Isolation and Characterisation of a Novel Dihydrolipoamide Dehydrogenase from Pea Chloroplasts

by

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A thesis submitted for the degree of Ph.D.

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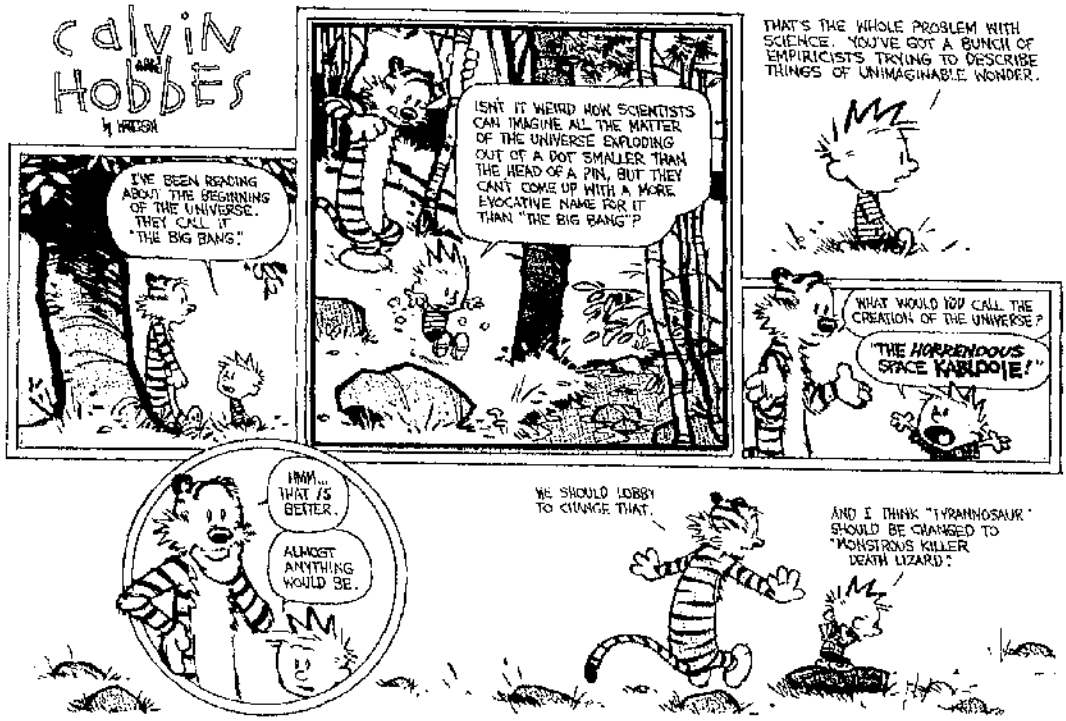
For Victoria and Nanna



Abstract

Dihydrolipoamide dehydrogenase (E3) is a common component of the three α-ketoacid dehydrogenase complexes oxidising pyruvate, 2-oxoglutarate, and the branched chain 2-oxoacids. It is also a component in the glycine cleavage system. E3 belongs to the family of pyridine nucleotide-disulphide oxidoreductases, catalysing electron transfer between pyridine nucleotides and disulphide compounds. It exists as a homodimer, with one flavin adenine dinucleotide (FAD) per subunit and a redox-active disulphide.

There is increasing evidence for the existence of multiple isoforms of E3 in mitochondria from bacterial and plant sources. Moreover, preliminary immunological analyses of pea mitochondria and chloroplasts have provided the first evidence of organelle-specific isoforms of dihydrolipoamide dehydrogenase existing in plants.

Utilising anion exchange chromatography, two distinct dihydrolipoamide dehydrogenases were isolated from a crude extract of pea leaves and eluted from the anion exchange column by differing concentrations of potassium phosphate. Isolation and purification of pea leaf chloroplasts and mitochondria revealed the location of the differing dihydrolipoamide dehydrogenases, with the major isoform located in mitochondria and the second, minor isoform located in the chloroplasts.

The chloroplast-specific dihydrolipoamide dehydrogenase was found to be a homodimer, with an apparent subunit molecular mass of 52 kDa, 4 kDa smaller than the apparent mass of pea mitochondrial E3. This polypeptide was not recognised by antiserum raised to the pea leaf mitochondrial isoform and antiserum against the chloroplastic dihydrolipoamide dehydrogenase failed to cross-react with the pea mitochondrial E3. Further analysis revealed that the N-terminal sequence was distinct from and did not correspond to any part of the mitochondrial sequence.

Kinetic studies demonstrated that the chloroplastic enzyme behaved as a conventional dihydrolipoamide dehydrogenase and contained a redox-active disulphide at its active site. However, the K_m for NAD^+ and DHL and the K_i for NADH of the chloroplastic isoenzyme was found to be significantly different from the mitochondrial E3. Moreover, the specific activity of the chloroplastic enzyme was found to be significantly lower than its mitochondrial counterpart.

Structural differences were revealed when guanidine hydrochloride, sodium chloride and elevated temperatures were used as denaturing agents. The chloroplastic dihydrolipoamide dehydrogenase was found to be more resistant to loss of activity in the presence of these agents than the mitochondrial isoform.

Chromatographic and immunological analyses were performed on leaf extracts of barley, spinach and oil seed rape and in each, an equivalent polypeptide was found to cross-react with antiserum raised to the pea chloroplastic isoenzyme.

An attempt was made to clone the cDNA corresponding to the chloroplastic dihydrolipoamide dehydrogenase protein using the available N-terminal sequence. This, however, proved difficult resulting in the amplification of a sequence which displayed only limited similarity to the N-terminal sequence of the chloroplastic enzyme outside the primer sequences.

The protein-biochemical data reveal the existence of a pea chloroplastic dihydrolipoamide dehydrogenase which is distinct from the mitochondrial isoform, and this enzyme may be the first component of a chloroplast-specific pyruvate dehydrogenase complex to be isolated and characterised to date.

Acknowledgements

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Last and certainly not least, a very big thanks to my partner, Victoria, whose help, support and sacrifices made throughout my PhD and in writing this thesis mean that this is as much hers as it is mine.

