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mutants with those of the wild type enzyme will help to determine the importance of the residue (which has been replaced by mutagenesis) to catalysis.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

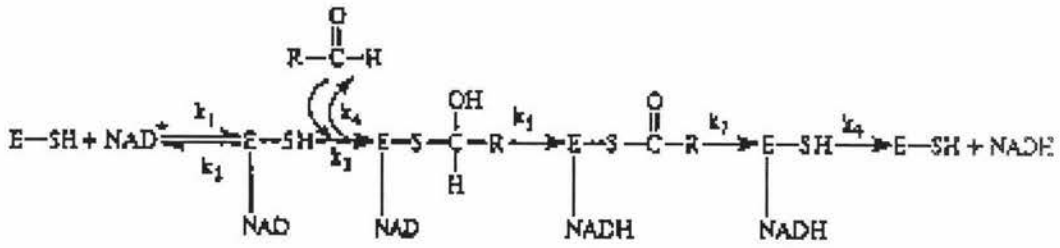

Figure 1.1
Model showing the reaction pathway for the ALDH catalysed oxidation of an aldehyde (Weiner *et al.*, 1995).

ALDH active site base

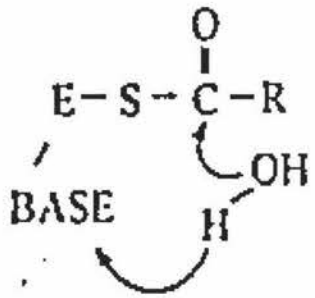

Figure 1.2

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.

Chapter Two

Materials and Methods

2.1 SOURCE OF MATERIALS AND REAGENTS

The following products were obtained from Life Technologies Ltd., MD, USA: T4 DNA ligase, BRL 1 kbp ladder, T4 polynucleotide kinase, T4 DNA polymerase and ammonium persulphate. Tryptone, agar and casamino acids were from Difco and NuSieve™ was obtained from FMC BioProducts, Rockland, ME, USA.

Methanol, acetic acid, TEMED and NADH were from BDH. Isopropanol, ethanol, sodium acetate, sodium chloride and ammonium acetate were from Ajax Chemicals. Magnesium chloride was from Riedel-de Haen, Tris (basic form) and dithiothreitol (DTT) were from Serva and boric acid from Prolabo Supplies. The Wizard™ PCR Preps and Magic Miniprep DNA Purification systems were from Promega Corporation, WI, USA. The Bresa Clean™ DNA purification kit was supplied by Bresatec Ltd., Adelaide, Australia.

The following were obtained form the Sigma Chemical Company, Saint Louis, MO, USA: Low EEO agarose, mineral oil, protein molecular weight markers, tetra sodium EDTA, PEG 8000, ampicillin, tetracycline, penicillin, Coomassie brilliant blue R-250, ethidium bromide and glycerol. All other compounds and chemical reagents used were of the highest grade available.

All water used in solutions was supplied from a NANOpure II water purification unit (Barnstead). This water is referred to as Milli-Q water in this work and is reagent grade deionised water with a resistivity greater than 16 MΩcm.

2.2 MOLECULAR BIOLOGY MATERIALS AND REAGENTS

TAE buffer: 40 mM Tris acetate pH 8.0, 1 mM EDTA.

TE buffer: 10 mM Tris-HCl pH 8.0, 1 mM EDTA.

TBE buffer: 90 mM Tris-borate pH 8.0, 90 mM borate, 2 mM EDTA.

5x TBE buffer: 450 mM Tris-borate pH 8.0, 450 mM borate, 10 mM EDTA.

20% Acrylamide solution: 96.5 g acrylamide, 3.35 g bis-acrylamide, 233.5 g urea, 100 ml 5x TBE and made up to a final volume of 500 ml using Milli-Q water.

10x Annealing sequencing buffer: 200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 500 mM NaCl, 0.15% (w/v) DTT.

Competency buffer one: 100 mM KCl, 30 mM potassium acetate, 60 mM $CaCl_2$, 15% glycerol pH 5.8.

Competency buffer two: 10 mM MOPS, 10 mM KCl, 75 mM $CaCl_2$, 15% glycerol pH 6.8.

2x Elongation buffer: 40 mM Tris-HCl pH 8.0, 4 mM DTT, 20 mM $MgCl_2$, 1 mM of each dNTP, 2 mM ATP.

Miniprep solution one: 75 µl 1 M Tris-HCl buffer pH 8.0, 60 µl 0.5 M EDTA, 75 µl 2 M glucose, 50 µl 50 mg/ml lysozyme, 2.7 ml Milli-Q water.

Miniprep solution two: 200 mM NaOH, 0.5 ml 1% SDS.

5x PEG NaCl: 15% polyethylene glycol 8000, 14.6% NaCl.

Phage elution buffer: 100 mM Tris-HCl pH 8.0, 300 mM NaCl, 1 mM EDTA.

2.2.1 Bacterial culture medium

Luria-Bertani (LB) medium: 1% tryptone, 1% NaCl, 0.5% yeast extract adjusted to pH 7.4 with NaOH and autoclauved.

LB agar: LB medium with 1.5% (w/v) agar.

M1 media: 15 g casamino acids, 5 g yeast extract, 10 ml 0.4 M sodium phosphate buffer pH 7.2, 10 ml solution A (1 M NH_4Cl, 50 mM K_2SO_4, 50 mM $MgSO_4$, 2 mM $CaCl_2$) and 1 ml of solution B (0.1 M HCl, 10 mM $FeSO_4$, 2 mM $MnCl_2$, 2 mM $ZnSO_4$, 0.2 mM $CoSO_4$, 0.1 mM $CuSO_4$, 1 mM $NiCl_2$) per litre. The pH was adjusted to 7.2 with NaOH before autoclauving the M1 media.

SOB medium: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ pH 6.8-7.4.

SOC medium: SOB medium containing 20 mM glucose.

Top agar: 1% tryptone, 0.8% NaCl, 0.4% (w/v) agar.

2TY medium: 1.6% tryptone, 1% yeast extract, 0.5% NaCl per litre pH 7.0

2TY agar: 2TY medium with 1.5% (w/v) agar.

2.2.2 Genotypes of bacterial strains

CJ236 *dut*1 *ung*1 thi-1 *rel*A1/pCJ105 [*cam*^{r} F'] (Sambrook *et al.*, 1989c).

SRP84 F' *ilv his strA sup*^{o} *gal* OP IS1 Δ(*lon*) *htpR*165 Tn10 Δ(*bio*) (λ B_{am} N^+ c1857 H1 [*cro-RAJ-bio*] *uvrB*).

TG1 *supE hsdΔ5 thi Δ(lac-proAB)* F' [*traD*36 *proAB*$^+$ *lacI*^{q} *lacZΔM15*] (Sambrook *et al.*, 1989c).

XL1 Blue *supE*44 *hsdR*17 *recA1 endA1 gyrA46 thi relA1 lac*$^-$ (Sambrook *et al.*, 1989c).

BL21 F-, *omp*T, *hsd*S(r_B^-, m_B^-), *gal.* (Studier & Moffatt, 1985; Grodberg & Dunn, 1988).

2.2.3 Plasmids

pGEX 4T-3: Pharmacia Biotech, Sollentuna, Sweden.

pGP1-2: Dr. Stan Tabor (Tabor *et al.*, 1985).

pThcAD: Dr. Henery Weiner, Prudue University (Zheng *et al.*, 1993).

pTscAD: Dr. John Tweedie, Massey University, N.Z.

2.3 BIOCHEMICAL MATERIALS AND REAGENTS

2.3.1 Protein purification buffers and reagents

CM-Sephadex starting buffer: 30 mM sodium phosphate pH 6.0, 1 mM EDTA, 3 mM DTT.

CM-Sephadex wash buffer: 30 mM sodium phosphate pH 6.0, 1 M NaCl, 3 mM DTT.

Coomassie brilliant blue stain: 0.125% Coomassie Blue R-250, 50% methanol, 10% glacial acetic acid.

DEAE starting buffer: 20 mM phosphate buffer pH 6.1, 1 mM EDTA, 3 mM DTT.

DEAE wash buffer: 20 mM phosphate buffer pH 6.1, 1 M NaCl.

p-Hydroxyacetophenone affinity column loading buffer: 30 mM sodium phosphate pH 7.4, 1 mM EDTA, 3 mM DTT.

p-Hydroxyacetophenone affinity column elution buffer: 30 mM sodium phosphate pH 7.4, 1 mM EDTA, 3 mM DTT, 50 mM NaCl, 10 mM *p*-hydroxyacetophenone.

p-Hydroxyacetophenone affinity column wash buffer: 30 mM sodium phosphate pH 7.4, 1 M NaCl.

Protein storage buffer: 30 mM Bis-Tris-HCl pH 6.0, 1 mM EDTA, 3 mM DTT, 10% glycerol.

Reservoir buffer: 72 g glycine, 15 g Tris, 2.5 g SDS made up to a final volume of 2.5 litres using Milli-Q water.

Resolving gel acrylamide solution: 32 g acrylamide, 0.2 g bis-acrylamide in 100 ml Milli-Q water.

4x Resolving gel buffer solution: 0.4% SDS, 1.5 M Tris-HCl pH 8.7 at 20°C.

SDS-PAGE running buffer: 25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% SDS. Adjusted to a final pH of 8.3.

2x SDS-PAGE sample buffer: 15% (v/v) glycerol, 2% (w/v) DTT, 0.005% bromophenol blue, 6% (w/v) SDS, 0.125 M Tris-HCl pH 6.7.

Stacking gel acrylamide solution: 4 g acrylamide, 0.105 g bis-acrylamide made up to a final volume of 50 ml using Milli-Q water.

8x Stacking gel buffer solution: 0.8% SDS, 1 M Tris-HCl pH 6.7 at 20 °C.

2.4 MOLECULAR BIOLOGY TECHNIQUES

2.4.1 DNA manipulation

The general precautions for handling DNA described in Sambrook *et al.* (1989a) were observed.

2.4.1.1 *Restriction endonuclease digestions*

Restriction endonucleases were obtained from the following companies: Life Technologies Ltd., MD, USA; Promega Corporation, WI, USA; Boehringer Mannheim, West Germany; Stratagene, La Jolla, CA, USA; New England Biolabs Inc., MA, USA. Reactions were carried out using the reaction buffers supplied by the manufacturers under the conditions specified.

2.4.1.2 *Ligations*

DNA inserts and vectors were prepared and cloned according to standard protocols as described in Ausubel *et al.* (1989a). Ligation reactions were performed with T4 DNA ligase using the 5x reaction buffer (250 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 5 mM DTT, 25% (w/v) polyethylene glycol-8000) supplied by the manufacturer. Each reaction was incubated at 37 °C for 1-4 hr.

2.4.2 Agarose gel electrophoresis of DNA

Electrophoresis of DNA fragments was performed in low electroendosmosis grade agarose gels (type I-A:Low EEO, Sigma) containing ethidium bromide (0.5

μg/ml) and TAE buffer (Sambrook *et al.*, 1989a). The BRL 1 kbp ladder was used to determine the approximate size of DNA fragments. Unless otherwise stated, 0.8% agarose gels were used to separate DNA fragments. To separate low molecular weight bands a 3% low melting point NuSieve™ gel was used.

2.4.3 DNA purification

DNA purification was carried out as described in Sambrook *et al.* (1989a). Pure preparations of DNA have an A_{260}/A_{280} ratio ≥ 1.8. Contamination by protein or phenol decreases this ratio. The purity of the DNA was examined by agarose gel electrophoresis, overloading at least one lane to visualise trace contaminants.

2.4.3.1 *Protein removal*

To remove enzymes and other proteins from DNA solutions, an equal volume of Tris-saturated phenol:chloroform:isoamyl alcohol (25:24:1) was added to the nucleic acid sample and mixed using a vortex until an emulsion formed. The sample was then centrifuged at 12,000 x g for 15 s in a microcentrifuge at room temperature. A pipette was used to transfer the aqueous phase to a fresh tube and the organic phase was discarded. This was repeated until no protein was visible at the interface of the organic and aqueous phases. The mixture was then extracted twice using an equal volume of chloroform:isomamyl (24:1) to remove any residual phenol (appendix E, Sambrook *et al.*, 1989c).

2.4.3.2 *Sodium acetate precipitation*

For total DNA recovery a 1/10 volume of 3 M sodium acetate (pH 5.2) was added to the DNA solution, followed by 2.5 volumes of cold 99% ethanol. The DNA was recovered by centrifuging at 12,000 x g for 2 min. The precipitated DNA pellet was washed with 70% ethanol and dried in a Speed Vac (Savant). The pellet was resuspended to the desired concentration using TE buffer pH 8.0 or Milli-Q water.

2.4.3.3 *Purification of DNA fragments from low melting point agarose*

DNA bands separated by gel electrophoresis were excised under illumination by long wavelength (305 nm) UV light. Several techniques were employed to purify DNA fragments embedded in agarose. These included the Wizard™ PCR Preps System and the Bresa Clean™ DNA purification kit. The protocol used was determined by the size of the fragment to be purified and was performed according to the manufacturer's instructions.

2.4.4 Quantitation of DNA

Purified DNA was quantified by spectrophotometric measurement (section 2.6.1). DNA solutions were diluted in TE buffer and the absorbance was measured at both 260 nm and 280 nm. The concentrations of nucleic acid solutions were calculated using the following relationships (Appendix E, Sambrook *et al.*, 1989).

A 50 μg/ml solution of double stranded DNA has an A_{260} of 1 unit.
A 20 μg/ml solution of single stranded DNA has an A_{260} of 1 unit.

2.4.5 Synthetic oligonucleotides

Oligonucleotides were obtained from either Oligos Etc, Oregon, USA or Life Technologies Ltd., MD, USA. The lyophilised oligonucleotide pellet was resuspended in 1 ml of sterile Milli-Q water and the concentration determined spectrophotometrically at 260 nm (section 2.6.1). All oligonucleotide primer solutions were stored at -20°C.

2.4.5.1 *Phosphorylation of oligonucleotides*

A reaction mixture containing 3 μl of 1 M Tris pH 8.0, 1.5 μl 0.2 M $MgCl_2$, 1.5 μl 0.1 M DTT, 200 pmoles of oligonucleotide, 13 μl of 1 mM was added to a microcentrifuge tube and made up to a final volume of 30 μl using Milli-Q water.

T4 polynucleotide kinase (4.5 units) was added, the samples mixed well and incubated at 37 °C for 45 min. The T4 polynucleotide kinase was then inactivated by heating to 65 °C for 10 min.

2.4.6 Amplification of DNA sequences using the polymerase chain reaction

Specific DNA sequences were amplified by the polymerase chain reaction (PCR) using the protocol recommended by Cetus Corporation Ltd. Reactions were carried out in 0.5 ml sterile PCR tubes and solutions were overlayed with mineral oil to eliminate evaporation during the thermal cycling.

The optimal conditions for the DNA amplification were determined by trial PCR experiments in which several parameters were varied including the concentrations of the template DNA, $MgCl_2$, annealing temperature and the number of PCR cycles. PCR reactions were typically 20 µl in volume unless stated otherwise.

Reactions were performed in a DNA thermal cycler (Perkin-Elmer-Cetus DNA thermal cycler) programmed for an initial 3 min denaturation at 95 °C followed by 30 cycles consisting of a 1 min denaturation at 94 °C, annealing for 1 min at 40 °C and a 1 min extension at 70 °C. The PCR products were analysed by agarose gel electrophoresis. Fore more detail on the amplification of DNA sequences using PCR see section 3.10.4.

2.4.7 Site directed mutagenesis

The cDNA for human cytosolic ALDH was obtained from Professor H. Weiner, Prudue University. The cDNA was cloned into M13mp18 by Dr. Kerrie Jones, Massey University. This M13mp18-ALDH construct was used as a template for mutagenesis.

In vitro mutagenesis reactions were performed according to Kunkel *et al.* 1987. This was accomplished by priming synthesis from a mutant oligonucleotide,

annealed to a uracil enriched M13 single stranded DNA template. The reaction products were then transformed into the uracil *N*-glycosylase-containing strain XL1-Blue.

Reactions were set up using template:primer ratios of 1:10 and 1:2, that is 0.08 pmol template:0.8 pmol primer and 0.08 pmol template:0.16 pmol primer respectively. A control reaction mixture containing no primer was also prepared. The oligonucleotide was annealed to the template in a reaction mix containing 1 µl 10x annealing sequencing buffer and made to a final volume of 10 µl with Milli-Q water. The reaction was incubated at 70 °C for 2 min and then cooled to O °C over a 2 hr period.

T4 DNA polymerase (1 unit) was added to the reaction mix with 12 µl of 2x elongation buffer. The reaction was incubated for 5 min at 0 °C, 5 min at room temperature and 2 hr at 37 °C. The reaction was stopped with the addition of 1 µl of 0.5 M EDTA. The synthesised strand was then ligated as described in section 2.4.1.2.

The products were examined by subjecting 20 µl of reaction mix to electrophoresis in a 0.8% agarose gel. For comparison adjacent lanes contained the following standards: single stranded, circular viral DNA and double stranded replicative form I (supercoiled covalently closed circular DNA) and form II (nicked circular DNA).

After incorporation of the mutant oligonucleotide into double stranded DNA, the plasmid was transformed into the $CaCl_2$-competent *E. coli* strain XL1 Blue (Sambrook *et al.*, 1989b). As this strain is *ung*$^+$, the uracil enriched parent strand will be digested, thus selecting for the mutant strand.

2.4.8 DNA sequencing

DNA was sequenced using the dideoxy chain termination method originally developed by Sanger *et al.* (1977). This protocol was carried out using dATP-5-[α^{35}S] thiosulphate > 1000 Ci/mmol (Amersham) with modified T7 DNA polymerase (Sequenase version 2.0: 9th Ed., Untied States Biochemicals, OH, USA). M13 template was prepared for sequencing as described in Kunkel *et al.* (1987). Other single stranded DNA templates were prepared from infectious phage stock or from plasmid DNA by standard procedures (Ausubel *et al.*, 1989a).

2.4.9 Sequencing gel

Sequencing gels were prepared and electrophoresed as described in Sambrook *et al.* (1989b). Dried gels were autoradiographed overnight at room temperature and developed using an automatic Kodak X-Omat developer. The DNA sequence was manually read and compared to the known DNA sequence of human cytosolic ALDH.

2.5 BACTERIAL AND PHAGEMID TECHNIQUES

2.5.1 Growth of bacterial strains and phage

Bacterial strains and phage were cultured, maintained and stored using standard protocols (Miller, 1987).

2.5.2 Preparation of competent cells

Competent cells suitable for transformation were prepared using calcium chloride and transformed by heat shock according to Sambrook *et al.* (1989a). Caution was taken to keep the bacteria ice cold throughout the protocol except during the heat shock step.

2.5.3 Transformation of competent cells with ligation products

Frozen competent cells were thawed on ice just prior to use. A portion of the ligation mix (see section 2.4.1.2) was added to the 50 µl of cells and incubated on ice for 20-50 min. The transformation reactions were heat shocked in a 42 °C water bath for 90 s and chilled on ice for 2-3 min. SOC medium (800 µl) was added to each tube which was then incubated at 37 °C for 1 hr with moderate agitation. Approximately half of the transformation was spread over a LB agar plate supplemented with the appropriate antibiotic and incubated for 16 hr.

2.5.4 Electroporation of *E. coli* strains

E. coli cells were prepared and electroporated as described in Dower *et al.* (1988). The BioRad Gene Pulser™ apparatus with the settings 800 Ω, 25 µFD and 2.5 V was used for electroporation.

2.5.5 Small scale plasmid DNA isolation

Small amounts of plasmid DNA were prepared by extraction from a bacterial culture using the rapid-boil technique according to Holmes and Quigley (1981) or with the Magic™ Miniprep DNA Purification System.

2.5.6 Growth of M13 phage

Uracil-enriched ss DNA template was isolated from intact M13 phage produced in CJ236 (*E. coli dut⁻ ung⁻* strain) according to Kunkel *et al.* (1987). The phage supernatant was stored at 4 °C.

2.5.7 Titre of M13 ALDH phage containing uridine base

Before preparing ss template DNA, the phage titres were compared using *ung*$^-$ and *ung*$^+$ hosts as described in Kunkel *et al.* (1987). One plaque of M13 usually contains 10^9-10^{10} plaque forming units (PFU).

Once suitable phagemid stock of uracil-enriched template was obtained, the DNA was purified by extraction (see section 2.4.3) prior to its use as template in the *in vitro* mutagenesis reactions.

2.5.8 Preparation of ALDH template DNA

Template was extracted from the phage stock as described in Kunkel *et al.* (1987). The purity of the DNA was examined by gel electrophoresis and an A_{260}/A_{280} ratio calculated. The DNA concentration was determined using spectrophotometric methods (see section 2.4.4.)

2.5.9 Growth and expression of ALDH in the *E. coli* strain SRP84/pGP1-2

2.5.9.1 *Overview of the expression system*

The following expression system was used for the growth of SRP84/pGP1-2 transformed with a cytosolic ALDH expression plasmid. Three different expression plasmids were used in the work described by this thesis, the human recombinant wild type cytosolic ALDH pT7.7 plasmid (pThcAD), the human recombinant Cys302 mutant cytosolic ALDH pT7.7 plasmid (pThcCys302AD) and the sheep recombinant wild type cytosolic ALDH pT7.7 plasmid (pTscAD).

Each plasmid was prepared by cloning the ALDH cDNA into the pT7.7 vector under the control of the Φ10 promoter which is specific for the RNA polymerase from bacteriophage T7 (Tabor & Richardson, 1985). The cDNA was expressed from this vector after transformation into *E. coli* SRP84/pGP1-2. The resident

pGP1-2 plasmid carries the DNA for T7 RNA polymerase under the control of the strong λ promoter pL, together with the c*I*857 temperature-sensitive λ repressor expressed from the *lac* promoter (Tabor & Richardson, 1985). Induction of ALDH expression was accomplished by heat shock to inactivate the heat-sensitive λ repressor. Figure 2.1 gives an overview of this expression method.

Recombinant ALDH protein expression in *E. coli* SPR84/pGP1-2

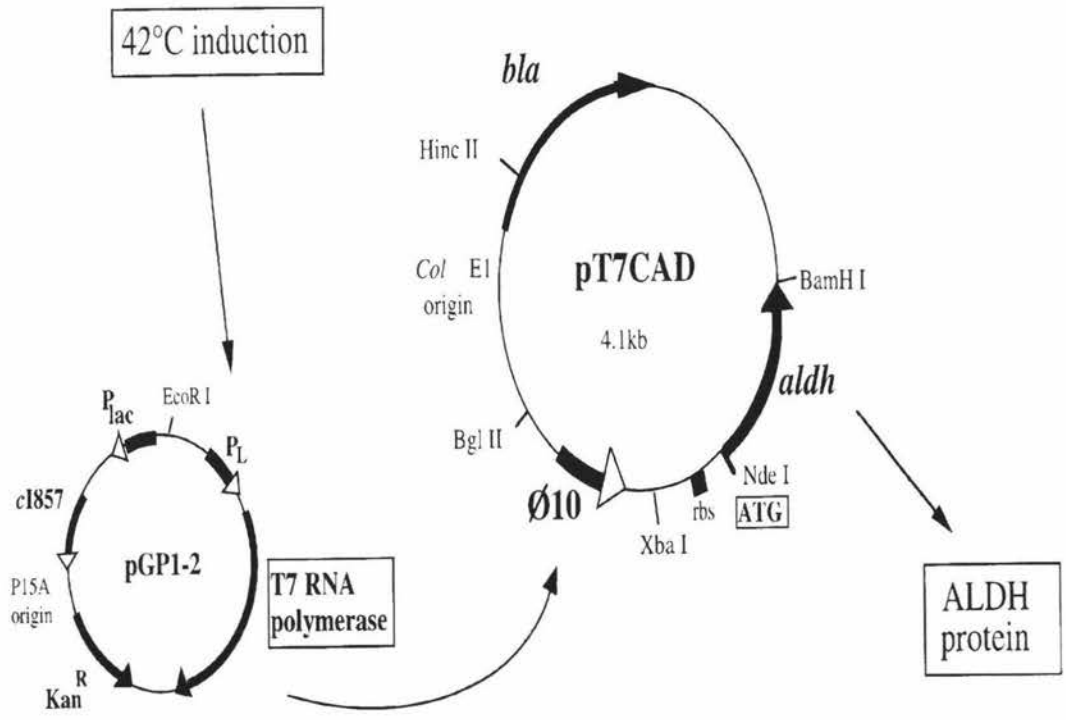

Figure 2-.1

An overview of the heat induction method used to express recombinant class 1 ALDH isozymes in *E. coli* SPR84/pGP1-2 (Tabour & Richardson, 1985).

2.5.9.2 *Large scale growth of E. coli strain SRP84/pGP1-2*

A 10 ml overnight culture of SRP84/pGP1-2 (containing the relevant expression plasmid) grown at 30 °C was used to inoculate four 2 l flasks containing 600 ml of Ml media supplemented with 10 mM glucose, 50 μg/ml kanamycin and 100 μg/ml ampicillin. The cultures were grown rapidly with shaking at 30 °C until the A_{650} reached at least 2.0.

