SITE DIRECTED MUTAGENESIS AND PURIFICATION OF THE cDNA FOR HUMAN CLASS I ALDEHYDE DEHYDROGENASE

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for the degree of Master of Science at Massey University

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DEDICATION

This thesis is dedicated to Wanny

(a patient man!)

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ABSTRACT

Aldehyde dehydrogenase (ALDH) is a key enzyme of alcohol metabolism, removing acetaldehyde which is formed as a product of the alcohol dehydrogenase reaction. If acetaldehyde is not effectively removed, acetaldehyde accumulates and produces an adverse reaction to alcohol, with nausea, flushing and increased heart rate and blood pressure.

ALDH is involved in the conversion of retinal to retinoic acid (RA). RA has recently been shown to bind to receptors, which then act as nuclear transcription factors and play important roles in foetal development and maintenance of the epithelial layer in the body. Interference by ethanol and perhaps by acetaldehyde with this process is probably the cause of Foetal Alcohol Syndrome.

In addition ALDH is also involved in the metabolism of catecholamine neurotransmitters, plays a role in the removal of toxic substances from the body and may have a role in protection against some chemical carcinogens.

Dr. Kerrie Jones had obtained moderate levels of expression of recombinant ALDH in *E. coli* and constructed a number of mutants chosen on the basis of chemical modification data and sequence alignment. Mutant proteins were also expressed and assayed for enzyme activity in crude extracts.

The aim of this thesis was to improve purification and yield of the expressed ALDH proteins. By the use of site-directed mutagenesis I attempted to mutate the amino acid residue Lys272 to either alanine, histidine or arginine. Future comparison of the properties of the site-directed mutants with those of the wild type enzyme will help to determine the importance of the residue (which has been replaced by mutagenesis) to catalysis.

ABBREVIATIONS

A	alanine
ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
ALDHs	aldehyde dehydrogenases
A_x	absorbance, eg. A_{600}
amp	ampicillin
ATP	adenosine triphosphate
bp	base pair
BRL	Bethesda Research Laboratories
C	cysteine
°C	degree Celsius
Cm	chloramphenicol
cDNA	complementary deoxyribonucleic acid
D	aspartic acid
Da	Dalton, the unit of molecular mass
dATP	deoxyadenosine triphosphate
DEAE	diethylamino...
dCTP	deoxycyteine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
ds	double stranded (as in ds DNA)
DTT	dithiothreitol
dUTP	deoxyuridine triphosphate
E	glutamic acid
E.NADH	enzyme with NADH bound
EDTA	ethylene diamine tetra-acetate
EEO	electroendosmosis
F	phenylalanine
F.A.S.	Foetal Alcohol Syndrome

g	gram
G	glycine
GST	glutathione-S-transferase
H	histidine
hr	hour/s
I	isoleucine
IEP	Isoelectric Point
IPTG	isopropyl-β-D-thiogalactopyranoside
K	lysine
kan	kanamycin
kbp	1000 basepairs
kDa	kiloDalton
l	litre
L	leucine
LB	Luria-Bertani bacterial growth medium
LMP	low melting point
M	methionine
M	molarity, moles of solute per liter of solution
min	minute/s
mol	mole
mmol	millimole
N	asparagine
NAD^+	nicotinamide adenine denucleotide
NADH	dihydronicotinamide-adenine denucleotide
ng	nanogram
N-terminal	amino terminal
OD	optical density
P	proline
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEI	polyethylenimine

PFU	plaque forming units
Q	glutamine
R	arginine
RA	retinoic acid
RF	replicative form
rpm	revolutions per minute
S	serine
s	second/s
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
ss	single stranded (as in ss DNA)
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TEMED	*N, N, N', N'*-tetramethylethylenediamine
T	threonine
Tris	Tris-(hydroxymethyl) aminomethane
TTP	thymidine triphosphate
µg	microgram
µl	microlitre
UV	ultraviolet
V	valine
(v/v)	volume per volume
W	tryptophan
wt	wild type
(w/v)	weight per volume
Y	tyrosine

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Chapter One

Introduction

1.1 OVERVIEW

Aldehyde dehydrogenase (E.C. 1.2.1.3) is an important enzyme in the pathway of alcohol metabolism and may be involved in the regulation of alcoholic drinking behaviour (see section 1.5.1). It also plays a more general protective role in the removal of toxic substances from the body and is involved in the conversion of retinol to retinoic acid.