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Abbreviations

In addition to the abbreviations recommended (Instructions to authors, *Biochem. J.* (1992) **281**, 1-19), the following were used throughout this thesis.

approx.	approximately
BCDC	branched chain 2-oxoacid dehydrogenase complex
BSA	bovine serum albumin
CAPS	3-[cyclohexylamino]-1-propanesulphonic acid
CAT	chloramphenicol acetyltransferase
CHES	2-[N-Cyclohexylamino]ethanesulphonic acid
Da	Daltons
DHL	dihydrolipoamide
DTNB	5,5'-dithio-bis (2-nitrobenzoic acid)
DTT	dithiothreitol
E3	dihydrolipoamide dehydrogenase
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)
ESMS	electrospray mass spectrometry
FPLC	fast protein liquid chromatography
GDC	glycine decarboxylase complex
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid
KP_i	potassium phosphate
MES	2-[N-Morpholino]ethanesulphonic acid
MOPS	3-(N-Morpholino) propane-sulphonic acid
MSUD	maple syrup urine disease
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide hydride
$NADP^+$	nicotinamide adenine dinucleotide phosphate

NADPH	nicotinamide adenine dinucleotide phosphate hydride
OGDC	2-oxoglutarate dehydrogenase complex
PAGE	polyacrylamide gel electrophoresis
PBC	primary biliary cirrhosis
PDC	pyruvate dehydrogenase complex
PEG	polyethylene glycol
PMSF	phenylmethylsulphonyl fluoride
PVDF	polyvinylidene difluoride
PVP	polyvinylpyrrolidine
SDS	sodium dodecyl sulphate
TCA cycle	tricarboxylic acid cycle
TEMED	NNN'N'-tetramethylethylenediamine
TES	(N-tris[hydroxymethyl]-methyl-2-aminoethane-sulphonic acid
TPP	thiamine pyrophosphate
Tricine	N-tris[Hydroxymethyl]methylglycine;N-[2-Hydroxy-1,1-bis(hydroxylmethyl)ethyl]glycine
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
Tween 20	polyethylenesorbitan monolaurate
Tx-100	Triton X-100
v/v	volume to volume
w/v	weight to volume
X	protein X

Declaration

I hereby declare that the work presented in this thesis is my own, except where otherwise cited or acknowledged. No part of this thesis has been presented for any other degree.

Mark Conner

May, 1997

Chapter 1

Introduction

1.1 Dihydrolipoamide Dehydrogenase

Dihydrolipoamide dehydrogenase (E3) is a member of the group of flavin-containing pyridine nucleotide-disulphide oxidoreductases (Carothers *et al.*, 1989), which includes glutathione reductase, mercuric reductase and tryptanothione reductase. These enzymes are composed of homodimers, containing one flavin adenine dinucleotide (FAD) molecule in each monomer and a redox active disulphide. In eukaryotes and eubacteria, E3 is an integral part of the 2-oxoacid dehydrogenase complexes and the related glycine decarboxylase complex (GDC) (Motokawa and Kikuchi, 1974), where it is responsible for the reoxidation of the lipoamide group and the subsequent transfer of reducing equivalents onto NAD^+ via the FAD co-factor. Research on E3 has focused on the enzyme from non-plant sources, particularly mammalian, yeast and specific bacterial sources, where it is present in high quantities. However, plant mitochondrial dihydrolipoamide dehydrogenase from the glycine decarboxylase complex (denoted the L-protein in this complex) has been studied fairly extensively due to the high levels of this complex in leaves, where GDC constitutes approx. 30% of the leaf mitochondrial matrix proteins. This chapter will present the current knowledge of the 2-oxoacid dehydrogenase complexes and the glycine decarboxylase complex, from mammalian and bacterial sources where they have been studied more extensively and from plants, where knowledge is more limited.

1.2 The 2-Oxoacid Dehydrogenase Complexes

The family of the 2-oxoacid dehydrogenase complexes comprises three related high molecular mass multienzyme assemblies. These are the pyruvate dehydrogenase complex (PDC), the 2-oxoglutarate dehydrogenase complex (OGDC) and the branched-chain 2-oxoacid dehydrogenase complex (BCDC). The mammalian complexes are found in the mitochondrial matrix, possibly associated with the inner

membrane. The molecular masses of these complexes are in the range 4-10 x 10^6 Da, comparable to the mass of ribosomes, and have been purified from a diverse range of organisms. The 2-oxoacid dehydrogenase complexes have been studied extensively as they represent classic examples of multienzyme complexes. These are self assembling, non-covalent aggregates of proteins which catalyse two or more consecutive chemical reactions in a metabolic sequence. The coupling of enzymatic reactions in multienzyme complexes has the advantage of substrate channelling, which enhances catalytic activity by increasing the concentration of the intermediates in the microenvironment, thereby reducing the effect of diffusion as a rate-limiting step, and the protection of intermediates where necessary (Perham, 1975; Reed, 1974).

1.2.1 Role of the 2-Oxoacid Dehydrogenase Complexes in Metabolism

The 2-oxoacid dehydrogenase complexes are of prime importance as they occupy key positions in intermediary metabolism, where they catalyse the irreversible oxidative decarboxylation of 2-oxoacids (see Figure 1.1). PDC oxidatively decarboxylates pyruvate to acetyl CoA with the generation of acetyl CoA and NADH, and the release of CO_2, committing the entry of two carbon units into the TCA cycle for subsequent oxidation. Acetyl CoA is also utilised in a range of biosynthetic pathways. OGDC is an integral component of the TCA cycle, where it converts 2-oxoglutarate to succinyl CoA, thus regulating the flux of carbon atoms around the latter stages of the cycle. This complex also supplies succinyl CoA for the biosynthesis of porphyrins, lysine and methionine (in mammalian cells). BCDC has a broad range of substrates, catalysing the committed step in the degradation of the branched-chain amino acids leucine, isoleucine and valine, and is also involved in the catabolism of methionine and threonine. This complex is of particular nutritional importance in mammals, where it controls the levels of branched-chain amino acids, converting excesses into acetyl CoA derivatives and thereby recycling carbon atoms back into central metabolism.