Each culture was then heat-induced with the addition of 400 ml Ml media (supplemented as above) which had been previously heated to 70 °C. The addition of hot media caused a rapid elevation of the temperature of the *E. coli* culture to approximately 45 °C. The mix was rapidly shaken by hand for 5 min and then cooled to 30 °C by shaking for 1 min in an ice bath. The cultures where then returned to 30 °C and grown for another 4 hr on a fast shaking platform.

Samples of 1 ml were removed from the culture at repeated intervals after the induction. The samples were centrifuged at 13,000 rpm and used to determine the amount of recombinant human cytosolic ALDH expression compared to background *E. coli* ALDH levels on a SDS-PAGE (see section 2.6.5).

2.5.10 Harvesting of *E. coli* SRP84/pGP1-2 large scale growth preparation

The cells where harvested by centrifugation in a H4000 rotor (Sorvall) at 4,000 rpm for 20 min. The supernatant was gently poured off and the cell pellet was resuspended in residual media. The bacterial cell suspension was spun in a SS-34 rotor (Sorvall) at 10,000 rpm for 10 min. The bacterial pellet was recovered, total yield wet weight of the cells determined and the cells were stored at 4 °C overnight.

2.5.11 Sonication of small scale growth preparation

A 1 ml volume of *E. coli* SRP84/pGP1-2 containing either the pThcCys302AD or the pTscAD plasmid was pelleted centrifuged at 12,000 x g in a microcentrifuge. The pellet was then resuspended in 1 ml of CM-Sephadex loading buffer and the cell suspension sonicated using a 1.5 cm diameter probe at an amplitude of 18 microns for a total of 5 min (5x 1 min bursts with intermittent cooling on ice). The cell suspension was judged to sufficiently disrupted when the suspension looked semi-transparent.

2.6 BIOCHEMICAL TECHNIQUES

2.6.1 Spectrophotometry

The CARY 1 UV-visible spectrophotometer (Varian) was used for all spectrophotometric readings recorded in this thesis unless stated otherwise. When wavelengths of up to 340 nm were required the UV lamp was switched on for at least 30 min before a reading was taken.

2.6.2 Activity assay for aldehyde dehydrogenase

ALDH activity was assayed by measuring the production of NADH during oxidation of acetaldehyde from the change in A_{340} values. To perform each assay 2.6 ml sodium pyrophosphate-HCl (0.1 M, pH 9.3 at 25 °C), 250 µl NAD^+ (20 mg/ml) and 50-200 µl enzyme sample were added to a curvette and mixed well by inverting several times.

The spectrophotometer was blanked at 340 nm, then 200 µl of acetaldehyde (200 mM) was added. The absorbance increase at A_{340} was recorded over a period of 10 min and the rate of increase was calculated and converted to the number of µmoles of acetaldehyde oxidised per minute per ml of original sample.

2.6.3 Purification of recombinant human cytosolic aldehyde dehydrogenase

The following purification of wild type human cytosolic ALDH was carried out at 4 °C by adapting the method described by Zheng *et al.* (1993). Samples of 1 ml were removed at different stages of the purification for analysis by SDS-PAGE (section 2.6.5).

2.6.3.1 *Cell lysis*

For every litre of SRP84/pGP1-2/pThcAD culture harvested 15 ml of the relevant starting buffer (see section 2.3.1) was added to the cell paste, to resuspend the cells. The cells were then lysed in a French press operating at 7,000 psi and the lysate was centrifuged at 12,000 rpm in a SS-34 rotor (Sorvall) for 15 min. The supernatant was then decanted into another chilled SS-34 tube and 125 mg of protamine sulphate was added and the lysed cells centrifuged at 12,000 rpm in a SS-34 rotor for 15 min. The supernatant was then decanted, the volume determined and a sample removed. The supernatant was dialyzed overnight at 4 °C against the appropriate starting buffer.

2.6.3.2 *CM-Sephadex ion exchange column*

The CM-Sephadex ion exchange column was prepared by degassing and packing 70 ml of CM-Sephadex into a 2.5 cm x 20 cm column. The column was equilibrated to pH 6.0 using CM-Sephadex starting buffer (see section 2.3.1).

The dialysed supernatant volume and ALDH activity were measured (section 2.6.2) and a 1 ml sample taken. The dialysed supernatant was loaded onto the prepared column and then eluted using CM-Sephadex starting buffer. The column was run at 1 ml per min and fractions of 8 ml were collected until the A_{280} reading of the eluate was approximately zero. Fractions were measured for ALDH activity as described in 2.6.2. The fractions containing enzyme activity were

pooled. The total volume and activity of the pooled fractions was determined and a 1 ml sample taken.

2.6.3.3 *DEAE-Sephacel ion exchange column*

The ion exchange column was prepared by degassing and packing 70 ml of DEAE Sephacel into a 2.5 cm x 20 cm column. The column was equilibrated to pH 6.1 using DEAE starting buffer (see section 2.3.1).

The dialysed supernatant volume and ALDH activity were measured (section 2.6.2) and a 1 ml sample taken. The dialysed supernatant was loaded onto the prepared ion exchange column. DEAE starting buffer was used to wash the column at a rate of 1 ml per min. Fractions (4 ml) were collected every 4 min until the A_{280} reading of the eluate was approximately zero. Fractions were measured for ALDH activity as described in 2.6.2.

A 400 ml gradient of 100 mM to 350 mM NaCl (in DEAE starting buffer) was run through the ion exchange column at 1 ml per min and 4 ml fractions were collected. Fractions were measured at A_{280} and assayed for ALDH activity (section 2.6.2). The fractions containing enzyme activity were pooled. The total volume and activity of the pooled fractions was determined and a 1 ml sample taken.

2.6.3.4 *Affinity column*

The affinity column was prepared by degassing and packing 50 ml of *p*-hydroxyacetophenone-substituted Sepharose into a 2.5 cm x 20 cm column and then equilibrating the column with *p*-hydroxyacetophenone affinity column loading buffer (section 2.3.1).

The pooled fractions from the ion exchange column were loaded onto the equilibrated affinity column. The affinity column was washed with *p*-

hydroxyacetophenone affinity column loading buffer at a rate of 1 ml per min. Fractions of 4 ml were collected until the A_{280} eluate reading was approximately zero. Fractions were assayed for ALDH activity (section 2.6.2).

A 400 ml elution gradient of 0 mM to 10 mM *p*-hydroxyacetophenone (in *p*-hydroxyacetophenone affinity column loading buffer) was run through the affinity column at 1 ml per min and 4 ml fractions were collected. Fractions were then measured at A_{280} and assayed for ALDH activity as described in section 2.6.2. The fractions containing enzyme activity were pooled, the total volume was determined and a 1 ml sample removed.

2.6.4 Concentration of active fractions

The pooled active fractions from the affinity column were concentrated using a Centriprep ultrafiltration unit (100 kDa molecular weight cut off) from Amicon in an SS-34 rotor at 1,500 x g. The final activity and concentration of purified ALDH was determined. The purified recombinant cytosolic ALDH was stored in aliquots at -20 °C.

2.6.5 Modified Laemmli discontinuous SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970) using the Ornstein (1964) and Davis (1964) buffer system with the addition of 0.1% SDS in gels and electrophoresis buffer. SDS-PAGE was performed using a mini gel vertical electrophoresis cell (Protean II, BIORAD). A 12.5% separating gel was prepared (30%:80% acrylamide:bisacrylamide ratio, 0.1% SDS, 0.375 M Tris-HCl pH 8.8) which was polymerised by the addition of 30 µl of ammonium persulphate and 4 µl of TEMED. A stacking gel (4.5% acrylamide as above) of approximately 5 mm was added on top of the polymerised separating gel. The final dimensions of the gel were 8.2 cm wide, 6.5 cm long and 0.075 cm thick.

The reservoirs for the electrophoresis apparatus were filled with SDS-PAGE running buffer (section 2.3.1) and the electrophoresis was carried out under constant current of 5 mA until the dye front had penetrated the resolving gel, and 15 mA thereafter.

Protein samples were mixed with an equal volume of 2x SDS-PAGE sample buffer (section 2.3.1), denatured by heating to 100 °C for 3 mins and then cooled to room temperature.

The gel was stained in a solution of 0.2% Coomassie Brilliant Blue R-250 in 30% methanol/10% acetic acid for 10 min and then destained in three 30 min washes in 30% methanol/10% acetic acid. During the staining and destaining process the gel was rotated at 30 rpm on a rotary shaker The gel was stored in Welcome solution (5% methanol/5% acetic acid) until it was dried onto 3MM paper under vacuum at 80 °C.

2.6.6 Determination of protein concentration of purified recombinant human cytosolic aldehyde dehydrogenase

A standard curve was prepared by adding 0.75 ml Biuret reagent (Scopes, 1987) to 0.15 ml of 0, 2, 4, 6 and 8 mg/ml bovine serum albumin in 1.5 ml microcentrifuge tubes. The same amount of Biuret reagent was also added to a 3x diluted ALDH preparation (50 litres ALDH + 100 litres H_2O).

The tubes were allowed to stand at room temperature for 30 min before the absorbance at 540 nm was measured using spectrophotometer (2.6.1). Concentrations of the ALDH protein were determined from the standard curve.

Chapter Three

Results and Discussion

PART I: SITE DIRECTED MUTAGENESIS

3.1 EXPERIMENTAL RATIONAL

By targeting conserved residues within hcALDH, site directed mutagenesis may help identify the active site residues important in substrate and cofactor binding and in the catalysis of aldehyde oxidation. The hcALDH amino acid Lys272 is totally conserved in all of the ALDH isozymes (numbering based on human Class I ALDH Hempel *et al.*, 1984) and could potentially act as the base in the acid-base reaction proposed by Weiner *et al.* (1991).

3.1.1 Establishing the orientation of the hcALDH cDNA insert within the M13mp18 vector

The first step in the Kunkel *et al.* (1987) method of mutagenesis is to clone the cDNA to be mutated into a M13 vector. Dr. Kerrie Jones at Massey University prepared this clone using standard protocols. The orientation of the ligated hcALDH gene within the M13mp18 vector was established using restriction enzyme analysis.

Each of the enzymes produced the expected restriction fragments (figure 3.1). The 7.2 kbp fragment in the *Xba* I digest represents the M13mp18 vector itself as the hcALDH cDNA was cloned into its unique *Xba* I site. The 1.4 kbp fragment represents the hcALDH cDNA the recognition sequence for *Xba* I does not occur in hcALDH cDNA. This was the expected result as Dr. Kerrie Jones cut the pT7.7 hcALDH vector at the two *Xba* I sites to remove the hcALDH cDNA and insert it into the M13mp18 vector.

The M13mp18 vector has only one *Bam*H I site and hcALDH cDNA does not contain a *Bam*H I site, therefore digestion with *Bam*H I linearises the M13mp18 hcALDH vector (figure 3.1). The M13mp18 vector contains one *Sst* I site and no *Nde* I sites whereas the hcALDH cDNA contains one *Nde* I site but no *Sst* I sites.

Therefore two fragments were expected to result from the *Nde* I/*Sst* I double digest, as observed in figure 3.1.

Restriction analysis of M13mp18 plasmid containing hcALDH cDNA

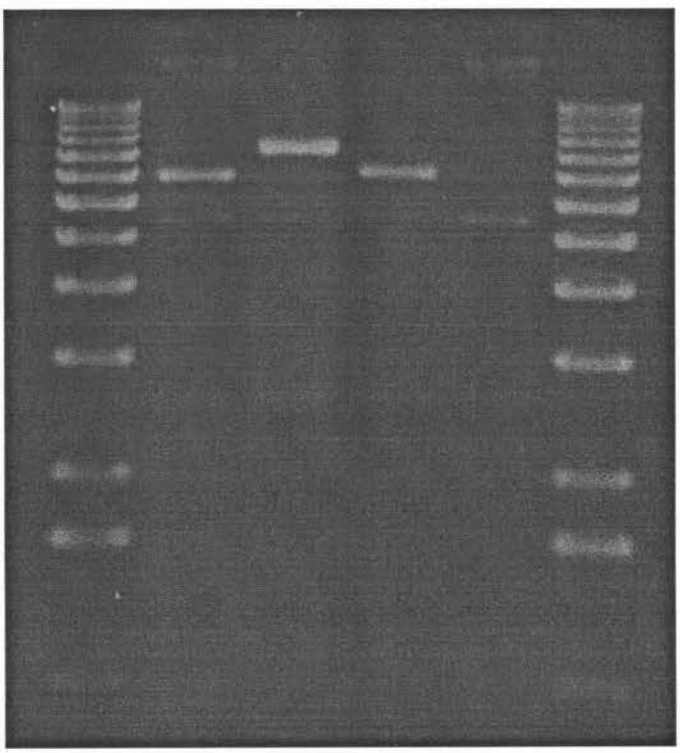

Figure 3.1

Aliquots of the M13mp18 hcALDH vector were digested with restriction endonucleases using the recommended React® buffers at 37 °C for 1 hr. Digests were analysed by gel electrophoresis using a 0.8% agarose gel (5.7 x 8.3 cm) in 1 x TAE at 80 V for 2 hr. (1) 5 µl BRL 1 kbp ladder; (2) 2 µl of the M13mp18 hcALDH vector digested with *Xba* I; (3) 2 µl of the M13mp18 hcALDH vector digested with *Bam*H I; (4) 2 µl of the M13mp18 hcALDH vector digested with *Nde* I and *Sst* I; (5) 2 µl of the M13mp18 hcALDH vector undigested; (6) 5 µl BRL 1 kbp ladder

Table 3.1 Restriction fragments of M13mp18 hcALDH

Restriction enzyme	Predicted fragment sizes (kbp)	Observed fragment sizes (kbp)
Xba I	7.2, 1.4	~7.2, ~1.4
Bam HI	8.6	~8.6
Nde I and Sst I	8.23, 0.37 or 7.2, 1.4 (desired orientation)	~7.2, ~1.4

The resulting 7.2 and 1.4 kbp fragment sizes indicate that the hcALDH cDNA is orientated 5' → 3' within the negative strand of the M13mp18 vector. If the cDNA was orientated 3' → 5' within the negative strand of the vector 8.23 and 0.37 kbp fragments would have resulted from the *Nde* I/*Sst* I digest reaction (table 3.1).

The positive DNA strand of the M13mp18 vector is packaged in M13 viral particles and shall be used as a template in future mutagenesis experiments. Hence mutagenic oligonucleotides must be designed to be almost identical in sequence to the negative strand of the M13mp18 hcALDH construct (the oligonucleotide will also contain deliberate mismatches for mutagenic purposes).

3.2 PREPARATION OF M13mp18 hcALDH TEMPLATE

3.2.1 Growth of M13mp18 hcALDH phage

The method of Kunkel *et al.* (1987) was used for the growth of M13mp18 hcALDH phage. Uracil-containing DNA was produced within the *E. coli* CJ236 strain which lacks the enzyme dUTPase and therefore contains an elevated concentration of dUTP which effectively competes with TTP for incorporation into DNA. This mutant strain also lacks the enzyme uracil *N*-glycosylase which normally removes uracil from DNA. Consequently uracil is incorporated into DNA in place of thymine and is not removed.

3.2.2 Assessment of uracil incorporation into M13mp18 hcALDH

M13mp18 hcALDH phage titres were compared using *ung*$^-$ and *ung*$^+$ hosts (table 3.2). Because the M13mp18 hcALDH phagemids contain uracil in their DNA, they survive far more readily in the bacterium without an active uracil *N*-glycosylase (CJ236) than in one with the active enzyme.

Table 3.2 Observed PFU/ml

***E. coli* strain**	**Observed phage titre**
XL1 Blue	1.09×10^3 PFU/ml
CJ236	1.11×10^{11} PFU/ml

3.2.3 Preparation and purification of M13mp18 hcALDH uracil template

M13mp18 hcALDH template was prepared and purified as described in Kunkel *et al.* (1987). The ss DNA purity was determined using spectrophotometric methods (table 3.3) and a sample of the purified template was examined by gel electrophoresis (figure 3.2).

Table 3.3 Purity and concentration of the uracil template

Spectrophotometric A_{260}:A_{280} ratio	1.06
Calculated DNA concentration	900 ng/µl

Purified M13mp18 hcALDH uracil-containing template

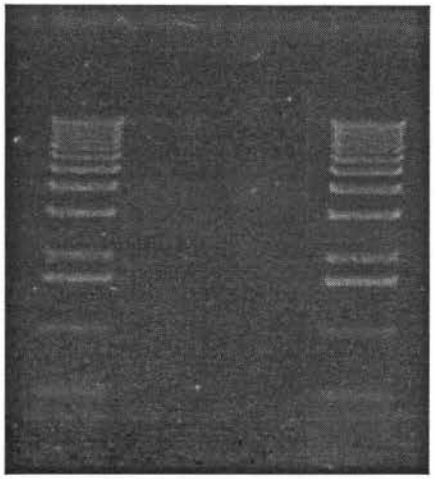

Figure 3.2

2 µl and 6 µl aliquots of the M13mp18 hcALDH uracil-enriched template were analysed by gel electrophoresis using a 0.8% agarose gel (5.7 x 8.3 cm) in 1 x TAE at 80 V for 2 hr. (1) 5 µl BRL 1 kbp ladder; (2) 2 µl of the M13mp18 hcALDH uracil template; (3) 6 µl of the hcM13mp18 ALDH uracil template; (4) 5 µl BRL 1 kbp ladder.

It appeared from the gel electrophoresis (figure 3.2) and spectrophotometric A_{260} reading that there was a good concentration of template DNA present in the preparation (table 3.3). However the prepared M13mp18 hcALDH uracil-containing template was calculated to have a purity ratio of only 1.06 (table 3.3) whereas a pure preparation of DNA would have an A_{260}/A_{280} ratio $\geq$ 1.8. The poor purity ratio suggested that the prepared DNA was contaminated by either protein or phenol.

Additional phenol:chloroform and chloroform:isoamyl alcohol extractions were performed on the M13mp18 hcALDH uracil-containing template in an attempt to improve the DNA purity (2.4.3.1). The DNA was precipitated after the extractions (2.4.3.2). The extractions caused only a slight improvement in DNA purity (table 3.4).

The DNA examined by gel electrophoresis (figure 3.3) demonstrated that the template ran at the expected molecular weight of 8.5 kbp and was not fragmented. Although the observed purity of the prepared uracil template was poor, the uracil content (table 3.2) and DNA concentration (table 3.3) of the template were adequate for the mutagenesis experimental protocol. This template was used for the following mutagenesis experiment, as several attempts at repeating this experiment failed to produce a template of higher purity.

Table 3.4 Observed template purity and concentration

Spectrophotometric A_{260}/A_{280} ratio	1.30
DNA concentration	655 ng/µl

Purified M13mp18 hcALDH uracil template

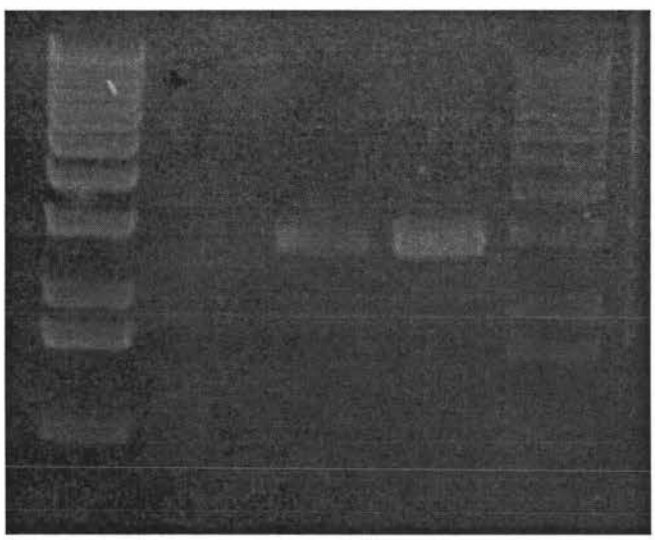

Figure 3.3

Aliquots of the M13mp18 hcALDH uracil-enriched template were analysed by gel electrophoresis using a 0.8% agarose gel (5.7 x 8.3 cm) in 1 x TAE at 80 V for 2 hr. (1) 4 µl BRL 1 kbp ladder; (2) 5 µl of the M13mp18 ALDH uracil template purified using phenol chloroform extractions; (3) 1 µl of ss M13mp18 control template; (4) 3 µl of the M13mp18 ALDH uracil template purified using phenol chloroform extractions; (5) 4 µl BRL 1 kbp ladder.

3.3 INTRODUCTION OF THE LYS272 MUTATION

3.3.1 Synthesis of the complementary DNA strand

The synthetic oligonucleotide Lys-272-SDM was designed to replace lysine with either histidine, alanine or arginine at the amino acid position 272 within the recombinant human Class I ALDH. All three changes were incorporated into the one oligonucleotide. Table 3.5 illustrates the possible sequences resulting from the degenerate oligonucleotide Lys-272-SDM. In addition to the desired amino acid mutations, replacement by asparagine, glycine and proline was also expected.

Table 3.5 Sequences resulting from the Lys-272-SDM mutagenic primer

Possible oligonucleotide sequence at position 1530 - 1557 within the hcALDH cDNA	**Resulting amino acid sequence**
5' GCTTGGAGGA**GAC**AGCCCTTGCATTGTG 3'	LGG**N**SPCIV
5' GCTTGGAGGA**GCC**AGCCCTTGCATTGTG 3'	LGG**A**SPCIV
5' GCTTGGAGGA**GGC**AGCCCTTGCATTGTG 3'	LGG**G**SPCIV
5' GCTTGGAGGA**CAC**AGCCCTTGCATTGTG 3'	LGG**H**SPCIV
5' GCTTGGAGGA**CCC**AGCCCTTGCATTGTG 3'	LGG**P**SPCIV
5' GCTTGGAGGA**CGC**AGCCCTTGCATTGTG 3'	LGG**R**SPCIV

The DNA contained within the Lys-272-SDM oligonucleotide was extended and ligated to produce covalently closed circular DNA as described by Kunkel *et al.*

(1987). Two reactions were set up using template:primer molar ratios of 0.08 pmol template:0.8 pmol primer and 0.08 pmol template:0.16 pmol primer.

A third reaction containing no primer and 0.08 pmol of template was prepared to test the nonspecific priming caused by contaminating nucleic acids in the template preparation. This control was important as nonspecific priming can result in a lowered mutation efficiency. Properly prepared templates should result in little, if any, synthesis of covalently closed circular DNA in the absence of added primer.

Reaction products from the three mutagenesis reactions were analysed by gel electrophoresis (fig 3.4). From this gel it was concluded that the elongation stage of the mutagenesis reaction had been unsuccessful as the reaction products appeared to migrate at the same rate as the ss template control. If the mutagenesis reaction had been successful the product from the *in vitro* DNA synthesis reaction would run at the same rate as the RF I standard, indicating that the DNA has been converted to duplex, covalently closed circular, relaxed DNA, by the combined action of DNA polymerase and ligase.

M13mp18 hcALDH products after the mutagenesis reaction

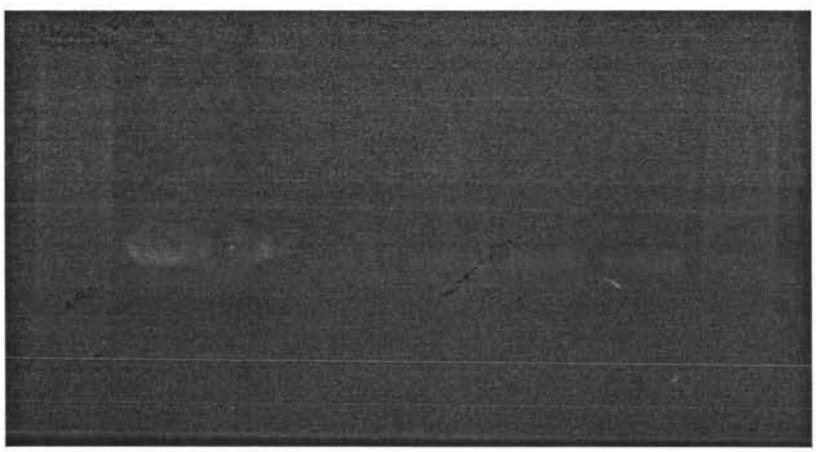

Figure 3.4

Aliquots of the mutagenesis reactions were analysed by gel electrophoresis using a 0.8% agarose gel (5.7 x 8.3 cm) in 1 x TAE at 80 V for 2 hr. (1) 4 µl BRL 1 kbp ladder; (2) 3 µl of the M13mp18 hcALDH ss uracil template; (3) 3 µl of M13mp18 hcALDH ds control template; (4) 12 µl of the 1:10 template:primer ratio reaction; (5) 12 µl of the 1:2 template:primer ratio reaction; (6) 12 µl of the 1:0 template:primer ratio reaction; (7) BRL 1 kbp ladder.

3.3.2 Investigation into the unsuccessful site directed mutagensis reaction

To determine what was at fault in the above mutagenesis experiment a variety of reactions were prepared to determine what component of the reaction was the preventing elongation of the DNA from the oligonucleotide. From these experiments the uracil template was shown to be the cause of the problems at the elongation stage. The concentration of ss M13mp18 hcALDH did not appear to be as high as initally calculated (contaminants possibily contributing to the A_{260} value used to calculate the concentration). A sample of the template was run on a gel to check its condition (figure 3.5) and this gel electrophoresis showed that the uracil template was actually lower in concentration than what was originally calculated.

