr at position 51. Lys- and Ser-51 are unable to form a direct hydrogen bond to the 2' ribose oxygen of the coenzyme because the distance is too great, but both could conceivably bind indirectly to the coenzyme via a water molecule, as is seen in

Figure 1.9.

Primary sequence comparison of the microbial NAD(P)H dependent group I alcohol dehydrogenases with horse liver alcohol dehydrogenase

Sequence alignment was done from sequences obtained through the VMS3 UNIX system, except TOL-BADH, TBADH, ECTH and KADHI which were obtained from Shaw *et al.*, 1993, Peretz & Burnstein, 1989, Aronson *et al.*, 1989 and Shain *et al.*, 1992, respectively. Abbreviations used are those quoted in table 1.1. Sequences were aligned using Pileup GCG package on the VMS3 UNIX system (Genetics Computer Group, Inc.). Gap weight=3.0. Gap weight length=0.1. Symbols: *, conserved residues.

```
VMS3 CODE              10         20         30         40
                        |          |          |          |
M84723    CBADH  .........M KGFAMLGIN. ..KLGWIEKE RPVAGSYDAI VRPLAVSPCT
          TBADH  .........M KGFAMLSIG. ..KVGWIEKE KPAPGPFDAI VRPLAVAPCT
M88600     EADH  .........M KGLAMLGIG. ..RIGWIEKK IPECGPLDAL VRPLALAPCT
J03362    AEADH  .........M KAAVFVEPG. ..RIELADKP IPDIGPNDAL VRITTTTICG
D90421    BSADH  .........M KAAVVEQFK. .KPLQVKEVE KPKISYGEVL VRIKACGVCH
M32100    ZMADH  .........M KAAVITK.D. .HTIEVKDTK LRPLKYGEAL LEMEYCGVCH
J01313    SADHI  ...MSIPETQ KGVIFYESHG ..KLEHKDIP VPKPKANELL INVKYSGVCH
V10293   SADHII  ...MSIPETQ KAIIFYESHG ..KLEHKDIP VPKPKANELL INVKYSGVCH
          KADHI  .MAASIPETQ KGVIFYENGG ..ELQYKDIP VPKPKANELL INVKYSGVCH
X64397   KADHII  ...MSIPETQ KGVIFYENGG ..ELQYKDIP VPKPKANELL INVKYSGVCH
X62766  KADHIII  .LATSVPETQ KGVIFYENGG ..KLEYKDIP VPKPKPNEIL INVKYSGVCH
K03292  SADHIII  QSTAAIPKTQ KGVIFYENKG ..NLHYKDIP VPEPKPNEIL INVKYSGVCH
X62767   KADHIV  NSSFAIPETQ KGVIFYENGG ..KLEYKDLP VPKPKANEIL INVKYSGVCH
X02764ANADHIII  ...MSVPEVQ WAQVVEKAGT ..PPVYKQVP VPKPGPDEIL VKMRYSGVCH
M16196  ANADHI  ...MCIPTMQ WAQVAEKVGG ..PLVYKQIP VPKPGPDQIL VKIRYSGVCH
J01341   SPADH  ...MTIPDKQ LAAVFHTHGG PENVKFEEVP VAEPGQDEVL VNIKYTGVCH
M64864   HLADH  MSTAGKVIKC KAAVLWEEKK ..PFSIEEVE VAPPKAHEVR IKMVATGICR
      TOL-BADH  .......MEI KAAIVRQKNG ..PFLLEHVA LNEPAEDQVL VRLVATGLCH
          ECTH  .........M KALSKLKAEE ..GIWMTDVP VPELGHNDLL IKIRKTAICG
                                                                    *

                    50         60         70         80          90
                     |          |          |          |           |
          CBADH  SDIHTV...F EGALGDERNM ILGHEAVGEV VEVGSEVK.. DFKPGDRVIV
          TBADH  SDIHTV...F EGAIGERHDM ILGHEAVGEV VEVGSEVK.. DFKPGDRVVV
           EADH  SDTHTV...W AGAIGDRHDM ILGHEAVGQI VKVGSLVK.. RLKVGDKVIV
          AEADH  TDVH.I...L KGEYPVARGL TVGHEPVGII EKLGSAVT.. GYREGQRVIA
          BSADH  TDLHAAHGDW ..PVKPKLPL IPGHEGVGVI EEVGPGVT.. HLKVGDRVGI
          ZMADH  TDLHVKNGDF ..GDET..GR ITGHEGIGIV KQVGEGVT.. SLKAGDRASV
          SADHI  TDLHAWHGDW ..PLPVKLPL VGGHEGAGVV VGMGENVK.. GWKIGDYAGI
         SADHII  TDLHAWHGDW ..PLPTKLPL VGGHEGAGVV VGMGENVK.. GWKIGDYAGI
          KADHI  TDLHAWKGDW ..PLPTKLPL VGGHEGAGVV VAMGENVK.. GWKIGDFAGI
         KADHII  TDLHAWKGDW ..PLPTKLPL VGGHEGAGVV VAMGENVK.. GWIIGDFAGI
        KADHIII  TDLHAWKGDW ..PLPTKLPL VGGHEGAGVV VAMGENVK.. GWNIGDFAGI
        SADHIII  TDLHAWHGDW ..PLPVKLPL VGGHEGAGVV VKLGSNVK.. GWKVGDLAGI
         KADHIV  TDLHAWKGDW ..PLPVKLPL VGGHEGAGIV VAKGENVK.. NFEIGDYAGI
       ANADHIII  TDLHAMKGDW ..PLPSKMPL IGGHEGPGVV VAKGELVKDE DFKIGDRAGI
         ANADHI  TDLHAMMGHW ..PIPVKMPL VGGHEGAGIV VAKGELV..H EFEIGDQAGI
          SPADH  TDLHALQGDW ..PLPAKMPL IGGHEGAGVV VKVGAGVTR. .LKIGDRVGV
          HLADH  SDDHVVS... .GTLVTPLPV IAGHEAAGIV ESIGEGVT.. TVRPGDKV.I
        TOL-BADH TDLVCRD... .QHYPVPLPM VFGHEGAGVV ERVGSAVK.. KVQPGDHV.V
          ECTH  TDVHIYNWDE WSQKTIPVPM VVGHEYVGEV VGIGQEVK.. GFKIGDRVSG
                 *                    ***  *      *  *
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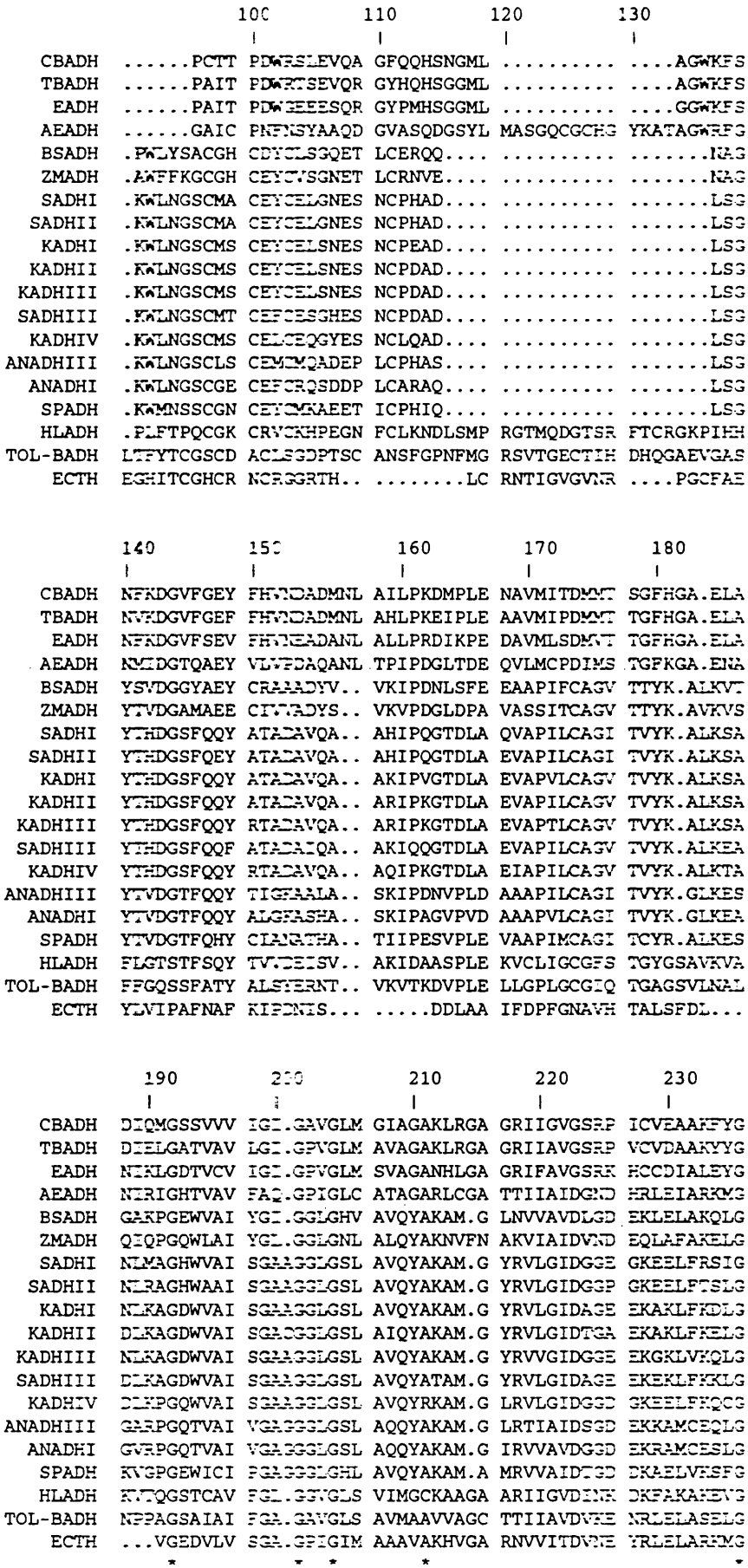
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TBADH ......PAIT PDWRTSEVQR GYHQHSGGML ....AGWKFS
EADH ......PAIT PDWEEESQR GYPMHSGGML ....GGWKFS
AEADH ......GAIC PNFNSYAAQD GVASQDGSYL MASGQCGCHG YKATAGWRFG
BSADH .PWLYSACGH CDYCLSGQET LCERQQ.... ....NAG
ZMADH .AWFFKGCGH CEYCVSGNET LCRNVE.... ....NAG
SADHI .KWLNGSCMA CEYCELGNES NCPHAD.... ....LSG
SADHII .KWLNGSCMA CEYCELGNES NCPHAD.... ....LSG
KADHI .KWLNGSCMS CEYCELSNES NCPEAD.... ....LSG
KADHII .KWLNGSCMS CEYCELSNES NCPDAD.... ....LSG
KADHIII .KWLNGSCMS CEYCELSNES NCPDAD.... ....LSG
SADHIII .KWLNGSCMT CEFCESGHES NCPDAD.... ....LSG
KADHIV .KWLNGSCMS CELCEQGYES NCLQAD.... ....LSG
ANADHIII .KWLNGSCLS CEMCMQADEP LCPHAS.... ....LSG
ANADHI .KWLNGSCGE CEFCRQSDDP LCARAQ.... ....LSG
SPADH .KWMNSSCGN CEYCMKAEET ICPHIQ.... ....LSG
HLADH .PLFTPQCGK CRVCKHPEGN FCLKNDLSMP RGTMQDGTSR FTCRGKPIHH
TOL-BADH LTFYTCGSCD ACLSGDPTSC ANSFGPNFMG RSVTGECTIH DHQGAEVGAS
ECTH EGHITCGHCR NCRGGRTH.. .....LC RNTIGVGVNR ....PGCFAE
140 150 160 170 180
CBADH NFKDGVFGEY FHVNDADMNL AILPKDMPLE NAVMITDMMT SGFHGA.ELA
TBADH NVKDGVFGEF FHVNDADMNL AHLPKEIPLE AAVMIPDMMT TGFHGA.ELA
EADH NFKDGVFSEV FHVNEADANL ALLPRDIKPE DAVMLSDMVT TGFHGA.ELA
AEADH NVIDGTQAEY VLVPDAQANL TPIPDGLTDE QVLMCPDIMS TGFKGA.ENA
BSADH YSVDGGYAEY CRAAADYV.. VKIPDNLSFE EAAPIFCAGV TTYK.ALKVT
ZMADH YTVDGAMAEE CIVTADYS.. VKVPDGLDPA VASSITCAGV TTYK.AVKVS
SADHI YTHDGSFQQY ATADAVQA.. AHIPQGTDLA QVAPILCAGI TVYK.ALKSA
SADHII YTHDGSFQEY ATADAVQA.. AHIPQGTDLA EVAPILCAGI TVYK.ALKSA
KADHI YTHDGSFQQY ATADAVQA.. AKIPVGTDLA EVAPVLCAGV TVYK.ALKSA
KADHII YTHDGSFQQY ATADAVQA.. ARIPKGTDLA EVAPILCAGV TVYK.ALKSA
KADHIII YTHDGSFQQY RTADAVQA.. ARIPKGTDLA EVAPTLCAGV TVYK.ALKSA
SADHIII YTHDGSFQQF ATADAIQA.. AKIQQGTDLA EVAPILCAGV TVYK.ALKEA
KADHIV YTHDGSFQQY RTADAVQA.. AQIPKGTDLA EIAPILCAGV TVYK.ALKTA
ANADHIII YTVDGTFQQY TIGFAALA.. SKIPDNVPLD AAAPILCAGI TVYK.GLKES
ANADHI YTVDGTFQQY ALGFASHA.. SKIPAGVPVD AAAPVLCAGI TVYK.GLKEA
SPADH YTVDGTFQHY CIADATHA.. TIIPESVPLE VAAPIMCAGI TCYR.ALKES
HLADH FLGTSTFSQY TVVDEISV.. AKIDAASPLE KVCLIGCGFS TGYGSAVKVA
TOL-BADH FFGQSSFATY ALSTERNT.. VKVTKDVPLE LLGPLGCGIQ TGAGSVLNAL
ECTH YLVIPAFNAF KIFDNIS... .....DDLAA IFDPFGNAVH TALSFDL...
190 200 210 220 230
CBADH DIQMGSSVVV IGI.GAVGLM GIAGAKLRGA GRIIGVGSRP ICVEAAKFYG
TBADH DIELGATVAV LGI.GPVGLM AVAGAKLRGA GRIIAVGSRP VCVDAAKYYG
EADH NIKLGDTVCV IGI.GPVGLM SVAGANHLGA GRIFAVGSRK HCCDIALEYG
AEADH NIRIGHTVAV FAQ.GPIGLC ATAGARLCGA TTIIAIDGND HRLEIARKMG
BSADH GAKPGEWVAI YGI.GGLGHV AVQYAKAM.G LNVVAVDLGD EKLELAKQLG
ZMADH QIQPGQWLAI YGL.GGLGNL ALQYAKNVFN AKVIAIDVND EQLAFAKELG
SADHI NLMAGHWVAI SGHAGGLGSL AVQYAKAM.G YRVLGIDGGE GKEELFRSIG
SADHII NLRAGHWAAI SGHAGGLGSL AVQYAKAM.G YRVLGIDGGP GKEELFTSLG
KADHI NLKAGDWVAI SGHAGGLGSL AVQYAKAM.G YRVLGIDAGE EKAKLFKDLG
KADHII DLKAGDWVAI SGHAGGLGSL AIQYAKAM.G YRVLGIDTGA EKAKLFKELG
KADHIII NLKAGDWVAI SGHAGGLGSL AVQYAKAM.G YRVVGIDGGE EKGKLVKQLG
SADHIII DLKAGDWVAI SGHAGGLGSL AVQYATAM.G YRVLGIDAGE EKEKLFKKLG
KADHIV DLKPGQWVAI SGHAGGLGSL AVQYRKAM.G LRVLGIDGGD GKEELFKQCG
ANADHIII GARPGQTVAI VGAGGGLGSL AQQYAKAM.G LRTIAIDSGD EKKAMCEQLG
ANADHI GVRPGQTVAI VGAGGGLGSL AQQYAKAM.G IRVVAVDGGD EKRAMCESLG
SPADH KVGPGEWICI PGAGGGLGHL AVQYAKAM.A MRVVAIDTGD DKAELVKSFG
HLADH KVTQGSTCAV FGL.GGVGLS VIMGCKAAGA ARIIGVDINK DKFAKAKEVG
TOL-BADH NPPAGSAIAI FGA.GAVGLS AVMAAVVAGC TTIIAVDVNE NRLELASELG
ECTH ...VGEDVLV SGA.GPIGIM AAAVAKHVGA RNVVITDVNE YRLELARKMG

```
                240         250         260         270         280
                 |           |           |           |           |
   CBADH  ATDILNY.KN GHIVDQVMKL TNG.EGVDRV IMAGGGSETL SQAVSMVKPG
   TBADH  ATDIVNY.KD GPIESQIMNL TEG.KGVDAA IIAGGNADIM ATAVKIVKPG
    EADH  ATDIINY.KN GDIVEQILKA TDG.KGVDKV VIAGGDVHTF AQAVKMIKPG
   AEADH  ADVVLNF.RN CDVVDEVMKL TGG.RGVDAS IEALGTQATF EQSLRVLKPG
   BSADH  ADLVVN.PKH DDAAQWIKEK VG...GVHAT VVTAVSKAAF ESAYKSIRRG
   ZMADH  ADMVIN.PKN EDAAKIIQEK VG...GAHAT VVTAVAKSAF NSAVEAIRAG
   SADHI  GEVFIDFTKE KDIVGAVLKA TDG..GAHGV INVSVSEAAI EASTRYVRAN
  SADHII  GEVFIDFTKE KDIVSAVVKA TNG..GAHGI INVSVSEAAI EASTRYCRAN
   KADHI  GEYFIDFTKS KNIPEEVIEA TKG..GAHGV INVSVSEFAI EQSTNYVRSN
  KADHII  GEYFVDYAVS KDLIKEIVDA TNG..GAHGV INVSVSEFAI EQSTNYVRSN
 KADHIII  GEAFVDFTKT KDMVAEIQEI TNG..GFHGV INVSVSEAAM NASTQFVRPT
 SADHIII  GEVFIDFTKT KNMVSDIQEA TKG..GFHGV INVSVSEAAI SLSTEYVRPC
  KADHIV  GEVFIDFRKS KDMVADIQEA TNG..GFHGV INVSVSEAAV SMSTEYLRPT
ANADHIII  AEVFIDFSKS ADVVADVKAA TPGGLGAHAV ILLAVAEKPF QQATEYVRSH
  ANADHI  TETYVDFTKS KDLVADVRHG .RGCLGAHAV ILLAVSEKPF QQATEYVRSR
   SPADH  AEVFLDFKKE ADMIEAVKAC TNG..GAHGT LVLSTSPKSY EQAAGFARPG
   HLADH  ATECVNPQDY KKPIQEVLTE M.SNGGVDFS FEVIGRLDTM VTALSCCQEA
TOL-BADH  ATHIINPAA. NDPI.EAIKE I.FADGVPYV LETSGLPAVL TQAILSSAIG
    ECTH  ITRAVNVA.. KENLNDVMAE LGMTEGFDVG LEMSGAPPAF RTMLDTMNHG

                290         300         310         320         330
                 |           |           |           |           |
   CBADH  GIISNINYHG SGDALLIPRV EWGCGMAHKT IKGGLCPGGR LRAEMLRDMV
   TBADH  GTIANVNYFG EGEVLPVPRL EWGCGMAHKT IKGGLCPGGR LRMERLIDLV
    EADH  SDIGNVNYLG EGDNIDIPRS EWGVGMGHKH IHGGLTPGGR VRMEKLASLI
   AEADH  GTLSSLGVYS SD..LTIPLS AFAAGLGDHK INTALCPGGK ERMRRLINVI
   BSADH  GAC.VL..VG LPPE.EIPIP IFDTVLNGVK IIGSIV.GTR ......KDLQ
   ZMADH  GRV.VA..VG LPPE.KMDLS IPRLVLDGIE VLGSLV.GTR ......EDLK
   SADHI  GTT.VL..VG MPAGAKCCSD VFNQVVKSIS IVGSYV.GNR ......ADTR
  SADHII  GTV.VL..VG LPAGAKCSSD VFNHVVKSIS IVGSYV.GNR ......ADTR
   KADHI  GTV.VL..VG LPRDAKCKSD VFNQVVKSIS IVGSYV.GNR ......ADTR
  KADHII  GTV.VL..VG LPRDAKCKSD VFTQVVKSVS IVGSYV.GNR ......ADTR
 KADHIII  GTV.VL..VG LPAGAVIKSE VFSHVVKSIN IKGSYV.GNR ......ADTR
 SADHIII  GTV.VL..VG LPANAYVKSE VFSHVVKSIN IKGSYV.GNR ......ADTR
  KADHIV  GLV.VL..VG LPADAYVKSE VFSHVVKSIS IKGSYV.GNR ......ADTR
ANADHIII  GSV.VA..IG LPANAFLKAP VFTTVVRMIN IKGSYV.GNR ......QDGV
  ANADHI  GTI.VA..IG LPPDAYLKAP VINTVVRMIT IKGSYV.GNR ......QDGV
   SPADH  STM.VT..VS MPAGAKLGAD IFWLTVKMLK ICGSHV.GNR ......IDSI
   HLADH  YGVSVI..VG VPPDSQNLSM NPMLLLSGRT WKGAIF.GGF KSKDSVPKLV
TOL-BADH  GEIG.I..VG APPMGATVPV DINFLLFNRK LRGIVE.GQS ISDIFIPRLV
    ECTH  GRIA.M..LG IPP..SDMSI DWTKVIFKGL FIKGIY.GRE MFETWYK..M
                                                      *