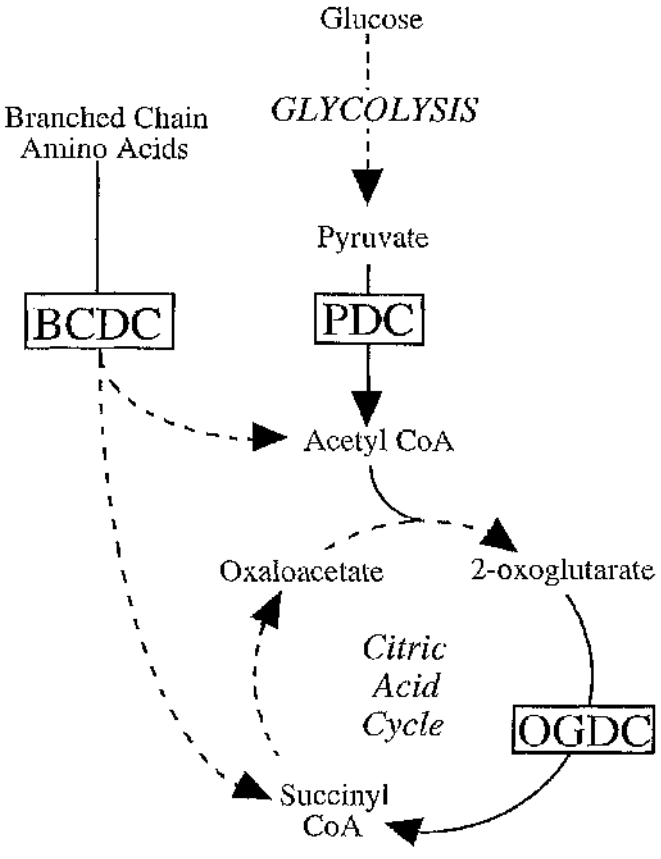

Figure 1.1: Partial representation illustrating the positions of the 2-oxoacid dehydrogenase complexes in the tricarboxylic acid cycle

PDC, pyruvate dehydrogenase complex; OGDC, 2-oxoglutarate dehydrogenase complex; BCDC, branched chain 2-oxoacid dehydrogenase complex; dotted lines indicate several steps.

1.2.2 Reaction Sequence of the 2-Oxoacid Dehydrogenase Complexes

The 2-oxoacid dehydrogenase complexes each consist of multiple copies of three component enzymes. These are E1, a substrate specific 2-oxoacid dehydrogenase which requires thiamine diphosphate (ThDP) as a catalytic cofactor; E2, a complex-specific dihydrolipoamide acyltransferase with a covalently bound lipoic acid prosthetic group; and E3, an FAD-requiring dihydrolipoamide dehydrogenase. Together these enzymes catalyse in concert the overall reaction of the multienzyme complexes of which they form an integral part (Reed, 1974; Yeaman, 1986). While E1 and E2 are complex-specific, it was previously thought that the same E3 was common to all of the 2-oxoacid dehydrogenase complexes. However, there is some evidence for isoforms of E3 in rat liver mitochondria (Carothers *et al.*, 1987), potato tuber mitochondria (R. Fullerton, Ph.D. thesis, Glasgow University, 1995) and bacteria (McCully *et al.*, 1986; Sokatch *et al.*, 1983).

The decarboxylation and dehydrogenation of 2-oxoacids is catalysed in a sequential and co-ordinated multi-step process involving the activities of multiple copies of the three separate enzymes to generate CO_2 and the corresponding acyl CoA (Reed, 1974; Yeaman, 1989) (see Figure 1.2). E1 catalyses the irreversible decarboxylation of its 2-oxoacid substrate via a covalent adduct of ThDP, with the formation of a 2-(1-hydroxyacyl)-ThDP intermediate and a molecule of CO_2 (see Figure 1.2). This is the overall rate-limiting step of the complexes as a whole (Walsh *et al.*, 1976). E1 then catalyses the reductive acetylation of the S^8-thiol groups of the lipoamide cofactor which is covalently bound to the lipoyl domains located in the N-terminal region of the oligomeric E2 enzyme to form an S^8-acyldihydrolipoamide intermediate. The interchange of the acyl group between the two sulphurs of lipoamide is known to occur in *E. coli* (Yang and Frey, 1986). However, the isomerisation constant was found to be at least three orders of magnitude lower than the overall turnover number for this complex indicating that this reaction is of no physiological relevance.

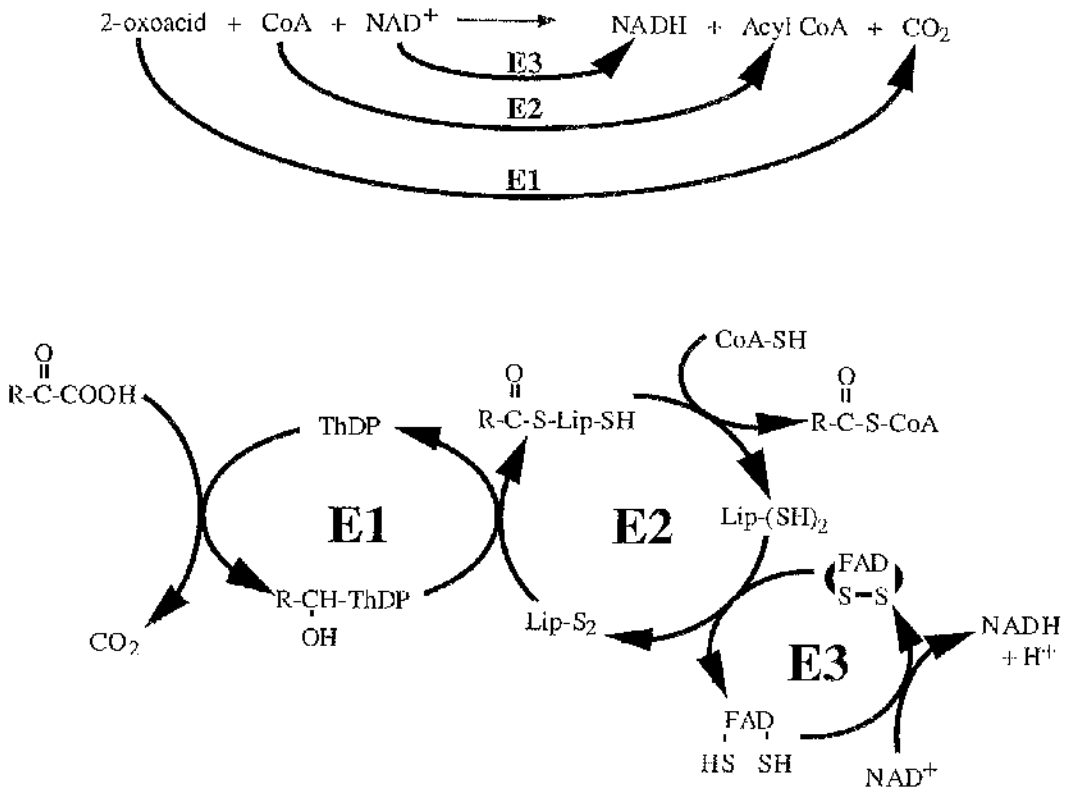

Figure 1.2: Reaction mechanism of the 2-oxoacid dehydrogenase complexes highlighting the specific reactions catalysed by the component enzymes

E1, 2-oxoacid dehydrogenase; E2, dihydrolipoamide acyltransferase; E3, dihydrolipoamide dehydrogenase; ThDP, thiamine diphosphate; Lip, lipoic acid; R=CH_3 for PDC, $COOHCH_2CH_2$ for OGDC, $(CH_3)_2CH$, $(CH_3)_2CHCH_2$ and $(CH_3)_2(C_2H_5)CH$ for BCDC.