Purified M13mp18 ALDH uracil containing template

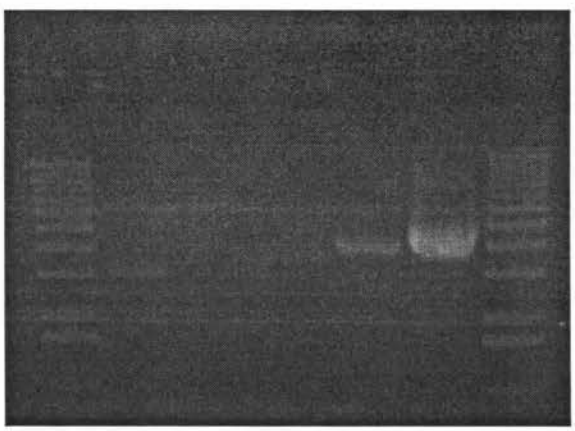

Figure 3.5

Aliquots of the M13mp18 hcALDH uracil containing template were analysed by gel electrophoresis using a 0.8% agarose gel (5.7 x 8.3 cm) in 1 x TAE at 80 V for 2 hr. (1) 4 µl BRL 1 kbp ladder; (2) 2 µl of the M13mp18 control marker; (3) 2 µl of the M13mp18 hcALDH uracil template prepared in section 3.2.3; (4) 6 µl of ss M13mp18 hcALDH uracil template prepared in section 3.2.3; (5) 2 µl of control M13mp18 hcALDH uracil template; (6) 6 µl of control ss M13mp18 hcALDH uracil template; (7) 4 µl BRL 1 kbp ladder.

3.3.3 Assessment of uracil incorporation and purity of the M13mp18 hcALDH template

The M13mp18 hcALDH template was prepared and purified once again as described in section 3.2. The phage titre indicated a high incorporation of uracil into the M13mp18 hcALDH template (table 3.2) which is a requirement of the mutagenesis protocol described by the Kunkel *et al.* (1987).

The observed purity of the prepared uracil template was shown to be 1.76 (table 3.7) which was a marked improvement in purity compared to the template prepared in section 3.2. Gel electrophoresis (figure 3.6) demonstrated that the uracil-containing template ran at the expected molecular weight of 8.5 kbp and was not fragmented. The DNA concentration calculated by spectrophotometric methods (table 3.7) was supported by the intensity of the DNA band observed in figure 3.6.

Purified M13mp18 ALDH uracil containing template

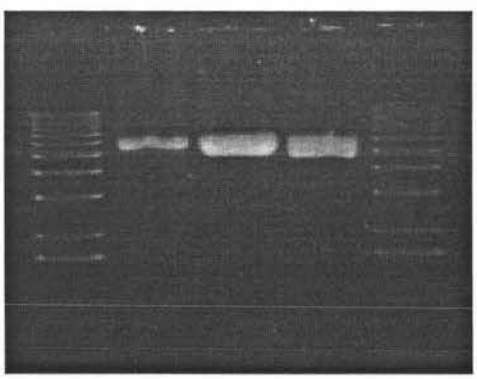

Figure 3.6

2 µl and 6 µl aliquots of the M13mp18 hcALDH uracil-enriched template were analysed by gel electrophoresis using a 0.8% agarose gel (5.7 x 8.3 cm) in 1 x TAE at 80 V for 2 hr. (1) 4 µl BRL 1 kbp ladder; (2) 2 µl of the RF M13mp18 hcALDH; (3) 6 µl of the ss M13mp18 hcALDH uracil template prepared by Dr. Kerrie Jones; (4) 6 µl of the ss M13mp18 hcALDH uracil template prepared in this section; (5) 4 µl BRL 1 kbp ladder.

Table 3.6 Observed PFU/ml

E. coli strain	Observed phage titre
XL1 Blue	2.88×10^5 PFU/ml
CJ236	1.15×10^{14} PFU/ml

Table 3.7 Purity and concentration of the uracil template

Spectrophotometric A_{260}:A_{280} ratio	1.76
Calculated DNA concentration	297 ng/µl

3.3.4 Synthesis of a complementary DNA strand

As the prepared template was shown by spectrophotometric measures and gel electrophoresis to have acceptable concentration and purity levels, this template was used to repeat the mutagenesis experiment.

The DNA contained within the Lys-272-SDM oligonucleotide was incorporated into covalently closed circular DNA by DNA synthesis and ligation as described in section 3.3.1. The experiment was performed in duplicate and the resulting products examined by gel electrophoresis (figure 3.7).

The results of analysis of products from the mutagenesis experiment were confusing because the RF M13mp18 hcALDH control marker appeared to be running at the same level as the ss M13mp18 hcALDH template. This was interpreted to mean that either the ss template was actually ds, or that the ds control marker was in fact ss.

Resulting M13mp18 hcALDH products after mutagenesis reaction

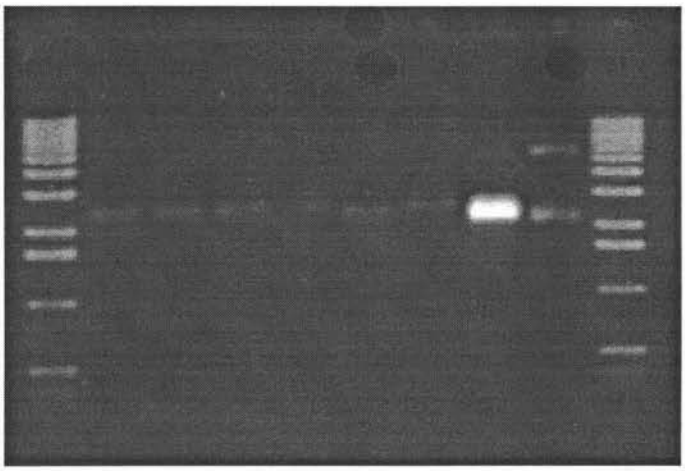

Figure 3.7

Aliquots of the resulting Lys272 mutants were analysed by gel electrophoresis using a 0.8% agarose (5.7 x 8.3 cm) in 1 x TAE at 80 V for 2 hr. (1) 4 µl BRL 1 kbp ladder; (2) 11 µl of the 1:0 reaction sample 1; (3) 11 µl of 1:0 reaction sample 2; (4) 11 µl of 1:2 reaction sample 1; (5) 11 µl of 1:2 reaction sample 2; (6) 11 µl of 1:10 reaction sample 1; (7) 11 µl of 1:10 reaction sample 2; (8) ss M13mp18 hcALDH marker; (9) RF M13mp18 hcALDH marker; (10) 4 µl BRL 1 kbp ladder.

3.3.5 Digest of M13mp18 hcALDH

Due to the problems encountered whlile trying to produce a mutant using the template prepared in section 3.3.4 and the observation that the prepared template may actually be ds DNA (figure 3.7), the M13mp18 hcALDH template was digested with *Hind* III. The restriction endonuclease *Hind* III only digests ds DNA and was therefore able to help determine whether the template was single or double stranded DNA.

A reaction containing 1 µl of prepared template (297 ng/µl) and 5 units of *Hind* III was prepared as described in table 3.8. The controls were prepared using RF M13mp18 hcALDH (positive control) and ss M13mp18 (negative control) in a

restriction enzyme digestion containing 5 units of *Hind* III. A fourth tube containing the prepared template but no enzyme was also prepared and incubated at 37 °C for 1 hr.

The resulting products from each *Hind* III reaction were examined by gel electrophoresis (figure 3.8). The RF M13mp18 hcALDH positive control demonstrated that the *Hind* III enzyme was digesting ds DNA efficiently. The ss M13mp18 negative control showed that ss DNA was not digested by the restriction endonuclease. Hence both of the control reactions produced the expected fragments from the *Hind* III digest.

The results from the *Hind* III digest with the M13mp18 hcALDH template lead to the conclusion that the prepared uracil-containing template was infact ds DNA. Unfortunatley this meant a third attempt at preparing ss M13mp18 hcALDH template using the Kunkel *et al.* (1987) method.

Hind III digest of M13mp18 hcALDH template

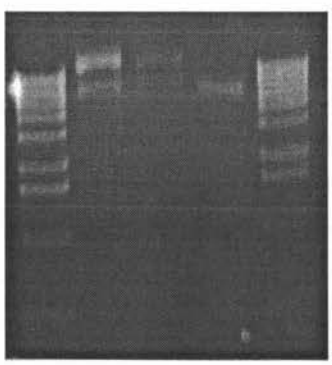

Figure 3.8

Aliquots of ss and RF M13mp18 hcALDH were digested with the restriction endonuclease *Hind* III using the recommended React® 2 buffer at 37 °C for 1 hr. Digests were analysed by gel electrophoresis using a 0.8% agarose (5.7 x 8.3 cm) in 1 x TAE at 80 V for 2 hr. (1) 5 µl BRL 1 kbp ladder; (2) 20 µl of the RF M13mp18 hcALDH *Hind* III digest; (3) 20 µl of ss M13mp18 hcALDH *Hind* III digest; (4) 20 µl ss M13mp18 *Hind* III digest; (5) 20ul M13mp18 subjected to digest conditions excluding *Hind* III; (6) 5 µl BRL 1 kbp ladder.

3.3.6 Third attempt at the preparation and purification of M13mp18 hcALDH uracil-containing template

The M13mp18 hcALDH template was prepared and purified in duplicate as described in section 3.2. One of the preparations was infected with M13mp18 hcALDH bacteriophage for 6 hours and the other preparation infected overnight. The M13mp18 hcALDH phage titres were compared using *ung*$^-$ and *ung*$^+$ hosts.

The template from the overnight infection appeared to be the most pure of the two prepared templates (table 3.8); a ratio of 1.85 was observed compared to only 1.55 for the 6 hour infection. The template from the overnight infection was calculated to have a DNA concentration of 967 ng/µl (table 3.8) and was shown to migrate at the correct rate under gel electrophoresis conditions (figure 3.9). Using the comparison of template purity, concentration and its appearance on the gel it appeared that the overnight template was the better of the two preparations. However before selecting the overnight template to use in the mutagenesis experiment the two templates were digested with *Hind* III to ensure that they were both in fact ss.

Table 3.8 Observed purity and concentration of the prepared M13mp18 hcALDH uracil template

	6 hour infection	**Overnight infection**
Spectrophotometric A_{260}:A_{280} ratio	1.55	1.85
Calculated DNA concentration	279 ng/µl	967 ng/µl

Purified M13mp18 hcALDH uracil-containing template

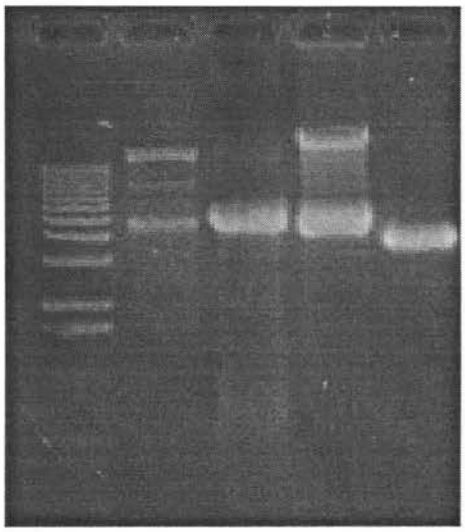

Figure 3.9

A 2 µl and 6 µl aliquot of the M13mp18 hcALDH uracil containing template was analysed by gel electrophoresis using a 0.8% agarose gel (5.7 x 8.3 cm) in 1 x TAE at 80 V for 2 hr. (1) 4 µl BRL 1 kbp ladder; (2) 2 µl of the RF M13mp18 hcALDH; (3) 1 µl of the overnight infection ss M13mp18 hcALDH uracil template preparation; (4) 4 µl of the 6 hour ss M13mp18 hcALDH uracil template preparation; (5) 2 µl of the ss M13mp18 hcALDH.

3.3.7 Digest of M13mp18 hcALDH

Digesting the prepared M13mp18 hcALDH templates with the restriction enzyme *Hind* III shall determine whether these are ss or ds DNA. A reaction containing approximately 1 µg of each template (table3.8) and 5 units of *Hind* III was prepared as described in table 3.10. The controls were prepared using RF M13mp18 hcALDH (positive control) and ss M13mp18 (negative control) in a restriction enzyme digestion containing 5 units of *Hind* III (table 3.10). Each reaction was made up to a final volume of 20 µl using Milli-Q water and incubated at 37 °C for 1 hr.

Table 3.10 Hind III reaction components

Components	**Overnight M13mp18 hcALDH template (μl)**	**6hr M13mp18 hcALDH template (μl)**	**Positive control RF M13mp18 hcALDH (μl)**	**Negative control ss M13mp18 (μl)**
10x buffer	2.0	2.0	2.0	2.0
DNA	1.0	4.0	2.0	1.0
Hind III	0.5	0.5	0.5	0.5
H_2O	16.5	13.5	15.5	16.5
Total volume	20.0	20.0	20.0	20.0

Each of the control reactions produced the expected fragments from the *Hind* III digest (figure 3.10). The RF M13mp18 hcALDH positive control demonstarted that the *Hind* III enzyme was digesting ds DNA efficiently. The ss M13mp18 negative control showed that ss DNA was not digested by the restriction endonuclease.

The template prepared from the 6 hr infection showed the prescence of double stranded DNA, possibly chromosomal (figure 3.10). Hind III did not digest the overnight ss M13mp18 hcALDH template. It was concluded from the results of this digest and the observations made in section 3.3.6, that the template produced from the overnight infection preparation was the better template to use for the following mutagenesis experiment.

Hind III digest of M13mp18 hcALDH template

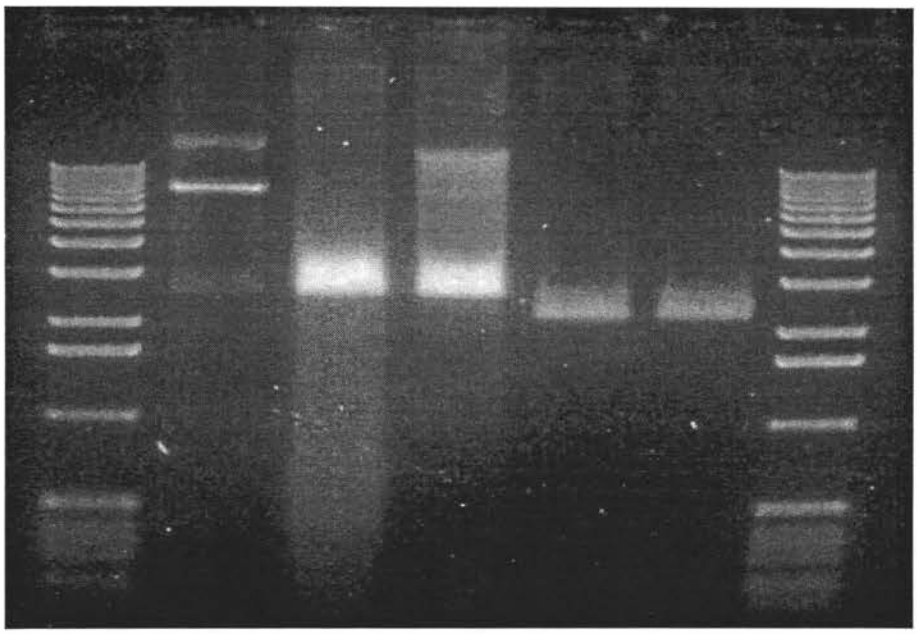

Figure 3.10

Aliquots of the prepared M13mp18 hcALDH templates were digested with the restriction endonuclease *Hind* III using the recommended React® 2 buffer at 37 °C for 1 hr. Digests were analysed by gel electrophoresis using a 0.8% agarose gel (5.7 x 8.3 cm) in 1 x TAE at 80 V for 2 hr. (1) 5 µl BRL 1 kbp ladder; (2) 20 µl of the RF M13mp18 hcALDH *Hind* III digest; (3) 20 µl of infected overnight ss M13mp18 hcALDH *Hind* III digest; (4) 20 µl 6 hour infected ss M13mp18 *Hind* III digest; (5) 20ul M13mp18 (2 µl 10 x buffer; 2 µl DNA; 16 µl water incubated at 37 °C for 1 hr) subjected to digest conditions excluding *Hind* III; (6) M13mp18 marker; (7) 5 µl BRL 1 kbp ladder.

3.4 SECOND ATTEMPT TO INTRODUCE THE LYS272 MUTATION INTO hcALDH

3.4.1 Synthesis of a complementary DNA strand

Using DNA synthesis and ligation I tried once again to extend and ligate the DNA contained within the Lys-272-SDM oligonucleotide, to produce covalently closed circular DNA as described by Kunkel *et al.* (1987). Reactions were set up as described in section 3.3.1 (table 3.11), using template:primer ratios of 0.08 pmol

template:0.8 pmol primer (tube 4) and 0.08 pmol template:0.16 pmol primer (tube 5). The mutagenesis experiment was performed in duplicate using the uracil-containing template prepared by overnight infection as described in section 3.3.7.

Three control reactions were also prepared, one to check that the modifying enzymes and buffers were performing properly (tube 1), the second to show that the ss uracil template was able to anneal to a control primer under the reaction conditions (tube 2) and the third control reaction (tube 3) contained no primer to test the nonspecific endogenous priming caused by contaminating nucleic acids in the template preparation (table 3.11).

Table 3.11 Components of mutagenesis reaction tubes

Components	**Tube 1 (µl)**	**Tube 2 (µl)**	**Tube 3 (µl)**	**Tube 4 (µl)**	**Tube 5 (µl)**
10x annealing sequencing buffer	1.0	1.0	1.0	1.0	1.0
ss M13mp18 (0.2 µg/µl)	2.0	-	-	-	-
M13mp18 hcALDH (966 ng/µl)	-	0.21	0.21	0.21	0.21
M13mp18 -40 primer	1.0	1.0	-	-	-
Lys-272-SDM primer	-	-	-	1.14	-
Lys-272-SDM primer	-	-	-	-	2.29
dH_2O	6.00	7.79	8.79	7.65	6.50
Total volume	10.0	10.0	10.0	10.0	10.0

The mutagenesis products from the control tubes tubes 1 and 2 demonstrated that the reaction buffer, enzyme and mutagenesis template were performing correctly under the reaction conditions due to the positive control being successfully elongated (figure 3.11) to form ds DNA. Reaction tube 3 did not show an elongation shift on the gel electrophoresis; this was expected as no primer was added to this reaction (figure 3.11).

Under gel electrophoresis conditions (figure 3.11) it was observed that the DNA from tubes 4 and 5 migrated at a different rate than the negative control (ss DNA) but at a similar rate to the positive control. I concluded from the observed migration rates that the DNA templates contained in these tubes had successfully elongated to produce ds DNA. The contents of tubes 4 and 5 where therefore selected for the transformation of the *ung*$^+$ *E. coli* strain XL1 Blue.

Resulting M13mp18 hcALDH products after mutagenesis reaction

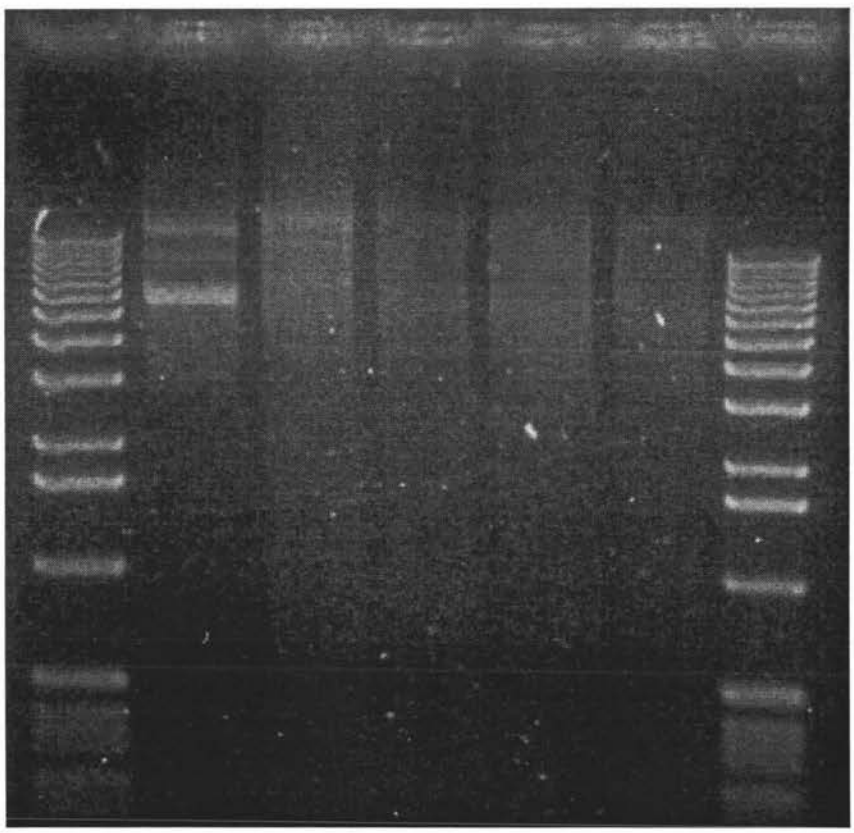

Figure 3.11

Aliquots resulting from the above mutagenesis experiment were analysed by gel electrophoresis using a 0.8% agarose gel (5.7 x 8.3 cm) in 1 x TAE at 80 V for 1 hr. (1) 4 µl BRL 1 kbp ladder; (2) 10 µl of tube 1 - enzyme buffer control; (3) 10 µl of tube 2 - uracil template control; (4) 10 µl of tube 3 - 1:0 template:primer reaction; (5) 10 µl of tube 4 - 1:10 template:primer reaction; (6) 10 µl of tube 5 - 1:2 template:primer reaction; (7) 4 µl BRL 1 kbp ladder.

3.4.2 Electroporation of the mutagenesis reaction products into XL1 Blue

The double stranded mutagenesis products from tubes 4 and 5 were introduced by electroporation into XL1 Blue, a *dut*$^{+}$ *ung*$^{+}$ strain of *E. coli*. The product of the *ung* gene (uracil-*N*-glucosidase) initiates a DNA repair process which causes extensive breakage of the uracil-containing (non-mutant) strand, but does not effect the mutant strand, which does not contain uracil. This results in a strong selection against the non-mutant strand and yields a very high proportion of clones which contain the mutant DNA.

Each mutagenesis reaction mixture was drop dialysed using a 0.025 μm filter (Millipore cat. no. VSWP 047 00) against 10% w/v glycerol for a period of 30 min, to remove salts that may impeed the electroporation reaction. Electroporation was performed as described in section 2.5.4 using 40 μl of electrocompetent XL1 Blue cells and 5 μl of each drop dialysed mutagenesis reaction. XL1 Blue was also subjected to electroporation conditions without the addition of DNA to act as a negative control. The addition of 5 μl of RF M13mp18 to the cells in the electroporation curvette acted as a positive control.

Both the positive and negative controls produced the expected results; that is the positive control produced PFU whereas the negative control did not. The electroporation of XL1 Blue with the ds DNA from tubes 4 (1:10 template:primer) and 5 (1:2 template:primer) yielded 5 and 4 plaques on their respective agar plates. Although the number of plaques was lower than what was expected, I decided to see if any of these samples contained one of the desired mutations.

3.5 SEQUENCING THE PLAQUE DNA

Due to the strong selection against the uracil-containing parental strand of the *in vitro* mutagenesis reaction, typically more than 50% of the plaques obtained from the transformation of XL1 Blue should carry mutant ALDH sequence. DNA from

the plaques prepared in section 3.4.2 was purified and isolated as described in section 2.4.3. The DNA pellet was redisolved in 30 µl TE pH 8.0 and an aliquot of each plaque solution was examined by gel electrophoresis (figure 3.12). Plaques 4.1, 4.2, 5.1 and 5.3 were selected for DNA sequencing as they appeared to have the highest purity of the samples loaded on the gel.

The DNA sequence surrounding the Lys272 region of hcALDH was sequenced as described in section 2.4.8 and 2.4.9 to determine if any of the desired mutations were present. The isolated DNA is equivalent in sequence to the positive strand M13mp18 hcALDH vector which contains the antisense strand of the ALDH sequence. The ALDH sequencing oligonucleotide (hALDHTop1441) was therefore designed to correspond to the sense strand of the hcALDH cDNA (table 3.12). This primer binds 100 bp upstream from the Lys-272 mutation region, see table 3.12.