                340         350         360         370
                 |           |           |           |
   CBADH  VYNRVDLSKL VTHVYH..GF DHIEEALLLM KDKPKDLIKA VVIL......
   TBADH  FYKRVDPSKL VTHVFR..GF DNIEKAFMLM KDKPKDLIKP VVILA.....
    EADH  STGKLDTSKL ITHRFE..GL EKVEDALMLM KNKPADLIKP VVRIHYDDEDTLH
   AEADH  ESGRVDLGAL VTHQYR...L DDIVAAYDLF ANQRDGVLKI AIKPH.....
   BSADH  EALQFAAEGK VKTIVEVQPL ENINDVFDRM .LKGQINGRV VLKVD.....
   ZMADH  EAFQFAAEGK VKPKVTKRKV EEINQIFDEM .EHGKFTGRM VVDFTHH...
   SADHI  EALDFFARGL VKSPIKVVGL STLPEIYEKM .EKGQIVGRY VVDTSK....
  SADHII  EALDFFARGL VKSPIKVVGL SSLPEIYEKM .EKGQIAGRY VVDTSK....
   KADHI  EAIDFFSRGL VKAPIHVVGL SELPSIYEKM .EKGAIVGRY VVDTSK....
  KADHII  EALDFFARGL VHAPIKIVGL SELADVYDKM .VKGEIVGRY VVDTSK....
 KADHIII  EAINFFANGH VHSPIKVVGL SELPKVYELM .EQGKILGRY VVDTSN....
 SADHIII  EALDFFSRGL IKSPIKIVGL SELPKVYDLM .EKGKILGRY VVDTSK....
  KADHIV  EATDLFTRGL VKSPIKIIGL SELPEAYALM .EQGKILGRF VVDTYK....
ANADHIII  EALDFFARGL IKAPFKKAPL QDLPQIFELM .GQGKIAGRY VLEIPE....
  ANADHI  EALDFFARGL IKAPFKTAPL KDLPKIYELM .EQGRIAGRY VLEMPE....
   SPADH  EALEYVSRGL VKPYYKVQPF STLPDVYRLM .HENKIAGRI VLDLSK....
   HLADH  ADFMAKKFAL DPLITHVLPF EKINEGFDLL .RSGE.SIRT ILTF......
TOL-BADH  ELYRQGKFPF DKLIL.FYPF DEINRAAEDS .EKGV.TLKP VLRIG.....
    ECTH  AALIQSGLDL SPIITHRFSI DDFQKGFDAM .RSGQ.SGKV ILSWD.....
```

glyceraldehyde-3-phosphate dehydrogenase (Skarzynski *et al.*, 1987). However, if the binding of coenzyme creates an anhydrous environment in the substrate cleft (Section 1.2.1.1), the indirect binding of a water molecule forming a proton-relay system would appears to be unlikely. Eklund *et al.* (1990) have also suggested that the hypothesis of an indirect water bond is unlikely because of the structure of human class II π and class III χ subunits. Val-51 in TOL-BADH is clearly unable to form any hydrogen bonds because it has an aliphatic side-chain. Site directed mutagenesis has already shown the importance of a Thr or Ser residue at position 48 in group I ADHs for catalysis (Section 1.3.3.3). Using a similar line of investigation it would be interesting to pursue the role of Ser/Thr-48 in the four enzymes lacking His-51 to see whether a hydroxyl group is required at position 48 for some sort of proton relay system.

Asp-223 is conserved in all microbial group I NAD(H)-dependent ADHs but is replaced by Gly-223 in all microbial group I NADP(H)-dependent ADHs (Section 1.2.3.1).

Two of the catalytic zinc ligands, Cys-46 and His-67, are conserved in all microbial ADHs. The third Cys-174 is said to be conservatively replaced by Asp in TBADH, AEADH and in the related *E.coli* threonine dehydrogenase (Aronson *et al.*, 1989), although in this sequence alignment, residue 174 in TBADH and CBADH is Ile and is Leu in EADH. In each of the three above mentioned NADP(H)-dependent ADHs there is a Asp conserved at position 176 which corresponds to the third catalytic zinc ligand as described by Aronson *et al.* (1989).

The zinc-containing polyol dehydrogenases have been judged to be related to the group I ADHs because of their primary sequence identity and functional similarities (Jörnvall *et al.*, 1981; Jeffery *et al.*, 1984). In the liver sorbitol dehydrogenases Cys-174 is replaced by Glu (Jörnvall *et al.*, 1981; Eklund *et al* ., 1985; Jeffery & Jörnvall, 1988; Karlsson *et al.*, 1991).

Other highly conserved residues include: Asp at position 87 and Gly 199 which are conserved in all microbial group I ADHs except for AEADH where they are Gln and Ala respectively. Arg or Lys is conserved in all sequences.

The large gap seen in the region of residues 120-130, (Figure 1.9) conserved to some degree in all sequences except AEADH, TOL-BADH, HLADH and ECTH, is one of the most striking features of the sequence alignment and is discussed in section 1.2.3.4.

1.2.3. Protein engineering

Conservation of a particular residue across all related enzymes indicates that it is essential for enzyme activity or for maintainance of structure, although these roles are not in any way exclusive of each other. Using site directed mutagenesis the importance of residues in the activity of a enzyme can be assessed and attempts can be made to answer questions such as : How do individual residues participate in catalysis ? Which residues determine the basis of the enzyme's substrate specificity ? Which features were selected for as enzymes evolved? This area has been previously reviewed by Plapp *et al.* (1990), and is discussed in the next four sections 1.2.3.1.-1.2.3.4. A summary of the site directed mutagenesis studies carried out on SADHI is presented in table 1.3.

1.2.3.1. Coenzyme requirement

All NAD(H)-dependent enzymes have the residue Asp-223. Using a three dimensional model of yeast alcohol dehydrogenase based on the HLADH structure (Jörnvall 1977; Ganzhorn *et al.*, 1987; Plapp *et al.*, 1990; Figure 1.10), Asp-223 was identified as the acidic residue responsible for interaction with the 2'- and 3'-hydroxyl groups of the adenosine ribose of the coenzyme. The ADHs without Asp-223 are the NADP(H)-dependent TBADH, CBADH and EADH which have a Gly residue corresponding to position 223 (Figure 1.9). The negative charge of the Asp carboxylate group sterically and electrostatically hinders the binding of NADP(H) in the group I NAD(H) dependent ADHs because of the coenzymes' 2'-phosphate group. Asp-223 is therefore essential for determination of coenzyme specificity. (Lamed *et al.*, 1987; Peretz & Burstein, 1990). Replacement of Asp-223 with Gly in SADHI (numbering

Table 1.3.

Mutations of yeast alcohol dehydrogenase I

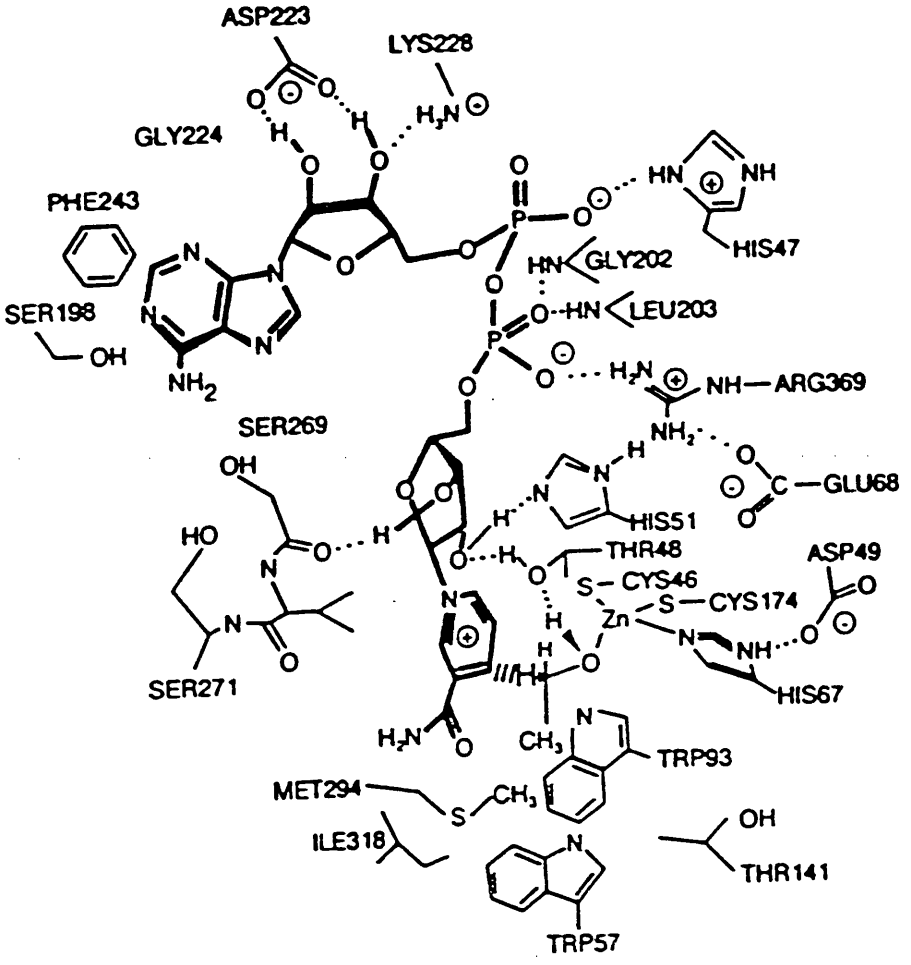
Relative change in kinetic constant, mutant: wild type. (Taken from Plapp *et al* ., 1990).

Mutation	K_d NAD^+	*V* Ethanol	V/K_m Ethanol	Other
Met294Leu	1.5	1.5	1.3	8 $V/K_{Butanol}$
Thr48Ser	1.3	0.60	0.60	10 2-Butanol *S*>*R*
Trp57Met	3.0	0.65	0.24	0.5 Hexanol
His47Arg	0.26	0.16	0.034	Slow E-NAD^+isom
Asp49Asn	6.3	0.022	0.001	$20K_d$ CF_3CH_2OH
Glu68Gln	3.8	0.029	0.012	$9K_d$ CF_3CH_2OH
His51Gln	0.5	0.079	0.071	$13K_d$ CF_3CH_2OH
His51Glu	0.08	0.005	0.015	$50K_d$ CF_3CH_2OH
Thr48Cys		<0.0001	No detectable activity	
Thr48Ala		<0.0001	No detectable activity	

Figure 1.10.

The active site model of *Sacchromyces cerevisiae* alcohol dehydrogenase I

Representation of the active site of SADHI based on the three dimensional structure of horse liver alcohol dehydrogenase. (Taken from Plapp *et al.*, 1990).



based on HLADH) removed the steric and electrostatic inhibition and allowed NADP(H) to bind (James *et al.*, 1991), although only 1% of the previous activity (V_{max}/K_m) with ethanol was seen with NAD(H). The Gly-223 mutant bound NAD^+ and $NADP^+$ equally well, but the dissociation constant for NAD^+ increased 17-fold compared with the wild type Asp-223 enzyme. The loss of 1.7-2.5 kcal/mol in the free energy of coenzyme binding correlates with the loss of two hydrogen bonds formed by Asp-223 to the 2' and 3'-hydroxyl groups of the adenosine ribose moiety of the coenzyme. These results may be explained by assuming that the Gly-223 mutant might find the transition from the open, inactive, form of the enzyme to the closed, active, form (Figure 1.2) less favourable than the wild type Asp-223 enzyme. Coenzyme requirements of the microbial group I ADHs are listed in table 1.4.

In the Gly-223 SADHI mutant it is possible that Lys-228 (Figure 1.10) could hydrogen bond with the 3'-hydroxyl.of the adenosine ribose moiety of the coenzyme. A substitution of a positively charged residue such as Arg could make NADP(H) binding even more favourable. In TBADH, the positively charged residue Arg-225 is in close proximity to the 2'-phosphate of the coenzyme. TBADH binds $NADP^+$ about 300-fold better than the Gly-223 mutant of SADHI, indicating that many residues contribute towards the specificity for the coenzyme. In order to completely reverse the coenzyme specificity more than one residue change would be required.

SADHI is a class-A enzyme. It binds coenzyme in the *anti* (rather than *syn*) conformation and transfers the hydrogen to the *Re* (pro-*R* or A position) -4 position carbon of the nicotinamide ring (Figure 1.11). It has been argued by Benner *et al.* (1985) and Glasfeld *et al.* (1990) that the stereospecificity of hydride transfer serves no selected function, but is a frozen evolutionary accident which was randomly selected during the early stages of evolution and once selected for could not be easily changed. Oppenheimer *et al.* (1984) argued that the reason for high the conservation of stereospecificity is that essential amino acids inhibit the binding of the coenzyme in the opposite conformation, either the *anti* or *syn* (depending on the enzymes class) because of steric hindrance. To accommodate the binding of the other conformation of the coenzyme would require a major reorganisation at the active site and cause dislocation

Table 1.4

Coenzyme specificities and quaternary structure of the microbial group I alcohol dehydrogenases

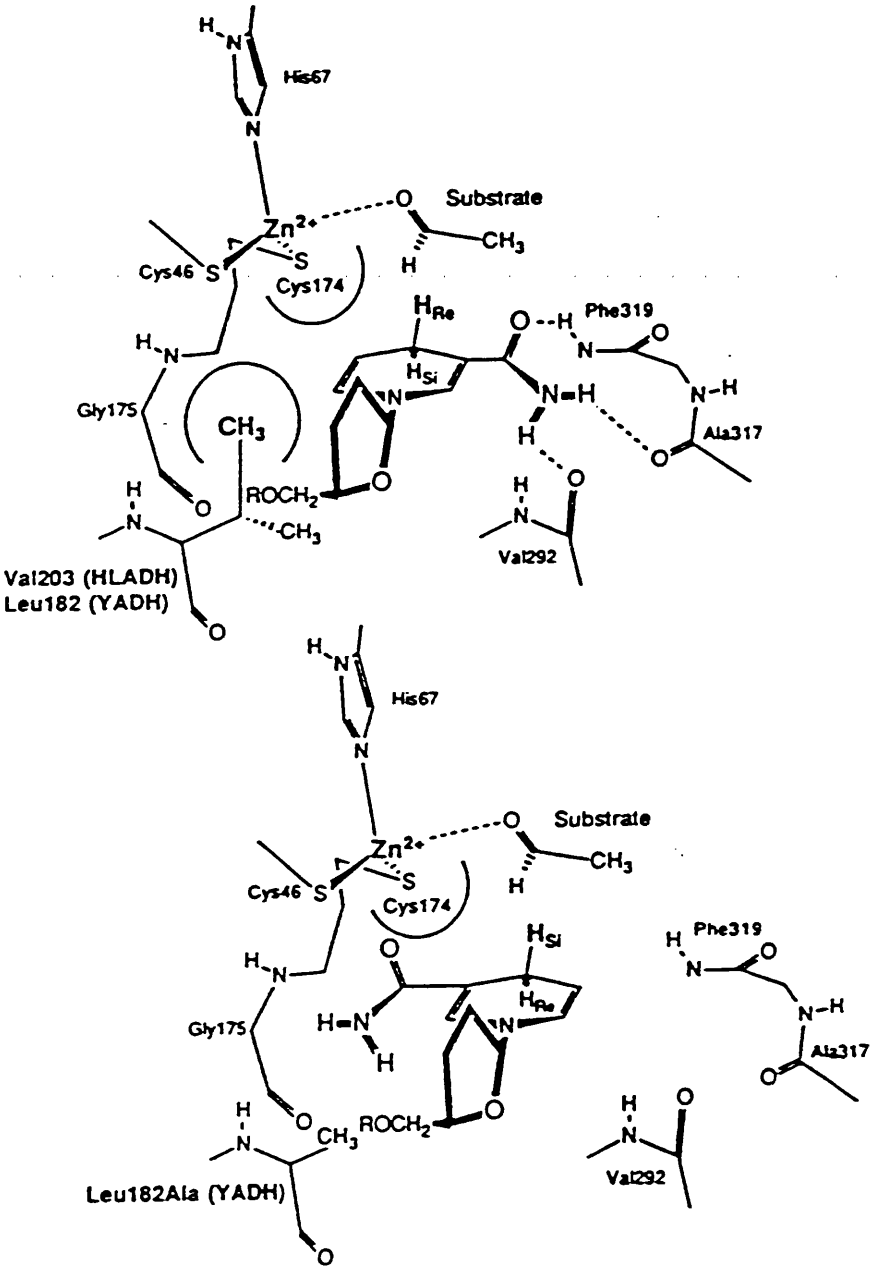
The abbreviations used are those quoted in table 1.1. The data contained in the table were taken from references listed: CBADH, Rifaat & Chen (1992); TBADH, Lamed & Zeikus. (1980;1981); EADH Lo & Reeves (1978); AEADH, Steinbüchel & Schlegal (1984), Jendrossek *et al.* (1988); BSADH, Sakoda & Imanaka (1992); ZMADHI Wills *et al* (1981); SADHI, Bennetzen & Hall (1982), Sun & Plapp (1992); SADHII, Russell *et al* .(1983), Sun & Plapp (1992); SADHIII, Young & Pilgrim (1985), Sun & Plapp (1992); KADHI, Shain *et al.* (1992), Saliola *et al* (1990); KADHII, Shain *et al.* (1992), Saliola *et al.* (1990); KADHIII, Saliola *et al.* (1990)I; KADHIV, Saliola *et al.* (1990); ANADHI, Creaser *et al.* (1985), Sun & Plapp (1992); ANADHIII, McKnight *et al.* (1985), Sun & Plapp (1992);SPADH, Russell & Hall (1983), Sun & Plapp (1992);HLADH, Brändén *et al.* (1975);TOL-BADH, Shaw & Harayama (1990). No evidence was found as to the quaternary structures of CBADH, EADH and BSADH.

Enzyme	Coenzyme Requirement	Quaternary structure
CBADH	NADP(H)	?
TBADH	NADP(H)	Tetrameric
EADH	NADP(H)	?
AEADH	NAD(H)	Tetrameric
BSADH	NAD(H)	?
ZMADHI	NAD(H)	Tetrameric
SADHI	NAD(H)	Tetrameric
SADHII	NAD(H)	Tetrameric
SADHIII	NAD(H)	Tetrameric
KADHI	NAD(H)	Tetrameric
KADHII	NAD(H)	Tetrameric
KADHIII	NAD(H)	Tetrameric
KADHIV	NAD(H)	Tetrameric
ANADHI	NAD(H)	Tetrameric
ANADHIII	NAD(H)	Tetrameric
SPADH	NAD(H)	Tetrameric
HLADH	NAD(H)	Dimeric
TOL-BADH (pWW0)	NAD(H)	Dimeric
ECTH	NAD(H)	Tetrameric

Figure 1.11.

Stereospecificity of coenzyme binding

Stereospecificity appears to be determined by conformation of the bound coenzyme. Numbers correspond to HLADH except where noted. The (Upper) normal cofiguration for NADH binding is in the *anti* conformation presenting the *Re* hydrogen to acetaldehyde above the cofactor. A 180° rotation around the glycosidic bond presents the opposite face with the *Si* hydrogen in the *syn* conformation (Lower) reversing stereospecificity. Thr-178 (Thr-157 in yeast) is not shown; The methyl group of this side chain lies below the left of the nicotinamide ring behind Val-203. (Taken form Weinhold *et al.*, 1991).



of a group (or groups) essential for catalysis. Weinhold *et al.* (1991) proposed that exchanging large residues in SADHI with smaller residues would remove the steric hindrance and allow binding of the coenzyme in either conformation. Leu-182 and Thr-157 of SADHI correspond to Val-203 and Thr-178 in HLADH. In the mutant enzymes, Leu-182 was replaced by Ala and resulted in an enzyme with 34% of its K_{cat} value and it bound the *syn* conformation of NAD(H). It makes a stereochemical "mistake" once every 850.000 turnovers (instead of one error every 700,000,000 turnovers in the native enzyme) by binding the coenzyme in the *anti* conformation. The double mutant in which Thr-157 was replaced by Ser also showed a decreased stereochemical fidelity, suggesting that stereospecificity is determined by the conformation of the nicotinamide ring of the coenzyme with respect to the adenosine moiety and the interactions at the active site.

The fact that these mutants still had catalytic activity, even though with decreased stereochemical fidelity, has obvious implications regarding ideas concerning why stereospecificity is so highly conserved in the ADHs. It raises questions about the hypothesis that stereospecificity is a frozen mistake and adds support to the notion that stereospecificity itself directly serves a selected function. The binding of the coenzyme is essential for catalysis as well as for the conformational change in HLADH. The conformational change increases the number of interactions formed between enzyme and coenzyme, optimising the binding of coenzyme (Eklund, 1988). For such a precise mechanism to work the steroespecificity of coenzyme binding is likely to have been directly selected for since binding of both *syn* and *anti* conformations is likely to be too loose and hence decrease catalytic activity.

In HLADH, a phosphate moiety of the coenzyme interacts with Arg-47, this interaction involves His-47 in SADHI. It is thought that Arg is the most suitable ligand for interaction with negatively charged groups because of its high *pK* value and its positive side chain. Replacement of His-47 with Arg-47 in SADHI (Gould & Plapp, 1990) did increase the strength of coenzyme binding in that the dissociation constants for NAD^+ and NADH decreased by factors of 4- and 2-fold respectively at pH 7.3 and turnover numbers decreased 4- and 6-fold for aldehyde reduction. Since the catalytic

process is thought to be an ordered one, limited by the rate of release of coenzyme, (Section 1.2.1.3) the observed decrease in coenzyme dissociation constants and decrease in turnover gives the expected outcome. The tighter binding of the coenzyme would account for the decrease in V_{max}. The change in magnitude of the rate constants suggest that the Arg-47 enzyme underwent a slower conformational change (believed to occur during catalysis), than the wild type, thus limiting turnover. The characterisation of the conformational change in HLADH has indicated that the loop containing residues 292-298 moves to accommodate residues 46-60 (Colonna-Cesari *et al.*, 1986). Arg-47 is flexible in the free enzyme but becomes fixed in its interaction with the pyrophosphate of coenzyme in the ternary complex. A substitution at this residue should have a pronounced affect on isomerisation process (Section 1.2.1.1).

Recently a trimeric aldehyde/alcohol oxidoreductase, of subunit size 39kD, has been isolated from *Amycolatopsis methanolica* and this enzyme has 56% identity with the *N*-terminus of HLADH. It is a nicotinoprotein which binds one NADH molecule per enzyme, permanently, as a cofactor and not as a coenzyme (van Ophem *et al.*, 1993). The oxidation of alcohols in assay conditions by the *A. methanolica* oxidoreductase is dependent on the presence of 4-nitroso-*N*,*N*-dimethylaniline (NDMA) and not the presence of NAD(P). After alcohol oxidation, it is thought that the internally bound NADH is regenerated through oxidation with NDMA and the enzyme does not directly communicate with the cystolic NAD/NADH pool (van Ophem *et al.*, 1993). It is possible that this oxidoreductase may be part of a complex which directly transfers its reducing equivalents to a component of the respiratory chain, as has been suggested with the NAD(P)-independent methanol dehydrogenase from *M. organophilum* (Section 1.5.1). Whether NDMA-dependent oxidoreductase forms one part of a complex is yet to be elucidated. There have been other nicotinoprotein identified which are are likely to be group III ADHs (Section 1.4.4).

1.2.3.2. Catalytic Zinc

The importance of the catalytic zinc ligands have been investigated in BSADH (Sakoda & Imanaka, 1992) by changing the ligand Cys-38 (corresponding to Cys-46 in HLADH) by site directed mutagenesis to a Ser (lacking a SH group but having an OH group). This enzyme had no catalytic activity, indicating that this residue is essential for activity in binding the catalytic zinc.

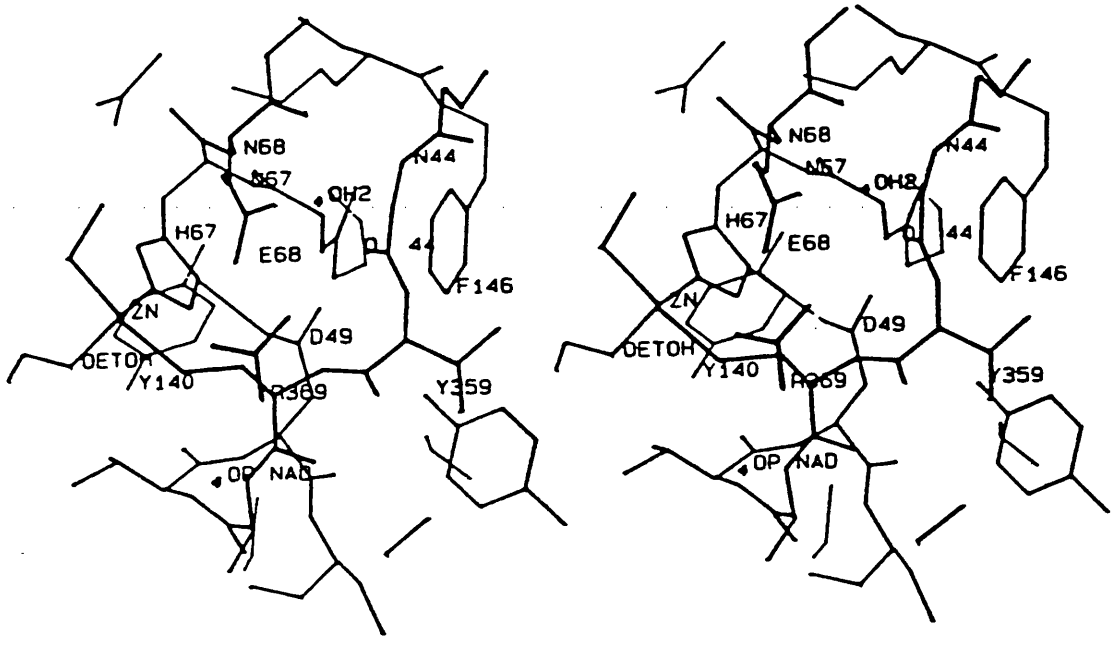
The conserved residues Asp-49 and Glu-68 are not directly ligated to the zinc but are second sphere ligands to the zinc, in that they interact with the zinc ligands themselves. The carboxyl group of Asp-49 forms a hydrogen bond with the imidazole group of the His-67 zinc ligand. The carboxyl group of Glu-68 has one oxygen bound to the the guanidino group of of Arg-369 by a hydrogen bond and the other oxygen is in close proximity to the zinc (Figure 1.12). The negative charges of these residues are believed to stabilise the metal by a polarising effect to facilitate hydride transfer. (Plapp*et al.*, 1990).

To investigate the importance of Asp-49, it was changed to Asn in SADHI so that there would no longer be an interaction with His-67 (Ganzhorn & Plapp, 1988). The Asn-49 mutant enzyme was stable and had essentially the same CD spectrum as the wild type enzyme. In another mutant, Glu-68 was replaced with Gln. Both Asn-49 and Gln-68 mutants showed a decrease in catalytic efficiency (V_{max}/K_m) of 1000 and 100-fold, respectively. The dissociation constant for NAD^+ in the Asn-49 mutant increased 7-fold where it increased by 4-fold in the Gln-68 mutant. It is thought that it is slow isomerisation of the ternary complex caused by lack of the carboxylate groups of Asp-49 and Glu-68 which accounts for the decrease in activity. The altered electrostatic environment in the mutant enzymes is thought to hinder the re-orientation of the residues in the isomerisation step. Although the 3-D structure of SADHI has not yet been solved, the conformational change is believed to follow similar structural changes to HLADH (Ganzhorn *et al.*, 1987).

Figure 1.12.

Stereo-diagram of the active site zinc atom and its environment in a model of yeast alcohol dehydrogenase

The model was based on the three dimensional structure of horse liver alcohol dehydrogenase complexed with NAD^+ and *p* -bromobenzyl alcohol by Eklund *et al.* (1982) The zinc is ligated by the sulphur atoms of Cys residues 46 and 174 and the imidazole group of His-67 and the hydroxyl of the ethanol substrate. Asp-49 is shown with its carboxylate hydrogen bonded to His-67, the carboxylate group of Glu-68 interacts with the guanidino group of Arg-369, which in turn binds an oxygen of the coenzyme pyrophosphate. (Taken from Ganzhorn & Plapp, 1988).



1.2.3.3. Substrate specificity

The substrate specificity of an enzyme is generally accepted to be determined by the size and shape of the reactive pocket. There are 12 differences in the residues lining the hydrophobic substrate binding pockets of HLADH and SADHI (Brändén *et al.*, 1975). These 12 differences can be assigned to three groups: (i) close to the substrate active site (ii) at the mouth of the pocket or (iii) in the walls of the pocket. HLADH is apparently able to oxidise larger alcohols because of the presence of smaller residues in the walls of the pocket compared to those found in SADHI (Brändén *et al.*, 1975). Creaser *et al.* (1990) generated a SADHI mutant in which Trp-93 and Thr-48 in were changed to Phe-93 and Ser-48 and also another mutant in which Ala-93 replaced Trp-93. Changing residues 93 and 48 to smaller residues allowed the oxidation of larger alcohols. Single and double mutants were made, containing Phe-93 and Ser-48 as well as Ala-93 and Ser-48. It has been shown that the ADHs containing Ser rather than Thr at position 48 are able to oxidise larger alcohols. The overall result of changes at positions 93 and 48 is that reducing the size of amino acids allows enhanced oxidation of alcohols larger than ethanol. Enlargement of the SADHI pocket allows oxidation of both optical isomers of 2-butanol or 2-octanol. The wild type SADHI will only oxidise one of the optical isomers. Dickenson & Dalziel, (1966) suggested that this was due to structural constraints in binding the L-isomer in the wild type SADHI. The simplest explanation for the observed increased activity is that the double mutants enlarged pockets remove these structural constraints (Creaser *et al.*, 1990).

Models of the yeast ternary complex indicate that the hydroxyl of the alcohol is linked to His-51 by a hydrogen bond through the hydroxyl group of Thr-48 forming a proton relay system in the ternary complex resulting in the formation of the zinc bound alcoholate ion (Sections 1.2.1.3 & 1.2.2.2). His-51 was replaced in two mutants by Gln and Glu, in order to investigate the role of this residue. (Plapp *et al.*, 1990). Gln and Glu were chosen because of their similar sized side chain to that of the His residue and they would therefore still form a hydrogen bond with the 2'-hydroxyl group of the nicotinamide ribose. However, Gln was unable to accept a proton and therefore unable

to participate in catalysis. Glu, however, could potentially accept a proton. Both mutants could bind the coenzyme as well as the wild type, if not better, because the dissociation constants for NAD^+ decreased 2-fold in the Gln-51 mutant and 12-fold in the Glu-51 mutant. In contrast to this, was the observation that the catalytic efficiencies (V_{max}/K_m) for ethanol oxidation were decreased 11-fold in the Gln-51 mutant and 65-fold in the Glu-51 mutant. These results suggest that the mutations hinder the deprotonation of the bound substrate. Glu has the greater effect, because of its negative charge opposing the development of a negative charge on the alcohol bound to the zinc. His-51, is maintained in all group I ADHs except the human classes II and III, and TOL-BADH (Section 1.2.2.2). Shaw *et al.* (1993) have suggested that the proton translocation system in TOL-BADH might be rearranged, connected to a water molecule or another general base. If the latter is the case the activity would be expected to be dependent on the pK_a of the external proton acceptor as was observed by the generation of a a Gln-51 HLADH mutant (Hurley *et al.*., 1990). However no dependency on the concentration and of the external proton acceptor for TOL-BADH activity was observed (Shaw *et al.*, 1993).

Residue 48 is also proposed to take part in the proton relay system, along with His-51 (Eklund *et al.*. 1982; Andersson *et al.*, 1981; Section 1.2.1.3). Ser-48 is conservatively replaced by Thr in all the microbial group I ADHs, except for TBADH, CBADH and EADH in which Ser is conserved. It has also been implicated in determination of substrate specificity (Creaser *et al.*, 1990). The importance of this residue in catalysis was spotlighted by the generation of SADHI mutants in which Thr-48 was replaced by Ala and Cys. The Ala-48 and Cys-48 mutants were found to have no detectable catalytic activity (Plapp *et al.*, 1990). This indicates that the presence of a hydroxyl group at position 48 for the proton relay system is essential in forming a productive ternary complex.

SADHI does not usually oxidise larger alcohols such as benzyl alcohol or cyclohexanol (Creaser *et al.*, 1990). However, changes at positions 93 and 48 to Phe/Ala and Ser respectively did not relieve this hindrance and allow oxidation of these larger alcohols. The changes at positions 93 and 48 were near the active site, at the

bottom of substrate binding pocket. Changes to widen the bottom of the pocket in order to attempt to allow larger substrates to bind may be ineffectual because of larger residues at the top of the pocket, blocking entry of large substrates. Plapp *et al.* (1987) suggest that Trp-57 at the top of the pocket in SADHI would protrude into the entrance of the active site.pocket and block entry of large alcohols. In HLADH, which is able to oxidise the large alcohols cyclohexanol and benzyl alcohol, a Leu residue (which has a smaller side chain than Trp) is located position 57, which could account for the access of larger alcohols to the active site (Creaser *et al.*, 1990).

Klinman (1976) found that a *S. cerevisiae* ADH would oxidise aromatic alcohols contradicting the evidence given above. The reason for the observed difference in catalytic activity can possibly be explained because commercially available yeast ADH was used as opposed to the SADHI enzyme cloned by Bennetzen and Hall (1982). There are five amino acid differences between the cloned SADHI and commercially available yeast ADH. One of these residue differences is Val-58 found in SADHI whereas in the commercially available yeast ADH a Thr residue is at position 58. It is thought that whereas the access of larger alcohols to the active site might be hindered by Val-58 in SADHI, this hindrance would be relieved by Thr-58 in commercially available yeast ADH. This hypothesis is yet to be investigated by site directed mutagenesis (Creaser *et al.*, 1990).

The isoenzymes SADHI and SADHII differ by only 23 amino acids out of 347 (Young & Pilgrim, 1985). Therefore it would be natural to assume that the differences seen in K_m values between SADHI and SADHII (Section 1.2.4) are a result of differences in the structures of the reactive pockets. SADHII has a K_m for ethanol which 20-fold lower than is the K_m of SADHI. The only difference between the residues which line the active site is that Met-294 in SADHI is replaced by Leu in SADH-II. A SADHI mutant produced with Leu-294 (Ganzhorn *et al.*, 1987) was found to have a K_m for ethanol which was unchanged. However, the catalytic activity (V_{max}/K_m) with butanol, pentanol and hexanol was increased 7- to 10-fold. This indicates that the residue at position 294 is in the active site and close enough to interact with butanol but not close enough to interact with the shorter chain substrate, ethanol. By replacing the larger Met

with the smaller Leu, longer chain substrates were able to gain access to the catalytic site. The unchanged K_m value for ethanol indicates that the differences of residues between SADHI and SADHII which do not line the active site must indirectly affect the binding affinity for ethanol as a substrate. Inspection of the three dimensional structure of SADHI based on that of HLADH leads to the hypothesis that the residue differences, other than those lining the active sight, might affect the conformational changes suggested to occur during coenzyme binding and catalysis and hence account for the different K_m values of SADHI and SADHII.

1.2.3.4. Structural zinc and quaternary structure

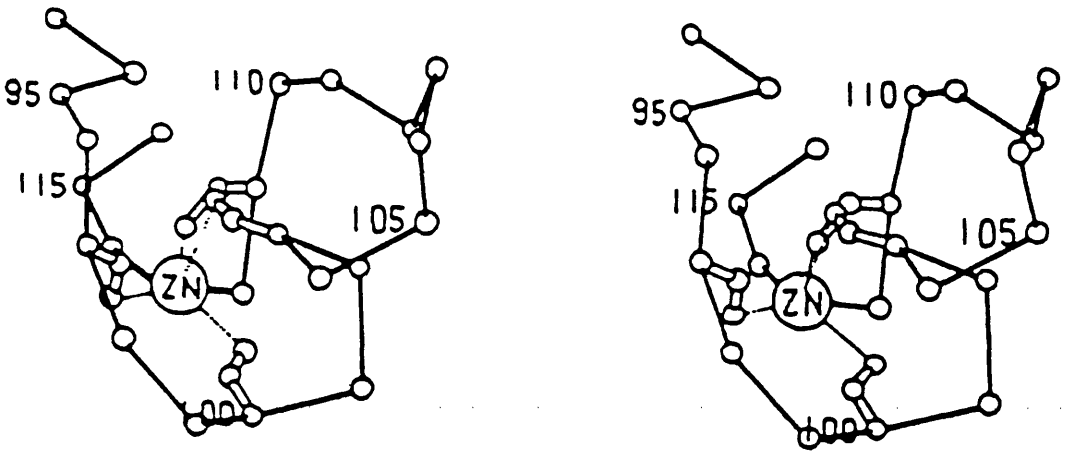
The structural zinc atom in HLADH is ligated by Cys-97, Cys-100, Cys103 and Cys-111 which part of a lobe (residues 95-113) projected out from the catalytic domain (Eklund *et al.*, 1976; Figure 1.13). This organisation of zinc ligands being within a short stretch of the polypeptide chain supports the idea that the role of the zinc is structural, since this pattern of zinc ligands is found in other proteins (Matthews *et al.*, 1974; Monaco *et al.*, 1978; Vallee & Auld, 1990; Kim & Wyckoff, 1991) whereas the ligands for the catalytic zinc atom are from different regions of the polypeptide chain. A similar pattern of the ligands to the structural zinc atom is found in the tetrahedral arrangement of Cys resides around the iron clusters in bacterial ferredoxins (Eklund *et al.*, 1976). The arrangement of these ligands follows the sequence pattern Xaa, Xaa+3, Xaa+6 and Xaa+N, where Xaa+N, the fourth ligand, is some distance away (Adman *et al.*, 1973). The similarity in structure may point to some relatedness between the bacterial ferredoxins and the long chain zinc dependent ADHs or, more likely, it could be a result of convergent evolution due to this conformation of ligands being energetically the most favourable.

The structural zinc ligands are conserved in alignment with HLADH in all microbial group I ADHs except CBADH, AEADH, TBADH, EADH, TOL-BADH and ECTH (Figure 1.9), although there are four Cys residues at positions 95, 98, 102 and 109 in TOL-BADH and in ECTH at positions 95, 98, 102 and 119 which follow a

Figure 1.13.

Stereo-diagram of the second structural zinc atom

Stereo diagram of the residues 95-113 showing the four Cys residues (Cys-97, Cys-100, Cys-103 and Cys-111) ligated to the structural zinc atom of HLADH. (Taken from Brändén *et al.*, 1975).



similar pattern to the structural zinc ligands and could easily bind a structural zinc atom (Shaw *et al.*, 1993). There is ambiguous evidence as whether or not microbial tetrameric enzymes, such as SADHI contain one or two zinc atoms per subunit (Veillon & Sytkowski, 1975; Klinman & Welsh, 1976). It has not yet been determined whether BSADH and ZMADHI contain one or two zinc atoms per subunit whereas the dimeric TOL-BADH has been identified as having two zincs per subunit (Shaw *et al.*, 1993). AEADH has been previously identified as having only one of the structural zinc ligands, Cys-97, and TBADH has none due to the deletion of an 18 residue stretch (96-113) corresponding to the region in which the zinc ligands are found (Sun & Plapp, 1992). In the progressive sequence alignment (Figure 1.9) this 18 residue gap is not present but there is a large gap running from residue 120 to 133 (corresponding to HLADH numbering) in the three NADP(H) dependent ADHs; TBADH, CBADH and EADH. There is a larger gap seen in 12 of the microbial ADHs corresponding to residues 115 to 137. This region is only conserved totally in AEADH and TOL-BADH, and partially in ECTH.

The tetrameric structural guinea pig eye lens ζ-crystallin has been proposed to be of common evolutionary history to the long chain zinc dependent ADHs although it lacks any catalytic activity (Borrás *et al.*, 1989; Sun & Plapp, 1992). Guinea pig eye lens ζ-crystallin lacks the region corresponding to residues 90 to 140 and hence lacks the ability to bind a structural zinc.

The loop formed by residues 113 to 128 in HLADH which lacks any notable secondary structure is suggested to be highly variable in its primary structure because of the lack of interaction with the rest of the protein. Borrás *et al.* (1989) proposed that the absence of the loop structure correlates with tetrameric structure and maintainance of it corresponds to dimeric structure. However, AEADH is tetrameric (Steinbuchel & Schlegel, 1984) and appears to maintain the loop structure (Figure 1.9) indicating that there is no definite correlation between the loop and quaternary stucture. Sheep liver sorbitol dehydrogenase dehydrogenase (a tetrameric enzyme) contains only one zinc atom per subunit but it contains all the necessary structural zinc ligands (Jeffery & Jörnvall, 1988), indicating there may be some relationship between quaternary structure

and ability to accommodate or even require a structural zinc atom, in contrast to the hypothesis that lack of a structural zinc atom correlates with tetrameric structure.

The quaternary structures and coenzyme requirements of the microbial group I ADHs are listed in table 1.4.

1.2.4. Isoenzymes and expression

HLADH exists in two forms, E and S isoenzymes, so denoted because of their different substrate specificities. Both E- and S-forms oxidise ethanol, but only the S-form oxidises 3-β-hydoxysteroids. The E isoenzyme was used in the primary structure and three-dimensional structural studies of Jörnvall (1970) and Eklund *et al.* (1976). The E and S subunits form heterodimers (Brändén *et al.*, 1975) indicating that their three-dimensional structures are similar. The primary structure of the S isoenzyme has been shown to differ from that of the E isoenzyme at 10 positions due to 9 residue substitutions and the deletion of Asp-115. This is brought about by 24 substitutions and 3 nucleotide deletions in the gene sequence (Park & Plapp, 1991; 1992). Leu -116 is thought to sterically hinder binding of steroids in the E isoenzyme, deletion of Asp-115 in the S isoenzyme relieves this hindrance.

Alcohol dehydrogenase isoenzymes are found in the yeasts *S. cerevisiae* , *K. lactis* and *Aspergillus nidulans*. These enzymes have different substrate specificities and are differentially expressed. related to the requirement of fermentation or oxidation of alcohols.

The three chromosomally encoded isoenzymes found in *S. cerevisiae* are differently expressed. Although SADHI and SADHII perform different metabolic roles, they do not differ greatly in their V_{max} values for both the forward and reverse reactions, but do differ in their affinity for ethanol as a substrate (Wills, 1976). SADHI is normally constitutive under laboratory conditions and has a high K_m ($1x10^{-5}$M) for ethanol. SADHII has a lower K_m ($1.4x10^{-7}$) for ethanol and is glucose repressible (Wills, 1976). SADHI is the fermentative cytoplasmic enzyme which, when the yeasts are grown on glucose under anaerobic conditions can account for up to 1% of cell

protein (Bennetzen & Hall, 1982). An opposing physiological role of ethanol oxidation is carried out by the SADHII which converts ethanol accumulated in anaerobic growth to acetaldehyde and it is expressed when ethanol is used as a carbon source (Wills, 1976). These two isoenzymes have 94% sequence identity (Table 1.3; Young & Pilgrim, 1985). It must be these few differences which account for the enzymes different catalytic specificities (Section 1.2.3.3).

The third *S. cerevisiae* isoenzyme, SADHIII, is mitochondrially located (Lutsdorf & Megnet, 1968) and has 79 and 78% amino acid identity with SADHI and SADHII, respectively (Table 1.3). It is proposed that a highly basic 27 amino acid leader sequence, not seen in SADHI or II, is responsible for mitochondrial targeting, and this leader sequence is proteolytically cleaved in the mitochondria to generate the mature enzyme. The function of this mitochondrial enzyme has not been identified although its mitochondrial location would indicate that it may have had a role in respiration. Cells lacking a functional SADHIII isoenzyme can survive aerobically or anaerobically (Wills & Phelps, 1975).

The fourth ADH found in *S. cerevisiae* (SADHIV) is an iron-activated enzyme and will be discussed in section 1.4.

The four isoenzymes expressed in *K. lactis* are all long chain zinc-dependent ADHs Both KADHI and KADHII have similar substrate specificity to that of SADHI. KADHI is thought to be the primary fermentative enzyme, which is preferentially expressed in yeasts grown on glucose (Saliola *et al.*, 1990). KADHII is expressed to what appears to be a lower level than KADHI when the yeasts are grown on both glucose and ethanol (Shain *et al.*, 1992). KADHIII and KADHIV are mitochondrially located (Saliola *et al.*, 1990; 1991) and are translated with a basic leader sequences, like SADHIII, to target them to the mitochondria. The two putative mitochondrial isoenzymes are thought to be responsible for the glucose-repressed ADH activities, which are essentially oxidative roles, opposing the fermentative activities of KADHI and KADHII (Saliola *et al.*, 1990).

When the substrate specificities of the four cytoplasmic yeast isoenzymes were compared, the most striking difference was that only SADHI was unable to oxidise long

chain alcohols that the others were able to use as substrates. Examination of the substrate binding cleft based on the HLADH structure, indicates that the only amino acid change which could account for the difference in substrate specificity is Met-294 which is substituted for Leu-294 in the other yeast ADHs (Shain *et al.*, 1992) The Met side chain is larger than that of Leu and this may prevent the binding of larger alcohols. However, the residues lining the substrate cleft cannot account completely for the different substrate specificities since the residues lining the the substrate binding pocket in SADHII and KADHI are identical, but their substrate specificities are not.

There are three ADHs in *A. nidulans*, ANADHI, ANADHII and ANADHIII. ANADHI and ANADHIII are isoenzymes (Gwynne *et al.*, 1987) The products of the *alcA* and*aldA* genes (which code for ANADHI and aldehyde dehydrogenases, respectively) are induced by ethanol, indicating that the role of the ANADHI is oxidative (Gwynne *et al.*, 1987). The expression of *alcA* and *aldA* have been shown to be under identical regulatory control by the *alcR* gene product. Mutations in the *alcR* gene lead to the pleiotropic loss of alcohol and aldehyde dehydrogenase activity (Pateman *et al.*, 1983).

The physiological role of the second ADH of *A. nidulans* (ANADHII) is unknown but it is repressed in the presence of ethanol, indicating that it may have an opposing role to that of ANADHI. Sealy-Lewis *et al.* (1984) have suggested that the *alcR* gene product functions as a repressor when ethanol is present. The expression of *alcA* , *aldA* and the gene encoding ANADHII are catabolite-repressed by the negative regulatory gene product from *creA* (Bailey & Arst, 1975), probably acting via repression of *alcR* expression. The isoenzyme, ANADHIII (McKnight *et al.*, 1985), is only partially repressed by the presence of glucose and is induced by ethanol, thus suggesting that like ANADHI it may also may have a fermentative role. Mcknight *et al.* (1985) have suggested that it may play some role in the detoxification of alcohols.

There have been two ADHs identified in the facultatively anaerobic bacterium Z. *mobilis* , ZMADHI and ZMADHII (Wills & Jörnvall, 1979; Wills *et al.*, 1981; Neale *et al.*, 1986). Primary structures of these enzymes have shown them to be genetically unrelated and to be members of separate ADH groups. ZMADHI is a member of the

group I ADHs and ZMADHII is a member of the group II ADHs (Keshav *et al.*, 1990; Conway *et al.*, 1987). Both enzymes are expressed during fermentative growth although the K_m value of ZMADHII for ethanol is high, ($1x10^{-1}M$) whereas the K_m value of acetaldehyde is only $1.5x10^{-3}M$, suggesting that its primary role is fermentative and not oxidative (Wills *et al.*, 1981) The rate of acetaldehyde reduction by ZMADHII is stimulated by ethanol accumulation, thereby counteracting any product inhibition or reverse reaction (Neale *et al.*, 1986). ZMADHI is inhibited by high levels of ethanol (Hoppner & Doelle, 1983),.but it has a lower K_m value for ethanol than ZMADHII ($1.7x10^{-3}M$) and its K_m value for acetaldehyde is $1.8x10^{-5}M$. It may have a role in the early stages of fermentation when acetaldehyde and ethanol concentrations are low and hence ZMADHII is not fully activated.

1.2.5. Evolutionary characteristics

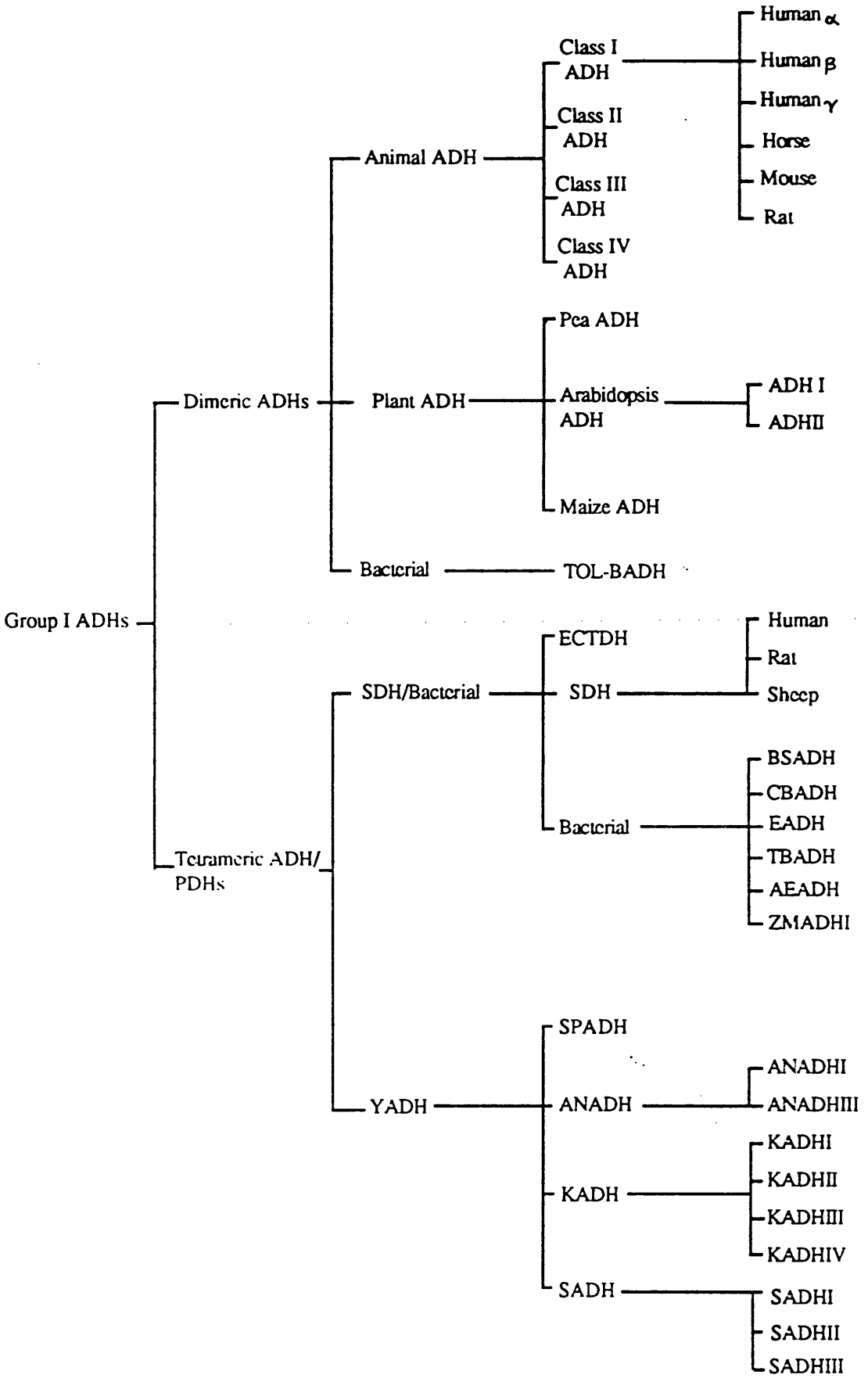
The group I ADHs contains highly divergent members including both structural proteins and enzymes. They have been classified into five sub groups by Jörnvall *et al.* (1987a): dimeric mammalian/plant ADHs, bacterial ADHs, tetrameric yeast ADHs, tetrameric polyol dehydrogenases and *E. coli* threonine dehydrogenase. (Figure 1.14). The mammalian/plant ADHs are further divided into four sub-classes, class I, II, III and IV Class I contains HLADH and the three human ADH subunit types α, β and γ. Class II contains two isoenzymes from maize and human ADH ϙ subunit. Class III contains avian ADH, the χ human ADH subunit and guinea pig eye lens ζ crystallin (Sun & Plapp, 1992). The recently discovered class IV is represented by human ADH6 (Yasunami *et al.*, 1991).

Characterisation of TOL-BADH by Shaw *et al.* (1993) have suggested that TOL-BADH pWW0 is more closely related to the mammalian/plant group of the group I ADHs. The sequence identity of TOL-BADH and HLADH with the other microbial ADHs (Table 1.3) indicates that they are of equal evolutionary distance from the microbial ADHs. The sequence alignment (Figure 1.9) helps to confirm the Shaw *et al.* (1993) observation. by aligning HLADH and TOL-BADH next to each to other at the bottom

Figure 1.14.

Evolutionary tree of the group I alcohol dehydrogenases

Proposed evolutionary tree of the group I ADHs based on information of Jörnvall *et al.*, 1987b, Sun & Plapp, 1992, Shaw *et al.*, 1993 and sequence comparisons in this study. Abbreviations: PDH, polyol dehydrogenase: SDH, sorbitol dehydrogenase: YADH, yeast alcohol dehydrogenase: ECTDH, *E. coli* threonine dehydrogenase. All other abbreviations are quoted in table 1.1.


Group I ADHs
Dimeric ADHs
Animal ADH
Class I ADH
Human α
Human β
Human γ
Horse
Mouse
Rat
Class II ADH
Class III ADH
Class IV ADH
Plant ADH
Pea ADH
Arabidopsis ADH
ADH I
ADHII
Maize ADH
Bacterial
TOL-BADH
Tetrameric ADH/ PDHs
SDH/Bacterial
ECTDH
SDH
Human
Rat
Sheep
Bacterial
BSADH
CBADH
EADH
TBADH
AEADH
ZMADHI
YADH
SPADH
ANADH
ANADHI
ANADHIII
KADH
KADHI
KADHII
KADHIII
KADHIV
SADH
SADHI
SADHII
SADHIII

(indicating they are the two least related sequences to the others of the alignment). Other evidence which aids this hypothesis is that both HLADH and TOL-BADH have dimeric quaternary structure compared to all other microbial group I ADHs which are tetrameric (Table 1.3). However, the evolutionary distance between TOL-BADH and mammalian/plant is much greater than the distance between the three mammalian/plant subclasses (Shaw *et al.*, 1993). The evolutionary grouping of TOL-BADH is unusual, in that analysis of the samilar protein sequences from different species generates an evolutionary tree that groups enzymes into phylogenic class; i.e. bacterial enzymes grouped together, mammalian enzymes grouped together etc. Anomalies such as a prokaryotic enzymes being grouped with mammalian/plant enzymes (Figure 1.14) suggests that horizontal gene transfer may have occurred (Smith *et al.*, 1992; Section 1.1.3).

The lack of His-51 in four functional group I ADHs, TOL-BADH, human class II π subunit, human class IV ADH6 and human class III χ, suggest that there is some proton release mechanism other than the proton-relay proposed by Eklund *et al.* (1982) and Andersson *et al.* (1981), (Section 1.2.1.3). Whether the catalytic mechanisms of these four dimeric enzymes operate in a similar fashion is yet to be deduced. It would be interesting to investigate whether these four enzymes are in any way more closely related to each other than to other group I ADHs.

The duplicatory steps by which long-chain zinc-dependent alcohol dehydrogenases might have evolved have been suggested by (Jörnvall *et al.*, 1987a). The first level of divergent duplication is that of the alcohol and polyol dehydrogenases. These two sub-groups now share about 25% identity. The second level of divergence gave the four classes of mammalian/plant long-chain zinc-dependent alcohol dehydrogenases; Class I the classical and most characterised (HLADH), class II (π), classIII(χ) (Vallee & Bazzone, 1983) and class IV (Yasunami *et al.*, 1991). These classes are defined on the basis of their differences in structure at the active site, subunit interactions, substrate specificity and the coordination of a structural zinc ligand, indicating that they are of separate enzyme class and not just isoenzymes (Jörnvall *et*

al.,1987a,b). The most recent level of divergence has resulted in the formation of isoenzymes, which have sequence identities of above 90% (Table 1.2).

NDMA-dependent oxidoreductase from *A. methanolicus* (Section 1.2.3.1) has a high *N* -terminal identity with HLADH (56%), which indicates that it is a member of the group I ADHs, although it is trimeric in structure and it is a nicotinoprotein. Another trimeric enzyme proposed to be a group I ADH on the basis of its 30% *N*-terminal identity with rat liver ADH is factor-dependent formaldehyde dehydrogenase, also from *A. methanolicus* (van Ophem *et al.*, 1992). However, the similarity in quaternary structure between the two *A. methanolicus* dehydrogenases is probably merely fortuitous; they are completely different in their catalytic and structural properties (van Ophem *et al.*, 1993). It could be suggested that NDMA-dependent oxidoreductase is a member of a nicotinoprotein group I ADH sub-group because of its striking catalytic differences from the other group I ADHs (Section 1.2.3.1). However, this suggestion of a sub-grouping cannot be confirmed until the complete NDMA-dependent oxidoreductase amino acid sequence is elucidated and at least one other example of such a sub-group found. Preliminary evidence suggests that another enzyme perhaps related to the group I ADHs is formaldehyde dismutase, a novel NAD-binding oxidoreductase from *P. putida* F61 (Kato *et al* ., 1986). Formaldehyde dismutase is composed of four identical subunits with a M_r of 44kD. Each subunit contains 2 zinc atoms and 1 NAD(H) molecule. The enzyme bound NAD(H) is reduced and oxidised only when alcohol and aldehyde substrates are added, respectively. Like NDMA-dependent oxidoreductase the redox state of the enzyme bound NAD(H) is not effected by exogenous electron acceptors such as NAD(H) (Kato *et al.*, 1986). Other examples of nicotinoproteins are may belong to the group III ADHs (Section 1.4.4).

The comparison of primary and quaternary structure of the microbial group I ADHs clearly demonstrates that these enzymes are structurally related. The identity and conservative replacement of residues extends throughout the whole length of the polypeptide chains. The relationships between structural genes encoding isoenzymes, especially those of the yeasts, help to provide a model for evolutionary events. Shain *et al.* (1992) have proposed an evolutionary model which accounts for the similarities

amongst the yeast group I ADHs (Figure 1.15). Shain *et al.* (1992) suggested that an ancestral gene coded for a cytoplasmically encoded ADH which functioned fermentatively before the appearance of oxygen in the atmosphere. Once oxygen appeared the ADH took on an oxidative role as well. Speciation occurred when *S. pombe* split in evolutionary terms, from the ancestor of *S. cerevisiae* and *K. lactis* and the ADH of *S. pombe* remained relatively unchanged in its current structure. However, the ADH systems in *S. cerevisiae* and *K. lactis* became highly complex in regulation and function. Firstly the gene was thought to duplicate and acquire a hydrophobic leader sequence resulting in mitochondrial targeting. So now there was the situation that one enzyme was located in the cytoplasm and the other in the mitochondria. This is thought to have occurred before speciation because both *S. cerevisiae* and *K lactis* have mitochondrially located ADHs.For this event to occur independently is not an impossibility but it is unlikely, especially because of the similarities observed between mitochondrial enzymes and their putative leader sequences (Figure 1.16). Following this duplication and separation into the two yeast species is suggested to have occurred with the cytoplasmic and mitochondrial coding genes both duplicating in *K. lactis*. The oxidative roles for the two mitochondrial isoenzymes in *K. lactis* (KADHIII and KADHIV) were maintained, whereas the putative oxidative role of the mitochondrial isoenzyme in *S. cerevisiae* was superseded by the duplication of the gene encoding the cytoplasmic ADH. This duplication event resulted in two cytoplasmically located isoenzymes with opposing physiological roles of fermentation and oxidation. The fermentative roles were taken over KADHI and KADHII in *K. lactis* and SADHI *S. cerevisiae*. The different physiological roles of the cytoplasmic isoenzymes indicates that these enzymes evolved separately, developing different strategies in their alcohol metabolism. *K. lactis* appears to keep oxidation and fermentation separate whereas *S. cerevisiae* maintains both in the cytoplasm (Shain *et al.*, 1992).

The high conservation of nucleotide sequence between SADHI and SADHII is probably due to a non-reciprocal cross over event. Between nucleotides 193 and 431 there is a difference of only two nucleotides, indicating that this event is fairly recent (Figure 1.17). The rate of evolutionary divergence must be calculated outside this

Figure 1.15.

Evolutionary tree of the group I yeast alcohol dehydrogenases

Evolutionary tree depicting the branching order among the ADHs from *S. cerevisiae*, *Kluyveromyces lactis* and *S. pombe* . Numbers represent average nucleotide differences with the 360 terminal nucleotides of KADHII. (Taken from Shain *et al.*, 1992).

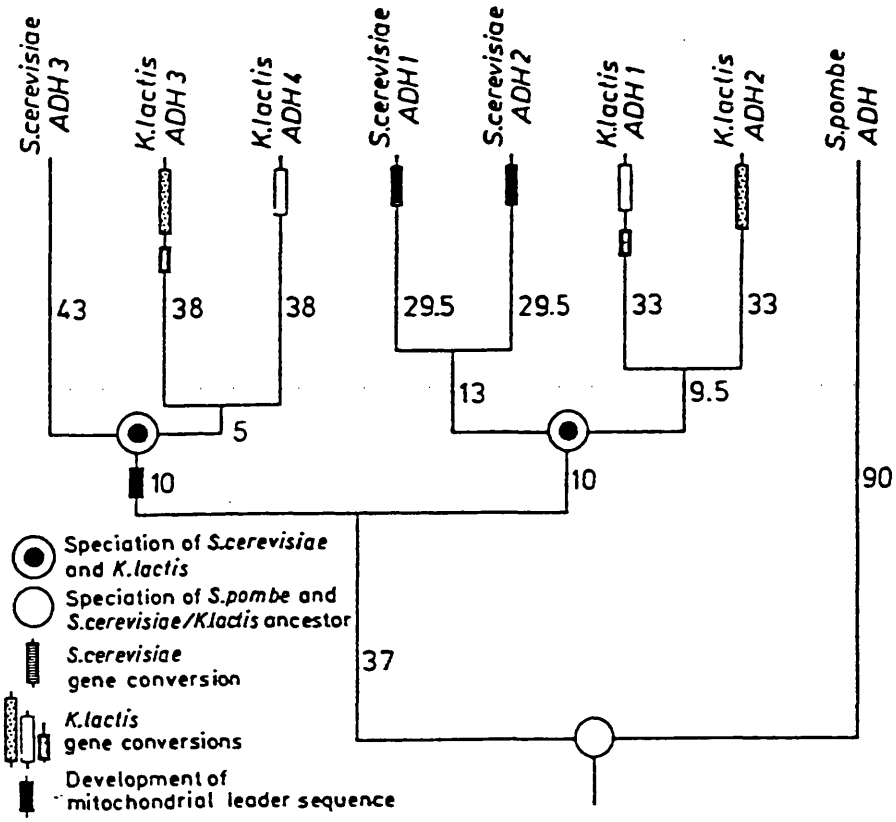


Figure 1.16.

The leader sequences of the mitochondrially located group I alcohol dehydrogenases from *S. cerevisiae* and *K. lactis*

Similarities seen amongst the mitochondrially located group I alcohol dehydrogenases from *S. cerevisiae* and *K. lactis* . (Adapted from Shain *et al.*,1992).1, refers to the start of the mature polypeptide. Symbols: *, conserved residues; +, chemically conserved residues.