The acyl group is then transferred by the E2 active site to the CoA acceptor, thus leaving the lipoic acid in a reduced state. This reduced cofactor is reoxidised by the FAD-containing E3 component which, in turn, becomes re-oxidised with NAD^+ as the final electron acceptor.

1.2.3 Structure of the 2-Oxoacid Dehydrogenase Complexes

Owing to the large size of the complexes, electron microscopy studies have been able to visualise the 2-oxoacid dehydrogenase complexes as particles of 30-40 nm (Henderson *et al.*, 1979; Oliver and Reed, 1982), making them comparable in size to ribosomes. The E2 component forms a symmetrical oligomeric core around which multiple copies of E1 and E3 bind non-covalently (Reed, 1974). Using electron microscopy, two distinct structures of the E2 core could be visualised, which were the cubic form and the dodecahedron, and these were found to be species-specific. All OGDCs and BCDCs studied to date have the cubic core comprising 24 identical subunits of E2 arranged with octahedral symmetry as does PDC from Gram negative bacteria such as *E. coli* and *Azotobacter vinelandii* (Oliver and Reed, 1982; Perham *et al.*, 1987) (see Figure 1.3a). Structural determination of the cubic core of *A. vinelandii* suggested that the building blocks were eight trimers of E2, forming a hollow cavity which was connected to the outside by large channels (Mattevi *et al.*, 1992b). In contrast, the pentagonal dodecahedron is composed of 60 E2 subunits arranged with icosahedral symmetry and is associated with PDC from Gram positive bacterial sources such as *B. subtilis*, yeast such as *S. cerevisiae* and also from mammalian, avian and fungal sources (Lowe *et al.*, 1983; Koha *et al.*, 1982; for review see Perham, 1991) (see Figure 1.3b).

Therefore, two structures of PDC exist, with the cubic E2 core associated with Gram negative bacteria, and the dodecahedral core associated with Gram positive bacteria

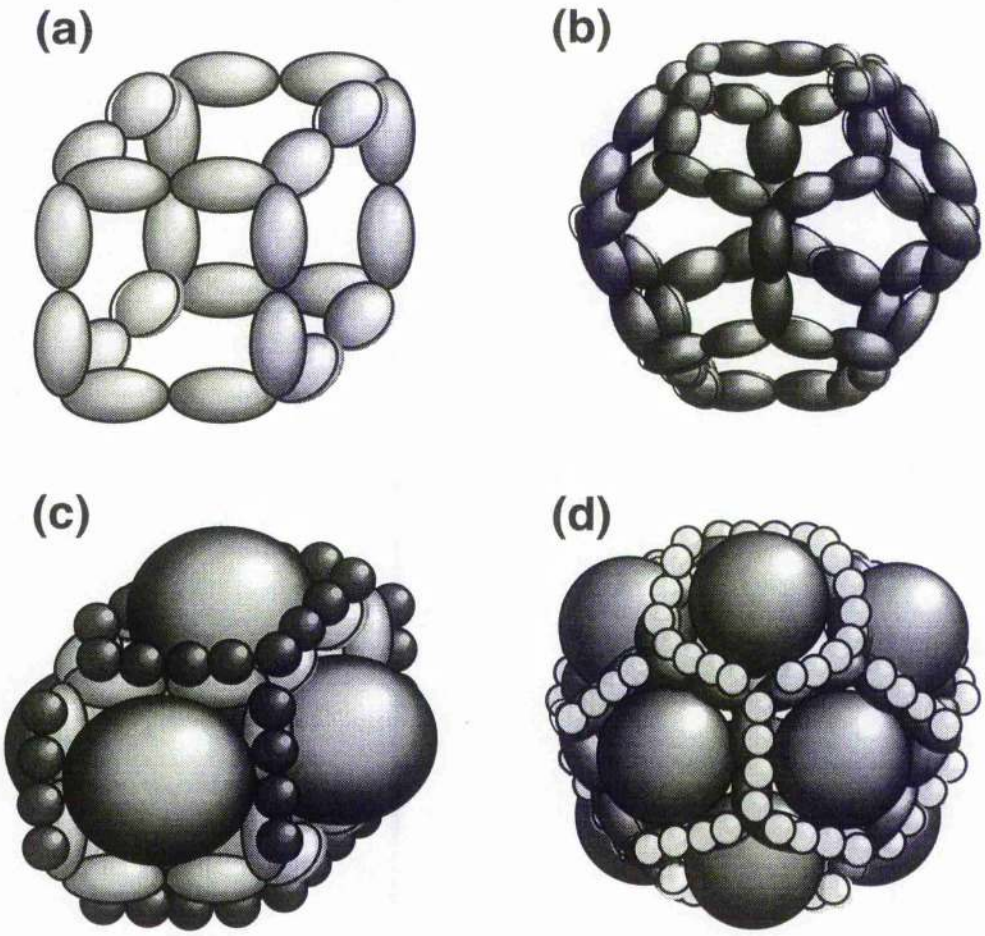

Figure 1.3: Models of the quaternary structure of dihydrolipoamide acyltransferase (E2)

(a) Model of the cubic core of 24 E2 subunits arranged in groups of three about the eight vertices of a cube.

(b) Proposed model of the pentagonal dodecahedral core of 60 E2 subunits arranged in groups of three about the twenty vertices.

(c) Model of the cubic core with 12 E1 dimers bound along the twelve edges of the E2 core and 6 E3 dimers bound along the six faces of the cube.