Purified DNA from 'mutant plaques'

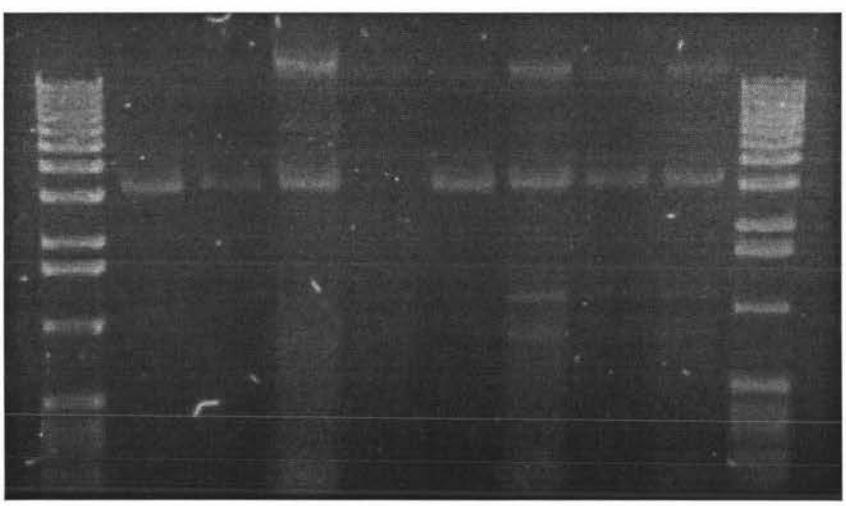

Figure 3.12

Aliquots of the purified DNA from the 'mutant plaques' were analysed by gel electrophoresis using a 0.8% agarose gel (5.7 x 8.3 cm) in 1 x TAE at 80V for 1 hour. (1) 4 µl BRL 1 kbp ladder; (2) 5 µl of plaque 4.1 DNA; (3) 5 µl of plaque 4.2 DNA; (4) 5 µl of plaque 4.4 DNA; (5) 5 µl of plaque 4.5 DNA; (6) 5 µl of plaque 5.1 DNA; (7) 5 µl of plaque 5.2; (8) 5 µl of plaque 5.3 DNA; (9) 5 µl of plaque 5.4; (10) 5 µl BRL 1 kbp ladder.

Tabe 3.12 Sequencing primer hALDHTop1441

Primer name	Sequence (5' → 3')	Position
hALDHTop1441	GAC AAA GTA GCC TTC ACA	1441 bp from ALDH start codon

3.5.1 Discussion

The developed film showed that no base pair changes where present in the Lys272 codon. Due to the amount of time that had been spent trying to create a mutation at Lys272 and time constraints, the construction of Lys272 mutants was abandoned.

A small amount of research time was spent investigating the Cys302Ala mutation created by Dr. Kerrie Jones when she left her post doctoral position at Massey University. Dr. Kerrie Jones had prepared the M13mp18 hcALDHC302A construct but had not gone any further into its investigation.

The M13mp18 hcALDHC302A was digested using *Nco* I and *Bst* I which cut around the the muatation region and the resulting 80 bp fragment was sequenced which confirmed that the mutation was present. The 80 bp fragment was then ligated into the pThcAD vector, transformed into *E. coli* SRP84/pGP1-2 and cultured in a 1 litre prepartion (section 2.5.9.2). The mutant protein was expressed by heat indution and purified using the DEAE ion exchnage and *p*-hydroxyacetophenone affinity columns (sections 2.6.3.3 and 2.6.3.4) The fractions were assayed for activity however no activity was detected (2.6.2).

PART II: PROTEIN PURIFICATION

3.6 EXPRESSION OF RECOMBINANT CLASS 1 ALDH

A culture of *E. coli* SRP84/pGP1-2/pThcAD was grown and recombinant ALDH was expressed by heat induction as described in section 2.5.9.2. Culture samples (1 ml) were removed at intervals during the induction for SDS-PAGE analysis of the recombinant protein expression.

Each culture sample was centrifuged (12,000 x g for 5 min) and each cell pellet resuspended in 100 µl of 1x SDS buffer. A 10 µl volume of dialysed protein sample (2.6.3.1) was mixed with 10 µl of 2 x SDS buffer (section 2.3.1). A sample of ALDH marker was prepared by adding 20 µg of purified native sheep ALDH (prepared by Treena Blythe) to 20 µl of 1 x SDS buffer. The above samples were then prepared for SDS-PAGE analysis as described in section 2.6.5. An SDS-PAGE gel of these samples (figure 3.13) showed that incubation overnight after heat induction lead to the highest level of protein expression.

SDS-PAGE of culture samples taken during the expression of the recombinant class 1 ALDH

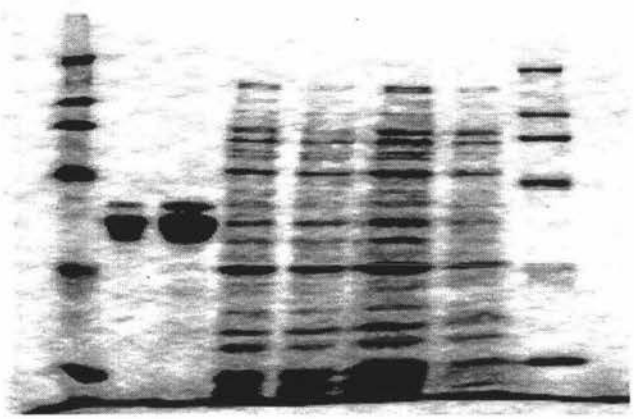

Figure 3.13

A 12% polyacrylamide-SDS gel was prepared, run and stained as described in section 2.4.4. (1) 5 µl SDS-6H ladder; (2) 2 µl native sheep ALDH marker (1 µg/µl); (3) 5 µl native sheep ALDH marker (1 µg/µl); (4) 5 µl 0 hr growth after induction; (5) 5 µl 1.5 hr growth after induction; (6) 5 µl overnight growth after induction; (7) 5 µl dialysed protein sample; (8) 5 µl SDS-6H ladder.

3.7 PURIFICATION OF RECOMBINANT HUMAN CLASS1 ALDH

3.7.1 Cell growth and lysis

E. coli SRP84/pGP1-2/pThcAD was grown and ALDH expressed during overnight incubation as described in section 2.5.9. The cells were then pelleted by centrifugation (2.5.10) and the resulting pellet was lysed using a French Press and prepared for loading onto a CM-Sephadex ion exchange column as described in section 2.6.3.1. Lane 7 on the polyacrylamide-SDS gel illustrated in figure 3.13 shows a 10µl sample of the dialysed ALDH protein (2.6.3.1) before loading onto the CM-Sephadex column. Many protein bands are present, including the one which has migrated at the rate expected of the ALDH protein.

3.7.2 CM-Sephadex column

Purification using the CM-Sephadex ion exchange column was performed as described in section 2.6.3.2. The sample was loaded onto the ion exchange column and 4 ml fractions were collected during elution and measured for ALDH activity as described in 2.6.2. The specific activity of the protein sample only increased a fraction after running through the CM-Sephadex column (table 3.13).

Table 3.13 Purification summary

Step	Activity (µmol/min/ml)	Total activity (µmol/min)	Protein (mg/ml)	Total protein (mg)	Specific activity (µmol/min/mg)
Cell lysate	1.11	25.0	37.0	855	0.03
CM-Sephadex	0.56	19.6	12.0	420	0.05
Affinity elution	0.21	3.50	0.33	5.40	0.65
Affinity wash	0.11	6.60	4.50	270	0.02
Concentrated	8.20	4.10	11.0	5.50	0.75

3.7.3 Affinity column

The next stage of purification was performed using the *p*-hydroxyacetophenone affinity column as described in section 2.6.3.4. This method involved the elution of bound ALDH from the column using a gradient containing the *p*-hydroxyacetophenone ligand. Fractions from the CM-Sephadex column that contained ALDH were pooled and loaded onto the affinity column, and 4 ml fractions were collected during the loading and elution of the column. These fractions were measured for ALDH activity as described in 2.6.2. The most pure fractions demonstrating ALDH activity (examined by SDS-PAGE) were pooled and concentrated to 0.5 ml as described in section 2.6.4. See table 3.13 for the detected ALDH activity.

3.7.4 Discussion of aldehyde dehydrogenase purification

It was observed in this preparation and several repeated attempts of recombinant ALDH purification that the quantity of active ALDH recovered was very low and a large percentage of enzyme was not binding to the affinity column but coming straight through the affinity column with the wash buffer (table 3.13).

The detection of significant amounts of ALDH activity in the affinity column wash fractions was unexpected as the above purification procedure had proven successful for the purification of native sheep liver class 1 ALDH. The partial binding of the recombinant protein to the affinity resin may be due to a variety of reasons, such as the recombinant human liver class 1 ALDH folding differently to the native form, or the presence of a component in the recombinant enzyme preparation which prevents the recombinant enzyme from binding to the affinity column. The observed difference in binding properties may also be due to the different species origin of ALDH.

3.8 INVESTIGATION OF THE POOR BINDING OF HUMAN RECOMBINANT CLASS 1 ALDH TO THE AFFINITY COLUMN

As discussed in section 3.7.4 the human recombinant class 1 ALDH bound poorly to the *p*-hydroxyacetophenone affinity column. However class 1 ALDH isolated from sheep liver has been shown to bind to this affinity column.

Cherie Stayner, in our laboratory, had successfully cloned the sheep liver class 1 ALDH cDNA into the pT7.7 vector under the control of the ϕ10 promoter (pTscAD). To determine whether the difference between native and recombinant enzyme would result in different binding properties I decided use this vector to determine if the expressed ALDH performed any differently to the native sheep ALDH in the purification proceedure.

Due to the use of an incorrect primer during the construction of the pTscAD vector, an error had been introduced into the cDNA that would result in an additional serine at the N-terminus of the enzyme. The possibility that this change may also affect the expressed enzyme activity made it necessary for me to determine that the pTscAD vector would result in expression of an active ALDH enzyme before using this vector as a tool in solving the problems that I had experienced.

3.8.1 Determining ALDH activity from the expression of enzyme from pTscAD

The pTscAD expression vector was transformed into *E. coli* SRP84/pGP1-2 by electroporation as described in section 2.5.4. A 5 ml culture was grown in M1 media supplemented with 10 mM glucose, 50 μg/ml kanamycin and 100 μg/ml ampicillin, at 30 °C until an A_{600} value of 1.0 was reached. The culture was heat-induced for 5 min at 42 °C to promote ALDH expression and then grown for 3 hr. The cells were then pelleted at 12,000 x g using a microcentrifuge and the supernatant discarded. The cell pellet was resuspended and sonicated as described

in section 2.5.11 to lyse the *E. coli* cells. The cell lysate was then assayed for ALDH activity as described in section 2.6.2.

ALDH enzyme activity was detected (table 3.14) and therefore the pTscAD vector was a valid tool for determining if a recombinant form of sheep liver ALDH, grown and expressed as described in section 2.5.9, could be purified as effectively as the native enzyme using the *p*-hydroxyacetophenone affinity column.

Table 3.14 Recombinant sheep liver Class I ALDH activity

Volume of assayed protein sample	100 µl
Total volume of protein sample	500 µl
Activity	16.0 µmol/min/ml
Total activity	8.0 µmol/min

3.8.2 Growth, expression and purification of recombinant sheep liver class 1 ALDH

A large-scale culture of *E. coli* SRP84/pGP1-2/pTscAD was grown and the recombinant ALDH expression induced as described in section 2.5.9.2. The cells were lysed and loaded onto the equilibrated CM-Sephadex ion exchange column as described in sections 2.5.10, 2.6.3.1 and 2.6.3.2. Fractions (8 ml) were collected at a flow rate of 1 ml per minute and assayed for ALDH activity as described in section 2.6.2 (table 3.15).

The ALDH isolated from the CM-Sephadex column was loaded onto the affinity column and 4 ml fractions were collected during the loading and elution steps (2.6.3.4). These fractions were assayed for ALDH activity as described in section 2.6.2 (table 3.15). The active fractions were pooled, then concentrated and the final activity of purified ALDH was determined as described in sections 2.6.4 and 2.6.2 respectively.

Table 3.15 Purification summary

Step	Volume (ml)	Activity (μmol/min/ml)	Total activity (μmol/min)
Cell lysate	23.5	7.50	176
CM-Sephadex column elution	34.5	0.60	20.7
Affinity column wash	30.0	0.30	9.00
Affinity column elution	34.0	0.08	2.75
Concentrated ALDH	0.50	0.10	0.05

3.8.3 Discussion of the recombinant sheep liver class 1 ALDH purification

Only 13% of the total activity loaded onto the affinity column (table 3.15) was eluted using the *p*-hydroxyacetophenone gradient (gradient contains *p*-hydroxyacetophenone elution ligand, section 2.3.1).

Although the total activity of the eluted recombinant sheep class 1 ALDH was only 2.75 μmoles/min I decided to concentrate this sample to determine if the large volume or the presence of *p*-hydroxyacetophenone may be inhibitory to the ALDH activity. This unfortunately showed no observable increase in ALDH activity. A sample of pure native sheep class 1 ALDH was also assayed for activity with and without the presence of *p*-hydroxyacetophenone and this also made little difference to the enzyme activity, suggesting that it is not an inhibitor of ALDH activity.

Almost half of the remaining activity (44%) did not bind to the affinity column and came through with the wash buffer (table 3.15). This may be due to some difference between the recombinant and native forms of the sheep liver class 1 ALDH. It may be that the enzyme has been folded or modified differently in the bacterial expression system or there is a component present in the bacterial preparation, but absent in the native preparation, that interferes with the binding of

ALDH to the affinity column. This supports the concern suggested in section 3.7.4, that there is some difference between native and recombinant preparations.

3.9 PURIFICATION OF THE RECOMBINANT HUMAN CLASS 1 ALDH USING A DEAE ION EXCHANGE COLUMN

The poor binding of recombinant ALDH to the *p*-hydroxyacetophenone affinity column may be due to an inhibitory component which is present in the recombinant but absent in the native ALDH preparation, as suggested in section 3.7.4. It was decided by Dr. Mike Hardman that the ion exchange column should be changed from a CM-Sephadex to a DEAE resin to see if this change would improve the ability of recombinant ALDH to bind to the affinity column, by the removal of the possible inhibitory factor .

3.9.1 Growth and expression of E. coli SRP84/pGP1-2/pThcAD

The growth of *E. coli* SRP84/pGP1-2/pThcAD culture and expression of recombinant human Class I ALDH by heat induction was prepared as described in section 2.5.9.2.

3.9.2 Ion exchange and affinity columns

The cells were harvested, lysed and loaded onto the equilibrated DEAE ion exchange column as described in sections 2.5.10, 2.6.3.1 and 2.6.3.3 respectively. The protein sample isolated from the DEAE column was loaded onto the *p*-hydroxyacetophenone column and left for 1 hr before running through the wash buffer (section 2.6.3.4). Fractions (4 ml) were collected during the loading and elution of both columns and assayed for ALDH activity as described in section 2.6.2.

The active fractions eluted from the affinity column were pooled, then concentrated and the final activity of purified ALDH was determined as described

in sections 2.6.4 and 2.6.2 respectively. Figures 3.14 through to figure 3.19 give an overview of the column loading and elution profiles, and what fractions contained ALDH activity in this preparation. See table 3.16 for a summary of the purification.

The protein fractions collected during the loading of protein sample onto the DEAE ion exchange column did not contain ALDH activity, indicating that ALDH had bound to the DEAE column. Fractions 4-15 were pooled from the elution of the DEAE column and the total activity determined to be 42.16 μmol/min (table 3.16).

ALDH activity was detected in the fractions collected during the loading of the affinity column (table 3.16). Active fractions 25-53 were pooled and found to contain 32% of the total activity loaded onto the affinity column. Although the activity losses at this step were still high, the amount of ALDH coming straight through the affinity column had reduced by the changing of ion exchange column (ie: change from CM-Sephadex to DEAE).

Fractions 13 to 18 were pooled from the affinity column elution. This pooled sample contained 62% of the total activity loaded onto the affinity column. The combination of activity that was detected in the elution of the affinity column (62%) and that which did not bind (32%) accounted for most of the activity loaded on the column (94%).

Although the total activity of the eluted recombinant human Class I ALDH was only 19.5 μmol/min I attempted to concentrate this protein as it was my most successful protein purification (section 2.6.4). Unfortunately the protein showed no observable ALDH activity which seemed a little strange.

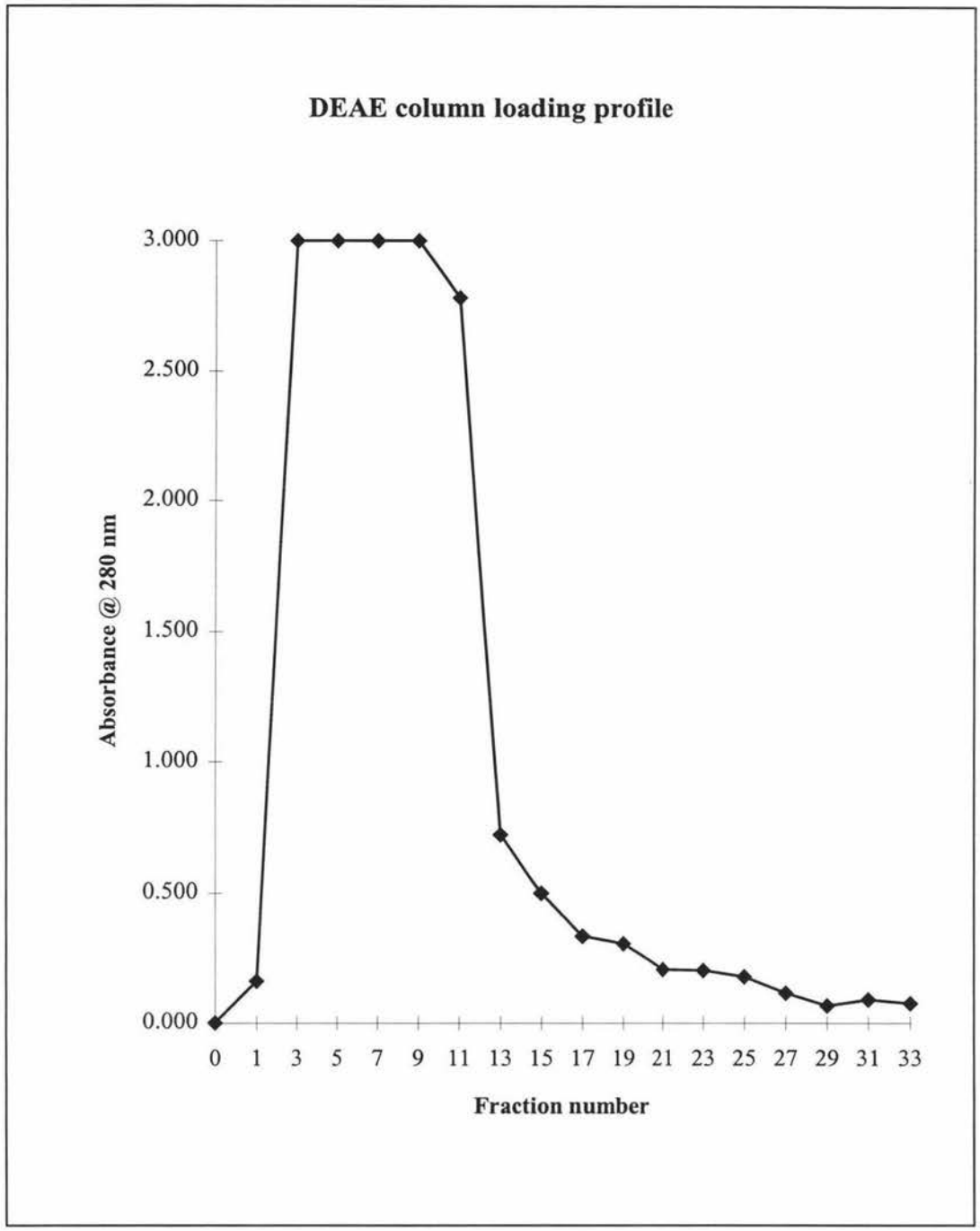

Figure 3.14

Human recombinant Class I ALDH purification. Loading profile from the DEAE ion exchange chromatography step. Fractions (8 ml) were collected and their absorbance read at 280 nm using the "CARY 1" UV-visible spectrophotometer (Varian).

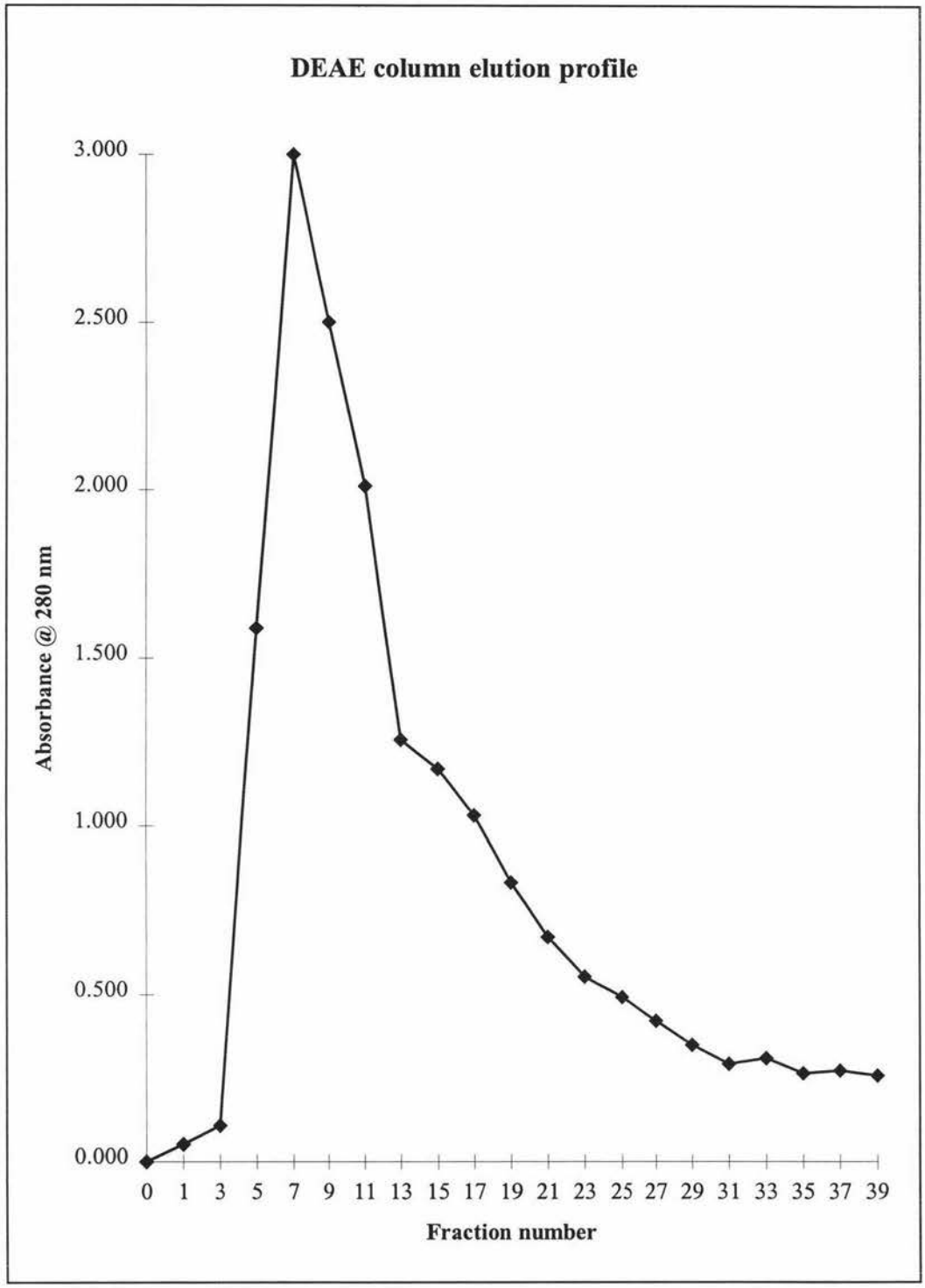

Figure 3.15

Human recombinant Class I ALDH purification. Elution profile from the DEAE ion exchange chromatography step; a 400 ml gradient of 0 mM to 10 mM *p*-hydroxyacetophenone was used to elute bound ALDH from the column. Fractions (8 ml) were collected and their absorbance read at 280 nm using the "CARY 1" UV visible spectrophotometer (Varian).