```
-29                                   1
MLRTSTLFTRRVQPSLFSRNILRLQST-AA
MLRLTSARSIVSPLRKGAFGSIRTLAT---
MFRLARAQTALANKASVSRSFLRLNSS-FA
*-*   + +       +     *   +
```

Figure 1.17.

Nucleotide sequence alignment between genes coding for isoenzymes SADHI and SADHII from *S. cerevisiae*

The nucleotide sequence comparison shows the 117 nucleotides differences between the two genes and shows the region of proposed non-reciprocal genetic exchange between nucleotides 193 and 431: a region which only differs by two nucleotides. The termination codon is indicated by ***.(Adapted from Russell *et al.*, 1983).

```
SADHII ATGTCTATTCCAGAAACTCAAAAAGCCATTATCTTCTACGAATCCAACGGCAAGTTGGAGCATAA 65
SADHI          C                 GTG                 C    T A     A C
       GGATATCCCAGTTCCAAAGCCAAAGCCCAACGAATTGTTAATCAACGTCAAGTACTCTGGTGTCT 130
             T                 G             G        T               T
       GCCACACCGATTTGCACGCTTGGCATGGTGACTGGCCATTGCCAACTAAGTTACCATTAGTTGGT 195
        T     T C              C                   GT    C          C
       GGTCACGAAGGTGCCGGTGTCGTTGTCGGCATGGGTGAAAACGTTAAGGGCTGGAAGATCGGTGA 260