(d) Model of the pentagonal dodecahedral core with 20 to 30 E1 tetramers bound to the thirty edges and 12 E3 dimers bound to the twelve faces of the pentagonal dodecahedral core.

and mammalian species. The cubic structure forms a complex with octahedral symmetry where 12 E1 dimers bind along the twelve edges of the E2 core and 6 dimers of E3 bind along the six faces of the cube (see Figure 1.3c). The dodecahedral core with 60 E2 subunits, however, forms a complex with 20 to 30 E1 tetramers ($\alpha_2\beta_2$) bound to the thirty edges. Previously, it was thought that 6 E3 dimers bound to the twelve faces of the pentagonal dodecahedral core (Reed *et al.*, 1975; Barcrra *et al.*, 1972) but recent evidence now suggests that 12 E3 dimers bind to the core (Wu and Reed, 1984; Sanderson *et al.*, 1996) (see Figure 1.3d). In mammalian PDC and BCDC, E1 is composed of two non-identical subunits (termed α and β) whereas the E1 component of OGDC is a homodimer (Koike and Koike, 1976).

Mammalian and yeast PDC also contain an additional component, termed protein X, which is found tightly associated with these complexes (De Marcucci and Lindsay, 1985; Jilka *et al.*, 1986). This, however, has not been identified in any other 2-oxoacid dehydrogenase complexes and does not appear to be essential to catalysis, but instead appears to bind E3 to the E2 core (Gopalkrishnan *et al.*, 1989). Protein X was thought to be present in 6 copies in bovine kidney PDC (Jilka *et al.*, 1986) but recent evidence suggests that there are 12 copies of X in PDC from *Saccharomyces cerevisiae* (Mang *et al.*, 1994) and bovine heart PDC (Sanderson *et al.*, 1996). Cross-linking data of the lipoyl domains from bovine heart PDC tentatively suggested that 12 monomers or 6 dimers of protein X bind 6 E3 dimers. In addition to protein X, bovine heart PDC possesses 1 to 3 molecules of a tightly bound kinase and approx. 5 molecules of a loosely associated phosphatase.

1.2.4 Disease States Associated with the 2-Oxoacid Dehydrogenase Complexes

Deficiencies in the activities of the 2-oxoacid dehydrogenase complexes caused by genetic defects can cause various forms of metabolic acidosis where substrates can accumulate to toxic levels. An example of this is "Maple Syrup Urine Disease" (MSUD) which is caused by a deficiency in BCDC (Chuang and Nin, 1981). It is an autosomal recessive inborn error of metabolism involving a dysfunction of the E1 and E2 subunits of BCDC and results in the accumulation of branched-chain amino acids and derived metabolites in the urine of sufferers which can cause severe mental retardation.

Another physical disorder linked to the 2-oxoacid dehydrogenase complexes is Primary Biliary Cirrhosis (PBC). This is a chronic autoimmune disease of the liver characterised by the inflammation of septal and interlobular bile ducts, leading to liver cell damage and cirrhosis (Kaplan, 1987). Patients with PBC are found to have anti-mitochondrial antibodies present in their sera. Six autoantigens have been identified as components of the 2-oxoacid dehydrogenase complexes, including the E2 component of PDC, OGDC and BCDC, component X and PDC E1α and E1β subunits (Yeaman *et al.*, 1988; Fussey *et al.*, 1988; Surh *et al.*, 1989a, 1989b; Fregeau *et al.*, 1989). Approximately 95% of patients with PBC possess autoantibodies against PDC-E2 with its inner lipoyl domain, or more specifically, the site of attachment of the lipoic acid cofactor, being the major site of immunoreactivity (Yeaman *et al.*, 1988; Bradford *et al.*, 1987; Surh *et al.*, 1990).

Deficiencies in OGDC activity have also been recently implicated in Alzheimers disease. Recent studies of Alzheimer's patients revealed both defective OGDC activity and an abnormality of the E2 component, whereas all other mitochondrial proteins were normal, including PDC (Sheu *et al.*, 1994).

1.3 Composition of the 2-Oxoacid Dehydrogenase Complexes

1.3.1 2-Oxoacid Dehydrogenase (E1)

The 2-oxoacid dehydrogenase (E1) enzymes catalyse the initial decarboxylation of the appropriate 2-oxoacid and reductive transfer of the acyl group to a lipoic acid moiety bound to their respective E2. This is the only irreversible reaction and is also the rate-limiting step in the multistep activity of the 2-oxoacid dehydrogenases (Walsh *et al.*, 1976). It is the main regulatory centre of the complexes from mitochondrial sources, via a phosphorylation (inactivation) and dephosphorylation (activation) cycle of the E1α subunit which is performed by a specific kinase and phosphatase (Linn *et al.*, 1972).

The E1 genes of many organisms have been cloned (Patel and Roche, 1990) and comparison of the sequences have revealed three groups. These groups were E1 from OGDCs, E1 from PDCs of Gram-negative bacteria and E1 from eukaryotic PDCs and BCDCs. Sequence comparison between the groups show little homology, and even E1 isolated from PDC and OGDC of the same organism have little homology (Darlinson *et al.*, 1984). The E1 component of the octahedral complexes, namely bacterial and mammalian OGDC and bacterial PDC exist as homodimers, with each subunit possessing a mass of 55,000 Da. The E1 components of the dodecahedral complexes, mammalian PDC and BCDC are, however, heterodimers ($\alpha_2\beta_2$) consisting of two copies of non-identical subunits termed α and β, with a mass of 41,000 Da and 36,000 Da, respectively in PDC (Reed, 1974; Perham, 1996).

In these tetrameric E1 components ($\alpha_2\beta_2$), a common sequence motif of approx. 30 amino acids was found in the α subunit beginning with the highly conserved sequence -GDG- and ending with another highly conserved sequence -NN-. This was thought

to be the binding site of thiamine diphosphate (ThDP), the cofactor required for decarboxylation of the 2-oxoacid (Stepp *et al.*, 1985) and has been confirmed by analysis of the crystal structures of related TPP-requiring enzymes. Two domains possessing the ThDP-binding motif are required for the binding of ThDP, with one binding the diphosphate group and the other the pyrimidine ring. Although each subunit contains both domains, ThDP is bound in a cleft between subunits so that the diphosphate domain belongs to one subunit and the pyrimidine domain to the other subunit (Lindqvist *et al.*, 1992) (see Figure 1.4). The function of the ThDP binding motif found by Hawkins *et al.* (1989) is to bind the diphosphate moiety. Further conserved sequences have been observed in both E1α and E1β, with an E1β binding site in E1α and an E2 binding site in E1β. It has been proposed that E1β is involved in the reductive acetylation of the dithiolane ring of the lipoyl group (Yeaman, 1989; Patel and Roche, 1990). This, however, has yet to be established.