Retinoic acid (RA) has been shown to bind nuclear receptors, which then act as nuclear transcription factors and play important roles in foetal development and in the maintenance of many tissues. Interference by ethanol and perhaps by acetaldehyde with this process, is probably the cause of Foetal Alcohol Syndrome (see section 1.5.2). Aldehyde dehydrogenase (ALDH) may play a role in protection against some chemical carcinogens but it has also been implicated in resistance to cytophosphamide anti cancer drugs (see section 1.5.3).

ALDH catalyses the $NAD(P)^+$ dependent irreversible oxidation of various aldehyde substrates to their corresponding acids. It exhibits a fairly broad substrate specificity with aldehydes of a variety of structures, including straight chain and branched, aliphatic and aromatic aldehydes being oxidised. ALDH primary endogenous substrates have yet to be identified and therefore the biological roles of ALDH are not clear. It seems likely that a particular form of ALDH may be able to oxidise efficiently a number of aldehyde substrates, the exact substrate varying with tissue and/or physiological situation.

ALDH was first isolated by Racker (1949) from bovine liver. In the late 1960s and during the 1970s ALDH was isolated from various other microbial and mammalian sources (see section 1.2). Research has since been directed towards identifying its physiological roles and understanding the structure and function relationships of the enzyme.

1.2 LOCALISATION OF ALDEHYDE DEHYDROGENASE

1.2.1 Isolation of aldehyde dehydrogenase

ALDH isozymes have been found in a variety of sites in the bodies of mammals, reflecting its role in the oxidation of aldehydes arising from other metabolic processes as well as those due to alcohol consumption. The highest levels of ALDH are found in the liver, which corresponds to the main site of ethanol oxidation in the body.

ALDH was first isolated by Racker (1949) from bovine liver. ALDHs from yeast (Steinman & Jakoby, 1967) and *Pseudomonas aeruginosa* (Tigerstrom & Razzell, 1968) were isolated and purified in 1967 and 1968, but these two non-mammalian ALDHs exhibit significantly different properties from those found in mammals.

ALDH has now been isolated and purified to homogeneity from a number of mammalian sources including horse liver (Feldman &Weiner, 1972; Eckfeldt & Yonetani, 1976), bovine liver (Sugimoto *et al.*, 1976; Leicht *et al.*, 1978), rat liver (Shum & Blair, 1972; Tottmar *et al.*, 1973), rabbit liver (Duncan, 1977), sheep liver (Crow *et al.*, 1974; MacGibbon *et al.*, 1979; Hart & Dickinson, 1977) and human liver (Greenfield & Pietruszko, 1977; Kraemer & Dietrich, 1968).

1.2.2 Tissue and subcellular distribution

Liver possesses the highest ALDH activity (Tottmar *et al.,* 1973; Crow *et al.*, 1974; Horton & Barrett, 1975; Siew *et al.,* 1976; Marjanen, 1972; Dickinson & Berrieman, 1979; Tipton *et al.*, 1981, 1989; Lindahl, 1979). A variety of isozyme forms of ALDH have been identified in the mitochondrial, microsomal and cytosolic fractions. Human liver contains multiple ALDHs distributed approximately equally between mitochondria and cytosol. Forms with both high and low-K_m values for substrate are known and different ALDH forms may have different substrate preferences. The low-K_m mitochondrial and high-K_m cytosolic

forms account for the majority of the acetaldehyde oxidising capacity of the human liver. A human microsomal ALDH has also been identified (Helander & Tottmar, 1986; Koivula, 1975; Santisteban *et al.*, 1985; Tipton *et al.*, 1989).

In all species examined tissues other than liver also possess significant ALDH activity (Deitrich, 1966; Simpson & Lindahl, 1979; Holmes, 1978; Rout & Holmes, 1985; Manthey *et al.*, 1990; Remond & Cohen, 1971; Petersen *et al.*, 1977; Smolen *et al.*, 1981; Harada *et al.*, 1978, 1980; Duley *et al.*, 1985; Yoshida, 1990; Santiseban *et al.*, 1985; Seeley *et al.*, 1984; Holmes & VandeBerg, 1986; Nilsson, 1988, 1989). For example, brain and kidney possess significant ALDH activity and the subcellular distribution and characteristics of these isozymes are generally similar to those of liver (Holmes, 1978; Rout & Holmes, 1985; Harada *et al.*, 1978; Holmes & VandeBerg, 1986; Cox *et al.*, 1975; Cederbaum & Rubin, 1977; Hjelle *et al.*, 1983; Erwin & Deitrich, 1966; Pettersson & Tottmar, 1982; Inoue & Lindros, 1982).