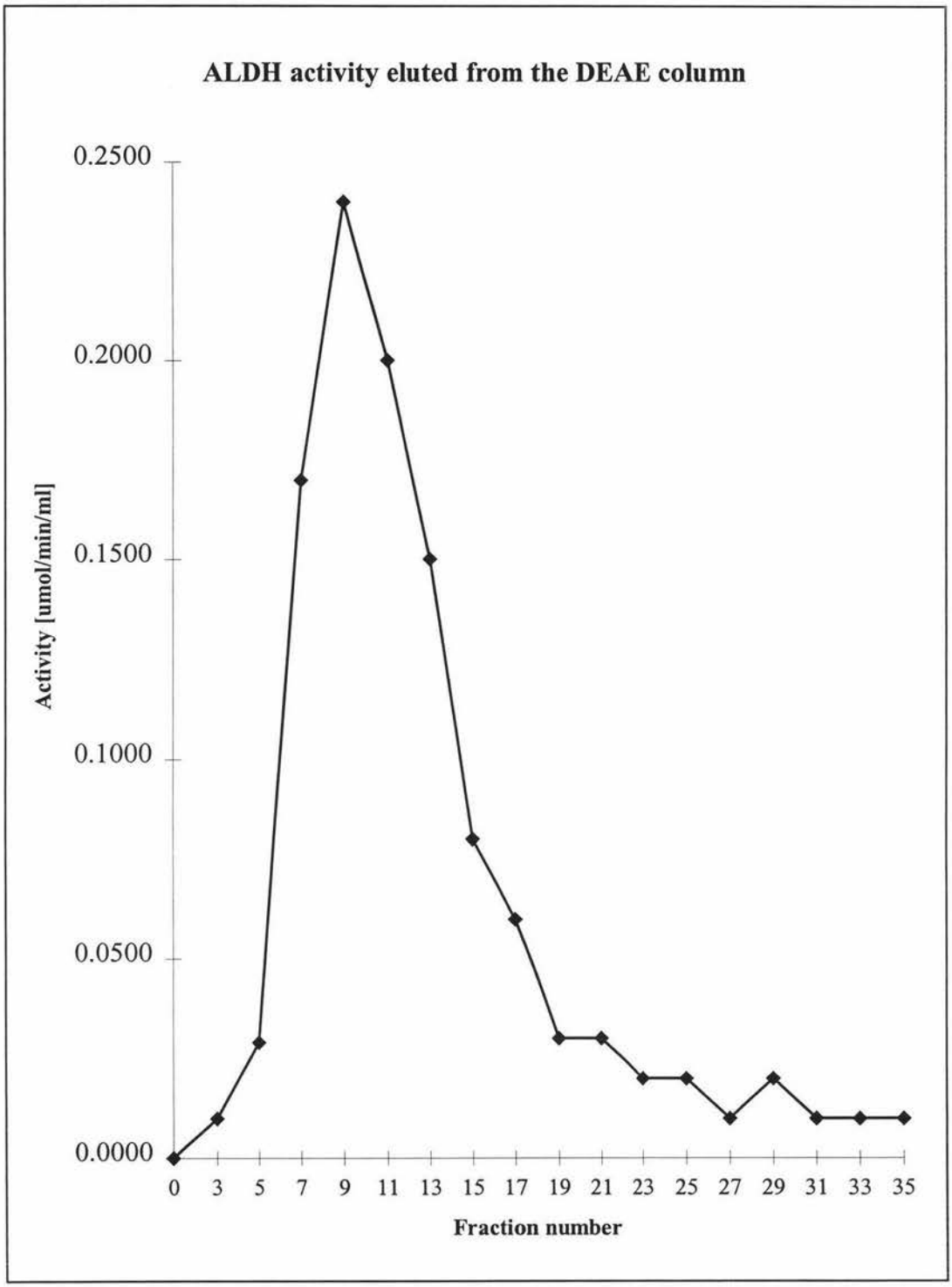

Figure 3.16

Human recombinant Class I ALDH purification. Fractions collected during the elution of the DEAE ion exchange column were assayed for ALDH activity at 340 nm as described in section 2.6.2. Fractions 4-15 were pooled.

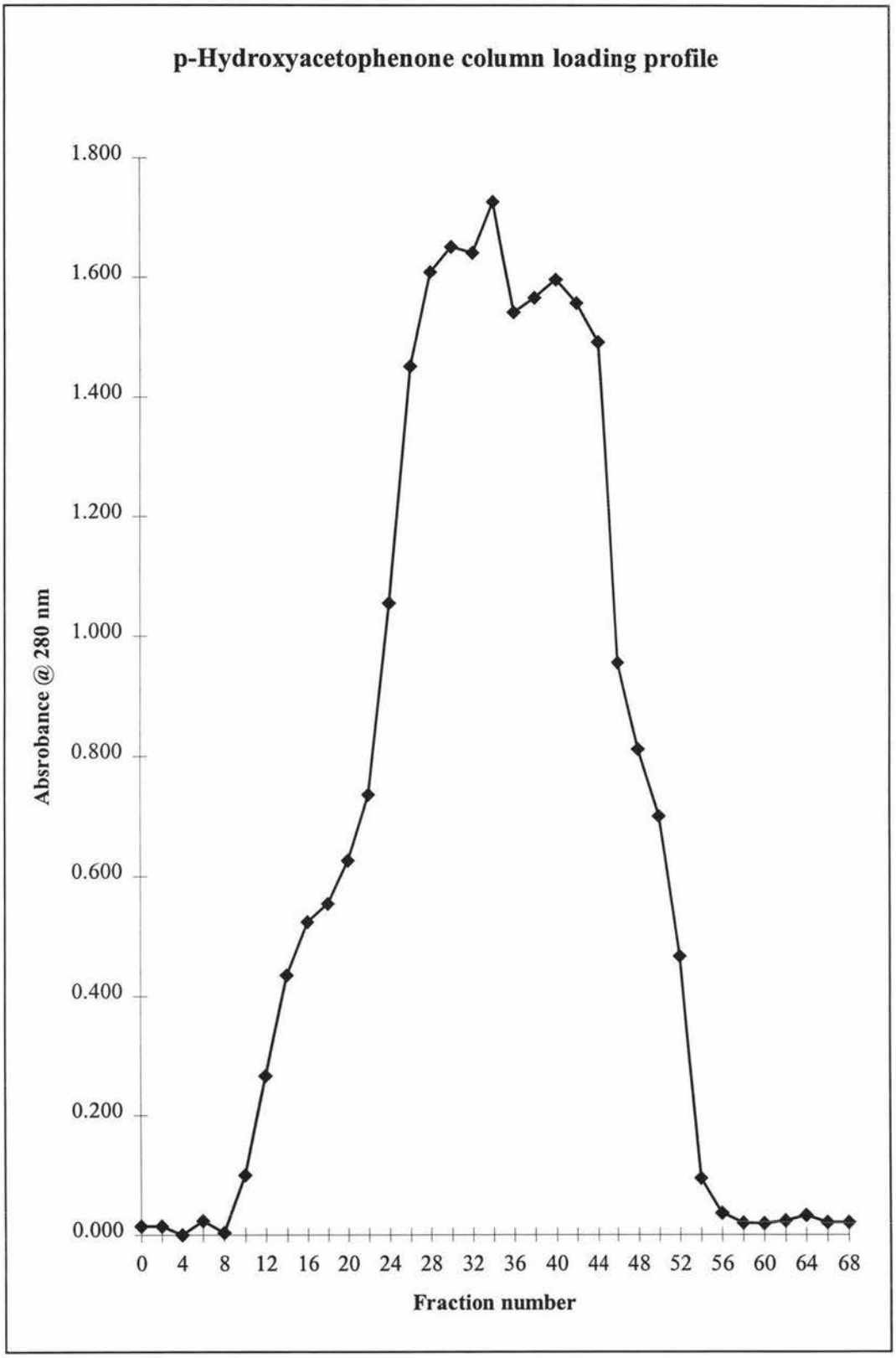

Figure 3.17

Human recombinant Class I ALDH purification. Loading profile from the *p*-hydroxyacetophenone affinity column. Fractions (8 ml) were collected and their absorbance read at 280 nm using the "CARY 1" UV-visible spectrophotometer (Varian).

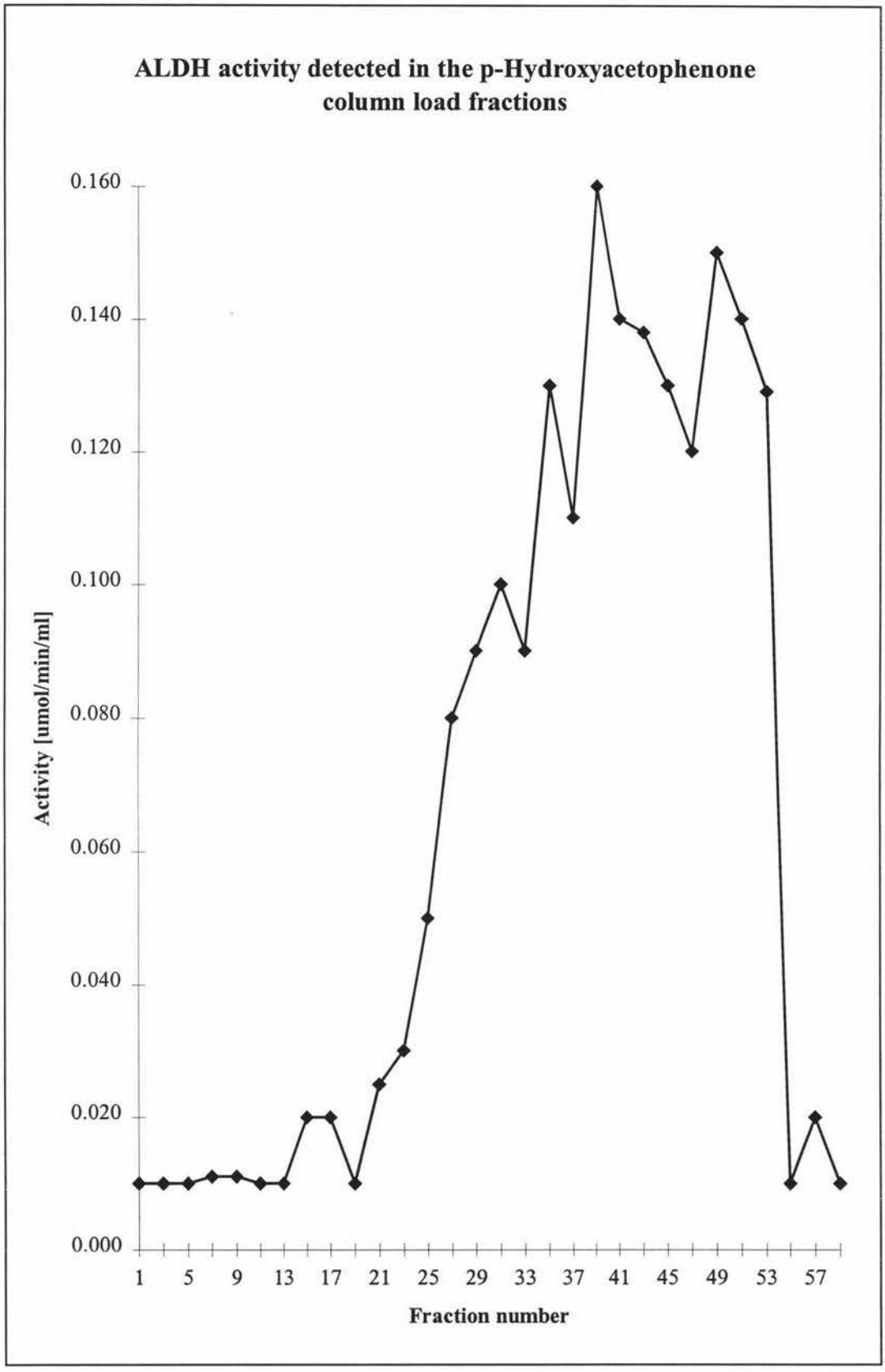

Figure 3.18
Human recombinant Class I ALDH purification. Fractions collected during the loading of the p-hydroxyacetophenone affinity column were assayed for ALDH activity at 340 nm as described in section 2.4.2. Fractions 25-53 were pooled.

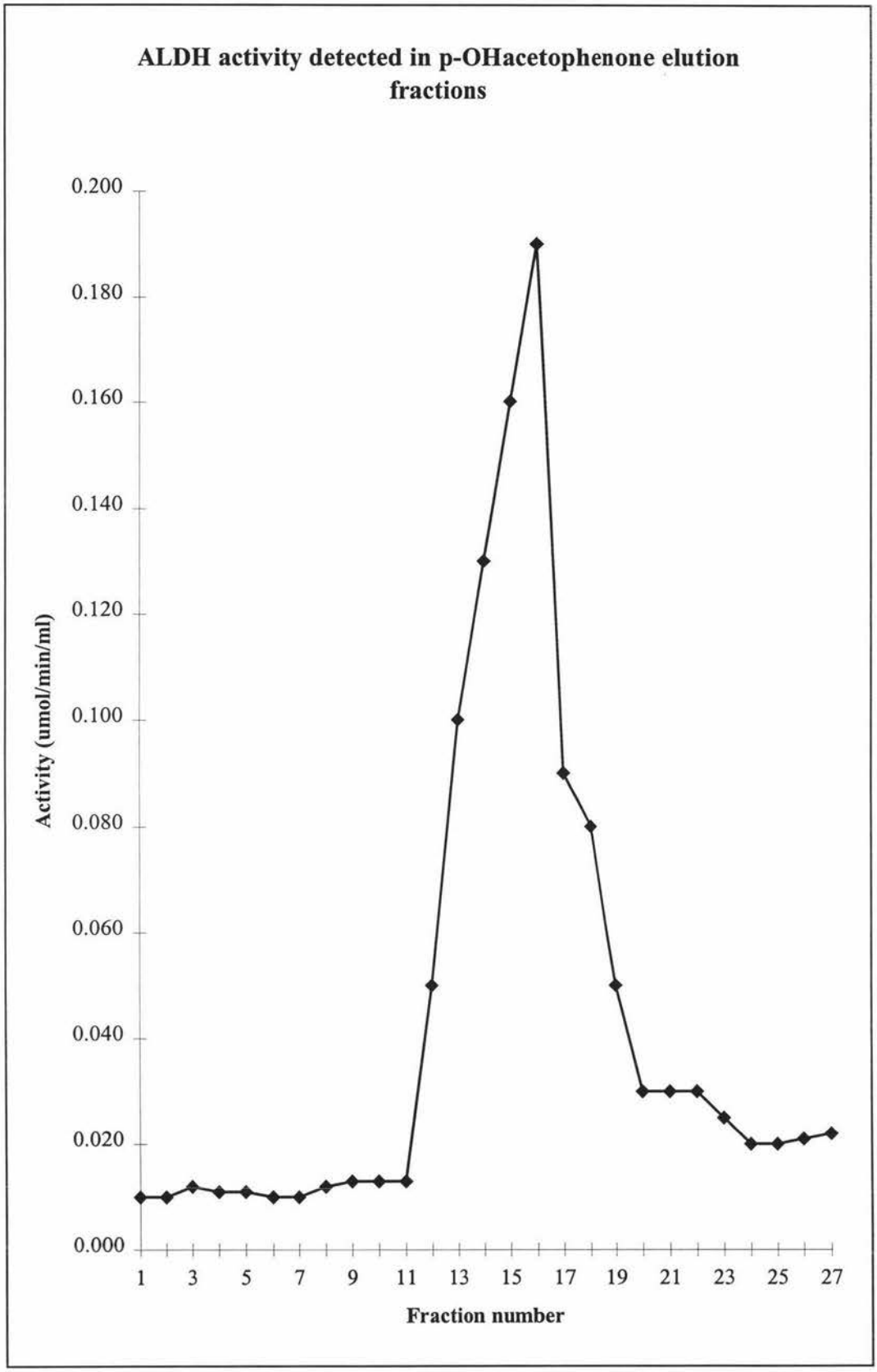

Figure 3.19

Human recombinant Class I ALDH purification. Fractions collected during the elution of the *p*-hydroxyacetophenone affinity column were assayed for ALDH activity at 340 nm as described in section 2.6.2. Fractions 13-18 were pooled.

SDS-PAGE analysis of the expression of the recombinant class 1 ALDH

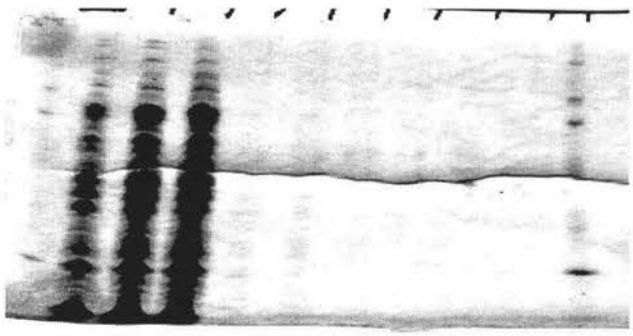

Figure 3.20

Human recombinant class 1 ALDH purification. A 12% polyacrylamide-SDS gel was prepared, run and stained as described in section 2.6.5. (1) 8 µl SDS-6H ladder; (2) 10 µl cell lysate from the French Press; (3) 10 µl cell lysate treated with protamine sulphate; (4) 10 µl dialysed protein sample; (5) 10 µl DEAE pooled fraction sample; (6) 10 µl p-hydroxyacetophenone pooled loading fractions sample; (7) 10 µl p-hydroxyacetophenone pooled elution fractions sample; (8) 10 µl concentrated human recombinant ALDH; (9) 8 µl SDS-6H ladder; (10) 2 µl SDS-6H ladder.

Table 3.16 Summary of human recombinant Class I ALDH purification

Step	Volume (ml)	Activity (µmol/min/ml)	Total activity (µmol/min)
Cell lysate	35.0	1.680	58.80
Protamine sulphate step	33.0	1.800	59.40
Dialysed cell lysate	31.0	1.360	42.16
DEAE column elution	98.0	0.320	31.36
Loading affinity column	86.0	0.117	10.07
Affinity column elution	30.0	0.650	19.50
Concentrated ALDH	1.50	0.000	0.000

3.10 RECOMBINANT HUMAN CLASS 1 ALDH PURIFICATION USING THE GLUTATHIONE S-TRANSFERASE GENE FUSION SYSTEM

3.10.1 Overview

The glutathione S-transferase (GST) gene fusion system was designed for inducible, high-level expression of a desired gene product as a fusion protein with the 26 kDa GST domain from *Schistosoma japonicum*. Because of the problems encountered using the *p*-hydroxyacetophenone affinity column to purify human recombinant class 1 ALDH protein I decided to try the GST gene fusion system as an alternative method.

ALDH cDNA was removed from the pThcAD vector in two fragments and ligated into the pGEX-4T-3 vector to produce the pGEX-4T-3-ALDH fusion construct. See figure 3.21 and 3.22 for the restriction maps of pThcAD and pGEX-4T-3.

Map of the pThcAD vector

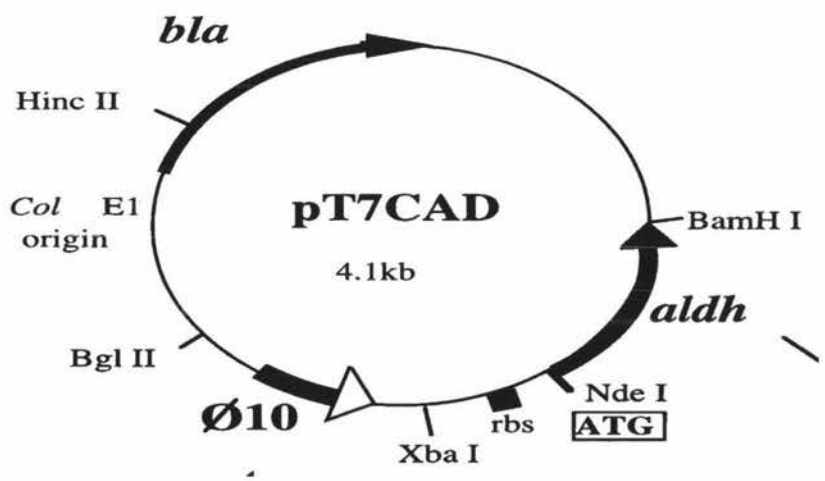

Figure 3.21

Map of the pThcAD vector showing its main features.

GST fusion vector pGEX-4T-3

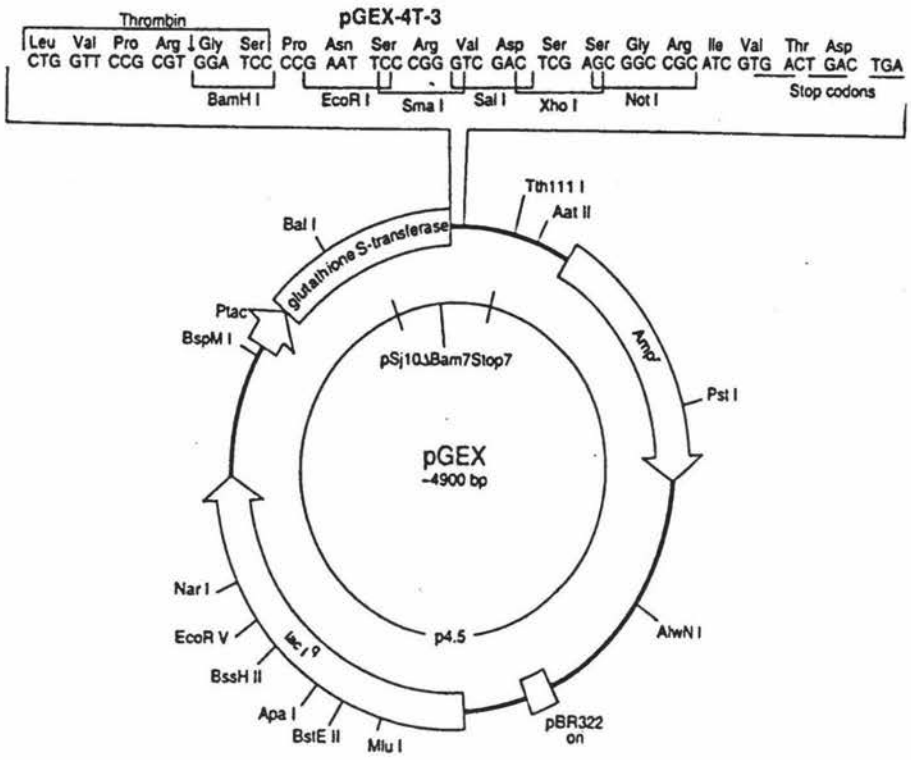

Figure 3.22
Map of the GST fusion vector pGEX-4T-3 showing the reading frames and main features. PGEX-4T-3 contains a thrombin cut site, inducible *tac* promoter and large multiple cloning site.

3.10.2 Large scale preparation of pThcAD and pGEX-4T-3 vectors

Electrocompetent XL1 Blue cells were transformed with 10 ng of pThcAD and 1 ng of pGEX-4T-3 (section 2.5.4) and overnight cultures (5 ml) were grown at 37 °C. The pTcAD and pGEX-4T-3 plasmids were purified from the XL1 Blue cells using the Wizard Midiprep (followed the protocol of manufacturer). The DNA concentration was calculated as described in section 2.4.4 (table 3.17).

Table 3.17 Concentration and purity of the pThcAD and pGEX-4T-3 plasmids

Vector	Concentration of DNA (ng/μl)	Purity of DNA (A_{260}/A_{280} ratio)
pThcAD	207	1.77
pGEX-4T-3	223	1.80

The purified plasmids were digested using restriction enzymes with unique recognition sequences (*EcoR* I for pGEX-4T-3 and *Nde* I for pThcAD). The products from each digests were examined by gel electrophoresis (figure 3.23).

Digestion of the pGEX-4T-3 produced the expected fragments but the digestion of pThcAD did not appear to have occurred efficiently. Zheng *et al.* (1993) cloned the ALDH cDNA into pT7.7 with *Nde* I and *BamH* I. This may explain why the digest with *Nde* I alone was not very successful rather than the plasmid being incorrect.

I decided therefore to digest the pThcAD plasmid with *Sal* I which also has a unique restriction enzyme site in the pThcAD plasmid. The resulting products were examined by gel electrophoresis (figure 3.24) and the expected fragments were observed, confirming the authenticity of the purified pGEX-4T-3 and pThcAD plasmids.

Products resulting from *EcoR* I and *Nde* I digest reactions

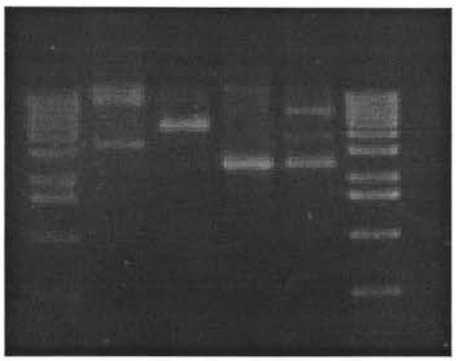

Figure 3.23

Ethidium bromide stained 0.8% agarose gel (5.7 x 8.3 cm), run at 80 V for 2 hr, showing aliquots of the pGEX-4T-3 digested with *EcoR* I and pThcAD vectors digested with *Nde* I. (1) 4 µl BRL 1kbp ladder; (2) 20 µl undigested pGEX-4T-3; (3) 20 µl *EcoR* I digested pGEX-4T-3; (4) 20 µl undigested pThcAD; (5) 20 µl *Nde* I digested pThcAD; (6) 4 µl BRL 1kbp ladder.