The mechanism of the decarboxylation and acetylation of the lipoyl group on E2 from PDC is reasonably well known, with the resultant production of 2-(1-hydroxyethylidene)-ThDP (see Figure 1.5). This product remains attached to E1 until it is reductively transferred as an acetyl group to the lipoyl moiety attached to E2. The mechanism of this transfer, however, is not clear and two pathways have been proposed for the reaction (Gruys *et al.*, 1989; Frey *et al.*, 1989). The interchange of the acyl group between the two sulphurs of the lipoamide prosthetic group is known to occur in *E. coli* after formation of the S^8-acetyldihydrolipoamide intermediate (Yang and Frey, 1986). As stated previously, this isomerisation has been found to be of no physiological relevance.

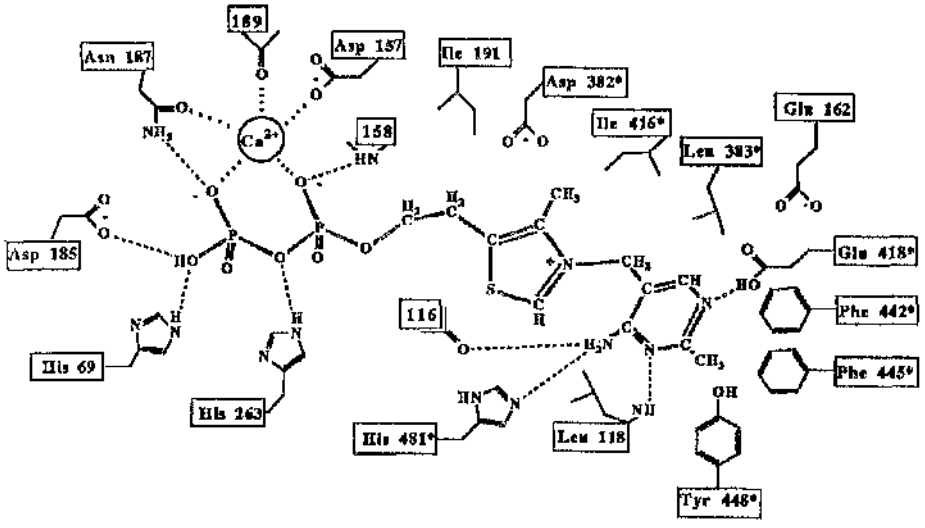

Figure 1.4: Schematic diagram of the interactions of ThDP at the cofactor binding site of transketolase

All residues within 4Å of the cofactor are included in the figure. Residues coming from the second subunit are indicated by an asterisk after the residue number. Possible hydrogen bonds are indicated by dashed lines. Reproduced from Lindqvist *et al.* (1992).

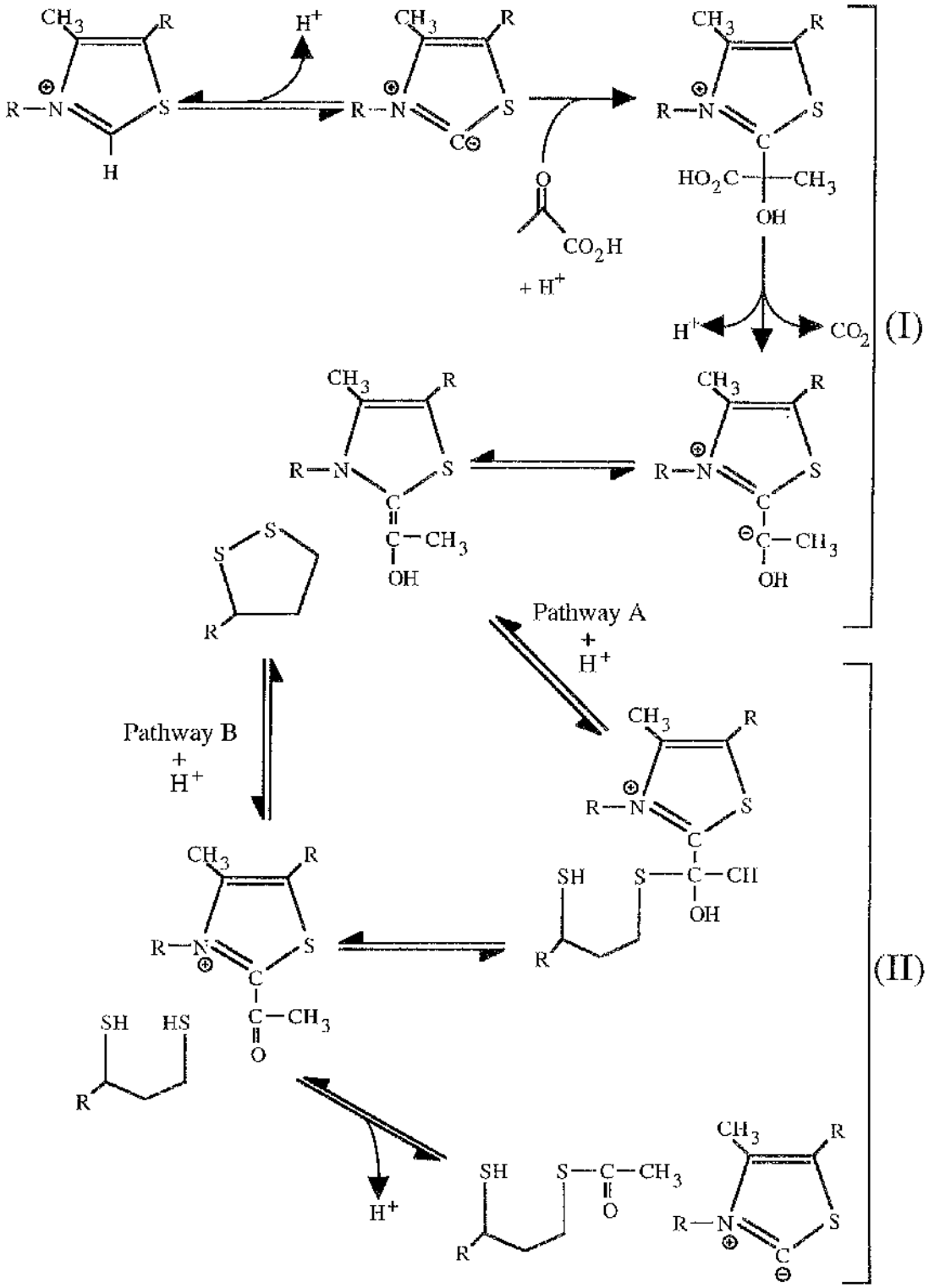

Figure 1.5: Mechanism of decarboxylation of pyruvate by the pyruvate dehydrogenase complex component, E1

(I) E1 forms 2-(1-hydroxyethylidene)-ThDP.