Other tissues, most notably cornea, lung, stomach and urinary bladder have a different subcellular distribution and activity profile from those of the liver (Manthey *et al.*, 1990; Dunn *et al.*, 1988; Evces and Lindahl, 1989; Lindahl, 1986; Teng, 1981; Yin *et al.*, 1989; Messiha, 1982). In these tissues the cytosol possesses most of the ALDH activity. In the cornea more than 90% of the total ALDH activity is cytosolic (Holmes & VandeBerg, 1986; Evces & Lindahl, 1989; Lindahl, 1986; Teng, 1981; Yin *et al.*, 1989; Messiha, 1982), where in stomach and urinary bladder the contribution of the cytosol to total ALDH approaches 50% (Remond & Cohen, 1971; Lindahl, 1986).

The distribution of the three classes of ALDH among mammalian tissues is complex. All tissues probably possess mitochondrial ALDH. However depending on the tissue and species they may also possess a constitutive cytosolic ALDH. In addition for some tissues other cytosolic forms of ALDH can be induced under certain conditions.

1.3 CLASSIFICATION

1.3.1 High-K_m and low-K_m aldehyde dehydrogenase groups

Eight different ALDH gene products have been identified from human DNA (Hsu *et al.*, 1995; Lin *et al.*, 1996). The best studied isozymes are the cytosolic and mitochondrial forms, ALDH1 and ALDH2 respectively (Greenfield & Pietruszko, 1977).

On the basis of the Michaelis constants for acetaldehyde the human ALDH forms can be divided into high-K_m (millimolar range) and low-K_m (micromolar range) groups, as proposed by Tottmar *et al.* (1973). The low-K_m forms comprise of ALDH1, ALDH2 and γ-aminobutyraldehyde dehydrogenase (Greenfield & Pietruszko, 1977; Kurys *et al.*, 1989). Human ALDH3 and ALDH4 (Yin *et al.*, 1989; Forte-McRobbie & Pietruszko, 1986) and rat microsomal ALDH (Lindahl & Evces, 1984) are the high-K_m forms.

The low-K_m mitochondrial and high-K_m constitutive cytosolic forms account for the majority of the acetaldehyde oxidising capacity of the human liver (Kraemer *et al.*, 1968; Blair *et al.*, 1969; Feldman & Weiner, 1972; Crow *et al.*, 1974; Eckfeldt & Yonetani, 1976; Eckfeldt *et al.*, 1976; Sugimoto *et al.*, 1976; Greenfield & Pietruszko, 1977).

1.3.2 Aldehyde dehydrogenase classes

ALDHs can be divided into five different classes based on the primary structure relationships of the various isozymes (Lindahl and Hempel, 1991). Class 1 (constitutive cytosolic) and class 2 (mitochondrial) share around 67% amino acid sequence identity and are homotetramers of 55 kDa monomer size (Hempel & Jörnvall, 1989; Yoshida *et al.*, 1991). Class 3 ALDH inducible forms of the enzyme (e.g. microsomal, stomach cytosolic and tumour-specific) are homodimers with 50 kDa monomer size and show less than 30% amino acid sequence identity

with classes 1 and 2 (Jones *et al.*, 1988; Hsu *et al.*, 1991; Hempel *et al.*, 1989). Other isozymes classified as class 4 and $ALDH_x$ have also been reported (for review, see Yoshida *et al.*, 1991).

1.4 ALDEHYDE DEHYDROGENASE STRUCTURE

At the time of the research component of this thesis little was known about the tertiary structure of the ALDH, although basic similarities had been shown to exist among various mammalian sources of ALDH. It was known that the class 1 and class 2 ALDH isozymes functioned as tetramer enzymes of approximately 220,000 to 250,000 Dalton's and that each isozyme was composed of identical subunits each containing approximately 500 amino acids. However the coenzyme-binding area known to be present in dehydrogenases, could not be identified from the primary structure.