Products resulting from the *Sal* I digest of the pThcAD plasmid

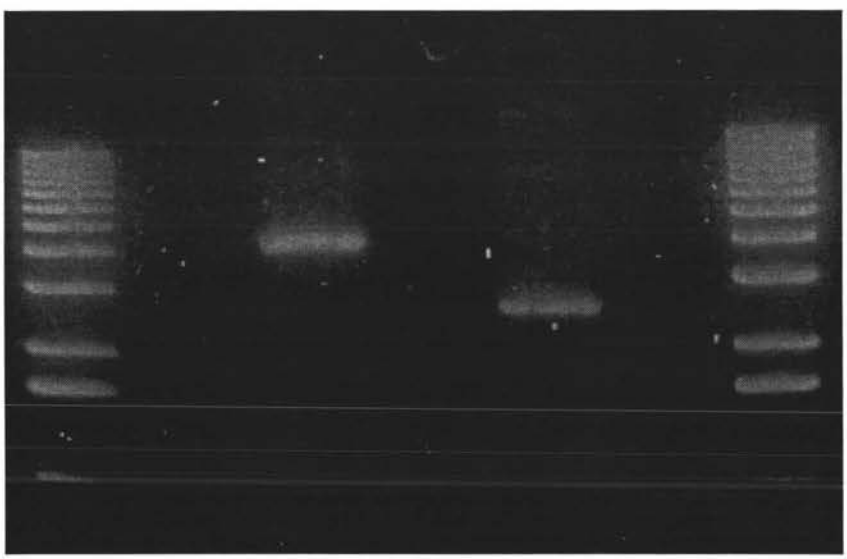

Figure 3.24

Ethidium bromide stained 0.8% agarose gel (5.7 x 8.3 cm), run at 80 V for 2 hr, showing aliquots of the *Sal* I digested pThcAD vectors. (1) 4 µl BRL 1kbp ladder; (2) empty; (3) 20 µl pThcAD digest with *Sal* I; (4) empty; (5) 20 µl pThcAD uncut; (6) empty; (7) 4 µl BRL 1kbp ladder.

3.10.3 Preparation of the pGEX-4T-3 vector for ligation

The pGEX-4T-3 vector was prepared for ligation with the first ALDH fragment by double digesting with the restriction endonucleases *Sma* I and *BamH* I. The resulting products were examined by gel electrophoresis as shown in figure 3.25. The 4.9 kbp fragment resulting from the *BamH* I/*Sma* I digest was gel purified using Bresa Clean™ (Bresatec) as described in section 2.4.3.3. The purified fragment was then examined by gel electrophoresis (figure 3.26)

Products resulting from the *Sma* I and *BamH* I digests of the pGEX-4T-3 plasmid

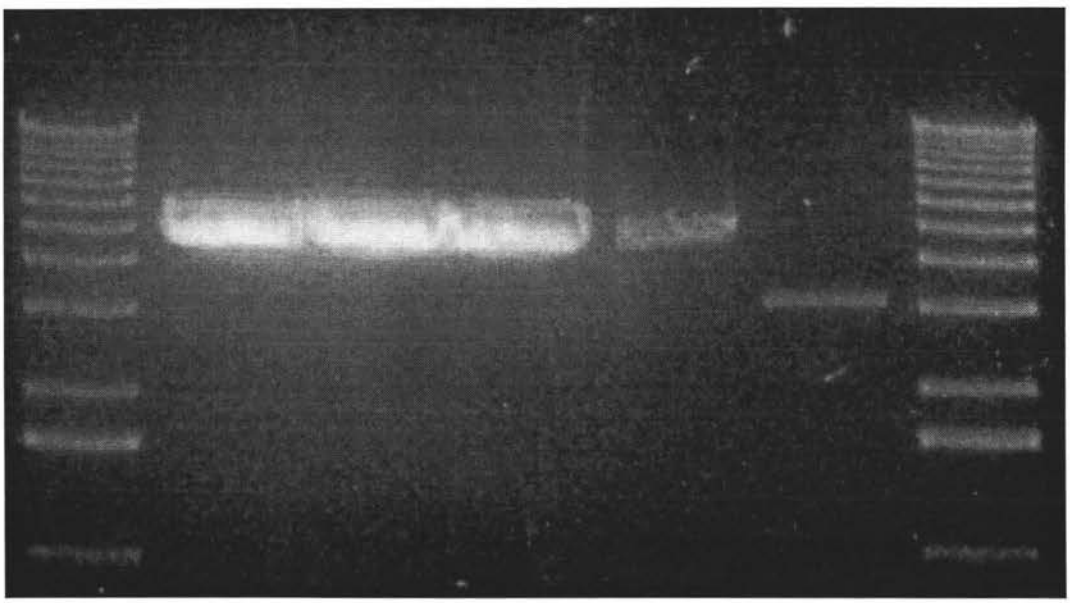

Figure 3.25

Ethidium bromide stained 1% agarose gel (5.7 x 8.3 cm), run at 80 V for 2 hr, showing aliquots of the *Sma* I/*BamH* I digested pGEX-4T-3 plasmid. (1) 4 µl BRL ladder; (2) 21.5 µl *BamH* I/*Sma* I digested pGEX-4T-3; (3) 1 µl *Sma* I digested pGEX-4T-3; (4) 1 µl pGEX-4T-3 plasmid before digest reaction; (5) 4 µl BRL ladder.

Examination of the purified pGEX-4T-3 *BamH* I/*Sma* I fragment

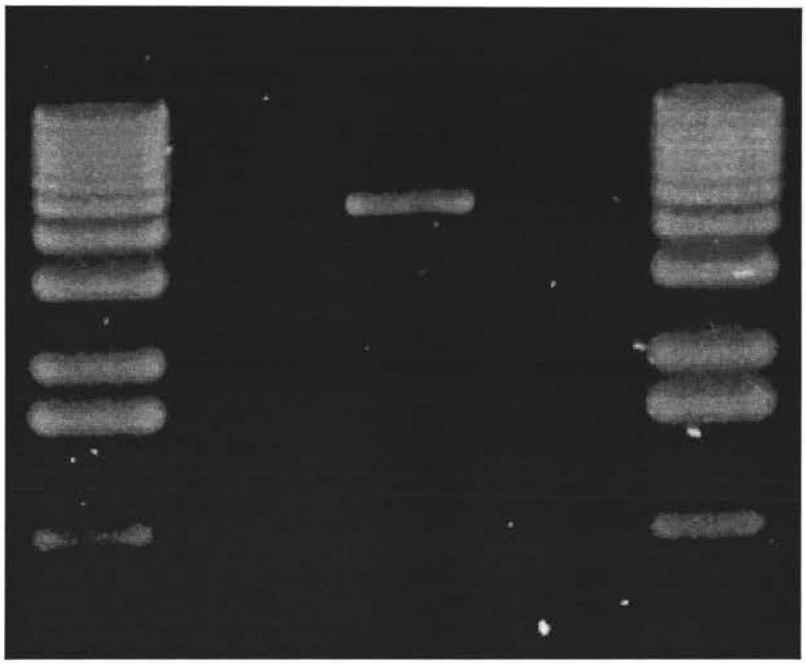

Figure 3.26

Ethidium bromide stained 0.8% agarose gel (5.7 x 8.3 cm), run at 80 V for 1 hr, showing an aliquot of the purified Bam HI/Sma I digested pGEX-4T-3 vector. (1) 4 µl BRL 1 kbp ladder; (2) 4 µl prepared pGEX-4T-3; (3) 4 µl BRL 1 kbp ladder.

3.10.4 Introduction of *BamH* I site by PCR on pThcAD template at the start of ALDH cDNA

A BamH I restriction site was introduced at the start of ALDH cDNA using PCR The *BamH* I mutagenic oligonucleotide (PCR F) and shALDH[20] (Ld-3) sequencing oligonucleotide were used as primers for the PCR reaction resulting in the amplification of a 295 bp product (figure 3.27).

PCR F and shALDH^{20} oligonucleotide sequences

PCR F'	5'-ATG *GG*A	TCC	TCA	GGC	ACG	CCA	GAC	CT...-3'
ALDH	3'-**TAC** AGT	AGG	AGT	CCG	TGC	GGT	CTG	GA...-5'

⇓ ALDH sequence becomes...

Mutated ALDH	5'-**ATG** *GG*A	TCC	TCA	GGC	ACG	CCA	GAC	CT...-3'
	3'-**TAC** *CC*T	AGG	AGT	CCG	TGC	GGT	CTG	GA...-5'

shALDH^{20} (Ld-3) primer 5'-CTC TTT CAA TTA AGT CAG CC-3'

Figure 3.27

The sequence of the PCR mutagenic oligonucleotide PCR F and the change in ALDH DNA sequence that will result. The ATG start codon of the ALDH gene is highlighted in bold letters and the *BamH* I recognition sequence is underlined. The shALDH^{20} (Ld-3) primer annealed to the ALDH sequence 300 bp downstream of the ATG start codon.

The optimum conditions for the amplification were obtained through preliminary PCR experiments in which the effect of variables such as annealing temperature, Mg^{2+} concentration, primer and template concentrations and number of cycles on PCR product yield were investigated. The optimum PCR conditions for the amplification of the fragment of ALDH cDNA were found to be: final concentrations of 2.5 mM $MgCl_2$, 6 ng of pTcAD template DNA, 5 pmol/µl of each oligonucleotide primer, 1x Taq polymerase buffer, 2.5 mM of each dNTP, 3 U Taq polymerase and preheating of the PCR machine heating block to 94 °C followed by program ERIN 14 (table 3.18). Seven PCR reactions were set up (all replicates) to increase the amount of 295 bp product. Each reaction was analysed by gel electrophoresis (figure 3.28).

Table 3.18 PCR program ERIN 14

Stage	Purpose	Conditions
1.1	Denature the template DNA	95°C for 2.5 min
2.1	Denature the template DNA	94°C for 30 s
2.2	Annealing of primers	40°C for 30 s
2.3	DNA extension from primers	70°C for 30 s
	Amplification	Stage 2 repeated for 30 cycles
3.1	Denature the template DNA	94°C for 30 s
3.2	Annealing of primers	40°C for 30 s
3.3	DNA extension from primers	70°C for 30 s

PCR reaction to introduce a *BamH* I site at the beginning of the ALDH cDNA.

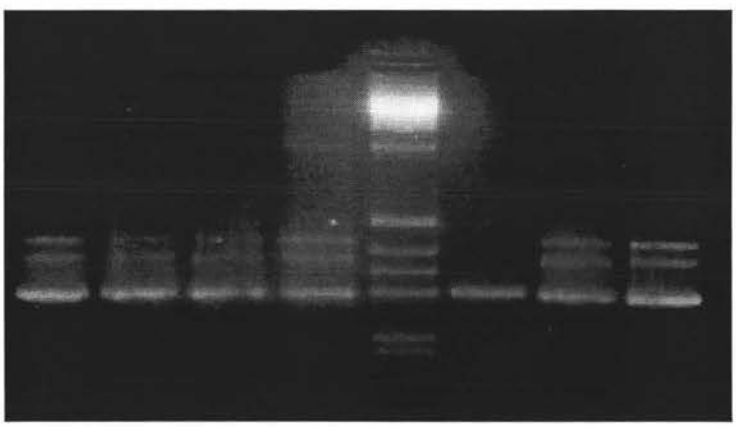

Figure 3.28

Ethidium bromide stained 3% NuSieve agarose gel (5.7 x 8.3 cm), at 64 V for 2 hr showing aliquots of the mutated ALDH cDNA fragment amplified by PCR. (1) 10 µl PCR reaction 1; (2) 10 µl PCR reaction 2; (3) 10 µl PCR reaction 3; (4) 10 µl PCR reaction 4; (5) 10 µl BRL 1 kbp ladder; (6)) 10 µl PCR reaction 5; (7) 10 µl PCR reaction 6; (8) 10 µl PCR reaction 7.

3.10.5 Digestion of PCR product with *Hinc* II and *BamH* I

The resulting 295 bp PCR product (as described in section 3.10.4) was digested with *BamH* I and *Hinc* II, resulting in 180 bp and 115 bp fragments. These products were separated by gel electrophoresis (figure 3.29).

Analysis of the *Hinc* II and *BamH* I digested PCR product

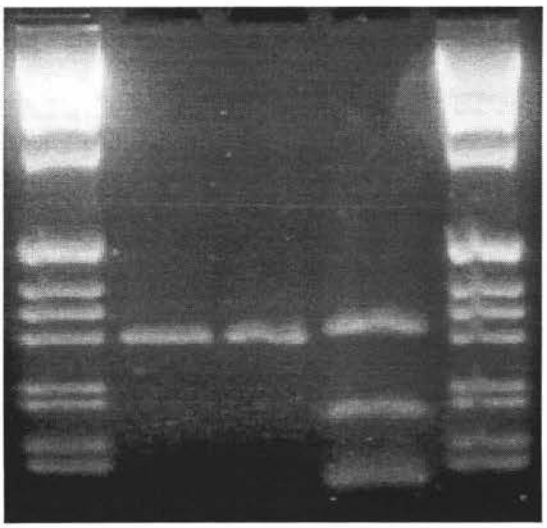

Figure 3.29

Ethidium bromide stained 3% NuSieve agarose gel (5.7 x 8.3 cm), at 64 V for 2 hr showing aliquots of the *BamH* I and *Hinc* II digested 295 bp PCR product. (1) 10 µl BRL 1kbp ladder; (2) 6 µl undigested 295 bp PCR product; (3) 6 µl 295 bp PCR product digested with *BamH* I; (4) 20 µl 295 bp PCR product digested with *BamH* I and *Hinc* II; (5) 10 µl BRL 1kbp ladder.

3.10.5.1 *Purification of the 180 bp fragment using Bresa Clean™*

The *Hinc* II reaction appeared to digest efficiently. I was unable to determine if *BamH* I had cut the 295 bp fragment, as the digested fragment was only a few nucleotides shorter that the undigested fragment. The 180 bp fragment was excised and gel purified using Bresa Clean™ (Bresatec) as described in section 2.4.3.3.

3.10.6 Ligation of 180 bp ALDH fragment into pGEX-4T-3

The 180 bp ALDH fragment (section 3.10.5) was ligated into the pGEX-4T-3 vector prepared as described in section 2.4.1.2. The ligation reaction mixture was digested with *EcoR* I, to linearise any pGEX-4T-3 that may have religated without the 180 bp insert, then transformed into XL1 blue cells (section 2.5.3), plated out and incubated overnight at 37 °C.

Cultures (5 ml) were prepared from isolated colonies grown on the pGEX-4T-3-180bp ALDH ligation plate; that is colonies that were successfully transformed with the pGEX-4T-3-180bp construct. The pGEX-4T-3-180bp ligation plasmid was isolated from each overnight culture using the rapid boil technique described in section 2.5.5.

3.10.6.1 *Digestion with Bgl II*

Each plasmid was checked to verify whether it contained the 180 bp ALDH insert by digesting with the restriction enzyme *Bgl* II. The 180 bp fragment contains a *Bgl* II restriction site whereas pGEX-4T-3 does not, therefore any plasmid that is successfully digested with *Bgl* II must contain the 180 bp ALDH fragment. Gel electrophoresis of the products of each *Bgl* II digest reactionshowed that all of the isolated plasmid samples contained the 180 bp insert except for HT2, L6, L3 and L1.

The first 100 bp of the resulting ligation products HT4, HT1, L8 and L5 were sequenced using the 5' pGEX sequencing primer to establish that no errors had been introduced between the fusion site and the unique *Bgl* II site, as described in section 2.4.8. Each ligation product was shown to be have the same DNA sequence as the wild type ALDH (Lindahl & Hempel, 1991).

3.10.7 Preparation of the remaining ALDH gene fragment from pThcAD

A 10 µg aliquot of the pThcAD plasmid (section 3.10.2) was digested with *Bgl* II and *Sal* I as described in section 2.4.1.1. After the digestion all of the reaction volume was analysed by gel electrophoresis as shown in figure 3.31. The 1.5 kbp fragment was excised from the gel and purified using Bresa Clean™ (section 2.4.3.3). An aliquot of the purified fragment was examined by gel electrophoresis (figure 3.32).

Products from the *Bgl* II/*Sal* I double digest of the pTcAD plasmid

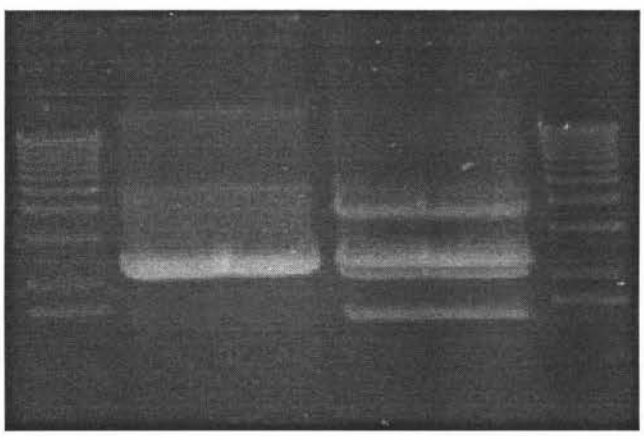

Figure 3.31

Ethidium bromide stained 1% agarose gel (5.7 x 8.3 cm) at 76 V for 1.5 hr showing the *Bgl* II/*Sal* I double digest of the pThcAD plasmid. (1) 4 µl BRL 1kbp ladder; (2) 60 µl undigested pThcAD; (3) 60 µl *Bgl* II/*Sal* I digested pThcAD; (4) 4 µl BRL 1 kbp ladder.

3.10.8 Double digest of the pGEX-4T-3-180bp ALDH construct

The pGEX-4T-3-180bp ALDH construct was digested with *Bgl* II and *Sal* I. The *Bgl* II restriction site, situated 80 bp from the 5' end of the ALDH fragment, is unique to the 180 bp ALDH fragment whereas the *Sal* I is one of the sites in the pGEX-4T-3 multiple cloning site. This double digest produced two products, a 5 kbp pGEX-4T-3-80 bp ALDH fragment and a 100 bp ALDH fragment. These products were separated on a gel as shown in figure 3.33.

Purification of the 1.5 kb pTcAD fragment resulting from a *Bgl* II/*Sal* I double digest

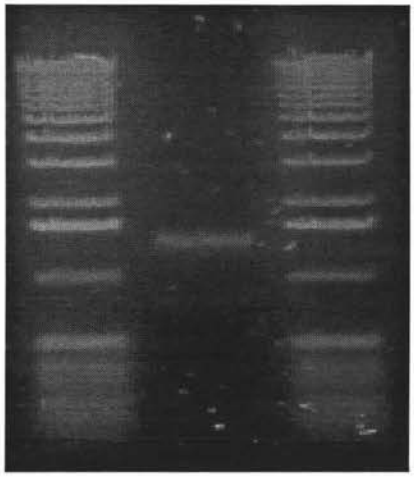

Figure 3.32

Ethidium bromide stained 1% agarose gel (5.7 x 8.3 cm) at 80 V for 1.5 hr showing an aliquot of the gel purified 1.5 kbp ALDH fragment. (1) 4 µl BRL 1 kbp ladder; (2) 2 µl 1.5 kbp ALDH fragment; (3) 4 µl BRL 1 kbp ladder.

The pGEX-4T-3-80 bp ALDH fragment was gel purified with Bresa Clean™ as described in section 2.4.3.3. An aliquot of the purified fragment was examined by gel electrophoresis alongside an aliquot of 1.5 kbp ALDH fragment (described in section 3.10.7). From this gel the concentrations of the 1.5 kbp ALDH and pGEX-4T-3-80 bp ALDH fragment were estimated to be 10 ng/µl and 5 ng/µl respectively (figure 3.34).

Sal I/*Bgl* II double digest of the pGEX-4T-3-180bp ALDH construct

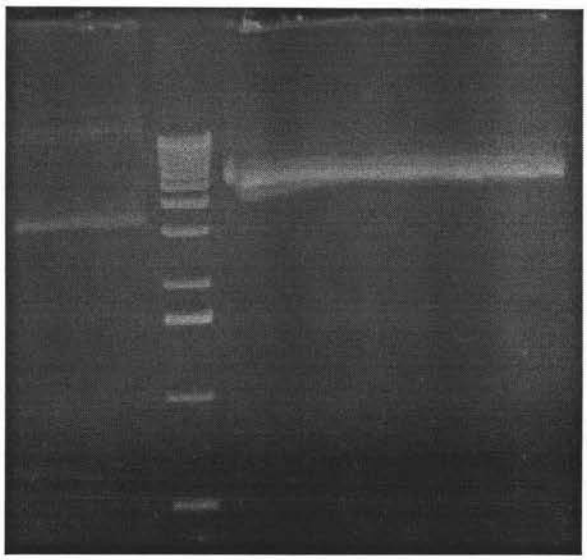

Figure 3.33

Ethidium bromide stained 1% agarose gel (5.7 x 8.3 cm) at 70 V for 1.5 hours showing the *Bgl* II/*Sal* I double digest of the pGEX-4T-3-180 bp ALDH construct. (1) 60 µl of undigested pGEX-4T-3-180 bp ALDH; (2) 5 µl BRL 1 kbp ladder; (3) 60 µl *Bgl* II/*Sal* I digested pGEX-4T-3-180 bp ALDH.

Purification of the 1.5 kb pTcAD fragment and 1.5 kb ALDH fragment resulting from separate from *Sal* I/*Bgl* II double digests

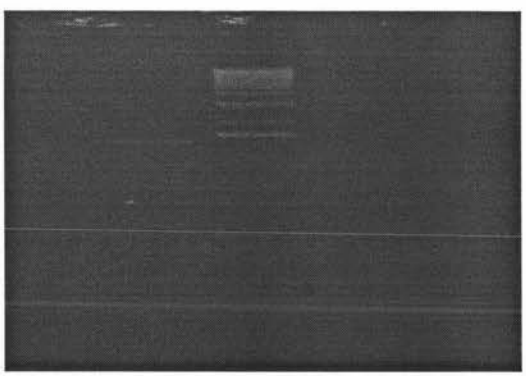

Figure 3.34

Ethidium bromide stained 1% agarose gel (5.7 x 8.3 cm) at 80 V for 1.5 hr showing the purified pGEX-4T-3-80 bp ALDH and 1.5 kbp ALDH fragments. (1) 2 µl 1.5 kbp ALDH; (2) 5 µl BRL 1 kbp ladder; (3) 2 µl pGEX-4T-3-80 bp fragment purified using Bresa Clean™ .

3.10.9 Ligation of 1.5 kbp ALDH and the pGEX-4T-3-80 bp fragments

The 1.5 kbp ALDH fragment (section 3.10.7) and the pGEX-4T-3-80 bp ALDH fragment (section 3.10.8) were ligated as described in section 2.4.1.2. The ligation reaction mixture was transformed into XL1 blue cells as described in section 2.5.3 and the cells were plated out and incubated overnight at 37 °C.

Cultures (5 ml) were prepared from isolated colonies grown on the pGEX-4T-3-ALDH ligation plate. The ligation plasmid was isolated from each overnight culture using the rapid boil technique described in section 2.5.5. An aliquot of each plasmid was examined by gel electrophoresis (figure 3.35).

Analysis of the pGEX-4T-3-ALDH plasmids isolated from XL1 Blue cells

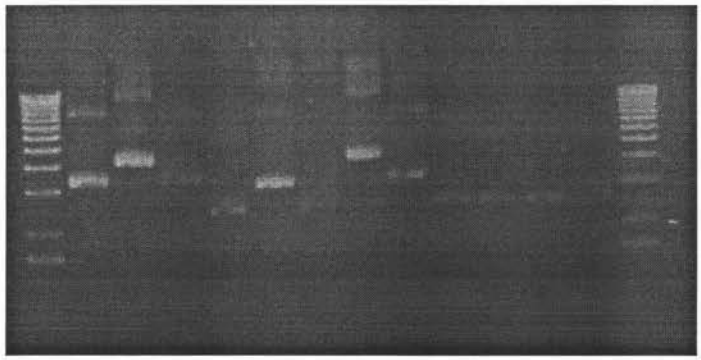

Figure 3.35

Ethidium bromide stained 0.8% agarose gel (11 x 14 cm) at 78 V for 2 hr showing aliquots of the isolated pGEX-4T-3- ALDH ligation constructs. (1) 4 µl BRL 1 kbp ladder; (2) 2 µl MP1 plasmid; (3) 2 µl MP2 plasmid; (4) 2 µl MP3 plasmid; (5) 2 µl MP4 plasmid; (6) 2 µl MP5 plasmid; (7) 2 µl MP6 plasmid; (8) 2 µl MP7 plasmid; (9) 2 µl MP8 plasmid; (10) 2 µl MP9 plasmid; (11) 2 µl MP10 plasmid; (12) 2 µl MP11 plasmid; (13) 2 µl MP12 plasmid; (14) 4 µl BRL 1 kbp ladder.

Each plasmid was checked by digesting with *Nco* I to verify whether it contained the 1.5 kbp ALDH insert. The 1.5 kbp fragment contains a *Nco* I restriction site whereas pGEX-4T-3-80 bp ALDH does not, and therefore any plasmid that is cut by *Nco* I must contain the 1.5 kbp ALDH fragment.

The product of each digestion was examined by gel electrophoresis as illustrated in figure 3.36. From this analysis it was decided that the MP7 plasmid was the a good plasmid to select for use in next stage of the preparation; *Nco* I digested this plasmid efficiently (therefore very likely to contain the 1.5 kbp ALDH fragment).