       CTACGCCGGTATCAAATGGTTGAACGGTTCTTGTATGGCCTGTGAATACTGTGAATTGGGTAACG 325

       AATCCAACTGTCCTCACGCGTGACTTGTCAGGTTACACCCACGACGGTTCTTTCCAAGAATACGC 390
                                T                                C
       TACCGCTGACGCTGTTCAAGCCGCTCACATTCCTCAAGGTACTGACTTGGCTGAAGTCGCGCCAA 455
                                                C        CC       C C
       TCTTGTGTGCTGGTATCACCGTATACAAGGCTTTGAAGTCTGCCAACTTGAGAGCAGGCCACTGG 520
                           C                         T       TG C T T
       AGCGGCCATTTCTGGTGCTGCTGGTGGTCTAGGTTCTTTGGCTGTTCAATATGCTAAGGCGATGG 585
         TT        C        C                              C C     T
       GTTACAGAGTCTTAGGTATTGATGGTGGTCCAGGAAAGGAAGAATTGTTTACCTCGCTCGGTGGT 650
                    G        C      GA   T           A  C GA  CA
       GAAGTATTCATCGACTTCACCAAAGAGAAGGACATTGTTAGCGCAGTCGTTAAGGCTACCAACGG 715
            C     T        T  G  A           CG T  T  TC A     C  TG
       CGGTGCCCACGGTATCATCAATGTTTCCGTTTCCGAAGCCGCTATCGAAGCTTCTACCAGATACT 780
       T     T      G       C                          T                G
       GTAGGGCGAACGGTACTGTTGTCTTGGTTGGTTTGCCAGCCGGTGCAAAGTGCTCCTCTGATGTC 845
       T   A  T        CACC  T     C   A       T     C     T GT
       TTCAACCACGTTGTCAAGTCTATCTCCATTGTCGGCTCTTACGTGGGGAACAGAGCTGATACCAG 910
             A  C        C     T     T  T        C  T        C  C
       AGAAGCCTTAGATTTCTTTGCCAGAGGTCTAGTCAAGTCTCCAATAAAGGTAGTTGGCTTATCCA 975
            T  G  C     C         T G              C     T  C     G  T
       GTTTACCAGAAATTTACGAAAAGATGGAGAAGGGCCAAATTGCTGGTAGATACGTTGTTGACACT
       1040
       CC  G                        A     T     C T
       TCTAAATAA
            ***
```

region. However it does give some indication of high sequence identity previously since this cross over event was allowed to occur.

1.3. The group II alcohol dehydrogenases

The group II, or short chain, alcohol dehydrogenase family is not as well characterised as group I. The first two members to be identified were the alcohol dehydrogenase from *Drosophila melanoganster* (Thatcher, 1980) and ribitol dehydrogenase from *Klebsiella aerogenes* (Jörnvall *et al.*, 1984). However, over the past few years there has been a great expansion in the known members of this group which now numbers over 20. Group II ADHs have been isolated mainly from *Drosophila* sp. and prokaryotes but recent studies have identified them in mammalian liver (Stolz *et al.*, 1991), placenta (Krook *et al.*, 1990; Peltoketo *et al.*, 1988) and other tissues (Marks *et al.*, 1992). Not all of the NAD(P)-dependent enzymes of this group that have been identified are alcohol dehydrogenases; rather they have wide range of substrate specificities and and metabolic roles. The group II ADHs include members as diverse as: glucose and hydroxysteroid dehydrogenases (Jany *et al.*, 1984; Yin *et al.*, 1991), acetoacetyl-CoA and keto-acetyl reductases (Peoples & Sinskey, 1989; Sherman *et al.*, 1989), polyol sugar dehydrogenases (Jörnvall *et al.*, 1981; Yamada & Saier, 1987), proteins whose function are unknown (Navre & Ringold, 1988; Thony *et al.*, 1987) and a nodulation protein (Debelle & Sharma, 1986). The active enzymes are dimers or tetramers, each subunit size is approximately 27kDa with around 250 residues in the polypeptide chain, apart from human 17β-hydroxysteroid which is 327 residues long (Peltoketo *et al.*, 1988). There is no evidence for a metal requirement in any of the enzymes so far characterised.

1.3.1. Conserved residues

There are six strictly conserved residues in all group II ADHs that have been sequenced (Persson *et al.*, 1991). These are Gly-14, Gly-19 and Gly-132 (numbering based on *D. melanoganster*) which are believed to have structural roles, and Asp-64, Tyr-152 and Lys-156. In addition to the strictly conserved residues there are a further seven residues conserved in all but one or two members. These are: Thr-13, Gly-17, Asp-87, Ala-93, Gly-94, Asn-114, and Ser-139. As in other related protein groups, Gly residues

Table 1.5.

Microbial group II alcohol dehydrogenases

Enzyme	Organism	Abbreviation
20β-Hydoxysteroid dehydrogenase	*Streptomyces hydrogenas*	2βDH
Glucose dehydrogenase	*Bacillus megateruim*	GLDH
Acatoacetyl-CoA reductase	*Alcaligenes eutrophus*	AEDH
2,3-Dihydro-2,3-dihydoxybenzoate dehydrogenase	*Escherichia coli*	DBDH
3β-Hydroxysteroid dehydrogenase	*Pseudomonas testosteroni*	3βDH
Ribitol dehydrogenase	*Klebsiella aerogenes*	RIDH
1,2-Dihydroxycyclohexa-3,5-diene carboxylate dehydrogenase	*Acinetobacter calcoaceticus*	ABDH
Cis -toluene dihydrodiol dehydrogenase	*Pseudomonas putida*	PTDH
Sorbitol-6-phosphate dehydrogenase	*Escherichia coli*	SPDH
Biphenyl dihydrodiol dehydrogenase	*Pseudomonas pseudoalcaligenes*	BPDH

are the most conserved because of their importance in bend forming for the tertiary structure.

1.3.2.Secondary structure predictions

The secondary structure of the first short chain alcohol dehydrogenase to be determined, *D. melanoganster* alcohol dehydrogenase, was predicted by Thatcher and Sawyer (1980) using a Chou & Fasman (1974) type analysis. The predicted structure obtained was that of an alternating βαβ pattern in the *N* -terminal half of the molecule, suggesting the existence of a nucleotide-binding domain (Rossman *et al.*, 1974; Richardson, 1981). This evidence was also strengthened by the observation of a Gly-Xaa-Xaa-Gly-Xaa-Gly fingerprint region [found in many NAD(H) binding domains (Wierenga *et al.*, 1985)] from position 14 to 19. However, when aligned with other members of the group II alcohol dehydrogenases (Persson *et al.*, 1991) this pattern is conserved as Gly-Xaa-Xaa-Xaa-Gly-Xaa-Gly in all but sorbitol-6-phosphate dehydrogenase from *E. coli* , the NodG protein from *Rhizobium meliloti* (Yamada & Saier, 1987; Debelle & Sharma, 1986) in which neither fingerprint sequence is conserved. This is because in the *D. melanoganster* ADH sequence described by Persson *et al.*, (1991) there is a deletion at position 15 relative to the other aligned sequences (a position within the fingerprint region). At this position in all other sequences this is a residue with a small side chain (Gly or Ala) except in ribitol dehydrogenase (Dothie *et al.*, 1985), glucose dehydrogenase (Jany *et al.*, 1984) and in 17β-hydroxysteroid dehydrogenase (Peltoketo *et al.*, 1988) in which it is Ser, Ser and Cys respectively. Small residues at this point may still allow, or partly allow, the conformation needed by this fingerprint pattern for coenzyme binding.

The location of the predicted coenzyme binding site contrasts with that of the long chain alcohol dehydrogenases where the coenzyme binding site is found in the *C*-terminal portion of the polypeptide. Observations of the coenzyme binding site being *N*-terminally located were confirmed using far-u.v. circular dichroism measurements (Benyajati *et al.*, 1981; Ribas de Pouplana *et al.*, 1991). Secondary structure prediction of 7-hydroxysteroid dehydrogenase *Eubacterium sp.* strain VPI12708 by Baron *et al.* (1991),

using the method of Garnier *et al*. (1978), has also confirmed that the *N*-terminal region contains alternate β sheet structures similar to those predicted in the *D. melanoganster* alcohol dehydrogenase and hence it appears to be a general feature of the group II alcohol dehydrogenases that the coenzyme binding domain is *N*-terminally located.

Prediction of *D. melanoganster* ADH secondary structure by Benyajati *et al.* (1981) concerning the percentage of α–helix in the protein gave results which conflicted with those presented by Ribas de Pouplana *et al.* (1991) and Thatcher & Sawyer (1980). Benyajati *et al.* (1981) predicted the enzyme to have 50% α–helix structure. Whereas, using the circular dichroism measurement and a Chou & Fasman analysis, Ribas de Pouplana *et al.* (1991) and Thatcher & Sawyer (1980) predicted approximately 29% α–helix. The reasons for this discrepancy in secondary structure prediction remains unclear but it may be a result of differences in primary sequence, since Benyajati *et al.* (1981) predicted a primary structure for which differed for 25 residues out of 251 to that published by Thatcher (1980).

1.3.3. Group II alcohol dehydrogenase secondary and tertiary structure

Preliminary work has been done to solve the structure of structure of ADH from *D. melanoganster* by Gordon *et al.* (1992). However, the best group II ADH three-dimensional structure currently available is that of the tetrameric 3α,20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans*, which has been solved to a resolution of 2.6Å (Ghosh *et al.*, 1991; Figure 1.18). The subunit consists of a single domain, unlike the long chain zinc-dependent dehydrogenases, and it contains a β-sheet generated by seven parallel strands with three parallel α-helices on either side (Figure 1.19). These structures are named βA-G and αB-G (Figure 1.19).The coenzyme binding site is generated by the structures in the *N* -terminal half of the polypeptide, as was predicted for the alcohol dehydrogenase from *D. melanoganster* (Benyajati *et al.*, 1981; Ribas de Pouplana *et al.*, 1991), the βA-αB-βA-αC-βC fold being characteristic of NAD(H) binding regions. The tight turn of βA-αB is facilitated by Gly residues in the fold area. The NAD(H) binding cleft is formed by the amino-terminal ends of α-helices B, C and D

Figure 1.18.

Stereo-diagram of the three dimensional structure of 3α,20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans*

Numbering based on 3α,20β -hydroxysteroid dehydrogenase (Taken from Ghosh *et al.*, 1991).

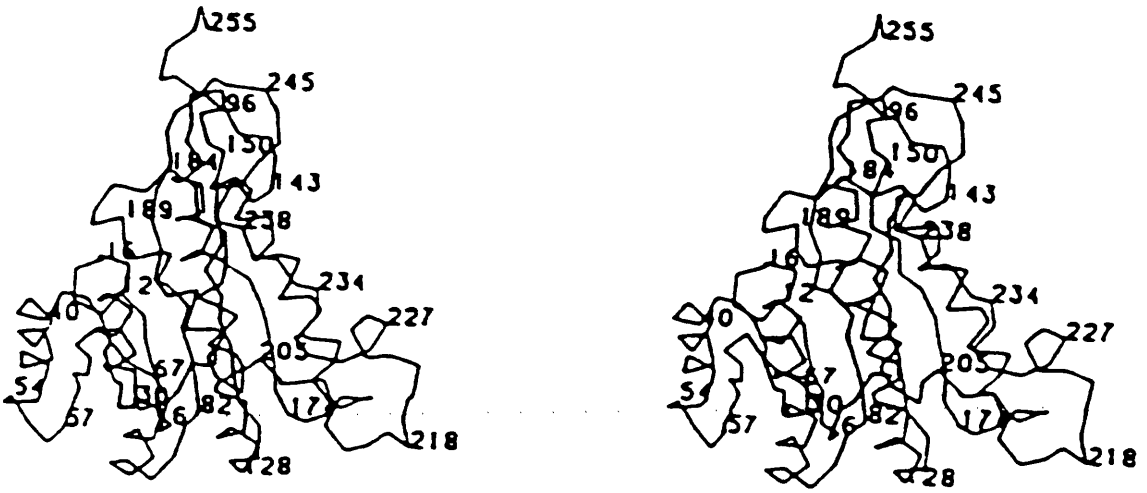
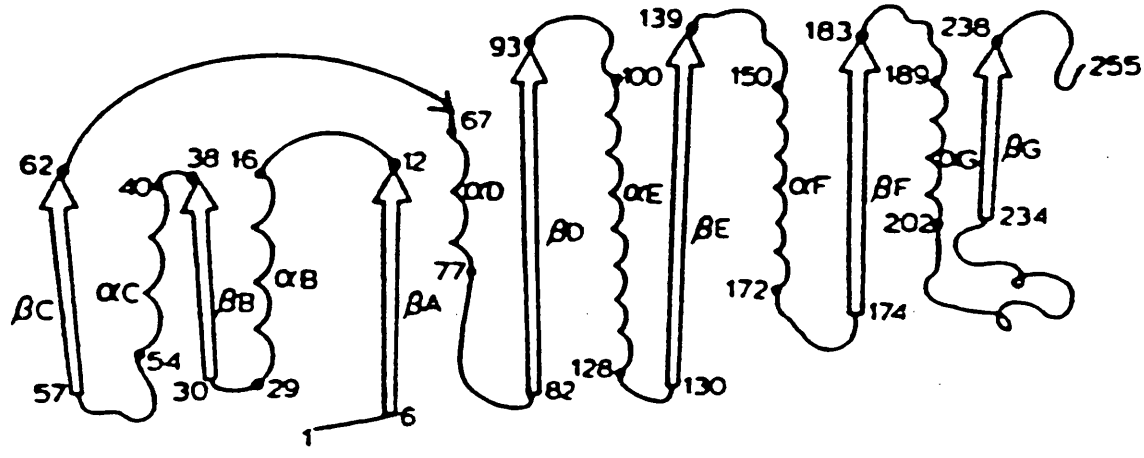


Figure 1.19.

The secondary structure of 3α,20β-hydroxysteroid dehydrogenase from *S. hydrogenans*

Representation of the secondary structure made up from alternating 7 β-strands and 6 α–helix structures. The NAD(H) binding cleft is made up from the amino termini of α–helices B, C and D and carboxyl termini of β-strands A, B and C. Numbering based on 3α,20β -hydroxysteroid dehydrogenase. (Taken from Ghosh *et al.*, 1991).



and the *C*-terminal ends of β-strands A, B and C (Figure 1.20). The carboxamide group of the NAD(H) is close enough to the side chain of Asp-37 (corresponding to Asp-38 in *D. melanoganster* ADH) to form a hydrogen bond. The highly conserved Thr-12 (corresponding to Thr-13 in *D. melanoganster*) adjacent to the strictly conserved Gly-13, may be involved in hydrogen bonding to the ribose moiety of the coenzyme, similar to that in the conserved threonine ferredoxin-NADP(H) reductase (Karplus *et al.*, 1991). Arg-16, corresponding to Gly -16 in *D. melanoganster* ADH, is also in close contact with the 4-carbon of the nicotinamide ring and may be involved in coenzyme binding. Site directed mutagenesis of Gly-14 to Asp of *Drosophila* alcohol dehydrogenase (Thatcher, 1980) has been seen to affect the affinity of the enzyme for the coenzyme. This indicates that Gly-14 is not essential for direct interaction with the coenzyme but it is probably important in forming the correct conformation of fold to bind the coenzyme.

The adenine moiety of the cofactor is sandwiched between Trp-67 and Ile-117. Other charged side chains seen in the coenzyme binding region are residues Asp-109 and Glu-65, corresponding to Lys-112 and Ile-69 in *D. melanoganster*. Persson *et al.* (1991) noted that Asp-38 was conserved in over half of the group II ADH members aligned. Asp-38 was thought to correlate to the choice of coenzyme, NAD(H) or NADP(H). However, the conservation of Asp-38 does not exactly correlate with the choice of NAD(H) as opposed to NADP(H), as the conservation of Asp-223 does in the group I ADHs. Hence it is unlikely that Asp-38 is responsible for the interaction with the 2' and 3' hydroxyl group of the adenosine ribose in the same way that Asp-223 is in HLADH. Asp-64 is strictly conserved and is therefore is also unlikely to play a role in coenzyme binding specificity, because of differences of coenzyme requirement among the group II ADHs (Table 1.6). The maintainance of Asp-87, or of other highly conserved negatively charged residues, in the coenzyme binding region might correlate with coenzyme specificity. In contrast to the binding specificity of the group I ADHs, sequence analysis of the group II ADHs so far shows no clear pattern in residue conservations determining the choice of coenzyme. This ambiguity is exemplified by the use of both NADP(H) and NAD(H) by the glucose dehydrogenase of *Bacillus megaterium* which has only 16% lower rate of oxidation when using $NADP^+$ compared to NAD^+ (Pauly & Pfliederer, 1975). This coenzyme ambiguity is also seen in 3β-hydroxysteroid dehydrogenase from

Figure 1.20.

Stereo-diagram of the coenzyme binding region of 3α,20β-hydroxysteroid dehydrogenase from *S. hydrogenans*

The protein environment of the bound NAD(H) molecule in the *C* -terminal region of the subunit. Numbering based on 3α,20β-hydroxysteroid dehydrogenase. (Taken from Ghosh *et al.*, 1991).

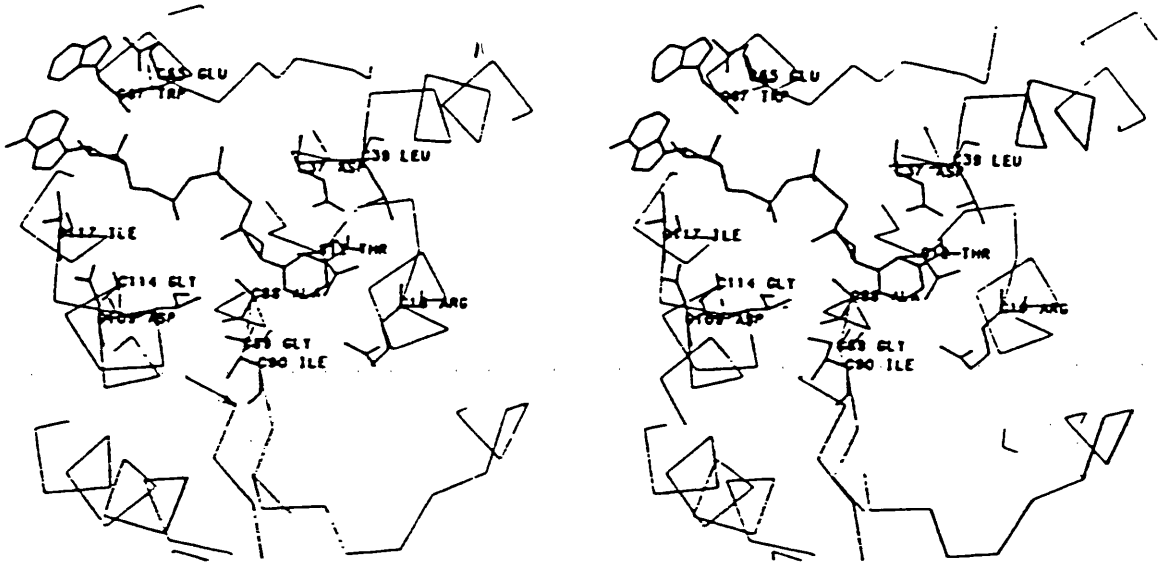


Table 1.6.

Coenzyme specificity of the microbial group II alcohol dehydrogenases

Information taken from: Ghosh *et al.*, 1991; Pauly & Pfeilderer; 1975, Peoples *et al.*, 1989; Liu *et al.*, 1989; Maser *et al.*, 1992; Taylor *et al.*, 1992; Neidle *et al.*, 1992; Irie *et al.*, 1987; Yamada *et al.*, 1987. Abbreviations used are those quoted in table 1.5.

Enzyme	Coenzyme requirement
2βDH	NAD(H)
GLDH	NAD(H) & NADP(H)
AEDH	NAD(H)
DBDH	NAD(H)
3βDH	NAD(H) & NADP(H)
RIDH	NAD(H)
ABDH	NAD(H)
PTDH	NAD(H)
SPDH	NAD(H)

P. testosteroni which has only an 11% lower V_{max} when using NADPH compared with NADH and similar K_m values of 0.64 and 0.56 mM respectively (Maser *et al.*, 1992; Table 1.6).

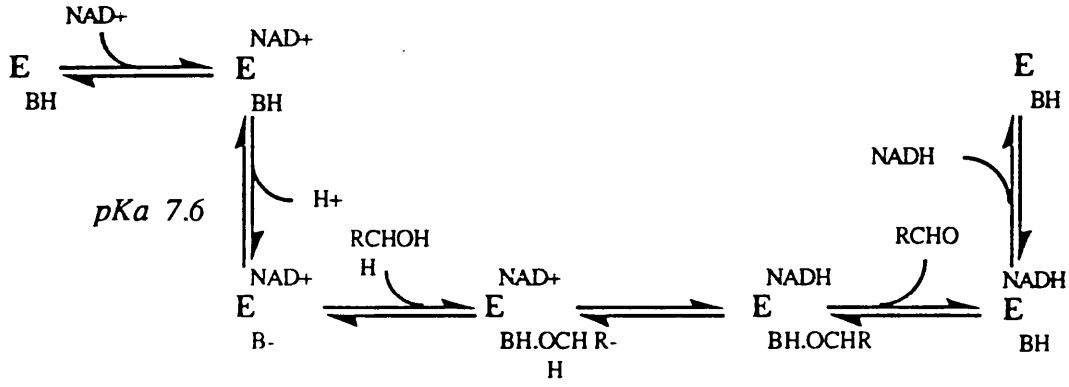
1.3.4. Catalytic mechanism

Oxidation of alcohols by the *D. melanoganster* alcohol dehydrogenase involves an ordered mechanism with cofactor binding first. Then subsequent binding of substrate requires the ionisation of a group of pK_a 7.6 in the binary enzyme-coenzyme complex (Mc Kinley-McKee *et al.*, 1991; Figure 1.21). The ionisation of the pK_a 7.6 group in the binary complex is thought to be an essential step required for formation of alcohol-enzyme-NAD^+ ternary complex. It is this ionisation step which is thought to be responsible for the pH dependence of coenzyme and alcohol binding. The binding of aldehyde as substrate is unaffected by pH. Proton release is associated with the formation of the binary complex and no proton release is seen with ternary-complex formation. It is therefore assumed that the proton release is from the ionisable group pK_a 7.6 and its release induced by the perturbation of the pK_a by binding of coenzyme, in a similar way to that which occurs in HLADH. Release of NAD^+ from the binary complex requires the protonation of the pK_a 7.6 group. The binding of alcohol is dependent on the ionisation of this group, indicating that it interacts directly with the substrate holding it in position to facilitate hydride transfer. The unprotonated form of the pK_a 7.6 group is thought to stabilise the ternary complex and may act as a nucleophilic catalyst for hydride transfer by increasing the negative charge on the bound substrate (Mc Kinley-McKee *et al.*, 1991).The ionisable group has been identified as most likely to be Tyr-152 (Krook *et al.*, 1990; Marekov *et al.*.1990; Ensor & Tai, 1991). Evidence of a charged group at the active site (Winberg & McKinley-McKee, 1988) did not, at first, indicate Tyr since this amino acid is only charged under certain circumstances; however, alteration of ionisation states can be caused by a conformational change as seen in the group I ADHs when coenzyme binds. The importance of the catalytic role of Tyr-151 in human placental 15-hydroxyprostaglandin dehydrogenase (corresponding to Tyr-152 in *D. melanoganster*) was determined by Ensor & Tai (1991) by replacing Tyr-151 with Ala, which resulted in

Figure 1.21.

Reaction scheme of the group II alcohol dehydrogenases

The mechanism proposed for *Drosophila* ADH catalysis. E denotes the enzyme and BH represents an ionising enzymic group tentatively identified as Tyr-152. (Taken from McKinley-McKee *et al.*, 1991).



removal of all catalytic activity. Cys residues 135 and 218 in *D. melanoganster* ADH, have been shown by site directed mutagenesis to have no catalytic relevance (Chen *et al.*, 1990).

Other resides believed to take part in the catalytic mechanism because of their location in the protein and putative ability to form interactions with the substrate have been identified by Persson *et al.*, (1991). Lys-156 is conserved in all sequences available, and Ser-139, is conserved in all except ribitol and glucose dehydrogenase which have Val residues corresponding to this position (Dothie *et al.*, 1985; Jany *et al.*, 1984). These residues are located in regions of hydrophilicity and higher than average residue conservation between the aligned sequences and with a predicted defined secondary structure (Persson *et al.*, 1991).

The affinity labelling of the active site 20β–hydroxysteroid dehydrogenase (Sweet & Samant, 1980) indicated that Asp and Glu are in some way involved in the catalytic mechanism. Asp-87 is highly conserved in the sequences and so could be expected to be involved in the catalytic mechanism and would be a good target for future site directed mutagenesis studies (Niedle *et al.*, 1992). However, there is no indication for a highly conserved Glu in the group II ADHs at this position and so may be important in determining the substrate specificity in 20β–hydroxysteroid dehydrogenase and not in the catalytic mechanism itself.

The active sites of the group II enzymes are likely to vary widely in their shape and size because of this groups' wide substrate specificity, although the catalytic mechanism is probably common to all. Ghosh *et al.*, (1991) identified the active site as a deep cleft situated around the centre of the tetramer, comprised of residues from all subunits. This is consistent with the observation that quaternary structure formation is essential for activity (Carrea *et al.*, 1984).

Interactions with the cortisone substrate in 3α,20β-hydroxysteroid dehydrogenase, are proposed to involve Tyr-152, Ser-139, Thr-185 and Asp-227 (corresponding to Gly-232 in *D. melanoganster*). No mention is made of Glu residues or of Asp-87 and Lys-156 by Ghosh *et al.* (1991), apart from the fact that the latter it is present in lining the active site cleft.

When bound to the enzyme, the coenzymes' nicotinamide B-face is open to the

cleft. A novel mechanism for hydride transfer between the substrate and the nicotinamide 4-carbon was suggested to be mediated, directly or indirectly, by Arg-16 (Ghosh *et al.*, 1991). It is a novel mechanism in so far as there are no previous reports of an Arg residue being involved in a hydride transfer mechanism. It is suggested that there is a conformational change associated with binding the substrate, such that the Arg residue relocates to allow the coenzyme to be in much closer proximity to the substrate so that hydride transfer may occur directly. However, Arg-16 is not highly conserved (Niedle *et al.*, 1992) so until site-directed mutagenesis has been carried out on all the key residues proposed to be involved in formation of the ternary complex and the catalytic mechanism the functional significance of each will remain undefined.

1.3.5. Evolutionary characteristics

The extent of amino acid identity between the group II microbial ADHs ranges from 18% to 58%, but is typically around 25% (Table 1.7).These identities are clustered, indicating regions of similar functional and/or structural importance such as the coenzyme binding region and active site. Neidle *et al.* (1992) suggests that the wide distribution of this group of enzymes among many different organisms might be accounted for in two alternative ways: (i) The group II enzymes were present as an ancestral protein before separation of the prokaryotic and eukaryotic kingdoms and strong selective pressure kept the identity above 20%. (ii) The proteins of this group have spread horizontally through gene transfer between prokaryotes and eukaryotes. Evolution of the group II of ADHs might have involved horizontal gene transfer but it is unlikely to be solely responsible since gene transfer and evolution from a common ancestor are not mutually exclusive (Section 1.1.3).

Table 1.7.

The percentage amino acid sequence identity amongst microbial group II dehydrogenases.

Taken from Niedle *et al.* (1992). Abbreviations are those quoted in table 1.5.

	% IDENTITY									
	2βDH	GLDH	AEDH	DBDH	3βDH	RIDH	ABDH	PTDH	SPDH	BPDH
2βDH	100	27	22	22	30	21	22	35	22	25
GLDH		100	29	26	26	23	26	23	22	24
AEDH			100	23	23	19	22	21	22	21
DBDH				100	26	25	20	22	20	23
3βDH					100	25	20	27	24	25
RIDH						100	20	20	18	23
ABDH							100	23	23	22
PTDH								100	23	59
SPDH									100	21
BPDH										100

1.4. The group III alcohol dehydrogenases

All members of the group III, or iron activated ADHs, that have so far been identified on the basis of their primary structure and subunit size (approximately 385 residues) are all microbial. The first enzyme of this group to be sequenced was the *adhB* gene product from *Z. mobilis* which encodes alcohol dehydrogenase II (ZMADHII: Conway *et al.*, 1987). Once the sequence for the fourth alcohol dehydrogenase isoenzyme (ADHIV) from *S. cerevisiae* has been shown to have 53% identity with ZMADHII (Williamson & Paquin, 1987), it was proposed that they constituted a new group of ADHs, independent of the other established groups. Other members since identified include a NAD(H)-dependent propanediol oxidoreductase from *E. coli* (ECPOR: Conway & Ingram, 1989), NADP(H)-dependent butanol dehydrogenase from *Clostridium acetobutylicum* (CABDH: Youngleson *et al.*, 1989), methanol dehydrogenase from thermotolerant *Bacillus methanolicus* C1, (BMDH: de Vries *et al.*, 1992), a fermentative alcohol dehydrogenase from *E.coli* (ECADH: Goodlove *et al.*, 1989; Clark, 1992). The glycerol dehydrogenase from *Bacillus stearothermophilus* (BSGDH) has limited identity with the type III alcohol dehydrogenases and it may also be member of this group (Mallinder *et al.*, 1992; Table 1.9).

1.4.1. Substrate specificity

ZMADHII is very selective in its substrate specificity. In contrast to ZMADHI, which will oxidise a wider range of substrates, it is active only towards ethanol and shows little activity towards *n* -propanol and allyl alcohol. It does not oxidise methanol or butanol (Wills *et al.*, 1981). It can be "activated" to both reduce acetaldehyde and oxidise ethanol by ethanol itself (in contrast to ZMADHI which is inhibited by high levels of ethanol) and so counteracts any possibility of product inhibition during fermentation (Neale *et al.*, 1986).

Gram-negative methylotrophic usually employ a pyrroloquinoline quinone (PQQ)-containing methanol dehydrogenase for methanol oxidation (Section 1.5.). The methanol

Table 1.8.

Group III microbial alcohol dehydrogenases

Enzyme	Organism	Abbreviation
Alcohol dehydrogenase II	*Zymomonas mobilis*	ZMADHII
Alcohol dehydrogenase IV	*Saccharomyces cerevisiae*	ADHIV
Methanol dehydrogenase	*Bacillus methanolicus*	BMDH
Propanediol dehydrogenase	*Escherichia coli*	ECPOR
Alcohol dehydrogenase	*Escherichia coli*	ECADH
Butan-1-ol dehydrogenase	*Clostridium acetobutylicum*	CABDH
Glycerol dehydrogenase	*Bacillus stearothermophilus*	BSGDH

dehydrogenase from *B.methanolicus* C1 is unusual in that it is a NAD(H)-dependent enzyme which functions as a methanol oxidising enzyme, enabling growth on methanol as sole carbon source (Arfman *et al.*, 1989).

The NADP(H)-dependent butanol dehydrogenase from *C. acetobutylicum* has a wide range of substrate specificity in that it oxidises ethanol, propanol and butanol (Youngleson *et al.*, 1988). This enzyme is distinct from the other enzymes in its specificity for NADP(H) over NAD(H) and because it is involved in production of butan-1-ol as a major fermentation product (Gottschalk, 1979).

The ADHIV from *S. cerevisiae* has a K_m value of 16.7mM for ethanol (Drewke & Ciriacy, 1988). ADHIV also oxidises *n* -propanol but at only approximately 70% of the rate of oxidation of ethanol. The activity seen with *n* -butanol and *n* -pentanol is very low, 2% and 1% that of the rate with ethanol respectively (Drewke & Ciriacy, 1988). It is interesting to note that the percentage identity of 53% (Table 1.9) between ADHIV and ZMADHII is the highest amongst the type III alcohol dehydrogenases, and yet their respective substrate specificities are so different.

Propanediol oxidoreductase from *E. coli* is involved in the fermentation of deoxysugars. It oxidises 1,2-propanediol, glycerol, ethylene glycol, 1,3-propanediol, and ethylene glycol.(Sridhara *et al.*, 1969).

The *adhE* gene encodes the bifunctional fermentative alcohol/acetaldehyde dehydrogenase from *E. coli*. It is a highly expressed enzyme and is able to oxidise both 2- and 3-carbon substrates such as ethanol and *n* -propanol (Clark, 1992).

Glycerol dehydrogenase from *B. stearothermophilus* var.*non-diastaticus* has 25% identity over the entire length of the polypeptide with *C. acetobutylicum* NADP-dependent ADH and 18% identity with ZMADHII. The deduced amino acid sequence indicates that it is extremely similar to the NAD(H)dependent glycerol dehydrogenase from *B. stearothermophilus* RS93 (Spencer *et al.*, 1990).

1.4.2. Secondary structure of the group III alcohol dehydrogenases

There is currently no information about secondary and tertiary structure available for

any of the enzymes of this group, but attempts have been made by Youngleson *et al* ., (1989) to predict the secondary structures of CABDH, ADHIV and ZMADHII using Chou and Fasman analysis (1974) and hydropathy profiles (Kyte & Doolittle, 1982). Secondary structure analysis indicated that the iron activated enzymes are α-helix rich, averaging 53% α-helix: whereas the long chain, zinc-dependent and the short chain, zinc-independent enzymes contain roughly equal amounts of α-helix and β-pleated sheet structures. Hydropathy profiles of the three proteins predicted that there was a major hydrophilic region between residues 230 and 240, indicating that this region would be found on the surface of the native structure (Youngleson *et al.*, 1989). This region has also been predicted to have extended α-helical structures containing four conserved His residues, which may be involved in the binding of the metal (Youngleson *et al.*, 1989).

There have been 54 strictly conserved and 41 conservatively replaced residues identified in the six members of this group (excluding BSGDH) by sequence alignment (de Vries *et al* ., 1992: Figure 1.22a). Rather few residues are conserved in the *N* - terminal region as compared to the rest of the sequence. There are five highly conserved regions later in the sequence, at positions 93-103, 137-145, 182-201, 248-279 and 355-365 (numbering based on the BMDH). When BSGDH is included in the alignment, 22 residues are strictly conserved, indicating that BSGDH is a more distant relation to the other group III ADHs.

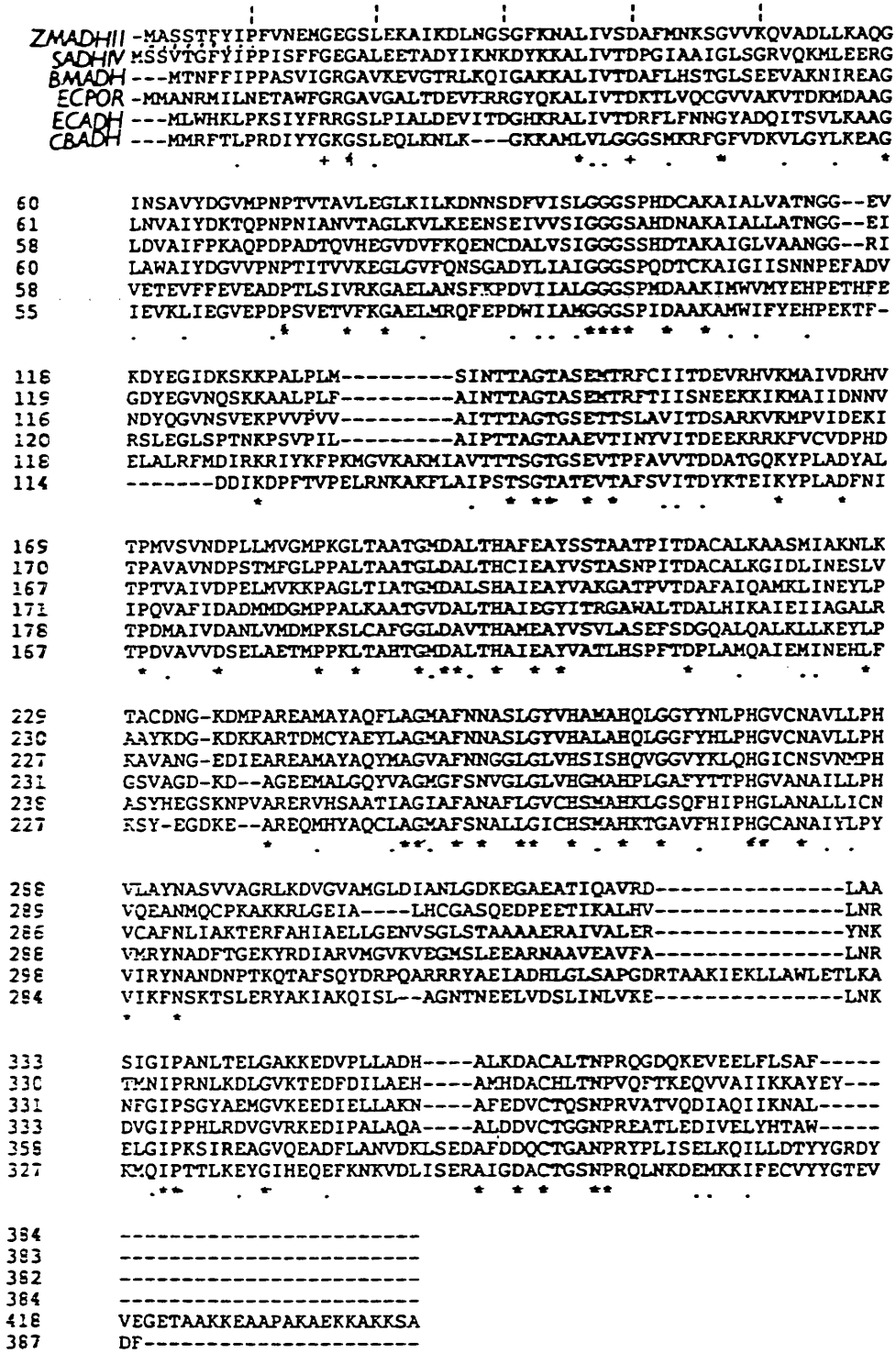
The subunit size for the group III ADHs is approximately 40 kDa except for the bifunctional fermentative alcohol dehydrogenase from *E. coli* which is a 96 kDa protein of 891 residues, being much larger that the members of the group III alcohol dehydrogenases (Goodlove *et al.*, 1989). The *C*-terminal third of the enzyme shows strong identity to the other iron activated alcohol dehydrogenases (Clark, 1992) and it has been shown to require ferrous ions for activity (Kessler *et al.*, 1991). It is thought that this gene may be the evolutionary result of fusion of separate genes encoding alcohol dehydrogenase and acetaldehyde dehydrogenase activities since the size of the enzyme is equivalent to the combined length of a typical iron activated ADH and aldehyde dehydrogenase (Clark. 1992).

Native quaternary structures of the group III enzymes differ. *S. cerevisiae* ADHIV

Figure 1.22a.

Primary sequence alignment of the group III alcohol dehydrogenases

Alignments of ZMADHII, SADHIV, BMADH, ECPOR, ECADH (*C*-terminal region of the protein coded for by the *adhE* gene starting at residue 449) and CBADH. Glycerol dehydrogenase from *Bacillus stearothermophilus* is not included. Symbols: *, exact match across all sequences; ·, conservative matches across all sequences; +, the *N*-terminal sequence, delimited by this symbol, resembles a βαβ-dinucleotide-binding fold. (Taken from deVries *et al.*, 1992).



and the fermentative bifunctional enzyme from *E. coli* are dimeric (Drewke & Ciriacy, 1988; Goodlove *et al.*, 1989). *Z. mobilis* ADHII is a tetramer (Wills *et al.*, 1981; Neale *et al.*, 1986) and the methanol dehydrogenase from *Bacillus* sp. C1 is a homo-decamer of approximately 430 kDa in its native state (Vonck *et al.*, 1991). The quaternary structures for the butanol dehydrogenase from *C. acetobutylicum*, glycerol dehydrogenase from *B. stearothermophilus* var *non-diastaticus* and the *E. coli* propane diol oxidoreductase have not yet been determined. However, the structure for the glycerol dehydrogenase from *B. stearothermophilus* RS93 is homo-tetrameric, approximately 180 kDa in its native state (Spencer *et al.*, 1989). This enzyme is very similar to glycerol dehydrogenase from *B. stearothermophilus* var *non-diastaticus* (Mallinder *et al.*, 1992).

1.4.3. Metal requirement

The presence of iron has been unambiguously identified only in ZMADHII using atomic absorption studies (Scopes, 1983; Neale *et al.*, 1986; Tse *et al.*, 1989). The Fe^{2+} can be replaced by Co^{2+}) to generate a more stable and still active form (Kintoshita *et al* ., 1985), but when replaced by Mn^{2+}, Ni^{2+}, Zn^{2+} or Cu^{2+} the enzyme is inactive (Tse *et al.*, 1989; Bakshi *et al.*, 1989). This inhibition by zinc was also reported by Neale *et al.* (1986). Spectroscopic and magnetic resolution investigations suggest that the native enzyme contains a six, co-ordinate ferrous binding site with three or four histidine ligands and the other ligands are most likely to be H_2O, Asp, Glu or Tyr residues (Bakshi *et al.*, 1989). Three histidine residues suggested to be involved in metal binding (Jörnvall *et al.*, 1987b) are conserved between residues 250 and 280 (Youngleson *et al.*, 1989; de Vries *et al.*, 1992). The requirement of Fe^{2+} by the bifunctional fermentative alcohol dehydrogenase from *E. coli* for all redox activity has been demonstrated by Kessler *et al.*, (1991).

Bacillus methanolicus C1 methanol dehydrogenase binds one zinc and one to two magnesium ions per subunit, but no iron (Vonck *et al.*, 1991). Activation of ADHIV by addition of Zn^{2+} and inhibition by Fe^{2+} has been reported by Drewke & Ciriacy (1988). However, it appears that the binding of zinc may be of a lesser magnitude than that of the

group I ADHs, since inactivation using EDTA requires a 10-fold higher concentration of EDTA to bring about similar levels of inhibition in the group I ADHs from *S. cerevisiae* compared to ADHIV (Drewke & Ciriacy, 1988).

The metal requirement of the glycerol dehydrogenase of *B. stearothermophilus* var. *non-diastaticus* has yet to be investigated (Mallinder *et al.*, 1992). However the glycerol dehydrogenase from *B. stearothermophilus* RS93 has been shown to require Zn^{2+} for activity (Spencer *et al.*, 1989).

It appears that ferrous and manganese ions re-activate propanediol oxidoreductase from *E. coli* whereas zinc causes what appears to cause complete inactivation of the enzyme (Sridhara *et al.*, 1969).

It thus appears, from current data, that there are different requirement for metal ions amongst the group III ADHs. In fact they are not, as their other name suggests, all iron activated, but rather they are activated by a range of divalent metal ions. Whether the metal ions are directly involved at the catalytic site, or in some other structural function, will have to be determined by X-ray crystallography, nuclear magnetic resonance and site directed mutagenesis.

1.4.4. Coenzyme binding

The NAD(H) binding fingerprint pattern (Gly-Xaa-Gly-Xaa-Xaa-Gly: Wierenga *et al.*, 1985) was identified only in *E. coli* propanediol oxidoreductase (de Vries *et al.*, 1992) and neither NADP(H) binding fingerprint region (Gly-Xaa-Gly-Xaa-Xaa-Ala: Scrutton *et al.*, 1990) or NAD(H) binding fingerprint region were identified in *S. cerevisiae* ADHIV, *Z. mobilis* ADHII or *C. acetobutylicum* (Youngleson *et al.*, 1989). The *N* -terminal regions in ADHIV, fermentative ADH from *E. coli* and BMDH exhibit limited resemblance to a βαβ-dinucleotide binding fold (Scrutton *et al.*, 1990), which is proposed to form part of a coenzyme-binding region. In contrast, it was predicted on the basis of primary sequence analysis that the leading β-peptide loop might well be absent from *E. coli* propanediol oxidoreductase, *Z. mobilis* ADHII and *C. acetobutylicum* butanol dehydrogenase (de Vries *et al.*, 1992).

Two nicotinoproteins, 4-nitroso-*N*,*N*-dimethlanaline- (NDMA-) dependent oxidoreductases, have been isolated and shown to be similar to BMADH. The NDMA-dependent methanol dehydrogenases from *Mycobacterium gastri* and *A. methanolicus* are both decameric, with subunit molecular weights of 50 and 49 kDa respectively, and partial amino acid sequences obtained share a high degree of identity with BMADH (Bystrykh *et al.*, 1993; Figure 1.22b). As previously mentioned with the group I nicotinoprotein aldehyde/alcohol oxidoreducase (Section 1.2.3.1), no alcohol dependent reduction of NAD(P) coenzyme in solution could be detected (Bystrykh *et al* ., 1993). The high sequence identity between BMADH and the two nicotinoproteins (Figure 1.22b) suggests that there may be evidence for a nicotinoprotein sub-group of the group III ADHs, as has been suggested with the group I ADHs (Section 1.2.5). This proposal will only be confirmed when the complete amino acid structures for the NDMA-dependent methanol dehydrogenases from *M. gastri* and *A. methanolicus* are completely determined.

1.4.5. Catalytic activity

The activity of *B. methanolicus* C1 methanol dehydrogenase relies upon the interaction of Mg^{2+} and a dimeric activator protein of native M_r =50 kDa (Arfman *et al.*, 1991), although Mg^{2+} is needed only for the oxidation of methanol and not for the reduction of formaldehyde (Arfman *et al.*, 1989). In the absence of the activator protein, the the enzyme has a low k_{cat}/k_m similar to the k_{cat}/k_m value for HLADH with methanol as a substrate. However, in the absence of activator protein BMDH has a 500-fold lower k_{cat}/k_m value for ethanol than that of HLADH (Sheehan *et al.*, 1988). In the presence of the activator, BMDHs methanol oxidising activity is raised 35- to 65-fold and its ethanol oxidising activity (k_{cat}/k_m) is raised 16-fold (Arfman *et al.*, 1991). The activator apparently raises the V_{max} and causes a slight drop in the K_m value. The properties of the enzyme are best explained by biphasic kinetics, indicating that the activator interaction is independent of the catalytic mechanism. Arfman *et al.* (1991) has suggested the activator acts fairly transiently as a release mechanism for NADH or formaldehyde. This transient interaction is suggested because there is an 18-fold molar excess of BMDH over the

Figure 1.22b

Alignment of nicotinoproteins and BMADH *N*-terminal and internal peptides

Two nicotinoproteins from *A. methanolica* (AMADH) and *M. gastri* (MGADH) with the group III ADH, BMADH. Symbols: *, conserved residues; +, conservative substitutions. Numbering based on BMADH. (Taken from Bystrykh *et al.*, 1993).