After the completion of the experimental work reported in this thesis the tertiary structures of the class 2 and class 3 ALDH enzymes were published (Steinmetz *et al.*, 1997; Sun *et al.*, 1995). Studies on the bovine liver class 2 isozyme by Steinmetz *et al.* (1997) observed that each subunit within the tetramer was composed of three distinct domains; two dinucleotide-binding domains and a small three-stranded β-sheet domain involved in subunit interactions in the enzyme. Although a recognisable Rossmann-type fold was also found, the coenzyme binding region of class 2 ALDH bound NAD^+ in a manner that had not been seen in other NAD^+ binding enzymes (Steinmetz *et al.*, 1997).

The structure of recombinant rat liver class 3 ALDH was resolved by Sun *et al.* (1995). This isozyme was found to be a dimeric rather than a tetrameric quaternary structure. It is composed of two sub-domains connected by a peptide hinge with the N terminal domain being somewhat larger than the C-terminal domain. The two monomers associate in the dimer in a head to tail manner (Sun *et al.*, 1995).

1.5 METABOLISM

1.5.1 Alcohol metabolism

Studies on the regulation of ethanol metabolism in mammalian liver have shown that the activity of alcohol dehydrogenase (ADH) is an important regulatory factor (Braggins & Crow, 1981; Crow & Hardman, 1989; Page *et al.*, 1991). ALDH probably plays a secondary regulatory role and the balance of activities between ADH and ALDH regulates the concentration of acetaldehyde in the liver (Crow *et al.*, 1982; Page *et al.*, 1991).

Because alcoholism is a significant health problem, major efforts have been made at understanding ethanol metabolism in the hope of gaining some insight into the pathophysiology of this widespread disease. The development of a clear understanding of the reaction mechanism of ALDH may be of use in the treatment of alcoholism.

As previously mentioned ALDH is an important enzyme in the pathway of alcohol metabolism, where it catalyses the oxidation of acetaldehyde in the liver to form acetate. As acetaldehyde is reactive and toxic, increased blood acetaldehyde levels are responsible for the symptoms of acute alcohol poisoning (i.e. flushing, headache and nausea) and for chronic damage to many organs, particularly the liver (Truitt and Walsh, 1971).

Acetaldehyde is thought to increase collagen gene transcription by binding to proteins that usually act as repressors, thereby causing de-repression of the gene and excessive collagen synthesis and deposition (Brenner & Chokier, 1987; Niemela *et al.*, 1990). This may be the first step in the development of alcoholic liver damage.

1.5.1.1 *Alcohol flush reaction*

Although there are multiple forms of ALDH in the liver, class 2 ALDH is believed to be responsible for the oxidation of most acetaldehyde generated during alcohol metabolism. Many Orientals and some South American Indians lack this mitochondrial ALDH activity and hence their ability to metabolise alcohol is impaired because acetaldehyde is degraded slowly and therefore accumulates.

The alcohol-flush reaction resulting from excessive acetaldehyde accumulation is believed to cause physiological responses unpleasant enough to serve as a deterrent to further drinking in both disulfiram-treated patients and in individuals who have inherited the atypical mutant allele of ALDH-1 (Dyck, 1990).

1.5.2 Foetal Alcohol Syndrome

Foetal Alcohol Syndrome (F.A.S.) consists of a varied group of neonatal malformations including brain, craniofacial, limb and growth abnormalities that are the result of excessive maternal ethanol consumption (Streissguth *et al.*, 1980). Despite numerous studies designed to research ethanol embryotoxicity in humans, rodents and other vertebrates, no single underlying mechanism for the teratogenic action of ethanol has been proposed (Schenkner *et al.*, 1990; Randall *et al.*, 1990).

Recent studies on the molecular basis of vertebrate embryonic development have revealed that RA plays a major role in the specification of spatial patterns during the morphogenesis of nervous system and limb tissues, both of which show abnormalities in cases of F.A.S. The controlled conversion of vitamin A into RA by specific embryonic tissues has been proposed as a major regulatory step in the morphogenic process (Duester, 1991; Durston *et al.*, 1989; Wagner *et al.*, 1990; Maden *et al.*, 1990).