Analysis of the products from the *Nco* I digest of the pGEX-4T-3-ALDH construct

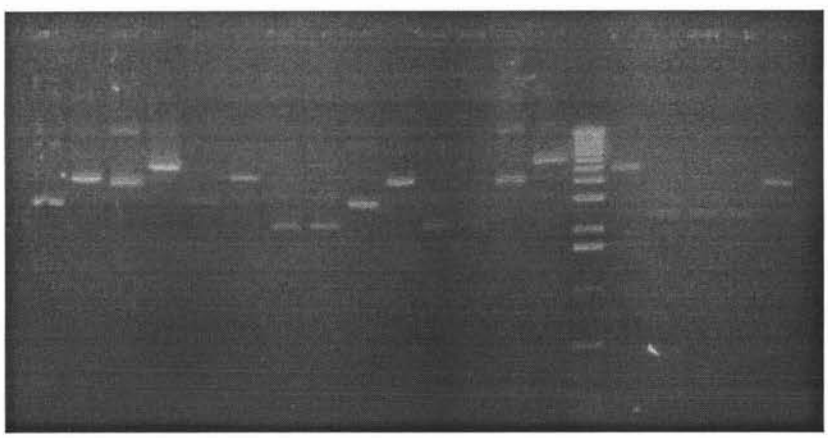

Figure 3.36

Ethidium bromide stained 0.8% agarose gel (11 x 14 cm) at 78 V for 2 hr showing *Nco* I digest of the pGEX-4T-3- ALDH ligation constructs. (1) 20 µl undigested MP1 plasmid; (2) 20 µl *Nco* I digested MP1 plasmid; (3) 20 µl undigested MP2 plasmid; (4) 20 µl *Nco* I digested MP2 plasmid; (5) 20 µl undigested MP3 plasmid; (6) 20 µl *Nco* I digested MP3 plasmid; (7) 20 µl undigested MP4 plasmid; (8) 20 µl *Nco* I digested MP4 plasmid; (9) 20 µl undigested MP5 plasmid; (10) 20 µl *Nco* I digested MP5 plasmid; (11) 20 µl undigested MP6 plasmid; (12) 20 µl *Nco* I digested MP6 plasmid; (13) 20 µl undigested MP7 plasmid; (14) 20 µl *Nco* I digested MP7 plasmid; (15) 5 µl BRL 1 kbp ladder; (16) 20 µl *Nco* I digested MP8 plasmid; (17) 20 µl *Nco* I digested MP9 plasmid; (18) 20 µl *Nco* I digested MP10 plasmid; (19) 20 µl *Nco* I digested MP11 plasmid; (20) 20 µl MP12 plasmid.

The MP7 plasmid was subjected to further restriction enzyme digests to verify that it did in fact contained the 1.5 kbp ALDH fragment. The restriction enzymes *Bgl* II and *Sal* I were chosen as these are unique sites in the expected pGEX-4T-3-ALDH construct and therefore if the MP7 plasmid is digested with these enzymes it must contain the desired 1.5 kbp ALDH fragment. These digests were analysed by agarose gel electrophoresis (figure 3.37) and from this gel it was observed that the MP 7 plasmid contained the 1.5 kbp ALDH insert.

Analysis of the products from the *Bgl* II/*Sal* I digest of the pGEX-4T-3-ALDH plasmid

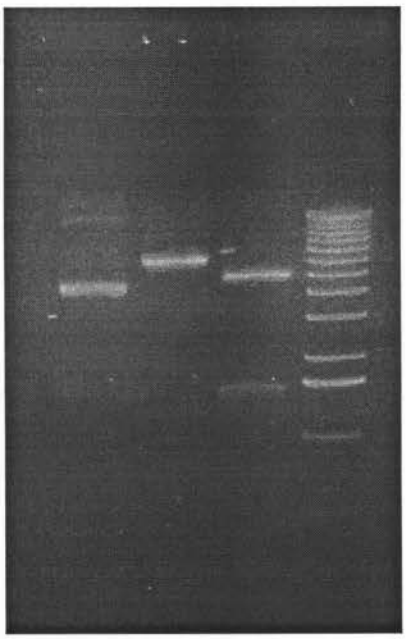

Figure 3.37

Ethidium bromide stained 0.8% agarose gel (5.7 x 8.3 cm) at 80 V for 2 hr showing the *Bgl* II and *Bgl* II/*Sal* I double digest of the MP7 plasmid. (1) 20 µl undigested MP7 plasmid; (2) 20 µl *Bgl* II digested MP7 plasmid; (3) 20 µl *Bgl* III/*Sal* I digested MP7 plasmid; (4) 5 µl BRL 1 kbp ladder.

3.10.10 Expression of the pGEX-4T-3 human ALDH fusion protein

The ligation of the remaining 1.5 kbp ALDH fragment into the expression vector pGEX-4T-3-80 bp ALDH gave the desired expression plasmid construct as suggested by the restriction digests carried out in section 3.10.9. This construct, named pGEX-4T-3-ALDH was transformed into the *E. coli* expression strain BL21 (section 2.5.3), and incubated overnight at 37°C on plates containing amp.

Cultures (3 ml) were prepared from isolated colonies grown on the BL21/pGEX-4T-3-ALDH transformation plate and grown to a turbidity of 2.0 at A_{600}. Two 1 ml aliquots were taken from each culture and 2.5 µl 0.4M IPTG was added to one of these aliquots to induce protein expression from the pGEX-4T-3-ALDH construct. The cultures were incubated at 37°C for 2.5 hr, pelleted by centrifugation and mixed with 100 µl of SDS sample buffer, see sections 2.3.1, 2.6.5 and figure 3.38.

The induction of *E. coli* BL21/pGEX-4T-3-ALDH resulted in the expression of the recombinant protein equal in molecular weight to the human recombinant class 1 ALDH. It was assumed that the expressed protein was in fact ALDH although further analysis of the gel using a western blot would confirm this assumption.

SDS-PAGE of culture samples taken during the IPTG expression of the GST-ALDH fusion protein

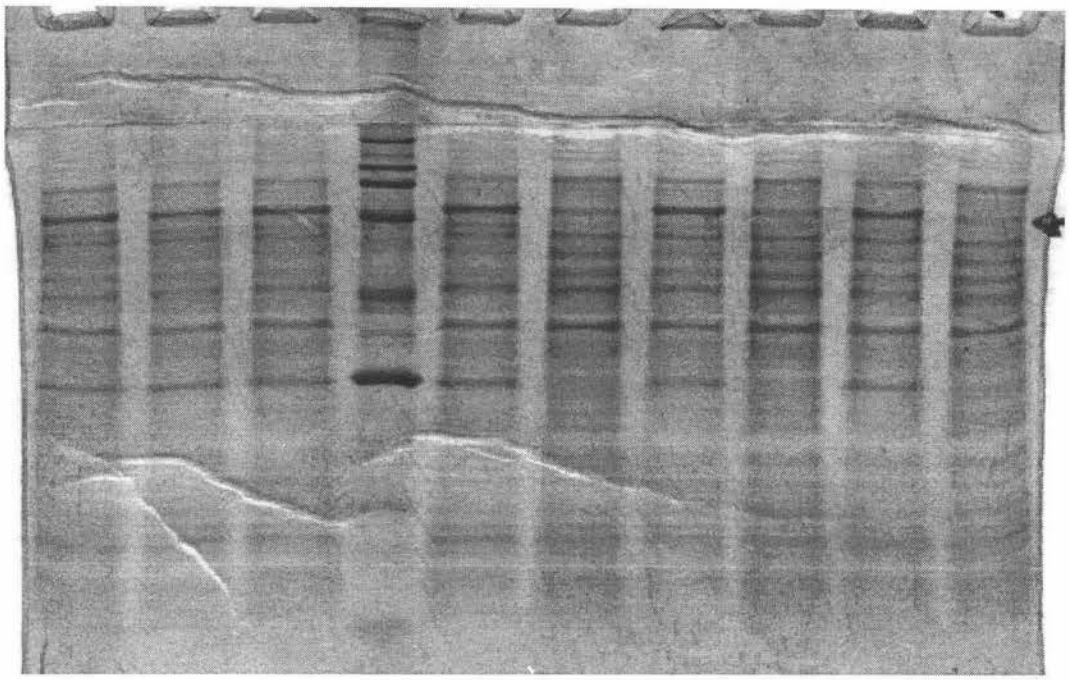

Figure 3.38

SDS-PAGE analysis of the BL21/pGEX-4T-3-ALDH growth and induction of ALDH expression using IPTG. A 12% polyacrylamide-SDS gel was prepared, run and stained as described in section 2.4.4. (1) 5 µl IPTG induced sample 6; (2) 5 µl IPTG induced sample 5; (3) 5 µl IPTG induced sample 4; (4) 8 µl Sigma SDS-6H marker; (5) 5 µl IPTG induced sample 3; (6) 5 µl uninduced sample 3; (7) 5 µl IPTG induced sample 2; (8) 5 µl uninduced sample 2; (9) 5 µl IPTG induced sample 1; (10) 5 µl uninduced sample 1.

Chapter Four

Summary

4.1 Summary of results

Through out the period of my thesis the purification of human recombinant class 1 ALDH proved to be some what of a head-ache. The recombinant ALDH was not purified successfully using the *p*-hydroxyacetophenone affinity column, unlike the native ALDH class 1 sheep isozyme. After one year of research into the purification of human recombinant class 1 ALDH with the purification protocol (utilising the *p*-hydroxyacetophenone affinity column), it was decided that perhaps the GST-fusion protein system may be more successful method for the purification. The GST-4T3-ALDH fusion protein construct was prepared using molecular biological techniques.

Despite several months of attempting to make a mutation at the Lys272 amino acid residue this was abandoned. A small amount of research was undertaken to further examine the C302A mutation that was prepared by Dr. Kerrie Jones although this was not conclusive.

4.2 Future directions

The prepared pGEX-4T-3-ALDH construct may prove to be a more successful method of purifying recombinant ALDH isozymes. The construct that was prepared in this thesis may be used in future research to express and purify recombinant wild type ALDH, however the sequence should be determined before any further research with this construct. If this expression system proves to be a better method for the purification of active recombinant ALDH this may be extended as a method of the purification for mutant proteins.

Chapter Five

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Appendix

Multiple Sequence Alignment of 28 Aldehyde Dehydrogenase Sequences

The alignment was created using the GCG programme PILEUP (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisc.) with default parameters. Amino acid sequences were obtained from the SwissProt, GenBank and PIR databases. Each row of amino acid sequence is preceded by a database accession number that identifies the sequence (see table below). Residues which are conserved in all 28 sequences are indicated in the consensus line at the bottom of the alignment (the residue in the consensus line that corresponds to Lys272 in human liver class 1 ALDH is underlined and in boldtype).

The identification of the proteins used in the multiple sequence alignment, in order of appearance in the alignment, is given in the following table.

Identification	Database Accession No.	No. of Amino Acids
Rat liver microsomal	a41028 (PIR)	484
Mouse liver microsomal (dioxin inducible)	u14390 (GenBank)	484
Rat class 3 (tumour-associated)	p11883 (SwissProt)	452
Mouse class 3 (dioxin inducible)	p47739 (SwissProt)	453
Human class 3	p30838 (SwissProt)	453
Human stomach ALDH7	p43353 (SwissProt)	468
Horse class 2	p12762 (SwissProt)	500
Bovine liver class 2	p20000 (SwissProt)	520
Rat liver class 2	p11884 (SwissProt)	519
Mouse class 2	p47738 (SwissProt)	519
Human class 2	p05091 (SwissProt)	517
Human stomach	m63967 (GenBank)	453
Rat retinal-specific, type 2	u60063 (GenBank)	499
Mouse retinal-specific, type 2	x99273 (GenBank)	499
Bovine class 1 from amacrine cells (eye)	136128 (GenBank)	501
Sheep liver class 1	p51977 (SwissProt)	500
Human liver class 1	p00352 (SwissProt)	500
Horse class 1	p15437 (SwissProt)	500
Mouse class 1	p24549 (SwissProt)	500
Rat kidney retinal-specific class 1, type 1	p51647 (SwissProt)	501
Rat class 1	p13601 (SwissProt)	500
Chicken class 1	p27463 (SwissProt)	509
Short-eared elephant shrew crystallin (eye)	u03906 (GenBank)	501
Human saliva precursor	a55684 (PIR)	512
Yeast mitochondrial 3 precursor	p40047 (SwissProt)	519
Yeast class 1 (*S. cerevisiae*)	u56604 (GenBank)	501
E. coli class 1	p23883 (SwissProt)	494
Human γ-aminobutyraldehyde oxidising	p49189 (SwissProt)	493

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          1                                                                60
a41028    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~
u14390    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~
p11883    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~
p47739    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~
p30838    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~
p43353    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~
p12762    ~~~~~~~~~~~~~~~~~~~~ ~~~~AAAATQAVPAPNQQPE VFYNQIFINNEWHDAVSKKT
p20000    ~~~~MLRAVALAAARLGPRQ GRRLLSAATQAVPTPNQQPE VLYNQIFINNEWHDAVSKKT
p11884    ~~~~MLRAALSTARRGPRLS .RLLSAAATSAVPAPNQQPE VFCNQIFINNEWHDAVSKKT
p47738    ~~~~MLRAALTTVRRGPRLS .RLLSAAATSAVPAPNHQPE VFCNQIFINNEWHDAVSRKT
p05091    ~~~~MLRAA...ARFGPRLG RRLLSAAATQAVPAPNQQPE VFCNQIFINNEWHDAVSRKT
m63967    ~~~~~~~MLRFLAPRLLSLQ GRTALYSSAAALPSPILNPD IPYNQLFINNEWQDAVSKKT
u60063    ~~~~~~~~~~~~~~~~~~~~ ~~~~~MASLQLLPSPTPNLE IKYTKIFINNEWQNSESGRV
x99273    ~~~~~~~~~~~~~~~~~~~~ ~~~~~MASLQLLPSPTPNLE IKYTKIFINNEWQNSESGRV
l36128    ~~~~~~~~~~~~~~~~~~~~ ~~~MSSSAMPDVPAPLTNLQ FKYTKIFINNEWHSSVSGKK
p51977    ~~~~~~~~~~~~~~~~~~~~ ~~~~SSSAMPDVPAPLTNLQ FKYTKIFINNEWHSSVSGKK
p00352    ~~~~~~~~~~~~~~~~~~~~ ~~~~SSSGTPDLPVLLTDLK IQYTKIFINNEWHDSVSGKK
p15437    ~~~~~~~~~~~~~~~~~~~~ ~~~~SSSGTPDLPVLLTDLK FQYTKIFINNEWHDSVSGKK
p24549    ~~~~~~~~~~~~~~~~~~~~ ~~~~SSPAQPAVPAPLADLK IQHTKIFINNEWHNSVSGKK
p51647    ~~~~~~~~~~~~~~~~~~~~ ~~~~SSPAQPAVPAPLANLK IQHTKIFINNEWHDSVSGKK
p13601    ~~~~~~~~~~~~~~~~~~~~ ~~~~SSPAQPAVPAPLANLK IQHTKIFINNEWHNSLNGKK
p27463    ~~~~~~~~~~~~~~~MKKQG SPSNPAPVLPALPEPLKDLK IKYTKIFINNEWHDSVSGKK
u03906    ~~~~~~~~~~~~~~~~~~~~ ~~~MSSSGMPDLPAPLTNIK IQHTKLFINNEWHDSVSGKT
a55684    ~~~~~~~~~~~~MATANGAV ENGQPDGKPPALPRPIRNLE VKFTKIFINNEWHESKSGKK
p40047    MLSRTRAAAPNSRIFTRSLL RLYSQAPLRVPITLPNGFTY EQPTGLFTNGEFVASKQKKT
u56604    ~~~~~~~~~~~~~~~~~~MT KLHFDTAEPVKITLPNGLTY EQPTGLFINNKFMKAQDGKT
p23883    ~~~~~~~~~~~~~~~~~~~~ ~~~~MNFHHLAYWQDKALSL AIENRLFINGEYTAAAENET
p49189    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~MSTGTFV VSQPLNYRGGAAGAGGRSGT
Consen    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~

          61                                                               120
a41028    ~~~~~~~~~~~~~~~~~~~~ ~~MERQVQRLRQTFRSGRSR PLRFRLQQLEALRRMVQERE
u14390    ~~~~~~~~~~~~~~~~~~~~ ~~MERQVLRLRQAFRSGRSR LLRFRLQQLEALRRMVQERE
p11883    ~~~~~~~~~~~~~~~~~~~~ SSISDTVKRAREAFNSGKTR SLQFRIQQLEALQRMINENL
p47739    ~~~~~~~~~~~~~~~~~~~M SNISSIVNRARDAFNSGKTR PLQFRVEQLEALQRMINENL
p30838    ~~~~~~~~~~~~~~~~~~~M SKISEAVKRARAAFSSGRTR PLQFRFQQLEALQRLIQEQE
p43353    ~~~~~~~~~~~~~~~~~~~M DPLGDTLRRLREAFHAGRTR PAEFRAAQLQGLGRFLQENK
p12762    FPTVNPSTGEVICQVAAGDK EDVDRAVKAARAAFQLGSPW RRMDASDRGRLLNRLADLIE
p20000    FPTVNPSTGDVICHVAEGDK ADVDRAVKAARAAFQLGSPW RRMDASERGRLLNRLADLIE
p11884    FPTVNPSTGEVICQVAEGNK EDVDKAVKAAQAAFQLGSPW RRMDASDRGRLLYRLADLIE
p47738    FPTVNPSTGEVICQVAEGNK EDVDKAVKAARAAFQLGSPW RRMDASDRGRLLYRLADLIE
p05091    FPTVNPSTGEVICQVAEGDK EDVDKAVKAARAAFQLGSPW RRMDASHRGRLLNRLADLIE
m63967    FPTVNPTTGEVIGHVAEGDR ADVDRAVKAAREAFRLGSPW RRMDASERGRLLNLLADLVE
u60063    FPVCNPATGEQVCEVQEADK VDIDKAVQAARLAFSLGSVW RRMDASERGRLLDKLADLVE
x99273    FPVCNPATGEQVCEVQEADK VDIDKAVQAARLAFSLGSVW RRMDASERGRLLDKLADLVE
l36128    FPVFNPATEEKLCEVEEGDK EDVDKAVKAVRQAFQIGSPW RTMDASERGRLLNKLADLIE
p51977    FPVFNPATEEKLCEVEEGDK EDVDKAVKAARQAFQIGSPW RTMDASERGRLLNKLADLIE
p00352    FPVFNPATEEELCQVEEGDK EDVDKAVKAARQAFQIGSPW RTMDASERGRLLYKLADLIE
p15437    FPVFNPATEEKLCEVEEGDK EDVNKAVAAARQAFQIGSPW RTMDASERGRLLYKLADLVE
p24549    FPVLNPATEEVICHVEEGDK ADVDKAVKAARQAFQIGSPW RTMDASERGCLLNKLADLME
p51647    FPVLNPATEEVICHVEEGDK ADVDKAVKAARQAFQIGSPW RTMDASERGRLLNKLADLME
p13601    FPVINPATEEVICHVEEGDK ADVDKAVKAARQAFQIGSPW RTMDASERGCLLNKLADLME
p27463    FEVFNPANEEKICEVAEGDK ADIDKAVKAARKAFELGSPW RTMDASERGRLLNKLADLVE
u03906    FPVFNPATEEKICEVEEADK EDVDKAVKAAREAFQMGSPW RTMDASERGQLIYKLADLIE
a55684    FATCNPSTREQICEVEEGDK PDVDKAVEAAQVAFQRGSPW RRLDALSRGRLLHQLADLVE
p40047    FDVINPSNEEKITTVYKAME DDVDEAVAALKKLLK.RSVY CRAG..VRAKALFNLADLVE
u56604    YPVEDPSTENTVCEVSSATT EDVEYAIECADRAFH.DTEW ATQDPRERGRLLSKLADELE
p23883    FETVDPVTQAPLAKIARGKS VDIDRAMSAARGVFERGD.W SLSSPAKRKAVLNKLADLME
p49189    EKAFEPATGRVIATFTCSGE KEVNLAVQNAKAAFKI...W SQKSGMERCRILLEAARIIR
Consen    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~
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          121                                                                 180
a41028    KDILAAIAADLSKSELNAYS HEVITILGEIDFMLGNLPEL ASARPAKKNLLTMMDEAYVQ
u14390    KEILAAIAADLSKSELNAYS HEVITILGEIDFMLGNLPEL ASARPAKKNLLTMMDEAYVQ
p11883    KSISGALASDLGKNEWTSYY EEVAHVLEELDTTIKELPDW AEDEPVAKTRQTQQDDLYIH
p47739    KGISKALASNLRKNEWTSYY EEVAHVLDEIDFTIKGLSDW AEDEPVGKTRQTQEDDLYIH
p30838    QELVGALAADLHKNEWNAYY EEVVYVLEEIEYMIQKLPEW AADEPVEKTPQTQQDELYIH
p43353    QLLHDALAQDLHKSAFESEV SEVAISQGEVTLALRNLRAW MKDERVPKNLATQLDSAFIR
p12762    RDRT.YLAALETLDNGKPYV ISYLVDLDMVLKCLRYYAGW ADKYHGKTIPIDGDFFSYTR
p20000    RDRT.YLAALETLDNGKPYI ISYLVDLDMVLKCLRYYAGW ADKYHGKTIPIDGDYFSYTR
p11884    RDRT.YLAALETLDNGKPYV ISYLVDLDMVLKCLRYYAGW ADKYHGKTIPIDGDFFSYTR
p47738    RDRT.YLAALETLDNGKPYV ISYLVDLDMVLKCLRYYAGW ADKYHGKTIPIDGDFFSYTR
p05091    RDRT.YLAALETLDNGKPYV ISYLVDLDMVLKCLRYYAGW ADKYHGKTIPIDGDFFSYTR
m63967    RDRV.YLASLETLDNGKPFQ ESYALDLDEVIKVYRYFAGW ADKWHGKTIPMHGQHFCFTR
u60063    RDRA.TLATMESLNGGKPFL QAFYIDLQGVIKTLRYYAGW ADKIHGMTIPVDGDYFTFTR
x99273    RDRA.TLATMESLNGGKPFL QAFYIDLQGVIKTLRYYAGW ADKIHGMTIPVDGDYFTFTR
l36128    RDHL.LLATMEAMNGGKLFS NAYLMDLGGCIKTLRYCAGW ADKIQGRTIPMDGNFFTYTR
p51977    RDRL.LLATMEAMNGGKLFS NAYLMDLGGCIKTLRYCAGW ADKIQGRTIPMDGNFFTYTR
p00352    RDRL.LLATMESMNGGKLYS NAYLNDLAGCIKTLRYCAGW ADKIQGRTIPIDGNFFTYTR
p15437    RDRL.ILATMESMNGGKLFS NAYLMDLGGCLKTLRYCAGW ADKIQGRTIPSDGNFFTYTR
p24549    RDRL.LLATMEALNGGKVFA NAYLSDLGGCIKALKYCAGW ADKIHGQTIPSDGDIFTYTR
p51647    RDCL.LLATIEAINGGKVFA NAYLSDLGGSIKALKYCAGW ADKIHGQTIPSDGDIFTFTR
p13601    RDRV.LLATMESMNAGKIFT HAYLLDTEVSIKALKYFAGW ADKIHGQTIPSDGDVFTYTR
p27463    RDRL.TLATMEAIDGGKLFS TAYLMDLGACIKTIRYCAGW ADKIHGRTVPMDGNFFTFTR
u03906    RDRL.LLATLESINAGKIFA SAYLMDLDYCIKVLRYCAGW ADKIQGRTIPVDGEFFSYTR
a55684    RDRA.TLAALETMDTGKPFL HAFFIDLEGCIRTLRYFAGW ADKIQGKTIPTDDNVVCFTR
p40047    KHQE.TLAAIESMDNGKSL. FCARGDVALVSKYLRSCGGW ADKIYGNVIDTGKNHFTYSI
u56604    SQID.LVSSIEALDNGKTLA FKARGDVTIAINCLRDAAAY ADKVNGRTINTGDGYMNFTT
p23883    AHAE.ELALLETLDTGKPIR HSLRDDIPGAARAIRWYAEA IDKVYGEVATTSSHELAMIV
p49189    ERED.EIATMECINNGKSIF EARL.DIDISWQCLEYYAGL AASMAGEHIQLPGGSFGYTR
Consen    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~