(II) The possible pathways for the reductive transfer of an acetyl group from ThDP to lipoic acid. Pathway A requires acetyl-ThDP as an intermediate, whereas pathway B does not.

1.3.2 Dihydrolipoamide Acyltransferase (E2)

The E2 component of the 2-oxoacid dehydrogenase complexes forms the structural core to which E1 and E3 are able to bind, coupling their activities by providing a flexible lipoyl group and domain which can visit the various active sites, and is also responsible for catalysing the acyltransferase reaction.

Analysis of the various proteolytic cleavage sites in E2 has enabled the approximate limits and functions of the domains and inter-domain linker regions to be defined. This has revealed a highly segmented structure consisting of several functional domains (Bleile *et al.*, 1979; Reed and Hackert, 1990; Perham, 1991) (See Figure 1.6).

1.3.2.1 The Lipoyl Domains

The N-terminal region of the E2 polypeptide consists of one to three highly homologous lipoyl domains of approx. 80 amino acids each, and are joined by flexible linker peptides, approx. 25-30 amino acids long and rich in alanine, proline and various charged amino acids (Dardel *et al.*, 1991). These lipoyl domains contain the lysine groups to which the lipoyl cofactors are covalently bound by an amide linkage, while the linker regions are essential for PDC activity by providing the lipoyl domain(s) with the mobility required for interaction with the active sites of the E1 and E3 components. The post-translational modification of the attachment of the lipoyl group to the E2 polypeptide is performed by specific ligases, which recognise not only the specific domain, but also the specific lysine residue (Brookfield *et al.*, 1991). No conformational changes are caused by lipoylation, which is in agreement with the idea that the lipoyl-lysine is a free-swinging arm in solution (Dardel *et al.*, 1990; Perham, 1996).

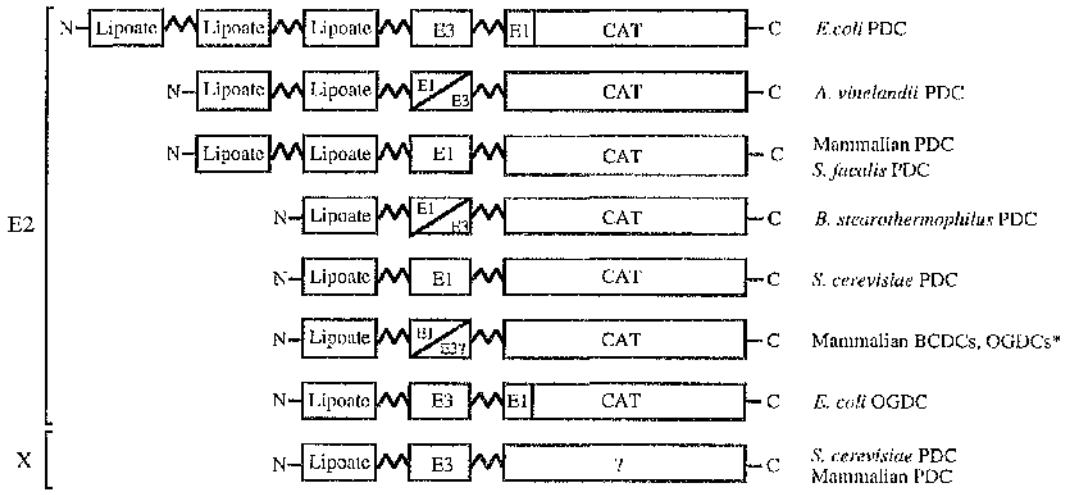

Figure 1.6: Schematic representation of the domain structure of the E2 and protein X components

E3/E1, E3 and/or E1 binding domain; CAT, catalytic core domain; Zigzags represent the linker regions. The domain structure of protein X is shown for comparison.

*Mammalian OGDC has been shown to lack the E3 binding domain. E3 binding sequences are found on E1 and the E1 binding sites are unknown as yet (Rice *et al.*, 1992).

The reason why some E2 polypeptides have three lipoyl domains (PDC of *E. coli* and *A. vinelandii*), others two (mammalian PDC) and some, for example BCDC, OGDC and yeast PDC, have only one domain remains obscure as there is no correlation with the source of the complex (for review, see Perham, 1991). Using site-directed mutagenesis and deletion analysis it has been shown that only one active lipoyl domain is necessary for complex activity in *E. coli* and it has been proposed that the extra domains are needed when substrate or cofactor concentrations become limiting. Those complexes containing one to three lipoyl domains have been shown to exhibit optimal PDC activity and linker mobility, both of which decreased in the presence of more than three lipoyl domains (Machado *et al.*, 1992, 1993). Linker mobility also decreased with one or two domains compared with three domains, although these complexes when purified possessed similar specific activities. It has been demonstrated that wild-type PDC (containing three lipoyl domains) confers a growth advantage in *E. coli* compared to strains expressing PDC with fewer lipoyl domains (Guest *et al.*, 1996). In fact, as long as the outer domain is lipoylated, the PDC activity is similar to the wild-type PDC, with three lipoylated domains, and higher than PDC with either of the other two lipoyl domains lipoylated.

The attachment of the lipoyl cofactor to the E2 polypeptide is an important structural feature for catalysis in all complexes. Its ability to act as a substrate for E1, when attached, is enhanced 10,000 fold, as judged by the K_m/K_{cat} factor, compared to the free substrate. Moreover, a lipoylated decapeptide with an amino acid sequence identical to that surrounding each of the three lipoyl-lysine residues in the *E. coli* E2 chain of PDC was ineffective as a substrate, indicating that a folded lipoyl domain is essential to the reaction (Graham *et al.*, 1988). The DKA motif, found on either side of the lipoylated lysine, is not required for recognition by the ligase enzymes, but is important for recognising E1, as replacement of the aspartic and alanine residues resulted in a decrease in the rate of reductive acetylation (Wallis and Perham, 1994).