One important aspect of RA induced differentiation that is not understood is the mechanism regulating the synthesis of RA from retinol (vitamin A). In mammals retinol in the liver or other tissues can be converted to RA via a two step oxidation

in which ADH (the rate limiting step) produces retinal and ALDH produces RA (Leo *et al.*, 1989; Napoli, 1986). Studies indicate that RA can also induce ADH gene expression suggesting a positive feedback mechanism for controlling RA synthesis (Duester, 1991).

Ethanol, a typical class 1 ADH substrate, acts as a competitive inhibitor of retinol oxidation in human liver extracts (Mezey & Holt, 1971) and it was proposed by Deuster *et al.* (1991) that a connection between F.A.S., RA homeostasis and ethanol-retinol metabolism catalysed by human ADH.

1.5.3 Lipid peroxidation and tumour cells

Peroxidation of lipids is a continuous process that can be stimulated by a number of factors that induce cellular oxidative stress. The reactions involved in lipid peroxidation are complex and a wide variety of products can be formed. Among the stable products of lipid peroxidation are a variety of aldehydes which comprise the majority of molecules produced from peroxidation of either linoleic or arachidonic acid (Canuto *et al.*, 1994).

Changes in the activities of enzymes metabolising aldehydes produced during lipid peroxidation have been reported in tumour cells. Consistent increases in ALDH and aldehyde reductase activities relative to normal liver have been reported in both primary hepatocellular carcinomas and hepatoma cell lines. It is well established that many types of tumour cells have a reduced lipid peroxidation capacity compared to their normal counterparts (Canuto *et al.*, 1994).

Class 3 ALDH is induced by a number of chemical carcinogens and this is also found in high levels in some tumours (Campbell *et al.*, 1989; Canuto *et al.*, 1989; Quemener *et al.*, 1990). The metabolic significance of these observations are not clear, but the enzyme may play a role in protection against some chemical carcinogens (Lindahl, 1992).

1.5.3.1 *Cancer treatment*

The presence of enzymes which metabolise and inactivate alkylating agents in tumour and normal cells, appear to play a major role in determining the effectiveness of using alkylating agents against human tumours and the toxicities of these agents to normal tissues. ALDH appears to protect bone marrow and the gastrointestinal tract against toxicity from cyclophosphamide and other closely related oxazophorine agents.

The resistance to the cyclophosphamide anti-cancer drugs demonstrated by one type of bone marrow tumour cells has been shown to be due to high levels of ALDH (Colvin *et al.*, 1988). If ALDH could be selectively activated in normal tissue cells as opposed to tumour cells, it would be a great advantage in cancer treatment (Colvin *et al.*, 1988; Kastan *et al.*, 1991; Koelling *et al.*, 1990).

1.5.4 Biogenic amines

Although ALDH is generally considered to function in detoxication, Ambroziak & Pietruszko (1991) suggest that it has an additional function in metabolism of biogenic aldehydes arising from biogenic amines and polyamines (Erwin & Dietrich, 1966; Ambroziak & Pietruszko, 1991).

In the brain the metabolism of biogenic amines is an important role. Among the biogenic amines found in the brain, high polyamine concentrations are found in brain tumour tissues and a correlation between the brain and the activity of enzymes involved in the polyamine pathway has been demonstrated. A relationship between alterations in ALDH isozyme activities and cytosolic aldehyde concentrations with respect to normal or tumour cell growth has been suggested by Quemener *et al.* (1990).

1.6 KINETIC STUDIES

Extensive studies of the kinetics of cytoplasmic ALDH have been carried out (MacGibbon *et al.*, 1977a,b,c, 1978b; Bennett *et al.*, 1982, 1983; Blackwell *et al.*, 1985; Hart & Dickinson, 1978a, 1982, 1983; Hart *et al.*, 1982; Dickinson, 1985; Buckley & Dunn, 1982, 1985). MacGibbon *et al.* (1977a,b,c) demonstrated that the enzyme operates by an ordered mechanism with NAD^+ binding before aldehyde. The steady state appears to be at least partially controlled by the rate of dissociation of NADH from the binary E.NADH complex.

From stopped-flow studies of the enzyme-catalysed reaction, Bennett *et al.* (1982) showed that a conformational change of the enzyme, which follows aldehyde binding (with simultaneous release of a proton), is rate limiting in the pre-steady-state phase of the reaction.