```
                                          1         5         10
                                          |         |         |
BMADH                                     M T N F F I P P A S V I G R
AMADH     A Q V D E L W K P F P I K E F H P F A R A L L G P
MGADH   A I E L N Q I W - D F P I K E F H P F P(R)A L L G V
          + + + + *     * * +   + *       +   + + + + *

        15        20        25        30        35
        |         |         |         |         |
BMADH   G A V K E V G T R L K Q I G A K K A L I V T D
AMADH   G A H(D)M I I P(D K L D Q)? F K(K A L V V)T ?
MGADH   G A(W D)I(A)G V L A(K)N L G F K(D)T L(L M G)D
        * *                   +   *   *   + * + + + *

        123               130                 140
        |                 |                   |
BMADH   S V E K P V V P V V A I T T T A G T G S E
AMADH   S E N P R N P P H I A V S T T A G T G S E
MGADH           Q N P P H I A V S T T A G(G)G S E
        *   -             *   + * + + * * * * + * * *

        237   240                     250
        |     |                       |
BMADH   A R E A M A Y A Q Y M A G V A F N N
MGADH   A R E G M X N A Q Y I A G Q A F N S
        * * * - *     * * * + * *   * * *

        361
        |
BMADH   T Q S N P R
AMADH(V)E S N P K
MGADH   V E S N P K
          + * * * +
```

activator protein in cells grown in methanol limiting conditions (Arfman *et al.*, 1992).

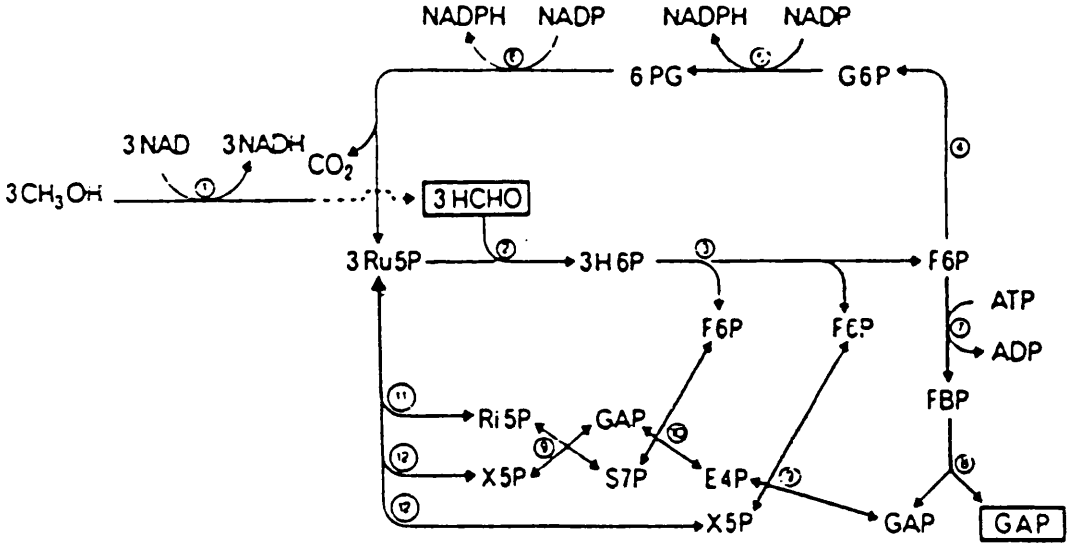
The *B. methanolicus* C1 methanol dehydrogenase is distinct from the other members of the group III ADHs in that it plays a major oxidative role. Carbon assimilation in methylotrophic *Bacillus* strains occurs by fixation of formaldehyde via the Ribulose monophosphate (RuMP) cycle (Roitosch & Stolp, 1985; Arfman *et al.*, 1989; Figure 1.23). The methanol dehydrogenase activity is ensured over that of formaldehyde reductase activity, by high levels of hexulose-6-phosphate synthase, the key enzyme in the RuMP cycle. Hexulose-6-phosphate synthase expression is probably induced by high concentrations of formaldehyde in order to protect against its toxic build up (Arfman *et al.*, 1992). The maintainance of a high internal pH also helps to pull the equilibrium towards the formation of NADH and formaldehyde (Arfman *et al.*, 1989). Cells with high BMDH activity and low hexulose-6-phosphate synthase activity immediately accumulate toxic formaldehyde when subjected to a rise in methanol (Arfman *et al.*, 1992). During growth on ethanol, cells have high BMDH activity, but there is no evidence for any additional ADH isoenzyme, therefore indicating that BMDH is also responsible for both ethanol and methanol oxidation (Arfman *et al.*, 1992).

Z. mobilis is obligately fermentative. Approximately 95% of the sugars that it ferments are converted to ethanol and carbon dioxide. ZMADHII is the dominant enzyme in fermentation, compared with the ZMADHII isoenzyme, accounting for 90% of the organisms ethanol oxidising activity (Neale *et al.*, 1986). The gene encoding ZMADHII is constitutively expressed and its activity is controlled by the presence of different amounts of available metal ions (MacKenzie *et al.*, 1989b). Growth under iron-limiting conditions leads to lowered ZMADHII activity; an inactive form of the enzyme is produced which is not reactivated by addition of Fe^{2+}, indicating that Fe^{2+} is needed for de novo synthesis of the enzyme (MacKenzie *et al.*, 1989b). High Zn^{2+} concentrations in the cell lowers the activity of ZMADHII and it is thought that there may be competition in metal binding when the enzyme folds and that the enzyme is inactive with zinc bound (MacKenzie *et al.*, 1989b). The presence in *Z. mobilis* of two ADHs with different metal requirements may allow the the organism some nutritional flexibility, maintaining essential fermentation under limiting metal and different growth conditions. The apparent dominance of

Figure 1.23.

The RuMP cycle

Schematic representation of methanol metabolism in thermotolerant methylotrophic *Bacillus* strains. Numbered reactions are catalysed by: 1, formaldehyde reductase: 2, hexulose-6-phosphate synthase: 3, hexulose-6-phosphate isomerase: 4, glucose-6-phosphate isomerase: 5, glucose-5-phophate dehydrogenase (NADP): 6, 6-phosphogluconate dehydrogenase (NADP): 7, 6-phosphofructokinase: 8, fructose-1, 6-bisphosphate aldolase: 9, transketolase: 10, transaldolase: 11, phosphoriboisomerase: 12, ribulose phophate 3-epimerase. Abbreviations: *DHAP*, dihydroxyacetone-3-phosphate: *GAP* ,glyceraldehyde-3-phosphate: *E4P*,Erythrose-4-phosphate: *Ru5P*, ribulose-5-phosphate: *Ri5P*, ribose-5-phosphate: *X5P*, xylulose-5-phosphate: *H6P*, hexulose-6-phosphate: *FBP*, fructose-1,6-bisphosphate; *S7P*, sedoheptulose-7-phosphate. (Taken from Arfman *et al.*, 1989).



ZMADHII in fermentation, the inhibition of ZMADHI by ethanol and the presence of ferrous sulphate in minimal media (MacKenzie *et al.*, 1989b), indicate that when iron is not limiting zinc is spared from ZMADHI for other essential cellular functions. It has been reported that the activity of ZMADHII in stationary phase cells grown on complex media is decreased (Kintoshita *et al.*, 1989), suggesting that the ZMADHI may have a more important role during the stationary phase and during the initial growth period.

In the facultative anaerobe *E. coli*, the activities of alcohol and acetaldehyde dehydrogenases for fermentation are induced only under anaerobic conditions and in the absence of nitrate (Clark & Cronan, 1980a,b; Clark, 1989).

Propane diol oxidoreductase of *E. coli* is part of the L-fucose pathway (Figure 1.24) and is induced during anaerobic growth. Acetaldehyde is reduced to 1,2-propanediol and excreted into the medium (Sridhara *et al.*, 1969). *E. coli* cannot to grow on 1,2-propanediol as a sole carbon source (Conway & Ingram, 1989) indicating that this enzyme's sole function is fermentative. It has not been shown whether it is induced by the *fnr* gene product which has been shown to be involved in the induction of a series of anaerobic functioning proteins and with repression of aerobic functioning proteins (Spiro & Guest, 1991).

The function of ADHIV from *S. cerevisiae* is unknown and is only expressed when the transposable element, Ty, is inserted at the *ADH4* locus of the yeast genome (Williamson & Paquin, 1987). It has been found recently that some yeasts used in brewing produce large amounts of ADHIV (Drewke & Ciriacy, 1988) and this may be due to multiple copies of the gene being expressed because of Ty insertions, suggesting that ADHIV is an enzyme which has undergone evolutionary changes and is no longer essential for growth (Drewke & Ciriacy, 1988). Its very low specific activity in comparison with the other *S. cerevisiae* isoenzymes, reflects its proposed minor or insignificant role in growth.

Figure 1.24.

The L-fucose pathway

The pathway for the degradation of L-fucose.(Taken from Mortlock, 1984).

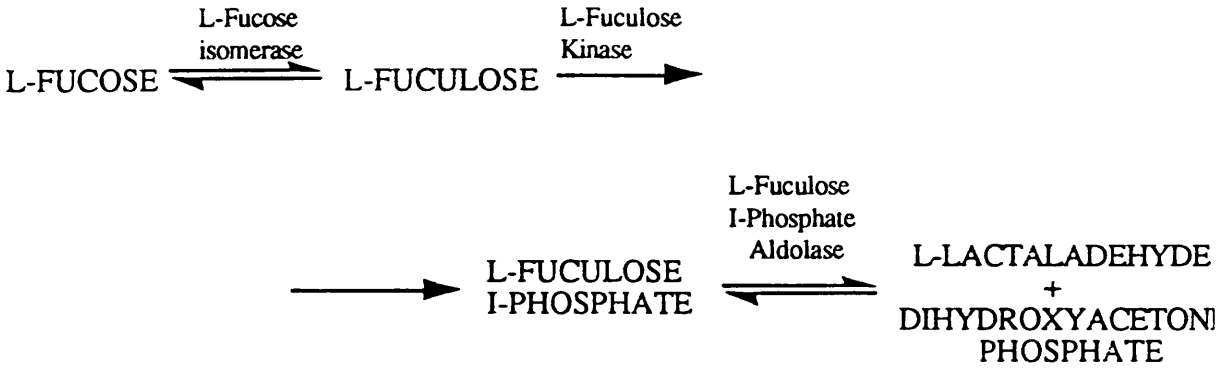


Table 1.9.

Percentage of amino acid sequence identity amongst type III microbial alcohol dehydrogenases

The data for the table was obtained from de Vries *et al.* (1992) and Mallinder *et al.* (1992). The comparisons between BSGDH have so far limited to ZMADHII and CABDH. Abbreviations used are those quoted in table 1.8.

	% IDENTITY						
TYPE III ADH	ZMADH II	ADHIV	BMDH	ECPOR	ECADH	CABDH	BSGDH
ZMADH II	100	53	44	42	33	37	18
ADHIV		100	46	39	32	37	-
BMDH			100	40	35	36	-
ECPOR				100	34	36	-
ECADH					100	46	-
CABDH						100	25
BSGDH							100

1.5. The NAD(P)-independent alcohol dehydrogenases

NAD(P)-independent, or group IV, alcohol dehydrogenases have been identified in a number of different bacteria and can be divided in to two functionally distinct groups, the methanol dehydrogenases (MDH) from methylotrophic bacteria, and the ethanol dehydrogenases (EDH) from *Pseudomonas* and *Acetobacter*, although the latter group is yet to be fully characterised.

The NAD(P)-independent alcohol dehydrogenases catalyse the reaction:

$$\text{ALCOHOL} + 2X_{(OX)} \rightleftharpoons \text{ALDEHYDE} + 2X_{(RED)} + 2H^+$$

Where $2X_{(OX)}$ and $2X_{(RED)}$ are representative of the oxidised and reduced form of the electron acceptor.

1.5.1. NAD(P)-independent methanol dehydrogenases

Methanol dehydrogenases (MDH) have highly conserved features in comparison to the EDHs. They have a high optimum pH, they are mostly homo-dimers and homo-tetramers of subunit size approximately 66 kDa and they oxidise most primary alcohols. Methanol dehydrogenase is required in methylotrophs to oxidise methanol to formaldehyde. Although the use of methanol is quite widespread in gram-negative bacteria, the first step of methanol utilisation is always the oxidation of methanol to formaldehyde (Harms *et al.*, 1987; Section 1.4.5). Because of its prominent role the MDH is a protein found to make up to 15% of the cellular protein when *Paracoccus dentrificans* is grown on methanol (Machlin & Hanson, 1988). The primary structure is known for two MDHs, from *P. dentrificans* (PDMDH; Harms *et al.*, 1987) and *Methylobacterium organophilum* (MOMDH; Machlin & Hanson, 1988). Both mature proteins contain 599 amino acid residues per subunit with subunit size of approximately 66 kDa. The identity between these two genes is very high (up to 82%) and is even higher in the protein primary structure (Machlin & Hanson, 1988). There is a putative 27 amino acid signal sequence observed in the MOMDH and also probably in the PDMDH

Table 1.10.

NAD(P)-independent alcohol dehydrogenases

Enzyme	Organism	Abbreviation
Methanol dehydrogenase	*P. dentrificans*	PDMDH
Methanol dehydrogenase	*M. organophilum*	MOMDH
Ethanol dehydrogenase	*A. polyoxogenes*	APEDH
Ethanol dehydrogenase	*A. aceti*	ACEDH

(Harms *et al.*, 1987). This was expected, since both of the MDHs are periplasmically located. The MDH protein is found to be loosely associated with the outer face of the inner membrane. In *M. organophilum* XX, 20% of the total MDH activity is membrane associated (Hancock & Williams, 1986). This association presumably provides the enzyme with a greater accessibility to the electron transport chain, especially cytochrome *c* which is believed to be responsible for accepting electrons from the oxidation of alcohol.

The synthesis of the MDHs in methylotrophic bacteria is a complex process which is thought to require at least 10 and 11 gene products in *P. dentrificans* and *M. organophilum* XX, respectively (Harms *et al.*, 1987; Machlin & Hanson, 1987). These genes are located in clusters in three separate chromosomal regions within 17 kilobases in *M. organophilum* XX with the *moxFG* cluster coding for MDH and cytochrome *c* respectively (Lidstrom *et al.*, 1987). It is proposed that cytochrome *c* and MDH are in the same operon and that cytochrome *c* is an electron acceptor from MDH, as it is for the EDH in the *Acetobacter* spp. (Harms *et al.*, 1987). This arrangement has also been proposed for the MDH and cytochrome *c* from *Methylobacterium* sp. strain AM1 (Nunn & Lidstrom, 1986a,b).

1.5.2. NAD(P)-independent ethanol dehydrogenases

The EDHs have a low pH optim and have very little or no activity with methanol (Inoue *et al.*, 1989). They form complexes with cytochrome *c* and pyrroloquinoline quinone (PQQ) in order to be active (Tamaki *et al.*, 1991). EDH from *Acetobacter* spp., along with aldehyde dehydrogenase, are important in acetic acid fermentation, where acetic acid is formed from ethanol via acetaldehyde. EDH has been found to located on the outer surface of the cytoplasmic membrane of *Acetobacter* spp and its oxidative activity is thought to be linked with the respiratory chain (Inoue *et al.*, 1989). The purified enzyme is a monomer of 63 kDa and it is associated with a 44 kDa cytochrome and two other proteins of 29 kDa and 13.5 kDa, whose functions are unknown (Adachi *et al.*, 1978).

The primary sequences for two ethanol dehydrogenases, from *A. aceti* (ACEDH) and *A. polyoxogenes* (APEDH), have been determined (Inoue *et al.*, 1989; Tamaki *et al.*, 1991). The predicted amino acid sequence of the 72 kDa APEDH has a about 80%

identity with the 72 kDa ACEDH and about 30% identity with the MDHs of methylotrophic bacteria (Tamaki *et al.*, 1991). Both of the EDHs were predicted to have a longer than normal *N* -terminal signal sequence of 35 amino acids which is thought to be cleaved from the pre-protein to generate the mature protein. The region coding for the 44kDa cytochrome *c* subunit is located in the same orientation and just downstream of the gene coding for the 72kDa APEDH with no obvious transcription termination signals. This indicates that these two genes are in the same operon and are co-transcribed (Tamaki *et al.*, 1991). The 44kDa cytochrome *c* appears also to be synthesized as a pre-protein, like the EDH, and the amino acid sequence around the putative leader sequence cleavage site resembles the cleavage site found in the cytochrome *c* of methylotrophic bacteria (Nunn *et al* ., 1989). The evidence for the need for cytochrome *c* in the EDH activity of *Acetobacter* spp.is strong, in that it appears that the cytochrome *c* is on the same operon as the EDH subunit and activity is only seen when both EDH and cytochrome *c* are expressed in transformed EDH negative mutants (Tamaki *et al.*, 1991).

Three possible haem-binding sequences were found within the 44kDa cytochrome *c*. The presence of three possible prosthetic haem groups in the 44kDa cytochrome *c* might account for its unusually large size because cytochromes *c* containing only one prosthetic haem group are usually only about 13kDa, approximately one third of the size of the cytochrome *c* from *A. polyoxogenes* . No similarity between this the cytochrome *c* from *A. polyoxogenes* and any other protein was observed (Tamaki *et al.*, 1991). It might be that the 44kDa cytochrome *c* is an evolutionary fusion product of three duplicated cytochrome *c* coding genes. Confirmation of this could be obtained by comparison of the 44kDa cytochrome *c* primary sequence with those of single haem-binding cytochrome *c* proteins.

One typical haem binding sequence was also identified in the *C* -terminal part of the 72kDa APEDH. No corresponding sequence has been seen in MDH sequences. This would suggest that the EDH should be classified as a quino-haem protein, similar to the alcohol dehydrogenase from *P. testosteroni* which contains one haem molecule per enzyme molecule and donates reducing equivalents to a quinoprotein (Groen *et al.*, 1986). The role of PQQ has been assumed to be involved in ethanol oxidation by receiving electrons, but there is no direct evidence for this as yet. The precise role of a possible

haem group in the 72kDa subunit is yet to be determined (Tamaki *et al.*, 1991).

1.5.3. Cofactor requirement

The cofactor in this group of enzymes appears to be mostly PQQ, located in the periplasmic space of gram-negative bacteria (de Vries *et al.*, 1990). The reducing equivalents are probably passed to PQQ from the enzyme via interaction with cytochrome *c*.

Evidence for a multienzyme MDH complex from the gram-positive *Nocardia* sp. 239 made up from three components; a methanol dehydrogenase, a NAD(H)-dependent aldehyde dehydrogenase and NAD(H)-dependent dehydrogenase, has been presented by Duine *et al.* (1984). The methanol dehydrogenase in its dissociated form shows no reductive activity, but this activity is restored when incubated with the corresponding NAD(H)-dependent dehydrogenase. It was proposed that methanol dehydrogenase transfers reducing equivalents generated from methanol oxidation directly to the NAD(H)-dependent dehydrogenase in a mechanism involving NAD(H) and PQQ (Duine *et al.*, 1984).

1.5.4. Evolutionary characteristics

The similar arrangement of genes within an operon and high gene identity indicates that there is an evolutionary link not just with the coding regions themselves but with the operon as a whole for both the MDH and EDH enzymes. It would be interesting to determine the extent of identity between the non-coding regions of the EDH/cytochrome genes from *Acetobacter* and the putative corresponding operon from *M. organophilum* XX and *Methylobacterium* sp. strain AM1. The non-coding regions are obviously not under the functional evolutionary pressures that the coding regions are under but comparisons of the non-coding regions can provide evidence of relationships in operon structure, and hence determine whether the operon structure is related by operon duplication, single gene acquisition or is the result of convergent evolution.

1.6 The alcohol oxidases

The oxidation of methanol to formaldehyde in methylotrophic yeasts and filamentous fungi is carried out by the alcohol oxidases a fundamentally different group of proteins from the alcohol dehydrogenases previously described in this introduction. The alcohol oxidases differ from alcohol dehydrogenases in that they oxidise alcohols to aldehydes irreversibly with the concurrent production of hydrogen peroxide, as shown below (Veenhuis *et al.*, 1983).

$$\text{ALCOHOL} + O_2 \longrightarrow \text{ALDEHYDE} + H_2O_2$$

The subunit M_r is typically 72-75kDa. They have been characterised as flavoproteins binding one FAD prosthetic group non-covalently per subunit. They are located in the peroxisomes, membrane bound organelles containing a matrix of proteins including catalase (Veenhuis *et al.*, 1992).

The first step in methanol metabolism in methylotrophic yeasts and filamentous fungi, as with methylotrophic bacteria (Section 1.4.5), is the oxidation of methanol to formaldehyde. Formaldehyde is either dissimilated to CO_2 by formaldehyde dehydrogenase and formate dehydrogenase or assimilated into carbohydrate via the xylulose monophosphate pathway (Figure 1.25). The H_2O_2 formed by methanol oxidation is converted to H_2O and O_2 by catalase. The methanol oxidases' affinity for oxygen is low, so to compensate for this poor catalytic ability the oxidase is synthesized in large amounts. The appearance of methanol oxidase, formaldehyde dehydrogenase and formate dehydrogenase, catalase and dihydroxyacetone synthase (an essential enzyme in the methanol assimilation process) is subject to repression by glucose. The induction of these enzymes means that methanol metabolism is carefully controlled and avoids the build up of toxic intermediates such as formaldehyde. When the organisms are grown on methanol, these enzymes are induced and under these conditions up to 80% of the cell volume can be taken up by the peroxisomes with methanol dehydrogenase accounting for up to 30% of soluble protein (Couderc & Baratti, 1980). In contrast, peroxisome volume fraction is very small in glucose grown cells (Ledeboer *et al.*, 1985).

Table 1.11.

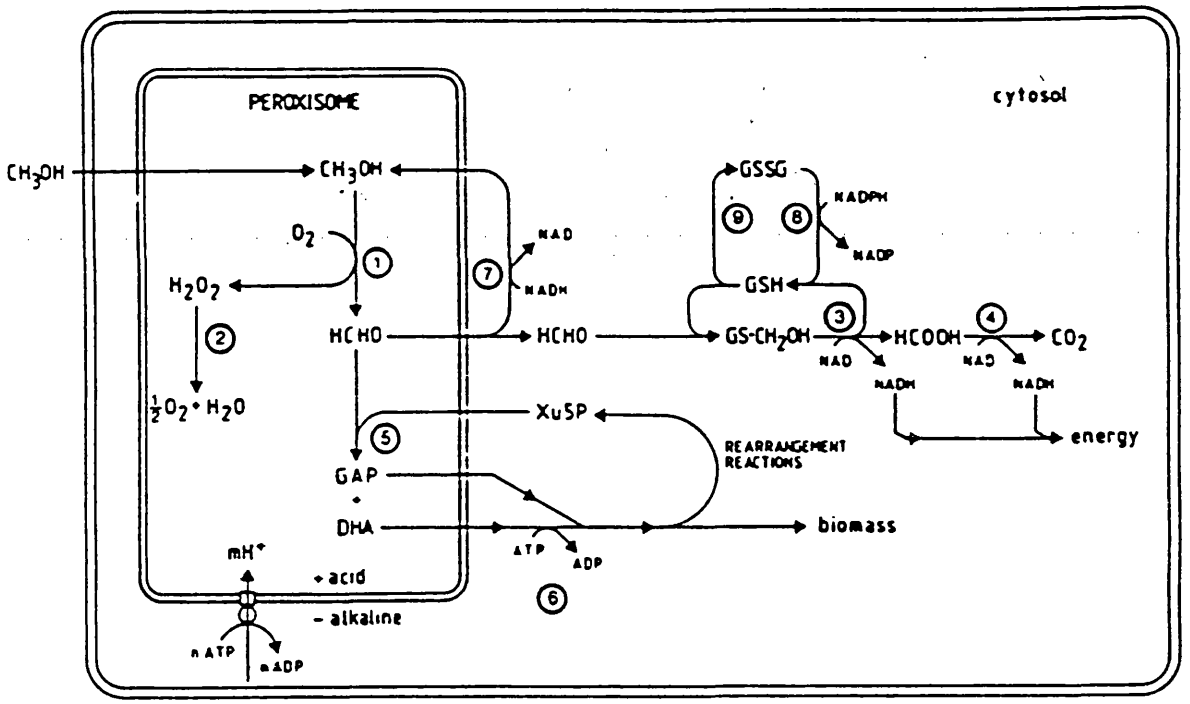
The microbial alcohol oxidases

Enzyme	Organism	Abbreviation
Methanol oxidase	*Hansenula polymorpha*	HPAOD
Alcohol oxidase 1	*Pichia pastoris*	PPAOD1
Alcohol oxidase 2	*Pichia pastoris*	PPAOD2
Alcohol oxidase 1	*Candida boidinii* S2	CBAOD1

Figure 1.25.

Methanol metabolism in methylotrophic bacteria

Schematic representation of the compartmentalisation and the function of peroxisomes in methanol metabolism in *Hansenula polmorpha* WT cells. Numbered reactions are catalysed by: 1, alcohol oxidase; 2, catalase; 3, formaldehyde dehydrogenase; 4, formate dehydrogenase; 5, dihydroxacetone synthase; 6, dihydroxyacetone kinase; 7, formaldehyde reductase; 8, glutathione reductase; 9, oxidation of glutathione. (Taken from Veenhius *et al.*, 1992).



1.6.1. The primary structure of the alcohol oxidases

cf p96

There are four alcohol oxidase sequences currently available from methylotrophic yeasts: methanol oxidase from *Hansenula polymorpha* (HPAOD; Ledeboer *et al.*, 1985), alcohol oxidases 1 and 2 from *Pichia pastoris* (PPAOD1, PPAOD2: Koutz *et al.*, 1989) and alcohol oxidase 1 from *Candida boidinii* S2 (CBAOD1; Sakai & Tani, 1992). The predicted polypeptide lengths for these proteins are 664 amino acid residues for HPAOD and 663 residues for the remaining three.

The degree of identity amongst the predicted enzyme primary structures is high. There is 92% identity between PPAOD1 and PPAOD2 nucleotide sequences and 97% identity at the predicted amino acid level (Koutz *et al.*, 1989). CBAOD1 has 77% identity with HPAOD and 73% with PPAOD1 and PPAOD2 (Sakai & Tani, 1992). HPAOD identity with PPAOD1 and PPAOD2 is greater than 85%.(Koutz *et al.*, 1989).

There are highly conserved regions within the predicted amino acid sequences of the alcohol oxidases. The first conserved region is found in the *N* -terminal region, from residues 8 to 83. This region is typical of the β-α-β motif for AMP binding. Three Gly residues between the first β-sheet and first α-helix in the Gly-Xaa-Gly-Xaa-Xaa-Gly consensus sequence are conserved in all alcohol oxidases and related flavoenzymes. This high conservation of predicted secondary structure suggests that the AMP moiety of the FAD binds near the *N* -terminus of the polypeptide (Sakai & Tani, 1992). The second highly conserved region, between residues 565 to 576 may form part of the active site and may be related to the active site of disulphydryl oxidases (Frederick *et al.*, 1990).

Previous work has suggested that CBAOD1 might be involved in mediating formaldehyde tolerance (Sakai & Tani, 1987; 1988; Tani, 1985). This is suggested to occur by the trapping of formaldehyde by binding to free Asp residues in the CBAOD1 subunit. Up to 26 molecules of formaldehyde are able to covalently bind to one subunit of CBAOD1 (Sakai & Tani, 1988). The sequence data show that Asn replaces a highly conserved Cys-572 residue in CBAOD1 and a further two Asn residues are present at positions 580 and 582.

1.6.2. Substrate specificity

Most of the MDHs characterised oxidise methanol and the lower primary alcohols such as ethanol, propan-1-ol and butan-1-ol. Very little, if any, activity is observed with benzyl alcohol or with lower secondary alcohols. However, two veratryl alcohol oxidases isolated from *Pleurotus sajor-caju* oxidise certain aromatic alcohols but not aliphatic alcohols (Bourbonnais & Paice, 1988) and the primary role of these oxidases is thought to be involved in the generation of H_2O_2 for lignin degradation, because lignin peroxidase, the first enzyme in the lignin degradation pathway, requires H_2O_2 for activity (Kirk & Farrell, 1987; Bourbonnais & Paice, 1988).

1.7. Industrial implications for biotransformations of aldehydes and alcohols

There are very few industrial chemical reactions which do not have an equivalent biological reaction. One of the main challenges for the chemical industry is to harness the natural activities of enzyme systems for the development of commercial processes. The interconversion of aldehydes, ketones and alcohols by alcohol oxidoreductases is an area which has generated a great deal of interest because of the huge potential for the production of pure optically active products. The wide substrate specificity of enzymes and the diversity of biochemical pathways derived from animal, plant and microbial systems, have enabled the production of important chemical intermediates by whole cell and isolated enzyme systems. Also, with the realisation of protein engineering the opportunities for the production of chemicals, which would otherwise be difficult and expensive to produce synthetically, have widened the opportunities available. Two different approaches are used in biotransformations; whole cell and isolated enzyme systems. The relative advantages of using either system has been discussed by Pugh *et al.* (1987). The relative advantages depend on a number of parameters such as the permeability of cells to product/substrate, the enzyme specificity and how many steps are involved in the transformation from substrate to product. It is difficult and impractical to re-use suspensions of biocatalysts or whole cells. However, this problem has been overcome by immobilisation of biocatalysts (whole cells or isolated enzymes) on solid supports, thus allowing reapplication in a continuous process. Co-immobilisation of coenzyme and enzyme has greatly improved the efficiency of the isolated cell system (Hartmeier, 1985).

1.7.1. Whole cell systems

The advantages of using whole cell systems are that they do not require the isolation and purification of enzymes, which is expensive and troublesome. Cofactor regeneration is continually carried out, sometimes by using a co-substrate in whole cells to maintain a

redox balance essential for cell viability. More complex transformations which involve a series of different enzyme reactions, requiring cofactors, are ideally suited to whole cell systems which contain the necessary metabolic pathways for the transformation.

The disadvantages of whole cell systems have become more apparent as more complex transformations using a wider range of substrates have been attempted. The toxic side affects of unique alien substrates and their corresponding products have proved to be of major consequence in biotransformations using whole cell systems (Long & Ward, 1989). The formation of L-phenylacetyl carbinol from benzaldehyde and acetaldehyde by pyruvate decarboxylase in *S. cerevisiae* is a key step in the manufacture of L-ephedrine (Table 1.12). Not all the benzaldehyde is quantitatively converted to L-phenylacetyl carbinol, usually some is reduced to benzyl alcohol and small amounts of benzoic acid. The production of alcohol dehydrogenase and benzoic acid have a pronounced effect on the viability of the cells (Long & Ward, 1989). As the benzaldehyde concentration was increased, the yeast growth rate was reduced at 0.5g/l, inhibited at 1-2g/l and cell viability was reduced at 3g/l. High concentrations of benzaldehyde appear to reduce the cells' permeability to benzaldehyde itself, exposing enzymes within the cell to the denaturing effects of high benzaldehyde concentrations (Long & Ward, 1989). Alcohol dehydrogenase is particularly susceptible to this effect of benzaldehyde. With inactivation of alcohol dehydrogenase, regeneration of NAD^+ no longer occurs. Hence a whole cell system for this process has no advantages over an isolated enzyme system since the increased permeability of the cells allows leakage of essential cofactors for pyruvate decarboxylase, such as thiamin pyrophosphate and Mg^{2+}, and NAD^+ is no longer regenerated.

Use of whole cells in aromatic biotransformation is used advantageously in the detoxification treatment of waste water. *Rhodotorula muculaginosa* has been used to produce the less toxic benzyl alcohol from the more toxic benzaldehyde (Wisnieski *et al.*, 1983; Table 1.12).

Methylotrophs have been identified as sources of high oxidative ability. *Methylosinus trichosporium* and *Methylococcus capsulatus* have been used to transform stereospecific substrates such as *m* -cresol to stereospecific products *m*- and *p* -

Table 1.12. Biotransformations of aldehydes, ketones and alcohols using isolated enzyme and whole cell systems

Organism/enzyme	System	Substrate(s)	Products
Saccharomyces cerevisiae	Whole cell	Benzaldehyde	L-Phenylacetyl carbinol
Rhoodotorula mucilaginosa	Whole cell	Benzaldehyde	Benzyl alcohol
Methylosinus trichosporium	Whole cell	*m* -Cresol	*m* & *p* -Hydroxybenzaldehyde
Methylococcus capsulatus	Whole cell	Toluene	Benzyl alcohol
Pichia pastoris /Alcohol oxidase	Isolated enzyme	(C_6-C_{11}) alcohols	(C_6-C_{11}) Aldehydes
Thermoanaerobium brockii/ /ADH	Isolated enzyme	Smaller ketones	*R* -Alcohols
Thermoanaerobium brockii/ /ADH	Isolated enzyme	Larger ketones	*S* -Alcohols
Methylophilus methylotrophus/ ADH	Isolated enzyme	Glycerol	L-Glyceraldehyde

hydroxybenzaldehyde and toluene to benzaldehyde (Pugh *et al.*, 1987; Table 1.12).

1.7.2. Isolated enzyme systems

Isolated enzyme systems have several advantages over whole cell systems. There is no requirement for a carbon and energy source, which is needed to make whole cell systems viable. Product is easily separated from the biocatalyst, thus avoiding the lysis steps which are often needed in whole cell systems. Isolated cell systems have an absolute requirement for redox power e.g. NAD(P)H. Redox power can be regenerated by chemical, enzymatic or electrochemical processes (Bowen & Pugh, 1985). Isolated enzyme systems confer greater specificity and so avoid the loss of redox power and energy in non-productive secondary reactions seen in whole cell systems. Also the direction of the reaction (oxidation/reduction) is more easily controlled by using redox availability. However, the main problems which exist with isolated enzyme systems are the availability, the stability, and cofactor requirements of the isolated enzymes.

Yeast ADH has been used extensively in biotransformations (Long *et al.*, 1989) because of its broad substrate specificity for aromatic alcohols. However, recently the availability of other alcohol dehydrogenases has generated the ability to metabolize a wider range of substrates and hence generate a wider range of products. The group I ADH from *T. brockii* has been used because of its reversal of substrate stereoselectivity. It will reduce smaller ketones (e.g. methyl ethyl, methyl isopropyl, methyl cyclopropyl) to *R* alcohols whereas larger ketones are reduced to the *S* enantiomers (Keinan, *et al.*, 1986; Table 1.12)

The oxidation of aliphatic and aromatic high molecular weight alcohols by *P. pastoris* has been explored by Murray & Duff, (1990). The alcohol oxidase from this organism (Section 1.6.) has been shown to be useful in the oxidation of large (C_6-C_{11}) alcohols which have low solubility in water (Table 1.12). By alteration of reaction parameters, the oxidation of large aliphatic and aromatic alcohols can be achieved. Using a two-phase system whereby the alcohol oxidase is in an aqueous phase and the substrate in an organic phase, the enzyme receives a constant flow of substrate and releases product

into the organic phase, thereby reducing product inhibition. This two phase system also facilitates the recovery of the biocatalyst and its re-use.

Substrate ambiguity of alcohol dehydrogenase from *Methylophilus methylotrophus* has allowed the stereospecific oxidation of glycerol to L-glyceraldehyde (Scott *et al.*, 1986; Table 1.12).

1.7.3. Future prospects

Perhaps surprisingly, protein engineering has yet to make a significant impact on the biotransformation of aldehydes, ketones and alcohols. High yields generated by plasmid encoded proteins should allow the easy purification for isolated cell systems or allow expression of a novel proteins in whole cell systems. It can also be assumed that genetic engineering will allow generation of enzymes with improved activity, increased stability and tailored substrate specificities.

1.8. The pathways for degradation of aromatic compounds may shed light on evolutionary relationships amongst enzymes

The primary sequence of enzymes, as well as information about their substrate specificity and other properties, can provide vital information about evolutionary relationships (Section 1.1.3). It can also help to characterise the metabolic pathways in which they operate. A great deal of work has been done to establish relationships among catabolic enzymes of prominent pathways, such as the Krebs cycle, as well as secondary pathways such as glycolysis. However, the peripheral pathways which convert a huge array of organic compounds into intermediates for secondary pathways have remained less studied. This is probably due to the vast number and variety of pathways with their constituent enzymes being seen as a huge task to characterise. However, studying these pathways can often provide evolutionary evidence which could not be obtained from the more central pathways, because under many growth conditions such peripheral pathways are not essential to the microorganism. This makes them more likely to undergo more rapid evolutionary changes, unlike the central pathways which are vital and therefore more constrained as to possible changes.

An ideal example of a such a peripheral catabolic pathway is the toluene- or TOL-degradative pathway found in some strains of *Pseudomonas putida*, which converts toluene and xylenes into intermediates for the central metabolic pathways (Figure 1.26). A very similar series of reactions to those of the TOL-degradative pathway are seen in the convergent benzyl alcohol and mandelate pathways of *A. calcoaceticus* which channel aromatics and key amphibolic intermediates into the central pathways of metabolism (Kennedy & Fewson, 1968; Figure 1.27). The TOL-degradative pathway and the convergent benzyl alcohol and mandelate pathways bear such similarities to each other, containing both benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase enzymes, that it is likely that they share a common evolutionary ancestry. Comparisons of genetic arrangement, expression and primary structure of enzymes common to these pathways can be used to help determine their putative shared evolutionary history.

1.8.1. The genus *Acinetobacter*

The genus *Acinetobacter* consists of gram-negative, oxidase-negative, non-motile bacteria which lack pigmentation (Ingram & Shewan, 1960) and are now classified as members of the family Neisseriaceae (Towner, 1991). They have been implicated in food spoilage and are quite often involved in nosocomial infections because of their high resistance to a wide range of antibiotics (Towner, 1991).

One of the most striking features of strains of *Acinetobacter* is their ability to grow on a wide range of aromatic compounds as sole carbon sources (Fewson, 1991). The dissimilation of aromatic compounds is achieved by a series of inducible and constitutive enzymes which feed the catabolites into the amphibolic pathways. One of the best-characterised of these aromatic dissimilatory pathways is that for mandelate and benzyl alcohol oxidation (Fewson, 1988).

1.8.2. The genus *Pseudomonas*

The genus *Pseudomonas* consists of a wide collection of gram-negative strains which occur in a diverse number of habitats and are involved in many important biological processes. Like acinetobacters, *Pseudomonas* spp. can grow on a wide variety of organic compounds as sole carbon sources. Some strains utilise over 100 different compounds, and only a few strains use less than 20 (Brock & Madigan, 1988).

Few pseudomonads are pathogenic, although those which are such as *P. aeruginosa* are frequently involved with infections of the respiratory and urine tracts.

The pseudomonads are ecologically important because of their probable role in the aerobic degradation of many soluble compounds generated in the breakdown of plant and animal materials. Interest in strains of *Pseudomonas* is high because of their nutritional versatility enabling them to produce useful industrial products and their ability to detoxify chemical waste through a wide range of metabolic peripheral pathways (Holloway, 1992).

1.8.3. The TOL-degradative pathway of *Pseudomonas putida*

The great majority of work that has been done on the microbial breakdown of aromatics has centred upon how the pathways are regulated and how they interact to produce the key secondary intermediates such as catechol and protocatechuate. The TOL-catabolic pathway is divided into two parts, the upper and lower pathways (Figure 1.26). The upper is concerned with the oxidation of aromatics to benzoates, and the lower (Figure 1.28) is concerned with the conversion of benzoate to central pathway metabolites. The TOL-plasmid gene products were identified as responsible for the catabolism of certain aromatic compounds by *P. putida* mt-2 (Williams & Murray, 1974; Wong & Dunn, 1974). The evidence was that a high frequency of conjugational transfer of the ability to utilise xylenes and toluene was seen between strains of *P. putida* and a loss of aromatic metabolism was induced by mitomycin C. The genes responsible for the upper pathway on *P. putida* mt-2 plasmid pWW0-161 have been shown to be arranged in the an operon in the following order: promoter-*xyl CMABN*, where *xylC* codes for benzaldehyde dehydrogenase (BZDH), *xylM* and *xylA* code for the xylene oxygenase (XMO) subunits, *xylB* codes for benzyl alcohol dehydrogenase (BADH) (Figure 1.26) and *xylN* codes for a 52kDa protein which is processed to a 47kDa protein whose function as yet is undetermined (Harayama *et al.*, 1989). The gene products responsible for catalysing the reactions in the lower pathway have also been well characterised (Harayama & Rekik, 1990). The lower pathway diverges at the ring-cleavage reactions into the *meta*-pathway and the β-ketoadipate or *ortho*-pathway (Figure 1.28) The characterisation of genes on the TOL-plasmid (pWW0) has been fairly thorough, especially those involved in benzoate catabolism via the *meta* -cleavage pathway (Horn *et al.*, 1991). The *meta* operon codes for the 13 genes involved in the lower pathway in the order *xyl XYZLTEGFJQKIH* (Harayama & Rekik, 1990; Figure 1.26), and is one of the longest prokaryotic operons known.

Table 1.13.

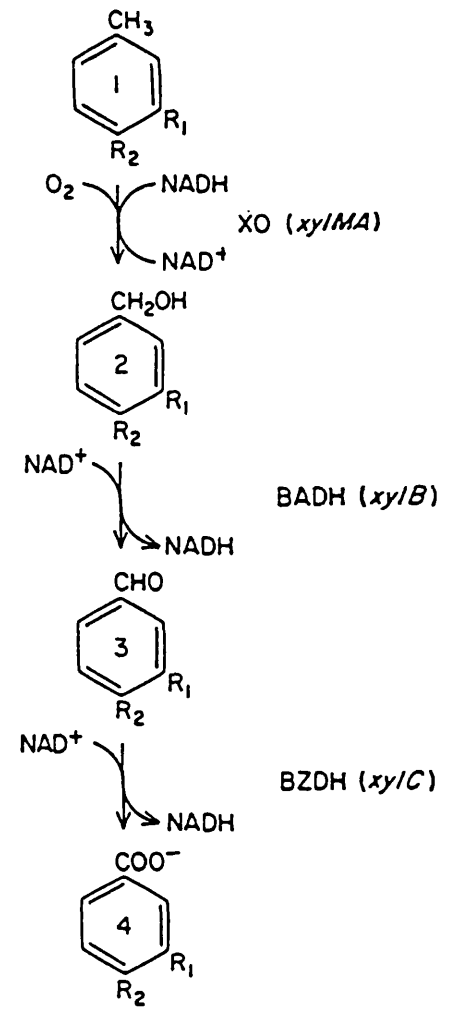
Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase from *A. calcoaceticus* and *P. putida* .

Enzyme	Organism/plasmid	Abbreviation
Benzyl alcohol dehydrogenase	*A. calcoaceticus*	BADH
Benzaldehyde dehydrogenase I	*A. calcoaceticus*	BZDHI
Benzaldehyde dehydrogenase II	*A. calcoaceticus*	BZDHII
Benzyl alcohol dehydrogenase	*P. putida* plasmid pWW0	TOL-BADH (pWW0)
Benzylaldehyde dehydrogenase	*P. putida* plasmid pWW0	TOL-BZDH (pWW0)
Benzyl alcohol dehydrogenase	*P. putida* plasmid pWW53	TOL-BADH (pWW53)
Benzaldehyde dehydrogenase	*P. putida* plasmid pWW53	TOL-BADH (pWW53)

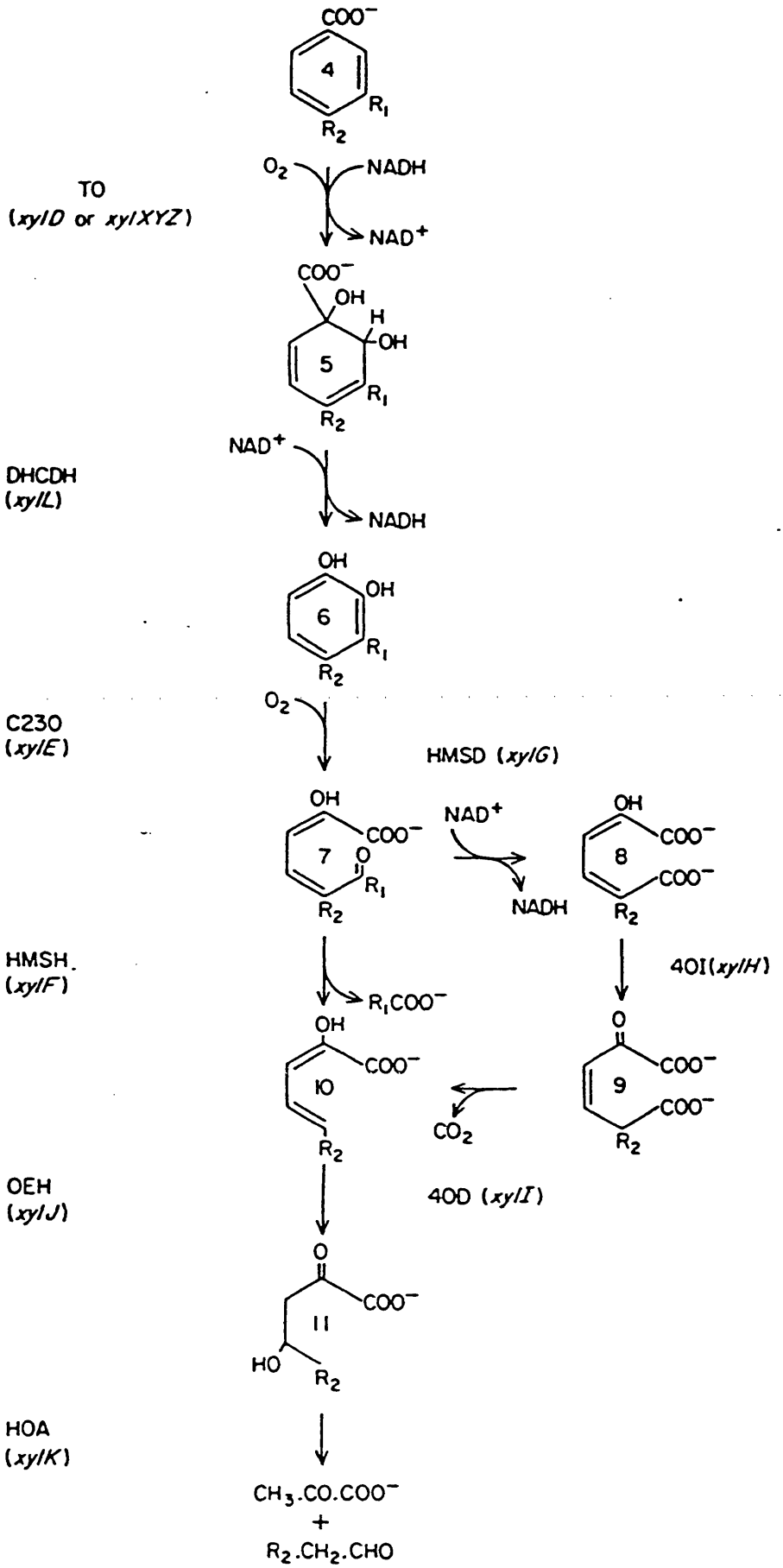
Figure 1.26.

The toluene- (TOL-) degradative pathway

The complete pathway encoded by TOL-plasmids. The following hydrocarbons (1) serve as growth substrates: toluene (R_1=R_2=H);*m* -xylene (R_1=CH_3, R_2=H); *p* -xylene (R_1=H, R_2=CH_3); 3-ethyltoluene (R_1=C_2H_5, R_2=H); 1,2,4-trimethylbenzene. Numbered compounds (toluene catabolism only) are: 2, benzyl alcohol; 3, benzaldehyde; 4, benzoate; 5, benzoate dihydrodiol; 6, catchol; 7, 2-hydroxymuconic semialdehyde; 8, 4-oxalocrotonate (enol); 9, 4-oxalocrotonate (keto); 10, 2-oxopentenoate (enol); 11, 4-hydroxy-2-oxovalerate. Enzyme abbreviations are: XO, xylene oxygenase; BADH, benzyl alcohol dehydrogenase; BZDH, benzaldehyde dehydrogenase; TO, toluate 1,2-dioxygenase; DHCDH, 1,2-dihyroxycatechol 2,3-oxygenase; HMSH, 2-hydroxymucoic-semialdehyde hydrolase; HMSD, 2-hydroxymucoic-semialdehyde dehydrogenase; 4OI, 4-oxalocrotonate isomerase; 4OD, 4-oxalocrotonate decarboxylase; OEH, 2-oxo-4-pentenoate; HOA, 4-hydroxy-2-oxovalerate aldolase. Genes encoding the enzyme subunits are designated. Their expression is regulated in two distinct operons, the upper-pathway operon and the *meta* -(or lower-) pathway operon. (Taken from Assinder & Williams, 1990).



Upper pathway operon



Meta-pathway operon

Figure 1.27

The mandelate and benzyl alcohol pathways of *Acinetobacter calcoaceticus*

Reactions are catalysed by: 1, D-mandelate dehydrogenase; 2,L-mandelate dehydrogenase; 3, phenylglyoxylate decarboxylase; 4, benzyl alcohol dehydrogenase; 5, benzaldehyde dehydrogenase I; 6, benzaldehyde dehydrogenase II. (Adapted from MacKintosh & Fewson, 1988a).

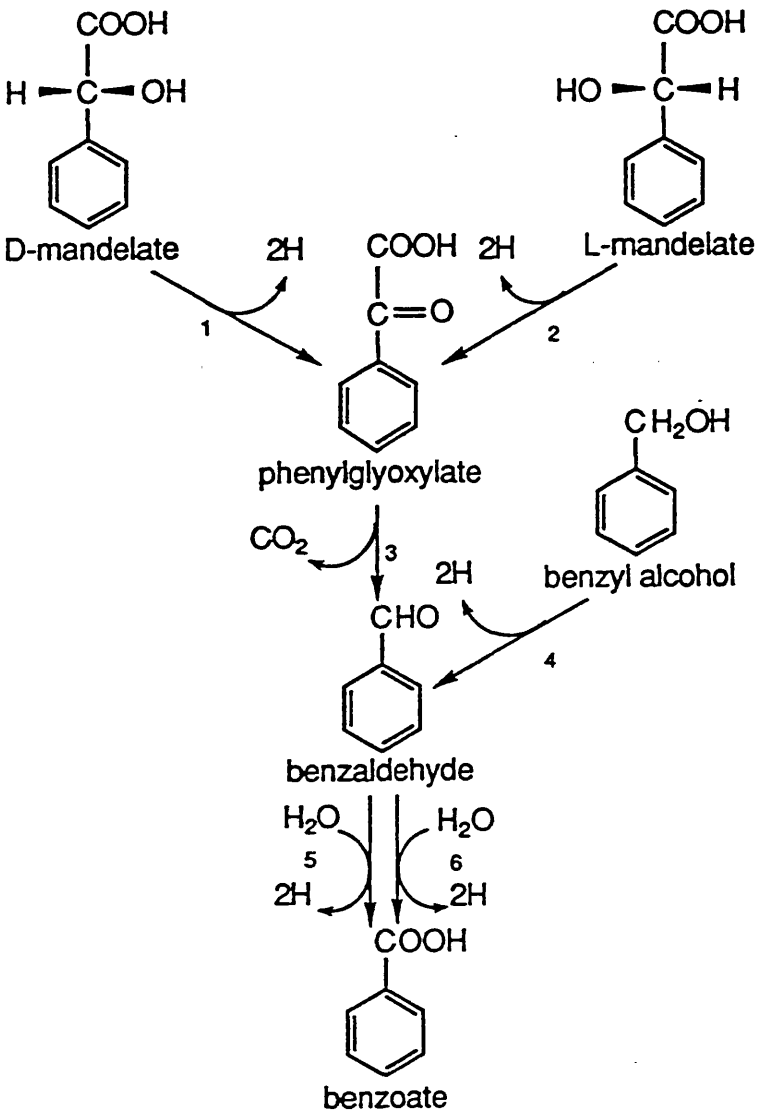
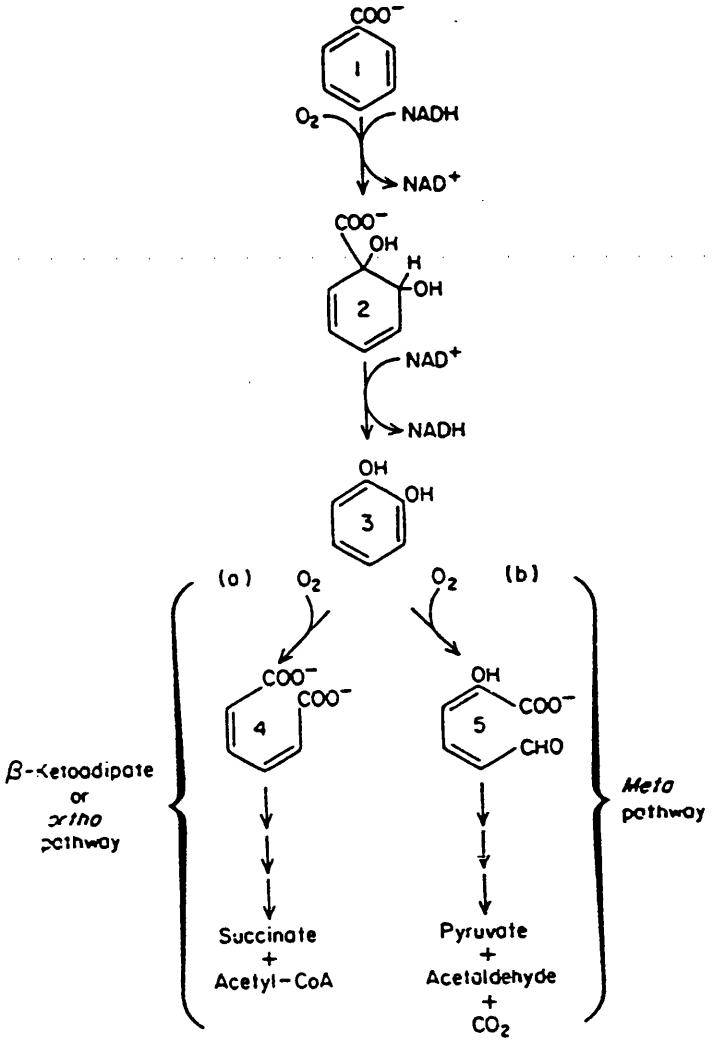


Figure 1.28.

The lower TOL-pathway

Alternative pathways for aromatic catabolism in *P. putida* . The first two common steps convert benzoate (1) to catechol (3). The pathways diverge in the ring-cleavage reactions catalysed by catechol 1,2-oxygenase (reaction a) and catechol 2,3-oxygenase (Reaction b). Names of compounds are: 1, benzoate; 2, benzoate dihydrodiol 1,2-dihydrocyclohexa-3,5-diene carboxylate); 3, catechol; 4, *cis,cis* -muconate; 5, 2-hydroxymuconic semialdehyde. (Taken from Assinder & Williams, 1990).



1.8.4. Mandelate and benzyl alcohol metabolism of *Acinetobacter calcoaceticus*

The mandelate pathway (Figure 1.27) is very common in microorganisms, which is not particularly surprising because it enables growth on a wide range of organic compounds since the enzymes involved have relaxed substrate specificities. This is exemplified by substitution on the 3-, 4-, and 5-positions of the benzene ring being tolerated and allowing metabolism or partial metabolism of many mandelate .analogues (Fewson, 1988). This type of substrate ambiguity is also seen with xylene oxygenase from *P. putida* (an analogous enzyme has not been observed in *A. calcoaceticus*) which catabolizes toluene and xylenes to benzyl alcohol but also has an affinity for benzyl alcohol as a substrate, mimicking the reaction catalysed by TOL-BADH (Harayama *et al.*, 1986). The physiological significance of this substrate ambiguity is unknown (Assinder &Williams, 1990).

The sequences of genes involved in the other branch of the lower *ortho* -cleavage pathway from *A.calcoaceticus* have been determined (Neidle & Ornston, 1986).There are three separate transcriptional units in a 16-kilobase supraoperonic cluster on the chromosome. The *ben ABCD* gene products are responsible for benzoate catabolism, *benABC* and *benD* coding for 1,2-dioxygenase and *cis*-diol dehydrogenase, corresponding to *xylXYZ* and *xylL* in the TOL-plasmid. The *ben ABCD* genes are independently regulated from the *cat ABCDEF* genes corresponding to the enzymes required for catechol dissimilation via the β-ketoadipate pathway (Figure 1.28).

In contrast to the well characterised lower pathway and to the upper pathway of the TOL-plasmid, relatively little is known about the structure and regulation of expression of the genes involved in the metabolism of mandelate and benzyl alcohol in *A.calcoaceticus*.

1.8.5. Regulation and expression of the mandelate and benzyl alcohol convergent pathways

The mandelate pathway enzymes are co-ordinately expressed in *A. calcoaceticus*. Induction is brought about by the presence of phenylglyoxylate and not by mandelate, as might be expected (Fewson *et al.*, 1978; Livingstone & Fewson, 1972). This poses a problem of how the inducer is formed. It might be that there is enough residual activity of non-induced mandelate dehydrogenase which can produce small amount of inducer. Alternatively, non-specific conversion of substrate by an unrelated enzyme with broad specificity may produce enough inducer to give rise to induction of expression. There is no evidence for any feedback inhibition by any of the the mandelate pathway metabolites (Hoey & Fewson, 1990). It is thought that regulation of the pathway occurs only at the level of gene expression (Fewson, 1991), hence showing the value in the characterisation of this pathway at the molecular level, to determine the processes by which this control is achieved.

Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II (BZDHII) expression is induced by the presence of benzyl alcohol. Benzaldehyde dehydrogenase I (BZDHI) is induced only in the mandelate pathway, so the expression of these two isofunctional benzaldehyde dehydrogenase enzymes is quite separate. The induction of the mandelate pathway is dominant over the benzyl alcohol pathway, even though benzyl alcohol supports a higher growth rate and yield (Beggs & Fewson, 1977; Beggs *et al.*, 1976; Fewson, 1985). Why this occurs is not clear.

1.8.6. Characterisation of enzymes involved in the TOL-plasmid upper pathway and mandelate and benzyl alcohol convergent pathways

Five enzymes from the mandelate and benzyl alcohol convergent pathways in *A. calcoaceticus* and the upper pathway in *P. putida* share two common substrates: NAD(H) and benzaldehyde, and carry out similar reactions: dehydrogenation. Such similarities provides a perfect opportunity to investigate the hypothesis of retrograde evolution and

gene recruitment (Section 1.1.3), within the pathway itself and between *P. putida* and *A. calcoaceticus*.