          181                                                                 240
a41028    PEPLGVVLIIGAWNYPFVLT LQPLVGAIAAGNAAIVKPSE LSENTAKILAELLPQY.LDQ
u14390    PEPLGVVLIIGAWNYPFVLT MQPLVGAIAAGNAAIVKPSE LSENTAKILAELLPQY.LDQ
p11883    SEPLGVVLVIGAWNYPFNLT IQPMVGAVAAGNAVILKPSE VSGHMADLLATLIPQY.MDQ
p47739    SEPLGVVLVIGAWNYPFNLT IQPMVGAIAAGNAVVLKPSE VSDHMADLLSTLIPQY.MDK
p30838    SEPLGVVLVIGTWNYPFNLT IQPMVGAIAAGNAVVLKPSE LSENMASLLATIIPQY.LDK
p43353    KEPFGLVLIIAPWNYPLNLT LVPLVGALAAGNCVVLKPSE ISKNVEKILAEVLPQY.VDQ
p12762    HEPVGVCGQIIPWNFPLLMQ AAKLGPALATGNVVVMKVAE QTPLTALYVANLTKEAGFPP
p20000    HEPVGVCGQIIPWNFPLLMQ AWKLGPALATGNVVVMKVAE QTPLTALYVANLIKEAGFPP
p11884    HEPVGVCGQIIPWNFPLLMQ AWKLGPALATGNVVVMKVAE QTPLTALYVANLIKEAGFPP
p47738    HEPVGVCGQIIPWNFPLLMQ AWKLGPALATGNVVVMKVAE QTPLTALYVANLIKEAGFPP
p05091    HEPVGVCGQIIPWNFPLLMQ AWKLGPALATGNVVVMKVAE QTPLTALYVANLIKEAGFPP
m63967    HEPVGVCGQIIPWNFPLVMQ GWKLAPALATGNTVVMKVAE QTPLSALYLASLIKEAGFPP
u60063    HEPIGVCGQIIPWNFPLLMF TWKIAPALCCGNTVVIKPAE QTPLSALYMGALIKEAGFPP
x99273    HEPIGVCGQIIPWNFPLLMF TWKIAPALCCGNTVVIKPAE QTPLSALYMGALIKEAGFPP
l36128    SEPVGVCGQIIPWNFPLLMF LWKIGPALSCGNTVVVKPAE QTPLTALHMGSLIKEAGFPP
p51977    SEPVGVCGQIIPWNFPLLMF LWKIGPALSCGNTVVVKPAE QTPLTALHMGSLIKEAGFPP
p00352    HEPIGVCGQIIPWNFPLVML IWKIGPALSCGNTVVVKPAE QTPLTALHVASLIKEAGFPP
p15437    HEPVGVCGQILPWNFPLLMF LWKIAPALSCGNTVVVKPAE QTPLSALHVATLIKEAGFPP
p24549    REPIGVCGQIIPWNFPMLMF IWKIGPALSCGNTVVVKPAE QTPLTALHLASLIKEAGFPP
p51647    REPIGVCGQIIPWEFPLLMF IWKIGPALSCGNTVVVKPAE QTPLTALHMASLIKEAGFPP
p13601    REPIGVCGQIIPWNGPLILF IWKIGAALSCGNTVIVKPAE QTPLTALYMASLIKEAGFPP
p27463    HEPVGVCGQIIPWNFPLVMF IWKIAPALCCGNTVVVKPAE QTPLSALYMGSLIKEAGFPP
u03906    HEPIGVCGQIFPWNAPMILL ACKIGPALCCGNTVIVKPAE QTPLTALHVASLIKEAGFPP
a55684    HEPIGVCGAITPWNFPLLML VWKLAPALCCGNTMVLKPAE QTPLTALYLGSLIKEAGFPP
p40047    KEPLGVCGQIIPWNFPLLMW SWKIGPALATGNTVVLKPAE TTPLSALFASQLCQEAGIPA
u56604    LEPIGVCGQIIPWNFPIMML AWKIAPALAMGNVCILKPAA VTPLNALYFASLCKKVGIPA
p23883    REPVGVIAAIVPWNFPLLLT CWKLGPALAAGNSVILKPSE KSPLSAIRLAGLAKEAGLPD
p49189    REPLGVCVGIGAWNYPFQIA SWKSAPALACGNAMVFKPSP FTPVSALLLAEIYSEAGVPP
Consen    ~EP~G~~~~I~~W~~P~~~~ ~~~~~~A~~~GN~~~~K~~~ ~~~~~~~~~~~~~~~~~~~~
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         241                                                                300
a41028   DLYMIVNGGVEETTELLRQR ..FDHILYTGNTAVGKIVME AAAK.HLTPVTLELGGKSPC
u14390   DLYAIVNGGIPETTELLKQR ..FDHILYTGNTAVGKIVME AAAK.HLTPVTLELGGKSPC
p11883   NLYLVVKGGVPETTELLKER ..FDHIMYTGSTAVGKIVMA AAAK.HLTPVTLELGGKSPC
p47739   DLYPVIKGGVPETTELLKEK ..FDHIMYTGSTAVGKIVMA AAAK.HLTPVTLELGGKSPC
p30838   DLYPVINGGVPETTELLKER ..FDHILYTGSTGVGKIIMT AAAK.HLTPVTLELGGKSPC
p43353   SCFAVVLGGPQETGQLLEHR ..FDYIFFTGSPRVGKIVMT AAAK.HLTPVTLELGGKNPC
p12762   GVVNVVPGFGPTAGAAIASH EDVDKVAFTGSTEVGHLIQV AAGRSNLKKVTLELGGKSPN
p20000   GVVNVIPGFGPTAGAAIASH EDVDKVAFTGSTEVGHLIQV AAGKSNLKRVTLEIGGKSPN
p11884   GVVNIVPGFGPTAGAAIASH EDVDKVAFTGSTEVGHLIQV AAGSSNLKRVTLELGGKSPN
p47738   GVVNIVPGFGPTAGAAIASH EGVDKVAFTGSTEVGHLIQV AAGSSNLKRVTLELGGKSPN
p05091   GVVNIVPGFGPTAGAAIASH EDVDKVAFTGSTEIGRVIQV AAGSSNLKRVTLELGGKSPN
m63967   GVVNIITGYGPTAGAAIAQH MDVDKVAFTGSTEVGHLIQK AAGDSNLKRVTLELGGKSPS
u60063   GVVNILPGYGPTAGAAIASH IGIDKIAFTGSTEVGKLIQE AAGRSNLKRVTLELGGKSPN
x99273   GVVNILPGYGPTAGAAIASH IGIDKIAFTGSTEVGKLIQE AAGRSNLKRVTLELGGKSPN
l36128   GVVNIVPGYGPTAGAAISSH MDVDKVAFTGSTEVGKLIKE AAGKSNLKRVSLELGGKSPC
p51977   GVVNIVPGYGPTAGAAISSH MDVDKVAFTGSTEVGKLIKE AAGKSNLKRVSLELGGKSPC
p00352   GVVNIVPGYGPTAGAAISSH MDIDKVAFTGSTEVGKLIKE AAGKSNLKRVTLELGGKSPC
p15437   GVVNIVPGYGPTAGAAISSH MDIDKVAFTGSTEVGKLIKE AAGKSNLKRVTLELGGKSPF
p24549   GVVNIVPGYGPTAGAAISSH MDVDKVAFTGSTQVGKLIKE AAGKSNLKRVTLELGGKSPC
p51647   GVVNIVPGYGPTAGAAISSH MDVDKVAFTGSTQVGKLIKE AAGKSNLKRVTLELGGKSPC
p13601   GVVNVVPGYGSTAGAAISSH MDIDKVSFTGSTEVGKLIKE AAGKSNLKRVTLELGGKSPC
p27463   GVVNIVPGFGPTAGAAISHH MDIDKVSFTGSTEVGKLIKE AAGKTNLKRVTLELGGKSPN
u03906   GVVNIVPGYGPTAGAAISSH MDVDKVAFTGSTEVGKMIQE AAAKSNLKRVTLELGAKNPC
a55684   GVVNIVPGFGPTVGAAISSH PQINKIAFTGSTEVGKLVKE AASRSNLKRVTLELGGKNPC
p40047   GVVNILPGSGRVVGERLSAH PDVKKIAFTGSTATGRHIMK VAADT.VKKVTLELGGKSPN
u56604   GVVNIVPGPGRTVGAALTND PRIRKLAFTGSTEVGKSVAV DSSESNLKKITLELGGKSAH
p23883   GVLNVVTGFGHEAGQALSRH NDIDAIAFTGSTRTGKQLLK DAGDSNMKRVWLEAGGKSAN
p49189   GLFNVVQG.GAATGQFLCQH PDVAKVSFTGSVPTGMKIME MSAK.GIKPVTLELGGKSPL
Consen   ~~~~~~~G~~~~~~~~~~~~ ~~~~~~~~TG~~~~G~~~~~ ~~~~~~~~~~~LE~G~K~~~

         301                                                                360
a41028   YIDRDC.DLDVACRRITWGK YMNCGQTCIAPDYILCEASS QDQIVQKIKDTVKDF..YGE
u14390   YIDRDC.DLDVACRRVAWGK YMNCGQTCIAPDYILCEASL QNQIVQKIKETVKDF..YGE
p11883   YVDKDC.DLDVACRRIAWGK FMNSGQTCVAPDYILCDPSI QNQIVEKLKKSLKDF..YGE
p47739   YVDKDC.DLDVACRRIAWGK FMNSGQTCVAPDYILCDPSI QNEIVEKLKKSLKDF..YGE
p30838   YVDKNC.DLDVACRRIAWGK FMNSGQTCVAPDYILCDPSI QNQIVEKLKKSLKEF..YGE
p43353   YVDDNC.DPQTVANRVAWFR YFNAGQTCVAPDYVLCSPEM QERLLPALQSTITRF..YGD
p12762   IIVSDA.DMDWAVEQAHFAL FFNQGQCCGAGSRTFVQEDV YAEFVERSVARAKSRV.VGN
p20000   IIMSDA.DMDWAVEQAHFAL FFNQGQCCCAGSRTFVQEDI YAEFVERSVARAKSRV.VGN
p11884   IIMSDA.DMDWAVEQAHFAL FFNQGQCCCAGSRTFVQEDV YDEFVERSVARAKSRV.VGN
p47738   IIMSDA.DMDWAVEQAHFAL FFNQGQCCCAGSRTFVQENV YDEFVERSVARAKSRV.VGN
p05091   IIMSDA.DMDWAVEQAHFAL FFNQGQCCCAGSRTFVQEDI YDEFVERSVARAKSRV.VGN
m63967   IVLADA.DMEHAVEQCHEAL FFNMGQCCCAGSRTFVEESI YNEFLERTVEKAKQRK.VGN
u60063   IIFADA.DLDYAVEQAHQGV FFNQGQCCTAGSRIFVEESI YEEFVKRSVERAKRRI.VGS
x99273   IIFADA.DLDYAVEQAHQGV FFNQGQCCTAGSRIFVEESI YEEFVKRSVERAKRRI.VGS
l36128   IVFADA.DLDNAVEFAHQGV FYHQGQCCIAASRLFVEESI YDEFVRRSVERAKKYV.LGN
p51977   IVFADA.DLDNAVEFAHQGV FYHQGQCCIAASRLFVEESI YDEFVRRSVERAKKYV.LGN
p00352   IVLADA.DLDNAVEFAHHGV FYHQGQCCIAASRIFVEESI YDEFVRRSVERAKKYI.LGN
p15437   IVFADA.DLETALEVTHQAL FYHQGQCCVAASRLFVEESI YDEFVRRSVERAKKYV.LGN
p24549   IVFADA.DLDIAVEFAHHGV FYHQGQCCVAASRIFVEESV YDEFVKRSVERAKKYV.LGN
p51647   IVFADA.DLDIAVEFAHHGV FYHQGQCCVAASRIFVEESV YDEFVRKSVERAKKYV.LGN
p13601   IVFADA.DLDSAVEFAHQGV FFHQGQICVAASRLFVEESI YDEFVRRSVERAKKYV.LGN
p27463   IIFADA.DLDEAAEFAHIGL FYHQGQCCIAGSRIFVEEPI YDEFVRRSIERAKKYT.LGD
u03906   IVFADA.DLDSAVEFAHQGV FTNQGQSCIAASKLFVEETI YDEFVQRSVERAKKYV.FGN
a55684   IVCADA.DLDLAVECAHQGV FFNQGQCCTAASRVFVEEQV YSEFVRRSVEYAKKRP.VGD
p40047   IVFADA.DLDKAVKNIAFGI FYNSGEVCCAGSRIYIQDTV YEEVLQKLKDYTES.LKVGD
u56604   LVFDDA.NIKKTLPNLVNGI FKNAGQICSSGSRIYVQEGI YDELLAAFKAYLETEIKVGN
p23883   IVFADCPDLQQAASATAAGI FYNQGQVCIAGTRLLLEERI ADEFLALLKQQAQNWQP.GH
p49189   IIFSDC.DMNNAVKGALMAN FLTQGQVCCNGTRVFVQKEI LDKFTEEVVKQTQ.RIKIGD
Consen   ~~~~~~~~~~~~~~~~~~~~ ~~~~G~~C~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~G~
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          361                                                                 420
a41028    NVKASPDYERIINLRHFKRI KSLL.....EGQKIAFGG.. .....ETDEATRYIAPTILT
u14390    NIKASPDYERIINLRHFKRL QSLL.....KGQKIAFGG.. .....EMDEATRYLAPTILT
p11883    DAKQSRDYGRIINDRHFQRV KGLI.....DNQKVAHGG.. .....TWDQSSRYIAPTILV
p47739    DAKQSHDYGRIINDRHFQRV INLI.....DSKKVAHGG.. .....TWDQPSRYIAPTILV
p30838    DAKKSRDYGRIISARHFQRV MGLI.....EGQKVAYGG.. .....TGDAATRYIAPTILT
p43353    DPQSSPNLGRIINQKQFQRL RALL.....GCGRVAIGG.. .....QSDESDRYIAPTVLV
p12762    PFDSQTEQGPQVDETQFNKV LGYIKSGKEEGAKLLCGGGA AADR......GYFIQPTVFG
p20000    PFDSRTEQGPQVDETQFKKV LGYIKSGKEEGLKLLCGGGA AADR......GYFIQPTVFG
p11884    PFDSRTEQGPQVDETQFKKI LGYIKSGQQEGAKLLCGGGA AADR......GYFIQPTVFG
p47738    PFDSRTEQGPQVDETQFKKI LGYIKSGQQEGAKLLCGGGA AADR......GYFIQPTVFG
p05091    PFDSKTEQGPQVDETQFKKI LGYINTGKQEGAKLLCGGGI AADR......GYFIQPTVFG
m63967    PFELDTQQGPQVDKEQFERV LGYIQLGQKEGAKLLCGGER FGER......GFFIKPTVFG
u60063    PFDPTTEQGPQIDKKQYNKI LELIQSGVAEGAKLECGGKG LGRK......GFFIEPTVFS
x99273    PFDPTTEQGPQIDKKQYNKV LELIQSGVAEGAKLECGGKG LGRK......GFFIEPTVFS
l36128    PLTPGVSQGPQIDKEQYEKI LDLIESGKKEGAKLECGGGP WGNK......GYFIQPTVFS
p51977    PLTPGVSQGPQIDKEQYEKI LDLIESGKKEGAKLECGGGP WGNK......GYFIQPTVFS
p00352    PLTPGVTQGPQIDKEQYDKI LDLIESGKKEGAKLECGGGP WGNK......GYFVQPTVFS
p15437    PLTPGVSQGPQIDKEQYDKI LDLIESGKKEGAKLECGGGP WGNK......GYFIQPTVFS
p24549    PLTPGINQGPQIDKEQHDKI LDLIESGKKEGAKLECGGGR WGNK......GFFVQPTVFS
p51647    PLTQGINQGPQIDKEQHDKI LDLIESGKKEGAKLECGGGR WGNK......GFFVQPTVFS
p13601    PLDSGISQGPQIDKEQHAKI LDLIESGKKEGAKLECGGGR WGNK......GFFVQPTVFS
p27463    PLLPGVQQGPQIDKEQFQKI LDLIESGKKEGAKLECGGGP WGNK......GYFIQPTVFS
u03906    PLTPGVNHGPQINKAQHNKI MELIESGKKEGAKLECGGGP WGNK......GYFIQPTIFS
a55684    PFDVKTEQGPQIDQKQFDKI LELIESGKKEGAKLECGGSA MEDK......GLFIKPTVFS
p40047    PFDEEVFQGAQTSDKQLHKI LDYVDVAKSEGARLVTGGAR HGSK......GYFVKPTVFA
u56604    PFDKANFQGAITNRQQFDTI MNYIDIGKKEGAKILTGGEK VGDK......GYFIRPTVFY
p23883    PLDPATTMGTLIDCAHADSV HSFIREGESKGQLLLDGRN. .AGL......AAAIGPTIFV
p49189    PLLEDTRMGPLINRPHLERV LGFVKVAKEQGAKVLCGGDI YVPEDPKLKDGYYMRPCVLT
Consen    ~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~G~~~ ~~~~~~~~~~~~~~~P~~~~

          421                                                                 480
a41028    DVDPNSKVMQEEIFGPILPI VSVKNVEEAINFINDREKPL ALYIFSHNNKLIKRVIDETS
u14390    DVDPNSKVMQEEIFGPILPI VSVKNVDEAINFINDREKPL ALYVFSRNNKLIKRVIDETS
p11883    DVDPQSPVMQEEIFGPVMPI VCVRSLEEAIQFINQREKPL ALYVFSNNEKVIKKMIAETS
p47739    DVDPQSPVMQEEIFGPVMPI VCVRSLDEAIKFINQREKPL ALYVFSNNDKVIKKMIAETS
p30838    DVDPQSPVMQEEIFGPVLPI VCVRSLEEAIQFINQREKPL ALYMFSSNDKVIKKMIAETS
p43353    DVQEMEPVMQEEIFGPILPI VNVQSLDEAIEFINRREKPL ALYAFSNSSQVVKRVLTQTS
p12762    DVQDGMTIAKEEIFGPVMQI LKFKTIEEVVGRANNSKYGL AAAVFTKDLDKANYLSQALQ
p20000    DLQDGMTIAKEEIFGPVMQI LKFKSMEEVVGRANNSKYGL AAAVFTKDLDKANYLSQALQ
p11884    DVKDGMTIAKEEIFGPVMQI LKFKTIEEVVGRANNSKYGL AAAVFTKDLDKANYLSQALQ
p47738    DVKDGMTIAKEEIFGPVMQI LKFKTIEEVVGRANDSKYGL AAAVFTKDLDKANYLSQALQ
p05091    DVQDGMTIAKEEIFGPVMQI LKFKTIEEVVGRANNSTYGL AAAVFTKDLDKANYLSQALQ
m63967    GVQDDMRIAKEEIFGPVQPL FKFKKIEEVVERANNTRYGL AAAVFTRDLDKAMYFTQALQ
u60063    NVTDDMRIAKEEIFGPVQEI LRFKTMDEVIERANNSDFGL VAAVFTNDINKALMVSSAMQ
x99273    NVTDDMRIAKEEIFGPVQEI LRFKTMDEVIERANNSDFGL VAAVFTNDINKALMVSSAMQ
l36128    DVTDDMRIAKEEIFGPVQQI MKFKSLDDVIKRANNTFYGL SAGIFTNDIDKAITVSSALQ
p51977    DVTDDMRIAKEEIFGPVQQI MKFKSLDDVIKRANNTFYGL SAGIFTNDIDKAITVSSALQ
p00352    NVTDEMRIAKEEIFGPVQQI MKFKSLDDVIKRANNTFYGL SAGVFTKDIDKAITISSALQ
p15437    NVSDEMRIAKEEIFGPVQQI MKFKSLDDVIKRANNTTYGL FAGSFTKDLDKAITVSAALQ
p24549    NVTDEMRIAKEEIFGPVQQI MKFKSVDDVIKRANNTTYGL AAGLFTKDLDKAITVSSALQ
p51647    NVTDEMRIAKEEIFGPVQQI MKFKSIDDVIKRANNTTYGL AAGVFTKDLDRAITVSSALQ
p13601    NVTDEMRIAKEEIFGPVQQI MKFKSIDEVIKRANNTPYGL AAGVFTKDLDRAITVSSALQ
p27463    NVTDDMRIAKEEIFGPVQQI MKFKTIDEVIKRANNTTYGL AAAVFTKDIDKALTFASALQ
u03906    NVTDDMRIAKEEIFGPVQQI MKFKSLDEVIKRANNTYYGL VAGVFTKDLDKAVTVSSALQ
a55684    EVTDNMRIAKEEIFGPVQPI LKFKSIEEVIKRANSTDYGL TAAVFTKNLDKALKLASALE
p40047    DVKEDMRIVKEEVFGPIVTV SKFSTVDEVIAMANDSQYGL AAGIHTNDINKAVDVSKRVK
u56604    DVNEDMRIVKEEIFGPVVTV AKFKTLEEGVEMANSSEFGL GSGIETESLSTGLKVAKMLK
p23883    DVDPNASLSREEIFGPVLVV TRFTSEEQALQLANDSQYGL GAAVWTRDLSRAHRMSRRLK
p49189    NCRDDMTCVKEEIFGPVMSI LSFDTEAEVLERANDTTFGL AAGVFTRDIQRAHRVVAELQ
Consen    ~~~~~~~~~~EE~FGP~~~~ ~~~~~~~~~~~~~N~~~~~L ~~~~~~~~~~~~~~~~~~~
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          481                                                                 540
a41028    SGGVTGNDVIMHFTVNSLPF GGVGASGMGAYHGKYSFDTF SHQRPCLLKGLKGESVNKLR
u14390    SGGVTGNDVIMHFTVNSLPF GGVGASGMGAYHGKYSFDAF SHQRPCLLKGLKGESVNKLR
p11883    SGGVTANDVIVHITVPTLPF GGVGNSGMGAYHGKKSFETF SHRRSCLVKSLLNEEAHKAR
p47739    SGGVTANDVIVHITVPTLPF GGVGNSGMGAYHGKKSFETF SHRRSCLVRSLRNEEANKAR
p30838    SGGVAANDVIVHITLHSLPF GGVGNSGMGSYHGKKSFETF SHRRSCLVRPLMNDEGLKVR
p43353    SGGFCGNDGFMHMTLASLPF GGVGASGMGRYHGKFSFDTF SHHRACLLRSPGMEKLNALR
p12762    AGTVWIN..CYDVFGAQSPF GGYKMSGNGRELGEYGLQAY TEVKTVTIKVPQKNS~~~~~
p20000    AGTVWVN..CYDVFGAQSPF GGYKLSGSGRELGEYGLQAY TEVKTVTVRVPQKNS~~~~~
p11884    AGTVWIN..CYDVFGAQSPF GGYKMSGSGRELGEYGLQAY TEVKTVTVKVPQKNS~~~~~
p47738    AGTVWIN..CYDVFGAQSPF GGYKMSGSGRELGEYGLQAY TEVKTVTVKVPQKNS~~~~~
p05091    AGTVWVN..CYDVFGAQSPF GGYKMSGSGRELGEYGLQAY TEVKTVTVKVPQKNS~~~~~
m63967    AGTVWVN..TYNIVTCHTPF GGFKESGNGRELGEDGLKAY TEVKTVTIKVPQKNS~~~~~
u60063    AGTVWIN..CYNALNAQSPF GGFKMSGNGREMGEFGLREY SEVKTVTVKIPQKNS~~~~~
x99273    AGTVWIN..CYNALNAQSPF GGFKMSGNGREMGEFGLREY SEVKTVTVKIPQKNS~~~~~
l36128    SGTVWVN..CYSVVSAQCPF GGFKMSGNGRELGEYGFHEY TEVKTVTIKISQKNS~~~~~
p51977    SGTVWVN..CYSVVSAQCPF GGFKMSGNGRELGEYGFHEY TEVKTVTIKISQKNS~~~~~
p00352    AGTVWVN..CYGVVSAQCPF GGFKMSGNGRELGEYGFHEY TEVKTVTVKISQKNS~~~~~
p15437    AGTVWVN..CYGVVSAQCPF GGFKMSGNGREMGEYGFHEY TEVKTVTVKISQKNS~~~~~
p24549    AGVVWVN..CYIMLSAQCPF GGFKMSGNGRELGEHGLYEY TELKTVAMKISQKNS~~~~~
p51647    AGVVWVN..CYMILSAQCPF GGFKMSGNGRELGEHGLYEY TELKTVAMKISQKNS~~~~~
p13601    AGTVWVN..CYLTLSVQCPF GGFKMSGNGREMGEQGVYEY TELKTVAMKISQKNS~~~~~
p27463    AGTVWVN..CYSAFSAQCPF GGFKMSGNGRELGEYGLQEY TEVKTVTIKIPQKNS~~~~~
u03906    AGTVWVN..CYLAASAQSPA GGFKMSGHGREMGEYGIHEY TEVKTVTMKISEKNS~~~~~
a55684    SGTVWIN..CYNALYAQAPF GGFKMSGNGRELGEYALAEY TEVKTVTIKLGDKNP~~~~~
p40047    AGTVWIN..TYNNFHQNVPF GGFGQSGIGREMGEAALSNY TQTKSVRIAIDKPIR~~~~~
u56604    AGTVWIN..TYNDFDSRVPF GGVKQSGYGREMGEEVYHAY TEVKAVRIKL~~~~~~~~~~
p23883    AGSVFVN..NYNDGDMTVPF GGYKQSGNGRDKSLHALEKF TELKTIWISLEA~~~~~~~~
p49189    AGTCFIN..NYNVSPVELPF GGYKKSGFGRENGRVTIEYY SQLKTVCVEMGDVESAF~~~
Consen    ~G~~~~N~~~~~~~~~~~P~ GG~~~SG~G~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~

          541                                                         585
a41028    YPPNSESKVSWSKFFLLKQF NKGRLQLLLLVCLVAVAAVI VKDQL
u14390    YPPNSESKVSWAKFFLLKQF NKGRLGMLLFVCLVAVAAVI VKDQL
p11883    YPPSPAKMPRH~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
p47739    YPPSPAKMPRH~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
p30838    YPPSPAKMTQH~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
p43353    YPPQSPRRLRMLLVAMEAQG CSCTLL~~~~~~~~~~~~~~ ~~~~~
p12762    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
p20000    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
p11884    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
p47738    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
p05091    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
m63967    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
u60063    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
x99273    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
l36128    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
p51977    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
p00352    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
p15437    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
p24549    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
p51647    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
p13601    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
p27463    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
u03906    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
a55684    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
p40047    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
u56604    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
p23883    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
p49189    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
Consen    ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~
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