1.7 CHEMICAL MODIFICATION STUDIES

Chemical modification of ALDH that leads to activation has also been investigated (Kitson, 1979, 1982a, b, 1986; Loomes & Kitson, 1989). The actions of disulfiram and related drugs have recently been reviewed (Kitson, 1989; Peachey, 1989). *p*-Nitrophenyl dimethylcarbamate was used to identify cysteine 302 as the active site nucleophile in the esterase action of cytosolic ALDH (Kitson *et al.*, 1991).

Class 1 ALDH is very sensitive to disulfiram *in vitro* and it is likely that the use of this drug causes significant inhibition of the enzyme *in vivo* (Kitson, 1989). Evidence suggests however that the mitochondrial isozyme is responsible for much of the acetaldehyde oxidation during ethanol metabolism *in vivo*. Therefore a question remains as to which isozyme is responsible for the 'disulfiram-ethanol reaction' (Kitson, 1989). Inhibition of the cytosolic enzyme may also be the cause of some of the unwanted side-effects of disulfiram, due to inhibition of natural substrates.

Modification of ALDH with iodoacetamide results in the labelling of cysteine 302 (Hempel *et al.*, 1985). Pre-exposure of ALDH to disulfiram decreased the rate of reaction with iodoacetamide, supporting the idea that iodoacetamide modifies a group at or near the active site. This conclusion was supported by the work of von Bahr-Lindstrom *et al.* (1985) and Kitson *et al.* (1991) who labelled cysteine 302 with the esterase substrate analogue 4-nitrophenyl dimethylcarbamate. Blatter *et al.* (1992) also labelled the cysteine 302 amino acid residue using trans-4-(N,N-dimethylamino)annamaldehyde and 4-trans-(N,N-dimethylamino) annamoyl-imidazole.

Abriola *et al.* (1987, 1990) showed that glutamic acid 268, as well as cysteine 302, was labelled by bromoacetophenone, an active site directed reagent that irreversibly abolishes both the dehydrogenase and esterase activities. Circumstantial evidence for the involvement of serine 74 as the active site nucleophile has been obtained from labelling studies using the chromophoric substrate *trans*-4-(N,N-diethylamino)cinnamaldehyde (Loomes *et al.*, 1990).

1.8 ALDHEHYDE DEHYDROGENASE ACTIVE SITE

1.8.1 Proposed reaction pathway

Unlike many other dehydrogenases the detailed mechanism for the oxidation of substrate is not known. Initial kinetic studies with the horse liver enzyme lead to the conclusion that ALDH functioned with ordered binding and that NAD^+ was the lead substrate. The rate limiting step for the enzyme was thought to be the acylaction step (Feldman & Weiner, 1972). It was also proposed that the active site should possess a general base to help in the deacylation step as illustrated in figure 1.2.

Other investigators studying the enzyme from different species concluded that the NADH dissociation could be rate limiting. Figure 1.1 illustrates the proposed active site reaction pathway proposed by Weiner *et al.* (1995).

Proposed ALDH active site reaction pathway

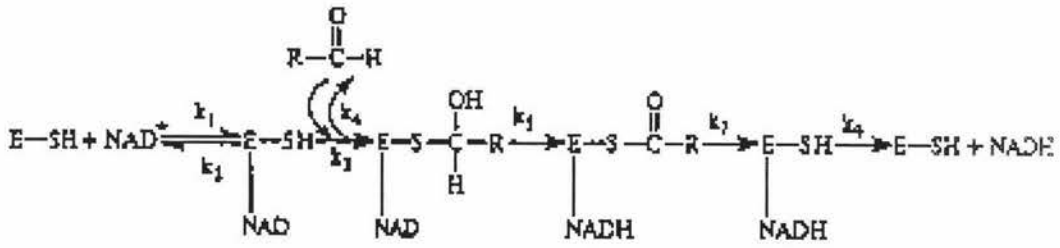

Figure 1.1
Model showing the reaction pathway for the ALDH catalysed oxidation of an aldehyde (Weiner *et al.*, 1995).

ALDH active site base

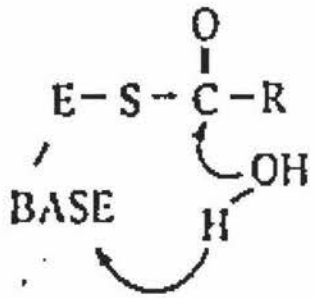

Figure 1.2

General base facilitated deacylation of the acyl intermediate (Weiner *et al.*, 1995).