Three enzymes, BADH, BZDH I and BZDH II from *A. calcoaceticus*, and the two analogous enzymes from *P. putida* mt-2 encoded by the TOL-plasmid (pWW53), TOL-BADH and TOL-BZDH, have been purified to homogeneity (Chalmers *et al.*, 1990; 1991). Two further analogous enzymes encoded by the TOL-plasmid (pWW0), TOL-BADH (pWW0) and TOL-BZDH (pWW0), have also been purified (Shaw *et al.*, 1990). Although a lot of preliminary characterisation substrate specificity and quaternary structure has been done on these enzymes only one has been fully sequenced: plasmid pWW0 encoded TOL-BADH (Shaw *et al.*, 1993). On the basis of its primary structure it has been characterised as a group I alcohol dehydrogenase.

The *A. calcoaceticus* enzymes BADH, BZDHI and BZDHII have been shown to be homo-tetramers of subunit size 39.7kDa, 56 kDa and 55 kDa respectively (Chalmers *et al.*, 1990; 1991; MacKintosh & Fewson, 1988a, b), Characterisation of plasmid pWW53 encoded TOL-BADH and TOL-BZDH showed their subunit sizes to be 43 kDa and 56 kDa respectively and to be tetrameric in structure (Chalmers *et al.*, 1991). These values seem to be very similar for equivalent enzymes. *N*-Terminal sequence analysis, immunological evidence, as well as amino acid composition, have been identified as similar among these enzymes (Chalmers *et al.*, 1991). The TOL-BADH and TOL-BZDH encoded by the TOL-plasmid (pWW0) have been found to have subunit sizes of 40 kDa and 57 kDa respectively (Harayama *et al.*, 1989) which agree well with the subunit sizes for the *A. calcoaceticus* and TOL-plasmid pWW53 encoded enzymes (Chalmers *et al.*, 1990). However, although there is this obvious similarity in subunit size the pWW0 encoded enzymes have been identified as dimeric (Shaw & Harayama, 1990; Shaw *et al.*, 1993), in contrast to the equivalent enzymes of *A. calcoaceticus* and TOL-plasmid (pWW53) which were characterised as tetrameric (Chalmers *et al.*, 1990). This difference in quaternary structure is unusual for what appear to be such closely related enzymes and also because all other microbial group I ADHs sequenced so far have been found to be tetrameric (Table 1.3). This poses interesting question in that why should such enzymes which appear to be very similar in function and and subunit size have such contrasting

quaternary structure? When the primary structures of the *A. calcoaceticus* BADH, BZDHI and BZDHII enzymes are determined they are likely to give an insight into the way in which primary structure can determine quaternary structure.

BADH from *A. calcoaceticus* and TOL-BADH (pWW53) have been tentatively classified as members of the group I ADHs, due to the *N*-terminal sequences of BADH and TOL-BADH (pWW53) having 26% identity with horse liver alcohol dehydrogenase. This proposal is strengthened by the primary stricture of TOL-BADH (pWW0) having been determined and classified as a member of the group I ADHs (Shaw *et al.*, 1993). The lengths of the polypeptide subunits of both TOL-BADH(pWW53) and BADH based on their respective molecular weights have been estimated as both 381 residues, a length compatible with that of a group I alcohol dehydrogenase (Chalmers *et al.*, 1991).The *N*-terminal sequences of BZDHI was shown to have 29% identity with both BZDHII and TOL-BZDH (pWW53). However, 37% identity was seen in between the *N*-terminus of BZDHII and TOL-BZDH (pWW53), which indicates a closer evolutionary relationship between BZDHII and TOL-BZDH (pWW53) than either have with BZDHI.

Antisera cross reactions occurred between both BZDHI and BZDHII using antisera raised against BZDHI. However, a greater cross reaction was seen between BZDHII and TOL-BZDH suggesting, again, that these two enzymes might be more closely linked in evolutionary terms than either is with BZDHI (Chalmers *et al.*, 1991).

Certain requirements for enzyme activity appear to conflict with the hypothesis that BZDHII and TOL-BZDH are more closely related to each other in evolutionary terms than either is to BZDHI. BZDHI has a dependence on the presence K^+, NH_4^+ or Rb^+ for maximum activity and this is also true of TOL-BZDH (pWW53). However, BZDHII does not appear to require cations ions for activity (Chalmers *et al.*, 1990). On the basis of these requirements it would appear that BZDHI and TOL-BZDH (pWW53) are more closely related than BZDHII. Hence the current picture is a complex one and will only be resolved once the primary structure of these enzymes has been determined.

The kinetic parameters amongst the BADH and BZDH enzymes from *A. calcoacteicus* and *P. putida* differ greatly (Table 1.14). Why there is such a large range of kinetic parameter is not clear. There is a definite correlation between quaternary structure

Table 1.14.

Kinetic parameters of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase from *A. calcoaceticus* and *P. putida* .

The information is taken from Chalmers *et al* . (1990) and Shaw *et al* . (1992). K_m and V_{max} values refer to benzaldehyde and benzyl alcohol as substrates for BZDH and BADH respectively. The abbreviations used are those quoted in table 1.13.

Enzyme	Apparent K_m (μM)	Apparent V_{max} (μmol. min^{-1}. mg protein)
BADH	121	351
TOL-BADH (pWW53)	233	96
TOL-BADH (pWW0)	220	330
BZDHI	0.69	106
BZDHII	0.63	64
TOL-BZDH (pWW53)	0.79	104
TOL-BZDH (pWW0)	460	17

of of the BZDHs and their kinetic parameters. The tetrameric TOL-BZDH (pWW53), BZDHI and BZDHII have apparent K_m and V_{max} values of a similar order, whereas the dimeric BZDH (pWW0) in contrast has a very high apparent K_m value and a much lower apparent V_{max} value. However, there is no correlation observed between quaternary structure and kinetic parameters of the BADH enzymes. The differences may be the result of some of the enzymes lacking a proton-relay mechanism as with TOL-BADH (pWW0) (Section 1.2.2.2) or differences in the substrate binding pocket (Section 1.2.3.3). However, the reasons for the anomalies in kinetic parameters of the three BADH enzymes will not become clear until primary sequence information is available and is related to enzyme function.

1.9. Aims and scope of this thesis

The original aim of this project was to clone and sequence the gene encoding BADH in *Acinetobacter calcoaceticus* NCIB 8250. This was to be done by generating a probe using PCR for the *N* -terminal region of the BADH gene which was to be used in screening a genomic DNA λ-library. The cloning and sequencing was to be done with a view to characterisation of the enzyme, its regulation and expression and its comparison with the equivalent TOL-plasmid encoded BADH. In the event, only about one year was available for this project and so it was decided to concentrate on producing a probe for the chromosomally encoded BADH from *A.calcoaceticus* NCIB 8250 with a view to identifying the BADH gene from a genomic DNA λ library.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

All chemicals and reagents used were obtained from BDH Chemicals Ltd, Poole, Dorset, U.K. unless otherwise stated.

Ampicillin, tetracycline, DTT, ethidium bromide, Mops buffer, ficoll, RNase A, BSA, Coomassie Brilliant Blue G250, Triton X-100 and Tris buffer were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K.

Bactotryptone, yeast extract and bactoagar (agar) were obtained from Difco, Detroit, U.S.A.

Oxoid No. 1 agar, nutrient agar and nutrient broth were obtained from Oxoid Ltd., London, U.K.

Urea (ultrapure) and phenol (ultrapure) were obtained from Gibco BRL Ltd., Paisley, U.K.

Xylene cyanol was obtained from IBI Ltd., Cambridge, U.K.

Sodium lauryl sulphate was obtained from Fisons Scientific Equipment, Loughborough, Leics., U.K.

Ethanol was obtained from James Burroughs (F.A.D.) Ltd., Witham, Essex, U.K.

Oligonucleotides were synthesized using an Applied Biosystems Model 280A DNA synthesizer using phosphoramidate chemistry by Dr. V. Math (University of Glasgow Department of Biochemistry).

Sequenase version 2.0 sequencing kit was obtained from U.S. Biochemical Corporation (distributed by Cambridge BioScience, Cambridge, U.K.).

[α–^{35}S]-dATP (code SJ.1304), [α–^{32}P]-dATP (PB10168) and Hybond nylon DNA filters were obtained from Amersham International p.l.c., Amersham, U.K.

All restriction enzymes, T4 DNA ligase and T4 polynucleotide kinase were obtained from Gibco BRL Ltd., Paisley, U.K.

Visking tubing was obtained from The Scientific Instruments Centre, Eastleigh, Hants, U.K.

Filter paper was obtained from Whatman International, Maidstone, Kent, U.K.

2.2. General biochemical methods

2.2.1. pH measurements

The pH values of solutions were routinely determined with a Kent Electronic Instruments Ltd. (Chertsey, Surrey, U.K.) #type 7010 meter calibrated at room temperature using standards of pH7 and pH4 prepared from tablets obtained from the manufacturer. Volumes of less than 3ml (e.g. enzyme assay mixtures) were measured with a Radiometer (Copenhagen, Denmark) type M26 pH monitor fitted with a GK 2302 micro pH electrode.

2.2.2. Glassware and plastics

Glassware was washed in Haemo-sol solutions [Alfred Cox (Surgical) Ltd., U.K.] according to the manufacturer's instruction, rinsed in tap water followed by distilled water and dried in an oven. Plastic tips for micropipettes and Eppendorf tubes were taken from a newly opened bag.

2.2.3. Dialysis

Dialysis tubing was prepared by boiling for 10 min in 1% (w/v) EDTA, then rinsed and boiled three more times for 10 minutes each in distilled water and stored in 20% (v/v) ethanol at 4°C until use.

2.2.4. Spectrophotometric determination of nucleic acid concentrations and purity

Nucleic acid concentrations were determined spectrophotometerically at 260nm (Sambrook *et al.*,1989). In a 1cm light path length quartz cuvette an OD of 1.0 corresponds to 50μg.ml^{-1} for double stranded DNA, 33μg.ml^{-1} for single stranded DNA and approximately 20μg.ml^{-1} for single stranded oligonucleotides. Purity was determined from the OD value of 260nm divided by that of 280nm (protein absorbance peak). Values above 1.8. were accepted as indicating sufficiently pure samples.

2.2.5. Buffers and other solutions

Glass distilled water stored in polythene containers was used in all experiments. All buffers were were prepared at room temperature. Solutions were made up to approximately nine-tenths volume and were adjusted with the appropriate acid or base for the correct pH.and then made up to the final volume. Buffers were sterilized before use if necessary.

Buffers commonly used:

TE buffer pH 7.4	10mM Tris HCl buffer (pH 7.4) 1mM EDTA (pH 8.0)
TE buffer pH7.6	10mM Tris HCl buffer (pH 7.6) 1mM EDTA (pH 8.0)

TE buffer pH 8.0	10mM Tris HCl buffer (pH 8.0) 1mM EDTA (pH 8.0)
GTE buffer pH 8.0	50mM Glucose 25mM Tris HCl (pH8.0) 10mM EDTA (pH8.0)
TBE buffer	22.5mM Tris-borate (pH 8.0) 5mM EDTA (pH8.0)
TAE buffer pH 8.0	40mM Tris-acetate (pH 8.0) 10mM EDTA (pH 8.0)
20xSSC buffer pH 7.0	3M NaCl 300mM $Na_3C_6H_5O_7$
50xDenhardt's solution	5g of Ficoll 5g polyvinylpyrrolidone 5g BSA

2.2.6 Sterilisation

Solutions for the manipulation of nucleic acids and growth media were autoclaved in a Dent and Hellyer Lab ClaveII Autoclave at 110°C for 30 minutes.

Because of heat lability or volatility, compounds such as antibiotics and alcohols were sterilised by filtration through 0.22μm pore-sized Millex-HV sterile filters (Millipore Ltd., Watford, U.K.)

2.2.7. Conductivity measurements

Conductivity measurements were made on a Type 5.10EB Mini-monitor (Mini Instruments, Burnham on Crouch, Essex). All work done using ionising radiation [α-^{35}S]-dATP and [α-^{32}P]-dATP was carried out as much as possible behind a 8mm perspex shield. All incubations and centrifugations were carried out in clearly labelled water baths and centrifuges.

2.3 Microbiological techniques

2.3.1 Source of micro-organisms and plasmids

Acinetobacter calcoaceticus NCIB 8250 was obtained from Professor C. A. Fewson, Department of Biochemistry, University of Glasgow. *Eschericha coli* JM109 was obtained from Dr. A. G. S. Robertson, Department of Biochemistry, University of Glasgow.

Plasmid pUC18 (Yanish-Perron *et al.*, 1985) carries the gene encoding ampicillin resistance and was obtained from BRL Gibco Ltd. Paisley, U.K.

2.3.2. Maintenance and storage of micro-organisms

Stock cultures of *A. calcoaceticus* NCIB 8250 were maintained in complex medium as described by Allison *et al.* (1985) at 4°C. Stock cultures *E. coli* JM109 were maintained in 50% glycerol, 50% L-broth at -70°C.

Short term storage was on suitably sealed L-agar inverted plates or as 10ml L-broth cultures at 4°C. Samples were checked for homogeneity and checked for appropriate antibiotic resistance.

2.3.3. Growth media

All amounts are per litre:

L-broth	Bactotryptone 10g Yeast extract 5g NaCl 10g [+5ml 20% (w/v) glucose] adjusted to pH7.5 with NaOH
L-agar	As for L-broth, +15g Difco-agar
Nutrient broth	13g Oxoid nutrient broth
Nutrient agar	28g Oxoid nutrient agar
L-Ampicillin broth	As for L-broth, +100mg ampicillin
L-Ampicillin agar	As for L-agar, +100mg ampicillin

Ampicillin was used in growth media at a final concentration of 100$\mu g.ml^{-1}$. A stock solution of 25$mg.ml^{-1}$. was filter sterilised and stored at -20°C. Hot molten L-agar was cooled to 55°C before ampicillin was added. L-ampicillin agar plates were stable for up to 4 weeks when stored at 4°C.

2.3.4. Growth of micro-organisms

2.3.4.1. Growth of *A. calcoaceticus* NCIB 8250

Stock culture (0.1ml) was transferred aseptically to 50ml of nutrient broth in a 250ml flask and grown on an orbital shaker for the appropriate length of time at 200 r.p.m. at 30°C.

2.3.4.2. Growth of *E. coli* JM109

Stock culture (0.1ml) was transferred aseptically to 50ml of nutrient broth in a 250ml flask and grown on an orbital shaker for the appropriate length of time at 200 r.p.m. at 37°C. Colonies picked from L-agar plates were transferred aseptically into a 10ml overnight L-broth culture containing 100$\mu g.ml^{-1}$ ampicillin (to maintain the plasmid) and incubated at 37°C for 12-15 hours.

2.3.5. Measurement of growth

Cell density was measured as apparent absorption (OD) at 600nm using a 1cm light path cuvette in a LKB Ultrospec spectrophotometer.

2.3.6.Harvesting of cells

All harvesting was carried out at 4°C by centrifugation at 10,000 r.p.m. for 15 minutes in a Mistral M.S.E. Highspeed 18 centrifuge (M.S.E. Ltd. London U.K.) The supernatant was decanted. the dry pellet weighed and either used immediately or stored at -20°C.

2.3.7. Cell disruption and isolation of DNA

High molecular weight DNA was isolated from *A. calcoaceticus* using a method based on that described by Borneleit & Kleber (1981). To prevent shearing of the DNA, all pipette tips were cut off to enlarge the orifices and all agitation and mixing were done as gently as possible.

A 1ml sample from a 10ml over-night nutrient broth culture of *A. calcoaceticus* NCIB 8250 grown at 30°C was used to inoculate a 50ml nutrient broth culture. This was grown overnight and used to inoculate a 500ml culture which was incubated for five hours and harvested whilst still in exponential phase [OD <0.850 (A_{600})] by centrifugation (Section 2.3.6). The supernatant was decanted and the pellet weighed. The pellet was washed with 0.15M NaCl and then resuspended in a conical flask containing 20ml 0.15M NaCl. 0.1M EDTA. A 1ml aliquot of 10% Triton X-100 was added to give a final concentration of 0.5% Triton X-100 and stirred at 4°C for 40 minutes to obtain complete lysis.The solution was then snap-frozen using dry-ice methanol; 80ml 1% SDS, 0.1M Tris, 0.1M NaCl was added and the mixture thawed to 65°C and then kept at that temperature with occasional stirring until the solution was clear of any debris. The freezing process was repeated on the mixture, then thawed again to 65°C and kept at 65°C overnight (11 hours) to ensure that a clear solution was obtained.

Phenol/chloroform extractions were done by adding an equal volume of phenol saturated with TE buffer (pH 8.0) and then mixing by gentle rolling for two hours at 4°C and left to stand overnight. Centrifugation at 3,000 r.p.m. (Beckman Model TJ-6 centrifuge) for 10 minutes was done to separate the organic and aqueous phases of the mixture allowing easy removal of the top aqueous phase. An equal volume of isoamyl alcohol/chloroform saturated with TE buffer (pH 8.0) was added and mixed by gentle rolling for 30 minutes. This was followed by centrifugation at 3,000 r.p.m. (Beckman Model TJ-6 centifuge) for 10 minutes to separate the two phases allowing removal of the top aqueous phase. The aqueous phase was dialysed against 4 litres of TE buffer (pH 8.0)

at room temperature for 1 hour, the buffer was then changed and left dialysing at 4°C for 12 hours during which the buffer was exchanged for fresh TE buffer (pH8.0) at 6 and 9 hours. The RNA in the sample was digested using Sigma RNAse A. RNAse free of DNAse activity was prepared by boiling RNAse A (10mg.ml^{-1}) in 0.01M sodium acetate buffer (pH 5.2) for 15 minutes. Following cooling to room temperature, the pH value was adjusted to 7.4 by adding 1M Tris HCl buffer (pH7.4). RNA was then removed by adding 0.5µl (10mg.ml^{-1}) RNAse A to the sample and incubating at 37°C for 30 minutes. The phenol/chloroform extractions were repeated as previously described to remove all remaining protein. DNA was precipitated by adding half the volume of 7.5M ammonium acetate and incubated at -20°C for 30 minutes followed by addition double the volume of ethanol at -20°C. DNA was spooled using a hooked glass rod and redissolved in 30ml TE (pH 8.0). Purity and DNA concentration were calculated according to section 2.2.4. The samples of DNA were stored at 4°C as 10 ml aliquots with 0.5ml isoamyl alcohol/chloroform saturated with TE buffer (pH 8.0) to prevent any bacterial growth.

2.4. General recombinant DNA techniques

2.4.1.Large-scale purification of plasmids

Harvested cell pellets were resuspended in 4ml solution containing 100µg.ml^{-1} RNase A, 50mM Tris.HCl, pH8.0, 10mM EDTA. A 4ml aliquot of 200mM NaOH, 1% (w/v) SDS was added gently mixed by rocking and incubated at room temperature for 5 minutes. A 4ml aliquot of 2.55M potassium acetate, pH4.8 was added to cause cell lysis. The lysate was centrifuged at 10,000 r.p.m. for 30 minutes at 4 C (JA-20, 8x50ml rotor, Beckman Model J2-21 centrifuge). The supernatant was removed and centrifuged again at 10,000 r.p.m. for 30 minutes at 4 C (JA-20, 8x50ml rotor, Beckman Model J2-21 centrifuge) to obtain a particle-free lysate. The lysate was loaded on to a QIAGEN-tip 100, pre-equilibrated with 3ml 750mM NaCl, 50mM Mops, 15% (v/v) ethanol, pH7.0,

0.15% (v/v) Triton X-100, and allowed to flow through the column by gravity. The QIAGEN-tip 100 was then washed with 10ml 1.0M NaCl, 50mM Mops, 15% (v/v) ethanol, pH7.0 and the plasmid DNA was eluted with 5ml 1.25M NaCl, 50mM Mops, 15% (v/v) ethanol, pH8.2. The QIAGEN-tip 100 was then re-equalibrated with 3ml 750mM NaCl, 50mM Mops, 15% (v/v) ethanol, pH7.0, 0.15% (v/v) Triton X-100 and the run-through from the first step was re-applied to the QIAGEN-tip 100. The QIAGEN-tip 100 was washed and the plasmid DNA eluted, as above, and the two eluates combined. The plasmid DNA was precipitated by the addition of 0.7 volumes of isopropanol and incubated at room temperature for 15 minutes followed by centrifugation at 25°C for 30 minutes (JA-20, 8x50ml rotor, Beckman Model J2-21 centrifuge). The DNA pellet was then washed with 2ml 70% (v/v) ethanol, allowed to dry at room temperature for 5-15 minutes and resuspended in 200µl TE (pH 8.0). The DNA concentration and purity were measured as according to section 2.2.4.

2.4.2 Small-scale purification of plasmids

The method used for small scale plasmid preparations of pUC18 was based on that of Birboim and Doly (1979). A 1.5ml sample of an overnight culture was harvested by centrifugation at 10,000 r.p.m. (Jouan MR14.11 cetrifuge) and resuspended in 100µl of GTE (pH 8.0) 10µl 10mg.ml^{-1} lysozyme was added and incubated at room temperature for 5 minutes. An 200µl aliquot of freshly prepared 0.2M NaOH, 1% (w/v) SDS was added to the resuspended pellet and lysozyme mixture, mixed gently and incubated at 4°C for 5 minutes. Addition of 150µl ice-cold 5M potassium acetate, pH4.8 caused lysis. The lysate was incubated at 4°C for 5-15 minutes and the debris removed by centrifugation at 4°C (Jouan MR14.11 centrifuge). The supernatant was removed into a fresh microfuge tube and the plasmid DNA was pecipitated by addition of 0.5 volumes of 7.5 ammonium acetate and 2.5 volumes of cold (-20°C) ethanol which was then incubated at -80°C for 30 -60 minutes. The precipitated DNA was recovered by centrifugation at 4°C for 30 minutes

(Jouan MR14.11 centrifuge). The pellet was washed with 200µl 70% (v/v) ethanol, dried at room temperature for 5-15 minutes and resuspended in 30µl TE (pH 8.0).

2.4.3. Digestion of DNA with restriction enzymes

The methods used were as described by Sambrook *et al.* (1989). Restriction digests were carried out using the BRL REact buffers which were provided for each enzyme. These buffers each have a different salt concentration, being suitable for a range of enzymes. Analytical digests of plasmid DNA samples were carried out using volumes of 20 or 40µl at 37°C for 1 to 2 hours where the maximum concentration of DNA used was 10µg.ml^{-1} with 0.5µl of restriction enzyme (between 10-20 units.µl^{-1}) and 20% final volume React buffer. Plasmid digestion with more than one enzyme used a React buffer suitable for both enzymes.

2.4.4. Agarose gel electrophoresis

DNA was separated on horizontal submerged agarose gels by the method described by Sambrook *et al.* (1989) . The buffers used in the agarose gels and as tank buffers were TBE buffer (pH 8.0) and TAE buffer (pH 8.0). Agarose gels of 0.8% (w/v) agarose were used to accurately separate size fragments of 0.8 to 10 kb, and 2% (w/v) agarose gels were used for separation of smaller fragments (100 base pairs or 0.1kb). Samples for agarose gel were prepared by the addition of 0.2 volumes of 10mM-Tris/HCl pH 7.2, 20% (w/v) Ficoll, 0.5% (w/v) Bromophenol Blue and ethidium bromide (10mg.ml^{-1}). Ethidium bromide was also present in agarose gels and tank buffers at 0.5mg.ml^{-1}. DNA was visualised on a long wave U.V. transilluminator (U.V. products Inc.). λ and pBR322 DNA digested *Hin*dIII and *Hae*III respectively, was run alongside the PCR products as marker fragments of molecular weight.

2.4.5. Recovery of DNA from agarose gels

The purification of a single DNA fragment was based on the method by Sambrook *et al* . (1989). Low melting point agarose gels were run at 4°C and at low current to prevent gels from melting.The DNA was visualised as described in section 2.4.4. The desired band was cut from the gel and placed in a sterile Eppendorf tube and incubated for 10-15 minutes at 65°C to melt the agarose.

2.4.6. Phenol/chloroform extraction of nucleic acids

Solutions containing DNA were deproteinised by extraction with phenol and chloroform. The volume of a sample was measured and adjusted to 100µl usually [except for chromosomal preparation from *A. calcoaceticus* which involved much larger volumes (Section 2.3.7)], with TE buffer (pH 8.0). An equal volume of a phenol: chloroform: isoamyl alcohol mixture in the ratios of 25:24:1, respectively saturated with TE buffer (pH 8.0) was added and then vortexed, allowed to stand and separate into two layers, and then vortexed again. The two layers were separated by centrifugation at room temperature by microfuge for 5 minutes. The upper, aqueous layer was removed into a fresh tube and 100µl TE buffer (pH8.0) added to the bottom, organic layer. The mixture was vortexed and separated again after centrifugation at room temperature. The two top layers were combined and extracted in identical manner but with an equal volume of TE (pH 8.0)-saturated chloroform:isoamylalcohol (24:1) removing any traces of phenol. DNA was recovered from the aqueous layer by ethanol precipitation (Section 2.4.7).

2.4.7. Ethanol precipitation

Aliquots of 0.1 volumes of 3M sodium acetate were added to the solution containing the DNA, followed by 2.5 volumes of ethanol at -20°C and mixed.When the DNA was to be treated with T4 polynucleotide kinase, 0.5 volumes of 3M sodium acetate were used instead of sodium acetate because ammonium acetate inhibits this enzyme. The mixture was incubated at -80°C for between 30 and 60 minutes and the DNA recovered by centrifugation in a microfuge at 4°C for 30 minutes. The pellet was washed in 70% (w/v) ethanol, allowed to dry and resuspended in a suitable volume of TE (pH 8.0).

2.4.8. Ligations of DNA

The plasmid pUC18 was linearised by digestion with *Bam*HI and *Eco*RI in a three-fold excess of DNA to that stated previously (Section 2.4.3), i.e. 1.5µl each. Once the digestion was complete, the linearised pUC18 was purified from a low melting point 0.8% agarose gel to separate the plasmid from the stuffer fragment (Sections 2.4.5-2.4.7). The DNA concentration and purity of the linearised pUC18 were determined (Section 2.2.4). The linearised plasmid was treated with calf intestine alkaline phosphotase to remove the 5'-phosphate groups from the linearised DNA and so prevent self ligation of the vector and ensure a low background of false positive clones.The 5'-phosphate groups were removed by incubation of the DNA with 10µl of 10x calf intestine alkaline phosphatase dephosphorylation buffer [10mM $ZnCl_2$, 10mM $MgCl_2$, 100mM Tris Cl (pH 8.0)] with 1 unit calf intestine alkaline phosphatase at 37°C for 30 minutes. Following incubation, the DNA was deproteinised (Sections 2.4.6 & 2.4.7), resuspended in TE buffer (pH8.0) and the DNA purity and concentration of the sample determined (Section 2.2.4).

For ligations with a purified single fragment, insert generated from PCR equal concentration of fragment DNA and linearised vector DNA was used. However, for

shotgun ligations a range of concentrations of foreign DNA was used, ranging from equal to four-fold the concentration of the linearised vector. Depending on the size of the insert, ligation mixtures contained 25ng vector and between 25 and 200ng insert in the final volume 5 to 10µl.

The linearised vector and fragment were pre-incubated at 45°C for 5 minutes in the ligation mixture less the ligase enzyme to melt any cohesive termini that might have reannealed. The ligation was carried out at 16°C for 16-20 hours in 66mM-Tris/Hcl pH7.6, 6.6mM-MgCl 0.5mM-ATP, 10mM-DTT, using 0.5 units of bacteriophage T4 DNA ligase (Weiss *et al* ., 1968).

2.4.9. Transformation of *E.coli* JM 109

2.4.9.1. Preparation of competent cells

Transformation was based upon the method of Cohen *et al.* (1972). A single colony of *E. coli* JM109 from an agar plate was used to inoculate a 10ml culture of L-broth and incubated at 37°C overnight on an orbital shaker. A 1ml aliquot of the overnight culture was used to inoculate a 100ml culture which was incubated at 37°C until the culture OD (A_{600}) was 0.4. The cells were chilled on ice for 10 minutes and then harvested by centrifuging at 4.000 r.p.m for 10 minutes (Beckman J2-21, 8x50ml rotor,4°C). The pellet was resuspended in 50 ml (half volume) of cold (4°C) 50mM-$CaCl_2$, 10mM-Tris/HCl buffer (pH8.0) and chilled on ice for 30 minutes. and then harvested at 4,000 r.p.m. for 10 minutes (Beckman J2-21, 8x50ml rotor,4°C). The cells were resuspended in 1/15th original culture volume (6.5ml) 50mM-$CaCl_2$, 10mM-Tris/HCl pH8.0 which was then mixed in the ratio 3:1 culture with glycerol. The glycerol stock culture was dispensed into chilled microfuge tubes in 300µl aliquots and stored at -80°C. The cells were used 24 hours after being prepared, when they were most viable (Dagert & Ehrlich, 1979).

2.4.9.2. Transformation of competent cells

Transformations were carried out in 1.5ml sterile Eppendorf tubes. A 10µl aliquot of the ligation mixture (containing up to 25ng of plasmid DNA) was added to 200µl of competent cells which were left on ice for one hour to allow absorption of the DNA. The cells were heat shocked by transfer to a 42°C incubation for 5 minutes followed by addition of 1 ml of pre-warmed L-Broth (37°C) and incubation at 37°C for 30 minutes. Aliquots of 100µl of the cells were spread on Amp^r selective L-agar plates (ampicillin 0.25mg.ml^{-1}) which were inverted and incubated at 37°C overnight. Amp^r selective plates were set up using 10µl of distilled water, 10µl of unligated phophotased pUC18 and 10µl intact pUC18 to assay for the frequency of revertants.

2.4.10 Generation of single stranded DNA

The host cells were prepared, infected and plated out as in section 2.4.9. Individual colonies were picked and used to inoculate a 10ml overnight L-broth (0.25mg.ml^{-1} ampicillin) culture. Large scale plasmid preparation from each of the selected colonies was done (Section 2.4.1.). Single stranded DNA was generated by incubation of approximately 4µg of DNA in 0.16mM EDTA, 0.16mM NaOH for 5 minutes at room temperature. This reaction was quenched by the addition of 0.1 volumes of ammonium acetate (pH4.6) and mixed by vortexing. DNA was then ethanol precipitated (Section 2.4.7).

2.5. Sequencing with Sequenase® Version 2.0

2.5.1. Annealing of primer to the template

7µl of single stranded DNA template (Section 2.4.10) was annealed for 2 hours at 55°C in a mixture containing:

1µl M13 sequencing primer, -40 primer (3ng/µl)

2µl 200mM Tris.HCl, pH7.5, 100mM $MgCl_2$, 250mM NaCl (5x concentrate Sequenase® buffer)

The M13-40 primer has the sequence 5'-GTT TTC CCA GTC ACG AC-3'.

2.5.2. Sequence reactions

To the annealed primer and template mixture was added; 1ml DTT (100µM), 2µl labelling mix (1.5M dGTP, 1.5µM dCTP, 1.5µM dTTP), 0.5µl (5µCi)[α-^{35}S]-dATP at >600Ci.m.mol^{-1} Amersham SJ.304) and 1.5 units of Sequenase Version 2.0. The labelling reaction was mixed and incubated at room temperature for 5 minutes. During this incubation 3.5µl aliquots of the labelling reaction were placed in the bottom of 4 Eppendorf tubes labelled A. C, G and T. In each of these four tubes there had previously been spotted on the underside if the lid 2.5µl of the appropriate termination mix [80µM dATP, 80µM dCTP, 80µM dGTP, 80µM dTTP, 8µM ddNTP(appropriate analogue), 50mM NaCl]. After the 5 minute incubation, and prior to mixing, the reagents were incubated for 1 minute at 37°C then mixed immediately by a brief microfuge spin. The mixtures were further incubated at 37°C for 5 minutes, followed by the addition to each of 4µl of stop mix (95% (v/v) formamide, 20mM EDTA, 0.05% (w/v) Bromophenol Blue, 0.05% (w/v) Xylene Cyanol FF). Samples were immediately prepared for loading on to the sequencing gel by heating to 75-80°C for 2 minutes or stored at -80°C.

For sequences hard to read because of G/C rich regions or compressions in the base ladder, the labelling mix contained (3.0μM dITP, 1.5μM dCTP, 1.5μM dTTP) and the termination mix contained (80μM dATP, 80M dCTP, 80μM dITP, 80μM dTTP, 8μM ddNTP(appropriate analogue), 50mM NaCl) except the ddGTPmix (80μM dATP, 80μM dCTP, 160μM dITP, 80μM dTTP, 8μM ddNTP (appropriate analogue), 50mM NaCl).

2.5.3.Polyacrylamide gel electrophoresis

The nested set of primer extended fragments produced by the sequencing protocols were resolved by electrophoresis on thin polyacrylamide gels using the BRL S2 sequencing gel apparatus (BRL-Gibco).Gels were composed of:

40% acrylamide	
(38% acrylamide, 2% bisacrylamide)	15ml
10xTBE	10ml
Urea	50g
Distilled water	35ml
10% (v/v) ammonium persulphate	350μl
TEMED	50μl

The solution was degassed before addition of TEMED and ammonium persulphate. Gels were pre-run for at least 15 minutes at 50mA before electrophoresis in TBE. Before loading pre-prepared samples, the top of the gel was cleared of any unpolymerised acrylamide or urea and the lanes formed using a sharks tooth comb. 5μl of the denatured samples were loaded to the top of the gel in the order of A, C, G, T (appropriate analogue) and electrophoresis carried out at 60W (constant power), 50mA, 2,000V for up to 5 hours.

Gels were fixed in 10% (v/v) acetic acid, 10% (v/v) methanol for 30 minutes following electrophoresis and were vacuum dried onto a sheet of Whatman 3MM paper using a Bio-Rad gel drier model 1125. The dried gel was autoradiographed using Fuji RX film at room temperature.

2.6. Polymerase chain reactions

2.6.1.Preparation of oligonucleotides

The oligonucleotides used for primers designed using the *N* -terminal amino acid sequence of benzyl alcohol dehydrogenase (Chalmers *et al.* 1988) and the Biosoft Gene-Jockey sequence processor (Taylor, 1991). A degenerate coding sequence was obtained and from this four oligonucleotide sequences of 22 bases with the lowest degeneracies were chosen as suitable to be used as primer pairs binding to complementary DNA strands. The oligonucleotides had the following sequences:

5'-CGG AAT TCC ACA TNC CNG TNG C (1108)	64
5'-CGG GAT CCT GYA ARG GNG CNG A (1109)	64
5'-CGG GAT CCA ARG AYA THA THG C (985)	48
5'-CGG AAT TCA CYT CRT CNC CYT G (986)	16

The numbers after each oligonucleotide refer to the number of degeneracies. Oligonucleotides 985 and 986 were designed by Dr. A. G. S. Robertson. The oligonucleotides were supplied in a solution of 35% (w/v) NH_4OH. The oligonucleotides were prepared by ethanol precipitation (Section 2.4.7.) and resuspended in TE (pH 8.0). The volume was adjusted to give a final concentration of $1 mg.ml^{-1}$.

2.6.2 Reaction parameters

Each 100µl reaction volume contained: approximately 1µg of target DNA (9µl of 119ng.µl^{-1}) 1µl of each primer at an assumed concentration of 1µg.µl^{-1}, all four dNTPs at a final concentration of 0.2 mM, 10µl of 10x Taq polymerase buffer, 2.5 units (0.5µl) Taq polymerase and made up to 100µl with distilled water. The reaction volume was overlaid with an equal volume of paraffin oil and air bubbles were removed by microfuging.

Reaction volumes containing only one primer, no target DNA and no enzyme were used as control reactions. Primers which had previously been successful in amplifying a sequence from benzaldehyde dehydrogenase II designed by Dr. A. G. S. Robertson were used in a positive control reaction.

PCR was set up as follows:

3 minutes 94°C [Melting]
Cycle x 30
1.5 minutes 94°C [Melting]
2 minutes 57°C [Annealing]
3 minutes 72°C [Polymerisation]
7minutes 72°C [Polymerisation]

The reaction tubes were immediately stored on ice (4°C) before removing a 1/10 volume aliquot to visualise run on a 2% agarose gel (Section 2.4.4).

2.7.Southern blotting

2.7.1. Radiolabelling of the probe

The probe was generated using PCR (Section 2.6.2) but using the sequenced clone pMR1 as target DNA in increasing amounts from 1ng to 2.75µg. The product was

purified from the target pMR1 by gel electrophoresis (Sections 2.4.5-2.4.7). This fragment was then used as target for further PCR (Section 2.6.2). This method meant that the probe generated was not likely to be contaminated with any pUC18 DNA. The product was cleaned using QIAEX and QIAGEN-spin 20 columns which removes oligonucleotides under 100 bases in length.

An equal volume of 400mM NaCl, 50mM Mops,pH7.0 was added to the sample and at the same time A QIAGEN-spin 20 column was equalibrated with 0.8ml 400mM NaCl, 50mM Mops,pH7.0. The sample was loaded onto the QIAGEN-spin 20 column and microfuged, the eluate discarded. The column was washed twice with 0.8ml 100% isopropanol to remove any traces of mineral oil, followed by two washes with 750mM NaCl, 50mM Mops, 15% ethanol pH7.0. The DNA was eluted with 0.8ml 7M $NaClO_4$, 10mMTris, 5% ethanol, pH7.0 and microfuged, the eluate collected in a fresh microfuge tube.

QIAEX was vortexed until a homogenate was obtained and then a 10µl aliquot was added to the DNA eluate and incubated at room temperature, vortexing briefly every 2 minutes. The sample was centrifuged at 7000 r.p.m. for 30 seconds and the supernatant removed. The pellet was washed twice with 0.5ml 100mM NaCl, 10mM Tris/HCl, 70% ethanol (pH7.5). The sample was again cetrifuged for 30 seconds at 7000 r.p.m.and any traces of ethanol removed and the pellet dried at room temperature for 10 minutes before being resuspended in 20µl TE buffer. The probe was labelled at the 5'-end using T4 polynucleotide kinase.

A reaction mixture was made containing:
1µl 5'-T4 polynucleotide kinase
5µl 10x5'-T4 polynucleotide kinase buffer
5µl probe
[α–^{32}P]-dATP 5µl (50µCi)
Final volume made up to 50µl with distilled water.

The reaction mixture was incubated at 37°C for 30 minutes. Incorporation of label and removal of unincorporated [α–^{32}P]-dATP was checked using a CHROMA Spin-100 column. The CHROMA Spin-100 column gel matrix was resuspended by several inversions of the column and then allowed to stand to drain the buffer. The CHROMA Spin-100 column followed was microfuged for 3 minutes at 700g until the column appeared semi-dry and the eluted buffer was discarded. This centrifugation was then repeated. A sterile microfuge tube was attached to the column the sample applied to the top and centrifuged for 5 minutes at 700g. The level of incorporation in the sample at the bottom of the tube was assessed using a series 900 mini-monitor (Section 2.2.7).

2.7.2.Transfer of DNA to filters

Transfer of DNA fragments from an agarose gel to a Hybond nylon filter was done using a method described by Sambrook *et al.* (1989). Aliquots containing 10µg of chromosomal DNA were digested with the appropriate restriction enzymes (Section 2.4.3.) and loaded onto an agarose gel. The fragments were separated (Section 2.4.4) on a 0.7% agarose gel cast in 0.5x TBE. After electrophoresis, the DNA was visualised under UV and photographed.

Capillary transfer of DNA to Hybond nylon filter was used (Southern, 1975). The gel was cut at the bottom left-hand corner to aid orientation in later operations. The DNA was denatured by soaking the gel in 3 volumes of 1.5M NaCl, 0.5N NaOH and gently agitated on a rotary platform (Luckham, model R100 rotary shaker) for 45 minutes. The gel was briefly rinsed in distilled water and then neutralised by soaking in 3 volumes of 1M Tris.HCl. (pH7.4), 1.5M NaCl for 30 minutes with constant agitation. This neutralisation step was repeated with fresh solution.A support for the gel was made by covering a piece of Plexiglass with Whatman 3MM paper which was then placing in a baking dish. The dish was filled, almost to the top of the support , with transfer buffer

(10x SSC). Any air bubbles under the wetted support were smoothed out with a glass rod.

A piece of Hybond nylon was cut to be about 1mm larger than the gel which was then thoroughly wetted by floating it on distilled water for 5 minutes before completely immersing it. A corner was cut from the nylon filter to match that cut from the gel. The gel was removed from the neutralisation solution and inverted so that its under side was uppermost when placed on the support. The gel was surrounded with Saran Wrap to serve as barrier to prevent any of the transfer buffer by-passing the gel to "short circuit" the transfer.

The wet nylon filter was placed on the gel so that the cut corners were aligned and any air bubbles removed. Two pieces of Whatman 3MM paper, the same size as the gel, were soaked in 2xSSC and placed on top of the filter avoiding any air bubbles. A stack of paper towels was placed on top of the Whatman 3MM paper 5-8cm high, and then a weight of approximately 500g was placed on top of that. The filter was left to absorb the DNA for 16 hours, allowing complete transfer to occur.

After transfer was complete, the filter was placed on Whatman 3MM paper, gel side up, and the gel slots marked on the nylon filter with a soft pencil. The gel was removed from the filter and viewed un U.V. light to ensure the DNA had transferred. The filter was soaked in 6xSSC for 5 minutes to remove any stuck agarose. The filter was removed and allowed to dry at room temperature for 30 minutes. To fix the DNA on the filter, the filter was wrapped between two sheets of Whatman 3MM paper and baked at 80°C for 2 hours. Following baking the filter was stored at room temperature in an air sealed bag.

2.7.3. Hybridisation of radiolabelled probe to filters

The pre-baked filter was placed on the surface of a tray of 2xSSC (DNA side up) until it had become thoroughly wetted from beneath, then submerged for 5 minutes. The filter was placed in a Salton heat-sealed plastic bag with 100ml pre-washing solution

(5xSSC,0.5%SDS. 1mM EDTA) and incubated and agitated for 2 hours at 42°C. The filter was gently removed and transferred to another plastic bag containing 40ml of pre-hybridisation solution (5xSSC, 5xDenhardt's solution, 0.5%[w/v]SDS) which was sealed and incubated at 68°C for 2 hours whilst being agitated. The filter was transferred to a bag containing 40ml of the solution above, less the Denhardt's solution. The ^{32}P-labelled probe was denatured by boiling for five minutes then placed on ice before adding 12.5µl-25µl to the hybridisation bag. Care was taken when sealing the bag that any air bubbles were kept to a minimum to ensure an even coating of the filter by the probe. The hybridisation bag was incubated at 68°C and agitated for 12 to 16 hours. When the hybridisation was complete the filter was removed and immediately and immersed in 300-500ml of 2xSSC and 0.1%SDS and agitated for 5 minutes then transferred to a fresh batch of solution and repeated. The washing of the filters was repeated a total of 6 times. Care was taken to at no point let the filter dry out. The filter was covered in Saran wrap and autoradiographed using Fuji RX film for 48 hours at -70°C with an intensifying screen.

CHAPTER 3

RESULTS

3.1. Introduction

The probing of a DNA library relies on a radio-labelled oligonucleotide annealing to a complementary region of DNA. This can be achieved by using a degenerate probe designed on the basis of the known amino acid sequence. The lower the proportion of degeneracy and the longer a probe is, the more specific is its annealing and thus the better it is for identification of a DNA sequence from a library. To generate a long probe with no degeneracies, PCR was used in this work.

3.2.Generation of a probe for the benzyl alcohol dehydrogenase gene from *A. calcoaceticus*

3.2.1. Oligonucleotide design

The *N*-terminal sequence of benzyl alcohol dehydrogenase (Figure 3.1.) which had been determined by Chalmers *et al.* (1991) was used to design a degenerate coding sequence (Figure 3.2.). Two sets of primer pairs were designed to anneal to the coding and non-coding strands within the *N*-terminal coding region of the sequence. Because of the nature of the genetic code, the oligonucleotide primers had also to be degenerate in order to ensure specific annealing to the target DNA. Sequences were chosen with minimum degeneracies to optimise specific annealing in the PCR reaction. Oligonucleotides 1108 and 1109 had 64 degenerate sequences, and 985 and 986 had 48 and 16 degenerate sequences respectively. The primer pairs 1108 and 1109, and 985 and 986 (Figure 3.2) were designed to incorporate restriction sites *Eco*RI and *Bam*HI, respectively, at their 3' ends to allow easy cloning of the product into a vector and determination of orientation in the vector. Two extra nucleotides (G and C) were incorporated at the ends of the restriction sites to increase the cutting efficiency of restriction enzymes.

It was predicted via *N*-terminal sequence alignments with other group I ADHs (Chalmers *et al.*, 1991) that the next amino acid residue in the sequence at position 43

Figure 3.1.

Amino acid sequence of the *N*-terminus of benzyl alcohol dehydrogenase from *A. caloaceticus*

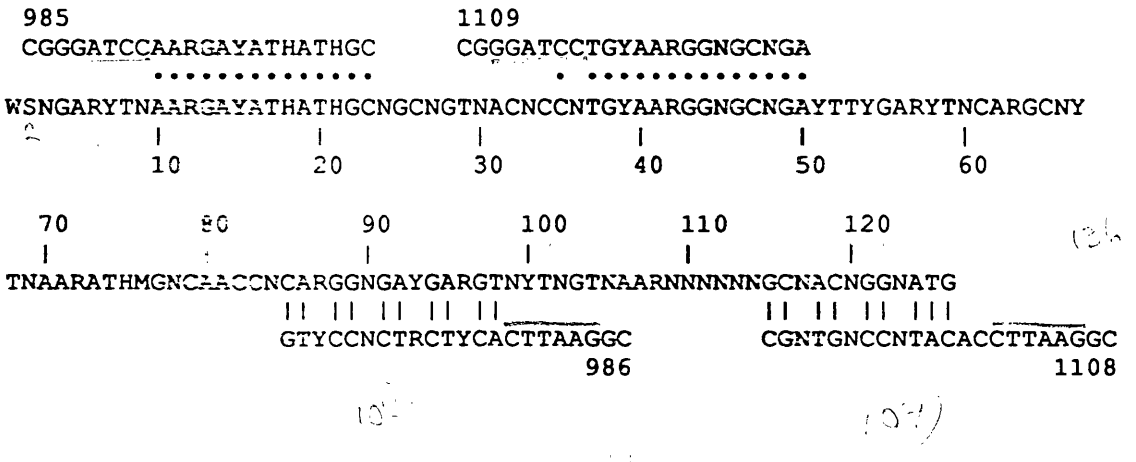
The first 42 *N*-terminal residues of benzyl alcohol dehydrogenase (BADH) as determined by Chalmers *et al.* (1991). X denotes undetermined residues. Aligned with HLADH Jörnvall. (1970); TOL-BADH(pWW0) Shaw *et al.* (1993); TOL-BADH(pWW53) Chalmers *et al.* (1991); BSADH Sakoda & Imanaka (1992); AEADH Jendrossek *et al.* (1988) and TBADH Peretz & Burstein. (1989). Numbering is based on HLADH.Symbols: *, conserved residues in most group I ADHs; +, conserved residues amongst the BADH enzymes. Abbreviations used are those quoted in table 1.13.