1.8.2 Esterase and dehydrogenase activity

ALDH has been shown to possess, in addition to dehydrogenase activity, an esterase activity (Feldman & Weiner, 1972; Blackwell *et al.*, 1983). Based on mechanistic considerations and inhibition studies, it was proposed that the oxidative and hydrolytic reactions catalysed by horse liver ALDH proceed via a common intermediate involving the active site cysteine (Feldman & Weiner,

1972). Kinetic arguments in support of a common active site have been presented (Duncan, 1985; Kitson, 1982, 1986; Loomes & Kitson 1986).

Mutational analyses of the enzyme including serine 74 (Rout & Weiner, 1994) and cysteine 302 (Farres *et al.*, 1995) as well as glutamate (Wang & Weiner, 1995) all showed that there was a parallel loss of dehydrogenase and esterase activity. Therefore it appears that the two reactions catalysed by ALDH require the same active site components and occur at the same site (see section 1.9).

However studies by Blackwell *et al.* (1983) indicate participation of separate sites in the oxidative and hydrolytic reactions catalysed by sheep liver cytosolic ALDH. This proposal was supported by chemical modification studies carried out by Tu & Weiner (1988), Deady *et al.* (1985) and Abriola & Pietruszko (1992).

1.9 MUTAGENESIS STUDIES

Information gained from kinetic and chemical modification studies (Hempel & Pietruszko, 1981; Tu & Weiner, 1988; Loomes *et al.*, 1990), combined with the identification of highly conserved amino acid residues across the various forms of ALDH (Hempel *et al.*, 1993), lead to numerous studies employing site-directed mutagenesis to further determine the components of the active site.

Sections 1.9.2 and 1.9.3 overview the amino acid residues that have been altered by site-directed mutagenesis to determine whether they played a role in the ALDH active site.

1.9.1 Conserved amino acid residues

Sequence alignment of the primary sequences of all know ALDHs revealed that cysteine 302, glutamic acid 268, glutamic acid 399, lysine 272 and serine 471 were the only residues with a potential catalytic function which are conserved

among all the know sequences (Hempel *et al.*, 1993; von Bahr-Lindstrom *et al.*, 1985). Refer to the appendix for a ALDH primary sequence alignment.

1.9.2 Active site nucleophile

1.9.2.1 *Cysteine*

Labelling by various substrates and substrate analogues implicated the cysteine residues 49 and 302 as probable active site nucleophiles (von Bahr-Lindström *et al.*, 1985; Kitson *et al.*, 1991; Pietruszko *et al.*, 1993). Sequence comparisons also demonstrated that cysteine 49, 301 and 302 where conserved across the various ALDH sequences and were therefore candidates for the active site nucleophile.

Weiner *et al.* (1995) altered cysteine 302 and cysteine 49 in the recombinant rat liver class 2 ALDH. They showed that converting cysteine 49 to alanine did not affect the activity of the enzyme. In contrast however they reported that changing cysteine 302 to alanine lead to an enzyme void of catalytic activity (Weiner *et al.*, 1995).

Cysteine 302 has been altered to serine in studies using both the class 1 and class 2 forms of the ALDH enzyme (Weiner *et al.*, 1995; Jones *et al.*, 1995). This change lead to an oxygen being substituted for sulphur at the postulated active site. This alteration caused the mutant enzyme to have greatly reduced catalytic activity. Jones *et al.* (1995) also altered cysteine 302 to alanine in the recombinant human liver class 1 ALDH and concluded from their research that cysteine 302 was likely to be the active site nucleophile, in agreement the mutagenesis studies of the mitochondrial enzyme (Weiner *et al.*, 1995; Jones *et al.*, 1995).

1.9.2.2 *Serine*

Circumstantial evidence for the involvement of serine 74 as the active site nucleophile has been obtained from labelling studies (Loomes *et al.*, 1990). Unlike cysteine 302, serine 74 is not a conserved residue in all species.

Rout & Weiner (1994) found that the replacement of serine 74 by an alanine residue caused the class 2 enzyme to have a Vmax of just 10%. The fact that the enzyme still maintained some activity and that the residue was not highly conserved across species suggested that serine 74 was not the essential nucleophile in the active site (Rout & Weiner, 1994). Weiner *et al.* (1995) constructed the corresponding cysteine and threonine mutant enzymes in recombinant rat liver class 2 ALDH. They found that both of the expressed enzymes behaved like the alanine mutant.

1.9.3 Active site base

1.9.3.1 *Histidine*

Histidine has been shown to be a general base catalyst in many enzymes including proteinases and dehydrogenases (Takahashi *et al.*, 1981; Fersht, 1985; Weiner *et al.*, 1985). The possibility of a histidine residue being involved in an acid base reaction was proposed by Weiner *et al.* (1991) however no direct evidence existed to suggest that the residue functioned in this capacity. Kinetic studies on horse liver mitochondrial ALDH which showed that a group with a pK_a of 7 (presumably histidine) maybe involved in the active site environment supported this proposal (Takahashi *et al.*, 1981).

Through chemical modification of the residue Weiner *et al.* (1985) demonstrated that histidine is not absolutely required for the enzyme to function. Attempts were made to chemically modify the residue with diethylpyrocarbonate but this modification only lead to partial inactivation of the enzyme (Weiner *et al.*, 1985).

Based on the comparison of the known mammalian ALDH sequences both histidine 235 and histidine 29 were found to be highly conserved. The mutation of histidine 235 and histidine 29 to alanine demonstrated that these residues were not essential for catalytic activity and that they may not function as a general base in the deacylation step as originally suggested by Zheng & Weiner (1993). Instead both the highly conserved histidines may be involved in obtaining and maintaining the stable native structure of the enzyme (Zheng & Weiner, 1993).

1.9.3.2 *Glutamic acid*

On the basis of chemical modification studies it was postulated that glutamic acid 268 was a component of the liver ALDH active site (Abriola *et al.*, 1987, 1990; Pietruszko *et al.*, 1991, 1993). Later it was found that all ALDHs had a conserved glutamate at position 268, supporting the suggestion that the residue could indeed be functioning as a component in the enzymes active site (Hempel *et al.*, 1993).

In human liver class 2 ALDH, the glutamic acid residue was mutated to aspartate, glutamine and lysine. The different mutations did not affect the K_m values for NAD^+ or propionaldehyde, but grossly affected the catalytic activity of the enzymes when compared to recombinantly expressed native enzyme. Furthermore both the dehydrogenase activity and esterase activity were essentially abolished when glutamate was changed to either aspartate, glutamine or lysine (Wang & Weiner, 1995).

These results can be interpreted as implying that glutamic acid 268 may function as a general base necessary for the initial activation of the essential cysteine residue, rather than being involved in only the deacylation or hydride transfer step. Alternatively glutamate 268 could function as a component of a charge relay triad necessary to activate the nucleophilic residue.

1.10 TERTIARY STRUCTURE STUDIES OF THE ACTIVE SITE

Since the completion of the research component the tertiary structure of the class 2 ALDH was resolved by Steinmetz *et al.* (1997). From this research a chemical mechanism was suggested whereby glutamic acid 268 functions as a general base through a bound water molecule. The side amide nitrogen of asparagine 169 and the peptide nitrogen of cysteine 302 were found to be in a position to stabilise the oxygen present in the tetrahedral transition state prior to hydride transfer. The functional importance of glutamic acid 487 now appears to be due to indirect interaction of this residue with the substrate-binding site via arginine 264 and arginine 475 (Steinmetz *et al.*, 1997).

1.11 AIM OF THE INVESTIGATION

The precise mechanism of action of ALDH has not been defined. Understanding the mechanism of action of the enzyme and the amino acid residues involved in the cofactor and substrates will provide valuable information for determining which metabolites are likely to be natural substrates for the enzyme.

During the research component of this thesis, studies on the tertiary structure of ALDH where not sufficiently advanced to suggest which amino acid residues where important in catalysis (Baker *et al.*, 1995; Hurley & Weiner, 1992). Therefore the primary aim of this project was to use SDM to help define if the amino acid lysine 272 was important for enzymatic activity.

Lysine 272 is another potential base that is totally conserved and therefore a strong candidate for an important role in the catalytic activity of ALDH. Subsequent comparisons of the properties of the mutants obtained with those of the wild type enzyme will help to determine the importance of the residues in the enzyme's structure and function.