```
1         10        20        30        40        50
|         |         |         |         |         |
MSTAGKVIKCKAAVLWEEKKPFSIEEVEVAPPKAHEVRIKMVATGICRSDDHVVSGTLVT  HLADH
    SELKDIIAAVTPCKGADFELQALKIRQPQGDEVLVKXXATGM                BADH
       MEIKAAIVRQKNGPFLLEXVALNEPAXDQVLVRLVATGLPCTDLVXRDQGYPV  TOL-BADH(pWW53)
       MEIKAAIVRQKNGPFLLEHVALNEPAEDQVLVRLVATGLCHTDLVCRDQHYPV  TOL-BADH(pWW0)
         MKAAVVEQFKKPLQVKEVEKPKISYGEVLVRIKACGVCHTDLHAAHGBWPV  BSADH
     MTAMMKAAV·FVEPGRIELADKPIPDIGPNDALVRITTTTICGTDVHILKGEYPV  AEADH
         MKGFA·MLSIGKVGWIEKEKPAPGPFDAIVRPLAVAPCTSDIHTVFEGAIG  TBADH
         + ++    +   + +       +  + +++  ++++ *  * *
```

Figure 3.2.

Degenerate nucleotide sequence of the *N*-terminus of benzyl alcohol dehydrogenase from *A. caloaceticus*.

The degenerate sequence was generated using the amino acid sequence determined by Chalmers *et al.* (1991) and the Biosoft, Gene-jockey Sequence processor (Taylor, 1991). Complementary and matching degenerate oligonucleotides 985, 986, 1108 and 1109 are depicted at their corresponding points of annealing.Degeneracies are marked by N,R and Y, where N corresponds to any base; R corresponds to an A or G; Y corresponds to a C or T; •, refers to base matching and |, refers to a complementary base.



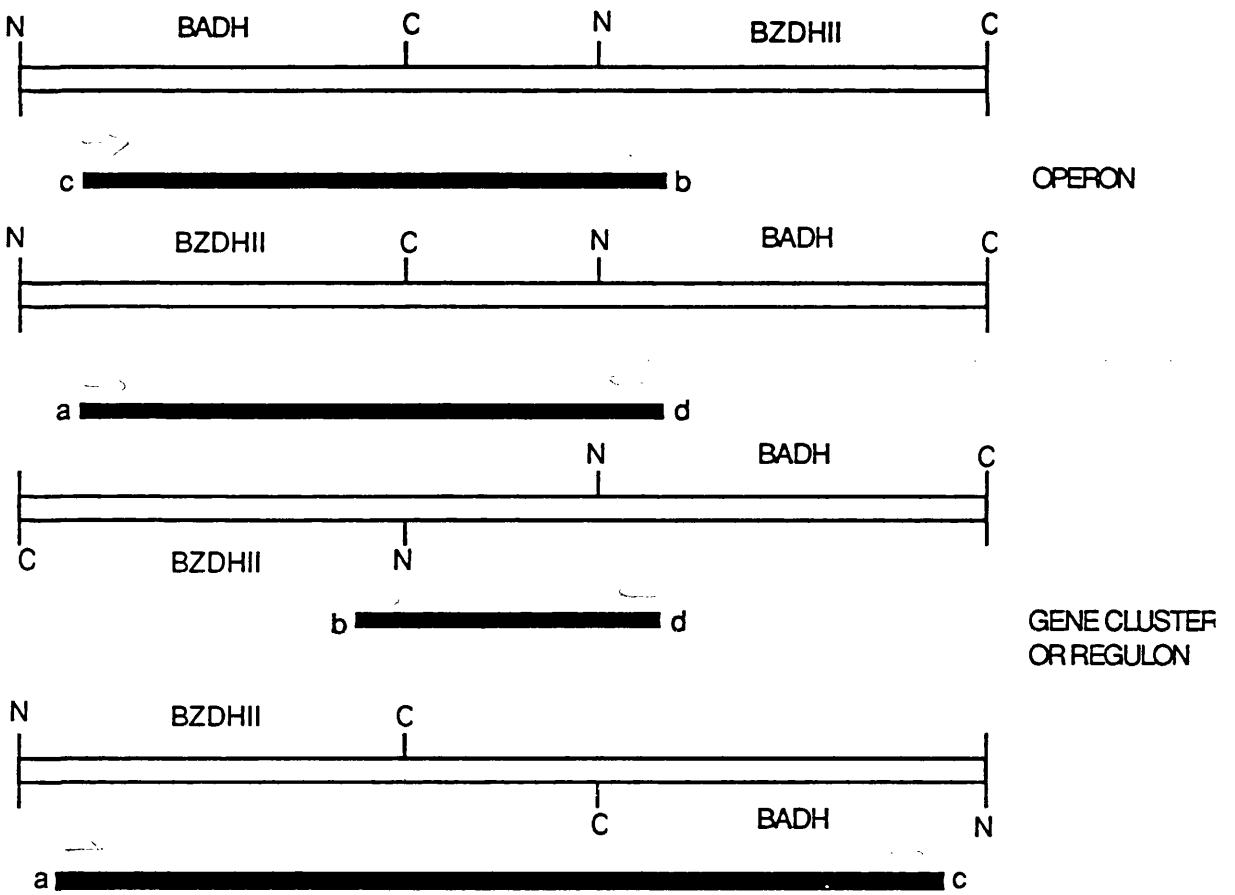
(Figure 3.1.) was likely to be a Cys residue, corresponding to position 46 (numbering based on HLADH) which is conserved in all group I ADHs, except the structural guinea pig lens ζ-crystallin (Sun & Plapp 1992; Section 1.2.3.4) and TOL-BADH (pWW53). However, the Pro residue corresponding to position 46 in TOL-BADH(pWW53) was not unambiguously identified by Chalmers *et al.* (1991), and the correlation between residues 34 to 44 in TOL-BADH(pWW53) with other group I ADH members are consistent with the prediction that it contains a Cys-46 rather than a Pro (Chalmers *et al.*, 1991). Because of the prediction that the Cys would also be conserved in BADH, two extra nucleotides were incorporated in the oligonucleotide 1108 (A and C) before the restriction site, *Eco*RI, and these correspond to the first two nucleotides in a Cys codon AC(A,C). Incorporation of these two extra nucleotides would increase the specificity of primer annealing if the Cys-46 is indeed conserved in BADH. If Cys-46 is not conserved in BADH, the two extra nucleotides at this point would not have too great an effect on the specificity of annealing since they do not increase the number of degeneracies and are adjacent to the restriction site which is also non-complementary to the target DNA. Using two sets of primer pairs meant that there were options to use nested PCR for probe generation if required.

Both BADH and BZDHII are induced together (Livingstone & Fewson, 1972; Beggs & Fewson, 1977) suggesting that they are under the same control system and therefore might be within the same operon, gene cluster or regulon, similar to the arrangement of analogous genes *xylCMABN* of the TOL-plasmid pWW161 (Section 1.8.4). Possible arrangements of BADH and BZDHII are shown in figure 3.3. The primer pairs for the BADH and BZDHII *N* -terminus were used in PCR in an attempt to identify the arrangement of these two genes. This method, if successful, might have resulted in an amplified sequence containing the *C*-terminal end of one of the genes which would have been very useful in PCR amplification of the whole gene as an aid to cloning and sequencing of the gene. The sizes of the fragments generated by this method were estimated to be at least 1.1 Kb for BADH and 1.6 Kb for BZDHII, since the predicted subunit sizes of BADH and BZDHII are 381 and 525 residues respectively (Chalmers *et al.*, 1991).

Figure 3.3.

Possible arrangements of genes coding for benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase from *A. caloaceticus*.

Oligonucleotides for both BZDHII and BADH were used to try and determine the possible arrangements of the two genes in relation to each other. The black bars refer to the expected PCR product sizes. Primers BZDHIIa and b for the *N*-terminal sequence of BZDHII are represented as a and b, respectively. Primers 985 and 1109 are represented by c, and primers 1108 and 986 are represented by d (Section 3.2.1).



The primer pairs BZDHIIa and BZDHIIb had been used to amplify a sequence of 124 base pairs from the *N*-terminus of the BZDHII gene, and were used in PCR reactions as a positive control (Figure 3.4; lane 15).

3.2.2. Optimisation of PCR

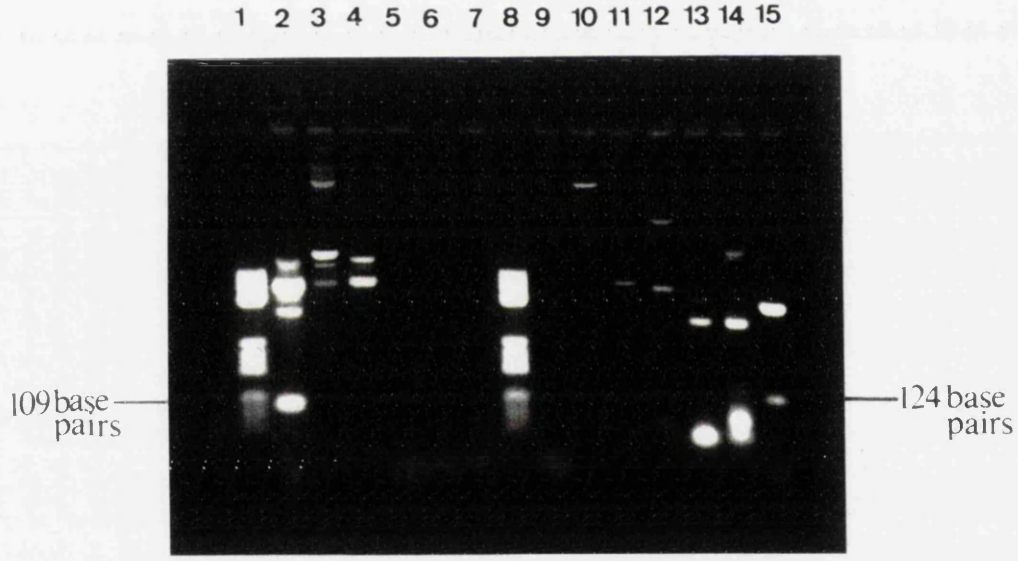
The degenerate oligonucleotide primers were prepared for use in PCR (Section 2.6.2). The concentration had been calculated previously and it was assumed that there was 100% recovery from the ethanol precipitation. The PCR system was set up with the reagents as described in section 3.6.2. The PCR cycle temperature parameters were chosen by examining the melting temperature of duplex degenerate oligonucleotide primers. The melting temperatures of double stranded DNA primer pairs 1108, 1109, 985 and 986 were determined using the Biosoft Gene-Jockey sequence processor (Taylor, 1991) as 62.8°C, 65.6°C, 61.9°C and 59.4°C respectively. The melting temperatures for the BZDHII *N*-terminus primer pairs were also determined as 59.7°C and 61.9°C. The annealing temperature in the PCR cycle had to be below the lower melting temperature to ensure annealing to the target DNA. The melting temperatures for the primer pairs were calculated for DNA complementary to the whole 22 bases of the primer, including the restriction sites. The restriction sites would not anneal to the target DNA in the first annealing step of the PCR cycle, so the melting temperature in the PCR would be some degrees below the calculated melting temperature of the primers. However, after the first PCR cycle there would be target DNA which is completely complementary to the whole primer sequences. The closer the annealing temperature is in a PCR cycle to the primer's melting temperature, the higher the specificity of primers annealing to the correct target DNA sequence. So during optimisation of PCR conditions the temperature of annealing was brought down from 62°C in decrements of 2°C (rather than increased) until the wanted PCR product (109 base pairs; Figure 3.4, lane 2)was visualised on an agarose gel (Section 2.4.4). Optimal PCR was carried out (Section 2.6.2).

When using primer pairs for BADH and BZDHII together, (Figure 3.4; lanes 9,

Figure 3.4.

PCR products using oligonucleotides 1108, 1109, 985 and 986 with chromosomal DNA from *A. calcoaceticus*

Notation: 1, Marker pBR322/*Hae* III ;2, All reaction substances; 3, control-1108 only; 4, control-1109 only; 5, control-No Taq polymerase; 6, control-no target DNA; 7, 1109 and 986; 8, marker-pBR322/*Hae* III ;9, 1109 and BZDHIIb primer; 10, 1108 and 985; 11, 1108 and BZDHIIa; 12, 986 and BZDHIIa; 13, 985 and BZDHIIb; 14, 985 and 986; 15, control-BZDHIIa and b primers.



11, 12, and 13) no prominent fragments of the correct length (above 1.1Kb and 1.6 Kb) were observed. Success or failure was likely to depended on the distance between the two genes. If the arrangement of the BADH and BZDHII genes are similar to that of the TOL-plasmid pWW161 (Section 1.8.4), where two gene products are coded for between the BADH and BZDHII genes it is unlikely that the sequence between would be amplified since *Taq* incorporates about 1000 base pairs per minute (Erlich, 1989), and the elongation time in the reactions used was only 3 minutes, giving a maximum amplification length of 3 kilobases.

Lanes 9 and 11 (Figure 3.4) using primer pairs of 985 and 1108, and 1109 and 986 were expected to give a fragments of 143 and 80 base pairs respectively, but neither was observed. This may have been due to primer pairs not have equal an equal number of degeneracies, or having such different melting temperatures.

3.3. Cloning and sequencing of probe

There was more than one product formed in each PCR reaction, most possibly because of nonspecific annealing of primers to target DNA (Figure 3.4.). The ligation of PCR product into cloning vector pUC18 was done in two ways; shotgun ligation of products and band purification followed by PCR amplification.

3.3.1. Shot gun cloning

The PCR products (Figure 3.4; lane 2) were digested using both *Bam*HI and *Eco*RI (Section 2.4.3) and ligated into pUC18 (Section 2.4.8). The cloned plasmids were then used to transform *E. coli* JM109 (Section 2.4.9). Cells were plated out on ampicillin plates and transformed ampicillin-resistant colonies were picked and grown up overnight along with controls to test for revertants (Section 2.3.4.2). Transformed cells were harvested (Section 2.3.6) and plasmids were isolated (Section 2.4.2). Digestion of plasmids with *Bam*HI (Section 2.4.3) and visualisation of the cut plasmids with inserts, using *Bam*HI digested pUC18 as a molecular weight marker, on a 2% agarose gel

allowed identification of the clone containing the insert of the required size (Section 2.4.4). Of 17 colonies picked (labelled A to Q), only 3 contained the correct size insert of 109 base pairs (Figure 3.5; Clones A,E and H; lanes 2,6 and 9).

3.3.2. Cloning of PCR generated fragment

This approach used the purification of the band of the correct size from the agarose gel (Figure 3.4; lane 2) as target DNA for PCR to increase the amount of DNA fragment available for cloning. The fragment labelled in figure 3.4 (lane 2) was purified from an agarose gel which had been loaded with a maximum volume of PCR product of 25µl (Section 2.4.5-2.4.7). The final volume of the resuspended DNA was 20µl, a volume too small to determine the DNA concentration or purity so it was used in PCR in increasing amounts 1µl, 2µl, 5µl, and 10µl as target DNA. PCR parameters were kept as before (Section 2.6.2). The amplified product was visualised on a gel (Section 2.4.4; Figure 3.6) and the DNA concentration of the remaining sample calculated (Section 2.2.4). The remaining product was cloned into pUC18 (Section 2.4.8). Transformation of JM109 and plasmid isolation were repeated, as described in section 3.3.1. Of four colonies picked using this method, all contained the correct size insert of 109 base pairs.

3.3.3.Sequencing of insert contained in pUC18

Two plasmids from each cloning strategy (Sections 3.3.1-3.3.2) were used for sequencing (Section 2.5). The sequences obtained from each of the four plasmids were aligned with the degenerate BADH oligonucleotide sequence using Biosoft Gene-Jockey sequence processor (Taylor, 1991). Each sequence showing a high degree of identity and exact match amongst themselves. Figure 3.7 shows the sequence obtained from the plasmid denoted as pMR1 which all subsequent work was done with. When the insert sequence of pMR1 was translated it was found to correspond to the *N*-terminus of BADH (Figure 3.8) and identified both the previously unknown two amino acid residues as Val. The transformed clone containing pMR1 was grown up and a large scale plasmid

Figure 3.5.

Single digestions of shot gun cloned fragments of lane 2 in Figure 3.4

Notation: 1,λ DNA/*Hin*dIII/*Eco*RI ; 2, clone A/*Bam*HI ;3, clone B/*Bam*HI; 4,clone C/*Bam*HI ; 5, clone D/*Bam*HI;6, clone E/*Bam*HI; 7, clone F/*Bam*HI ;8, clone G/*Bam* HI ; 9,clone H/*Bam*HI ; 10, pUC18/*Bam*HI ;11, pUC18/*Bam*HI ;12, λ DNA*Hin*dIII/*Bam*HI ;13, clone I/*Bam*HI ; 14,clone J/*Bam*HI 15, clone K/*Bam*HI ;16, clone L/*Bam*HI ; 17, clone M/*Bam*HI ; 18, clone N/*Bam*HI ; 19,clone O/*Bam*HI ; 20, clone P/*Bam*HI ; 21, clone Q/*Bam*HI . Positive clones were identified as A, E and H because of their size being slightly larger than that of pUC18/*Bam*HI.

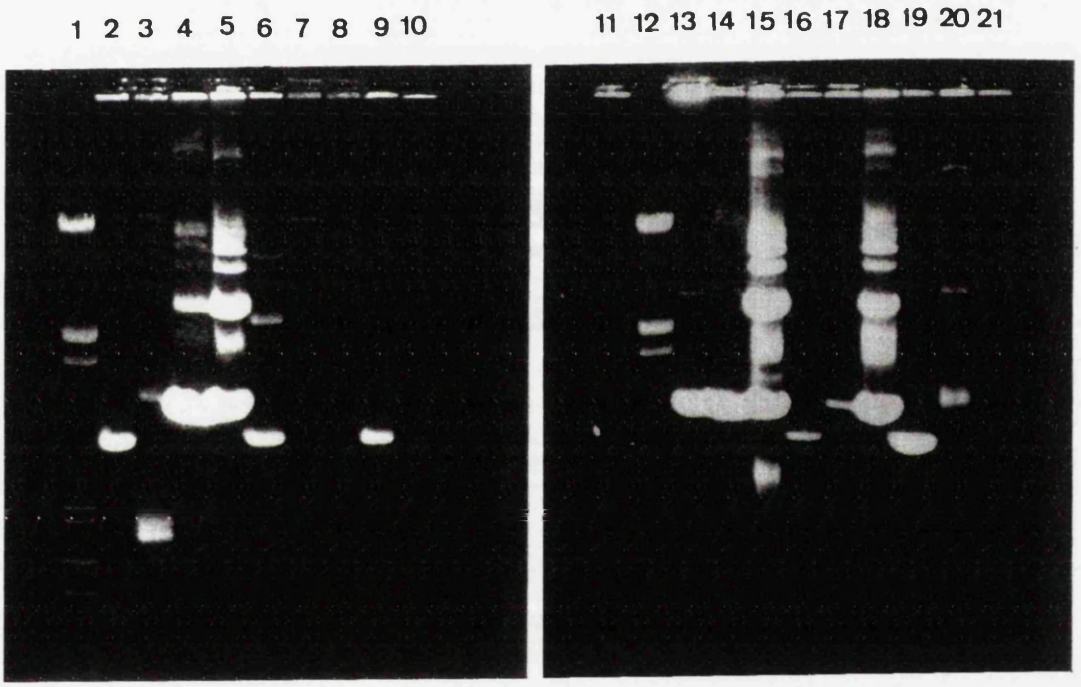


Figure 3.6.

PCR using purified PCR fragment denoted in Figure 3.4. as target DNA

Notation: 1, marker pBR322/*Hae*III ; 2, control no Taq. polymerase; 3, control-1108 only (chromosomal DNA); 4, control-1109 only (chromosomal DNA); 5, control-no target DNA; 6, 1µl target fragment; 7, all reaction substances (chromosomal DNA); 8, 2µl target fragment; 9, 5µl target fragment; 10, 10µl target fragment; 11, Marker pBR322/*Hae*III.

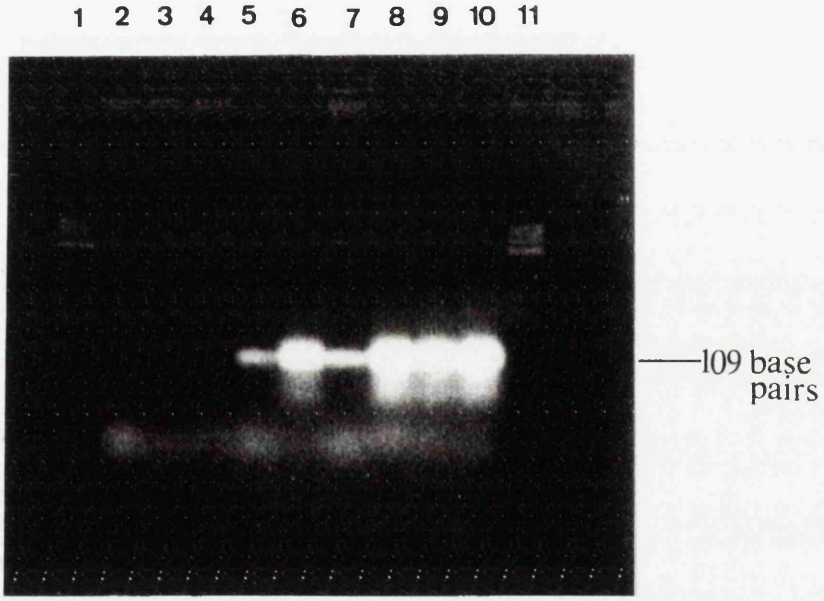


Figure 3.7.

Comparisons of the degenerate nucleotide sequence of the *N*-terminus of benzyl alcohol dehydrogenase from *A. caloaceticus* and the nucleotide sequence of pMR1.

Top line sequence refers to 109 base pair pMR1, bottom line refers to degenerate nucleotide sequence of BADH *N*-terminus. •, corresponds to matching bases.

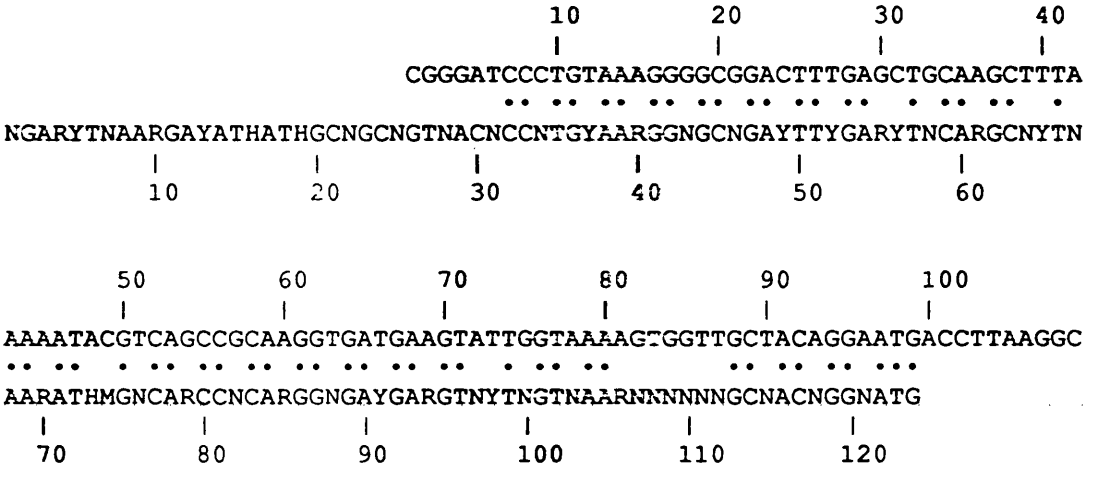


Figure 3.8.

Comparisons of the amino acid sequence of the *N*-terminus of benzyl alcohol dehydrogenase from *A. caloaceticus* and the translated amino acid sequence of pMR1

Top line sequence refers to translated sequence of pMR1, bottom line refers to BADH *N*-terminal sequence as quoted in figure 3.1. X, corresponds to unidentified residues; •, corresponds to matching amino acid residues.

```
            CKGADFELQALKIRQPQGDEVLVKVVATGM
            ••••••••••••••••••••••••••••••
SELKDIIAAVTPCKGADFELQALKIRQPQGDEVLVKXXATGM
         |           |           |           |
         10          20          30          40
```

preparation (Section 2.4.1) used to obtain a large quantity of pMR1 to be used in probe generation.

3.4. Probe generation

The generation of the probe was done using the sequenced pMR1 clone as target DNA for PCR reaction using the primers 1109 and 1108. The PCR parameters were as stated in section 2.6.2 except that the amounts of pMR1 used in the PCR were 1ng, 100ng, 1µg and 2.75µg. This gave a higher yield of probe than would be obtained by removing the insert from the plasmid.The PCR product was visualised as described previously (Section 2.4.4). The 109 base pair fragment was separated from the plasmid DNA (Sections 2.4.5-2.4.7) and then used again as target in order to remove all plasmid DNA.

3.5. Southern blotting

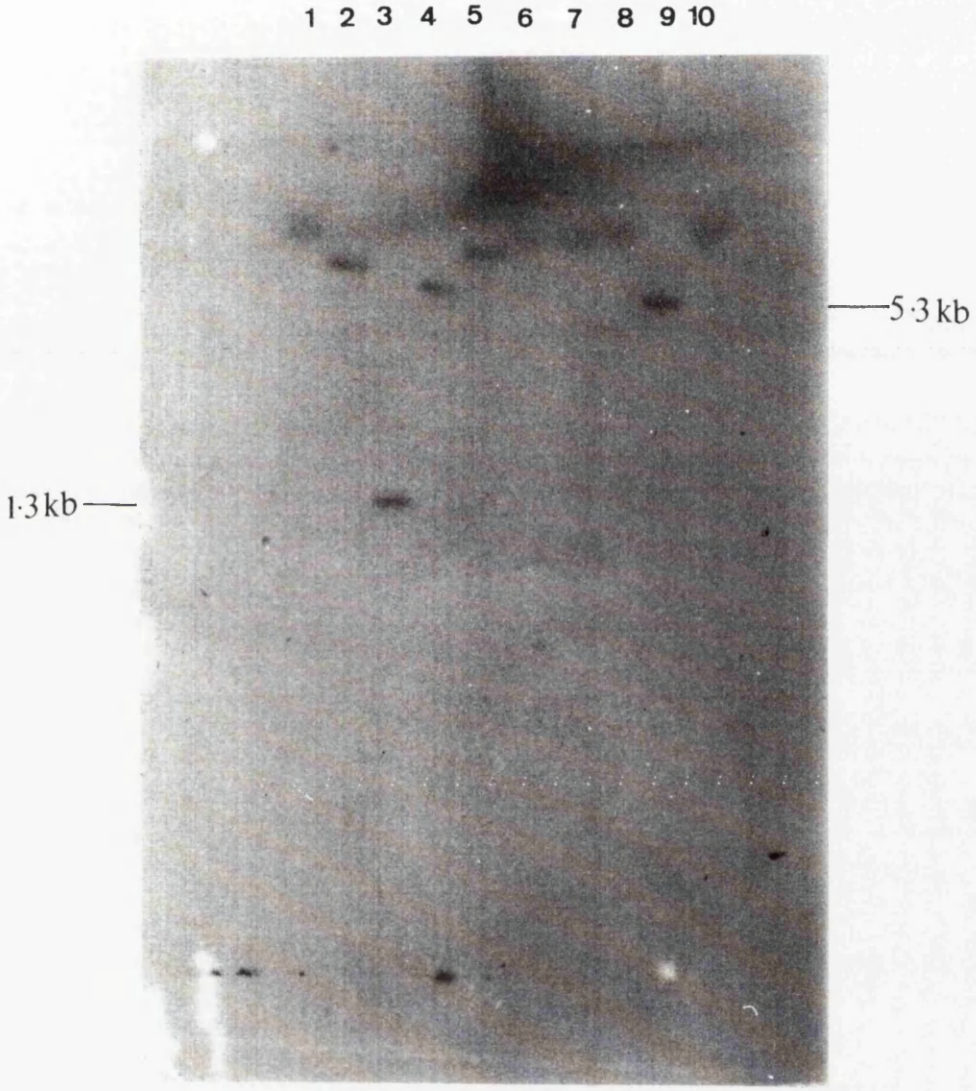
The probe was labelled at the 5' end rather than through incorporation of radio-active base into the PCR reaction mix since the procedure would require the manipulation of high activity probe during purification (Section 2.7.1). This method meant that labelling just before adding the probe to the filter would minimise exposure to ionising radiation. Southern blotting can be used to determine the location of of a particular gene sequence in relation to restriction enzyme sites. This provides vital information when probing a λ-library to identify whether the whole gene is contained within a λ-clone. Probing was carried out as described in sections 2.7.2-2.7.3. The annealing of the probe to digested *A. calcoaceticus* chromosomal DNA was seen on the x-ray film (Figure 3.9).

The two smallest fragments to which the probe anneals is the *Hin*dIII fragment of 1.3 Kb and the 5.3 Kb *Xba* I (Figure 3.9; lanes 3 and 9 respectively). By doing similar double digests using two restriction enzymes with chromosomal DNA and positive BADH clones from the λ–library it is possible to tell where abouts within the cloned sequence the probe anneals, thereby aiding sub-cloning and eventual sequencing.

Figure 3.9.

Southern blot restriction map of chromosomal DNA of *A. calcoaceticus* for the coding region of benzyl alcohol dehydrogenase

Notation: Digestion of *A. calcoaceticus* chromosomal DNA was done with; 1, *Bam* HI; 2, *Eco*RI; 3, *Hin*dIII; 4, *Kpn*I; 5, *Pst* I; 6, *Sac*I; 7, *Sal*I ; 8, *Sma*I; 9, *Xba*I; 10, *Xho*I.



Double probing of the λ–library using both BADH and BZDHII probes would enable mapping the locations of these two coordinately expressed genes in relation to each other.

CHAPTER 4

DISCUSSION

4.1. Production of a probe to identify the gene coding for benzyl alcohol dehydrogenase

The cloning technique using a PCR-generated fragment was the better of the two techniques used because of the way that the second PCR provided confirmation that the fragment was the correct size (Section 3.3.2). The second PCR cycle generated a high concentration of fairly pure product which made the actual cloning easier and generated all positive clones (plasmids containing the insert); this was in contrast to the shot-gun cloning, which provided only three positive clones from 17 colonies picked. When the sequence identified in pMR1 was translated it gave an amino acid sequence identical to that identified by Chalmers *et al.* (1991). The two residues unidentified by Chalmers *et al.*, 1991 were both identified as Val residues one of which (Val-41) is conserved in TOL-BADH and HLADH but in no other microbial group I ADHs (Figure 1.9). No conclusions can be made as to whether residue 43 of BADH, corresponding to residue 46 in HLADH, is a Cys or not.

The information contained in a Southern blot restriction map aids cloning when a λ-clone has been identified from a DNA library. The Southern blotting of chromosomal DNA from *A. calcoaceticus* NCIB 8250 indicated the size of restriction fragments which contained the sequence, or part of the sequence, which codes for BADH (Figure 3.8). Double digestion of chromosomal DNA would provide a more precise picture of the Southern blot restriction map. The low level of signal seen in figure 3.9 could be due to two factors: low level of labelling of the 5' end of the probe with α-^{32}P, and/or low levels of probe annealing to the complementary DNA in the hybridisation mixture. The lack of α-^{32}P labelling could be overcome in future work by the incorporation of [α-^{32}P]-dATP when the probe is generated using PCR rather than the 5'-labelling. Denhardt's reagent is used as a blocking agent in Southern blotting to prevent nonspecific annealing of the probe. However, lower levels of annealing than might be expected can been caused by interference of the protein in Denhardt's reagent with the probe. It was for this reason that the Denhartd's reagent was left out of the hybridisation mixture; however, it was included in the quenching solution which may have had an effect on the

probe annealing. Probes under 100 bases in length are particularly susceptible to this problem (Sambrook *et al.*, 1989). Lowering concentrations of Denhardt's reagent or its complete removal from the quenching solution may increase the signal, but it may also allow an increase in nonspecificity of probe annealing.

Attempts were made to identify positive clones from a phage-λ *A. calcoacteticus* chromosomal DNA library containing the *N* -terminal gene sequence of BADH, but no positive results were obtained (results not shown). However, since the work described in this thesis was completed the oligonucleotide primers 1108 and 1109 have been used by Dr A. G .S . Robertson (unpublished results) in PCR to identify the *N* -terminal sequence encoded for by pMR1 in a positive BZDHII phage λ-clone of 17.5 kilobases . This indicates that the BADH and BZDHII genes are in fairly close proximity on the *A. calcoaceticus* genome.

4.2. Further work

The determination of the primary sequence of BADH would be the next step of this work and would immediately determine to which ADH group BADH belongs. All indications so far point to it being a group I ADH (Section 1.8.6).

Once the primary stucture of BADH had been determined, one of the first aims will be to predict the three-dimensional structure of BADH based on its primary sequence and on comparison with the three-dimensional structure of HLADH (assuming the identity between the two sequences is high enough). This would provide information as to which residues are involved in coenzyme and substrate binding. Three dimensional modelling in conjunction with site directed mutagenesis would provide an essential insight into the roles of various residues in the catalytic mechanism. If Val-51 is conserved in BADH as in TOL-BADH (pWW0), it would be a prime target to determine whether this residue has a role in catalysis and thus determine whether a proton-relay system is essential for activity in the BADH enzymes. Comparison with other group I ADHs which lack a proton-relay mechanism may suggest that these enzymes constitute a sub-group which has a different catalytic mechanism from the rest of the group I ADHs. It would be

interesting to see if this lack of proton-relay system in these ADHs has come about through convergent evolution or perhaps through horizontal gene transfer (Smith *et al.*, 1992). Other targets for site directed mutagenesis would be those at the active site pocket to try to assign the roles of residues in determining substrate specificity.

If BADH and TOL-BADH (pWW0) are as closely related as their *N*-termini suggest, the paradoxical characteristics in their quaternary structures would provide an ideal opportunity to examine the way in which primary structure determines subunit interaction in the native enzyme. It will be interesting to see to which sub-group BADH belongs; whether it is similar to TOL-BADH, being more closely related to the mammalian/plant sub-group Shaw *et al.* (1993), or whether it should be classed as a tetrameric bacterial ADH (Section 1.2.5).

Other work should include the cloning and sequencing of the BZDHII gene to determine the evolutionary relationships (if any) between BADH and BZDHII, as well as mapping the location of their genes on the chromosome. Comparison of the analogous TOL-encoded enzymes, their genetic arrangements, and regulation of expression will provide more information about the possible evolutionary relationships between the enzymes involved in benzyl alcohol degradation in *A. calcoaceticus* and *P. putida* and about how these relationships came about.

Cloning the BADH gene into an expression vector could be used to generate large amounts of protein which may be used for various studies such as determination of the number of zinc atoms per subunit and crystallisation with a view to three-dimensional structure determination. Since TOL-BADH (pWW0) has been identified as dimeric and containing two zinc atoms per subunit (Shaw *et al.*, 1993), comparison of the number of zinc atoms per BADH subunit would provide information as to whether there is any correlation between tertiary structure and metal content. Three-dimensional structure determination of BADH would be extremely useful since the only group I ADH structure available is for the dimeric HLADH. The three-dimensional structure of tetrameric BADH would be useful as a model for other tetrameric ADHs, as would being useful in determining how tetrameric subunit interactions differ from those of a dimeric enzyme.

The high expression of cloned BADH could be used in isolated enzyme

biotransformation systems such as those discussed in section 1.7. BADH might perhaps be used in the treatment of waste water to produce the less toxic benzyl alcohol from benzaldehyde (Section 1.7.1). The use of BADH to generate optically pure aldehydes and alcohols could easily be exploited. BADH is a particularly attractive candidate for this because of its relaxed substrate specificity in tolerating substitutions on the 3-, 4- and 5-positions of the benzene ring (Section 1.8.4).

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