The lipoamide cofactor, covalently bound to a lysine residue, is very flexible, forming 14Å long 'swinging arms' which facilitate interaction between the three active sites (Reed, 1974). Extra flexibility is provided by the mobile linker regions, which give the lipoyl domains their extended conformation (Perham *et al.*, 1981). In the intact complex, the lipoyl domains and the subunit-binding domain extend outwards from the inner E2 core, interdigitating between the E1 and the E3 components allowing the E1 and E3 catalysed reactions to take place outside the E2 core, while the acyltransferase reaction takes place in the inner E2 core (Perham, 1996).

1.3.2.2 The Peripheral Subunit Binding Domain

Following the lipoyl domains, there is another domain of approx. 50 amino acids which appears to bind E3 to the E2 core, named the peripheral subunit binding domain. The degree of sequence conservation in this domain is consistent with the fact that E3 subunits from one source will often complement the overall enzyme complex activity of E1 and E2 subunits from a heterologous source and also form hybrid high mass complexes. In complexes such as *A. vinelandii* (Hanemaaijer *et al.*, 1988), *B. stearothermophilus* (Packman *et al.*, 1988) and mammalian BCDC (Lau *et al.*, 1992), this domain also functions as a major contributor to binding E1 to the E2 core. It has been shown in these complexes that E1 and E3 do not bind simultaneously but compete for the space on the binding domain of E2. In contrast, it is thought that the subunit binding domain of mammalian PDC and OGDC E2 binds only E1. The crystal structures of *B. stearothermophilus* unexpectedly revealed a molar ratio of 1:1 for E3 binding to the E2 core, where each subunit of the E3 component were both providing interacting surfaces (Hipps *et al.*, 1994; Mande *et al.*, 1996). There were actually two E3 binding sites on E2 but as they are located near the two-fold axis of symmetry, it is only possible for one site to bind E3 due to steric hindrance.

1.3.2.3 Acyltransferase Inner-Core Domain

The subunit-binding domain is linked, in turn, to the C-terminus of the E2 polypeptide, which consists of a compact inner domain containing the E2 binding sites and allowing the E2 polypeptides to interact with one another, thus forming the core of the complex. In addition, this domain contains the catalytic site of the acyltransferase (Packman *et al.*, 1988). Of the whole E2 polypeptide the catalytic domain contains the most conserved amino acids. His 602 in *E. coli* PDC E2 is highly conserved due to its importance to the active site and is surrounded by a highly conserved motif, -HXXXDG-, in a region that is shared by the active site region of chloramphenicol acetyltransferases (Guest, 1987; Russell and Guest, 1990). Analysis of the bovine acetyltransferase has suggested that the reaction proceeds via a random sequential (rapid equilibrium) mechanism (Butterworth *et al.*, 1975). X-ray crystallography of the cubic complex of *A. vinelandii* (Mattevi *et al.*, 1992a, 1993a, 1993b) and site-directed mutagenesis of E2 from *E. coli* (Russell and Guest, 1990; Russell *et al.*, 1992), ox (Griffin and Chuang, 1990; Meng and Chuang, 1994) and *S. cerevisiae* (Niu *et al.*, 1990) have revealed the important roles of histidine, aspartate, asparagine and serine in the catalytic site (see Figures 1.7 and 1.8).

1.3.2.4 Three-Dimensional Structure of E2

Owing to the highly segmented structure and the mobile lipoyl domains preventing growth of well-ordered crystals, the crystallisation of the intact E2 subunit has not been possible (De Rosier *et al.*, 1971; Fuller *et al.*, 1979). Therefore, structural determination has concentrated on separate domains and the linkers that join them together. The structures of the E3 binding domain (35 amino acids) of the E2 component of *E. coli* OGDC (Robien *et al.*, 1992), *B. stearothermophilus* PDC (Kalia *et al.*, 1993), the lipoyl domain of *B. stearothermophilus* (Dardel *et al.*, 1993) and *E. coli* PDC (Green *et al.*, 1995) have been solved using NMR spectroscopy. The lipoyl domain is composed of two β-sheets forming a flattened barrel which results in a core of hydrophobic residues with the lipoyl-lysine perched at the tip of an exposed β-turn

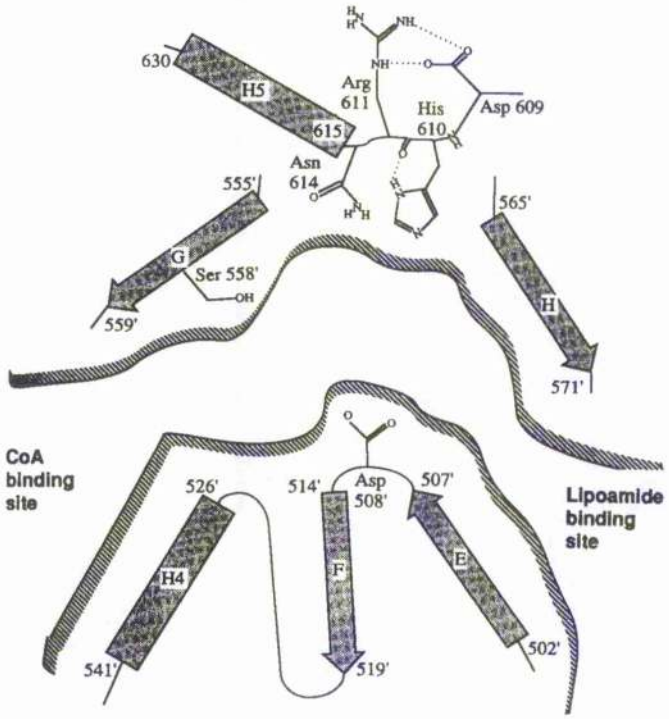

Figure 1.7: Schematic representation of the E2 catalytic core

The 29Å long channel goes across the subunit trimer, with the CoA and lipoamide binding sites located at the two opposite entrances, as derived by analogy with chloramphenicol acetyltransferase (CAT). Reproduced from Mattevi *et al.* (1992b).

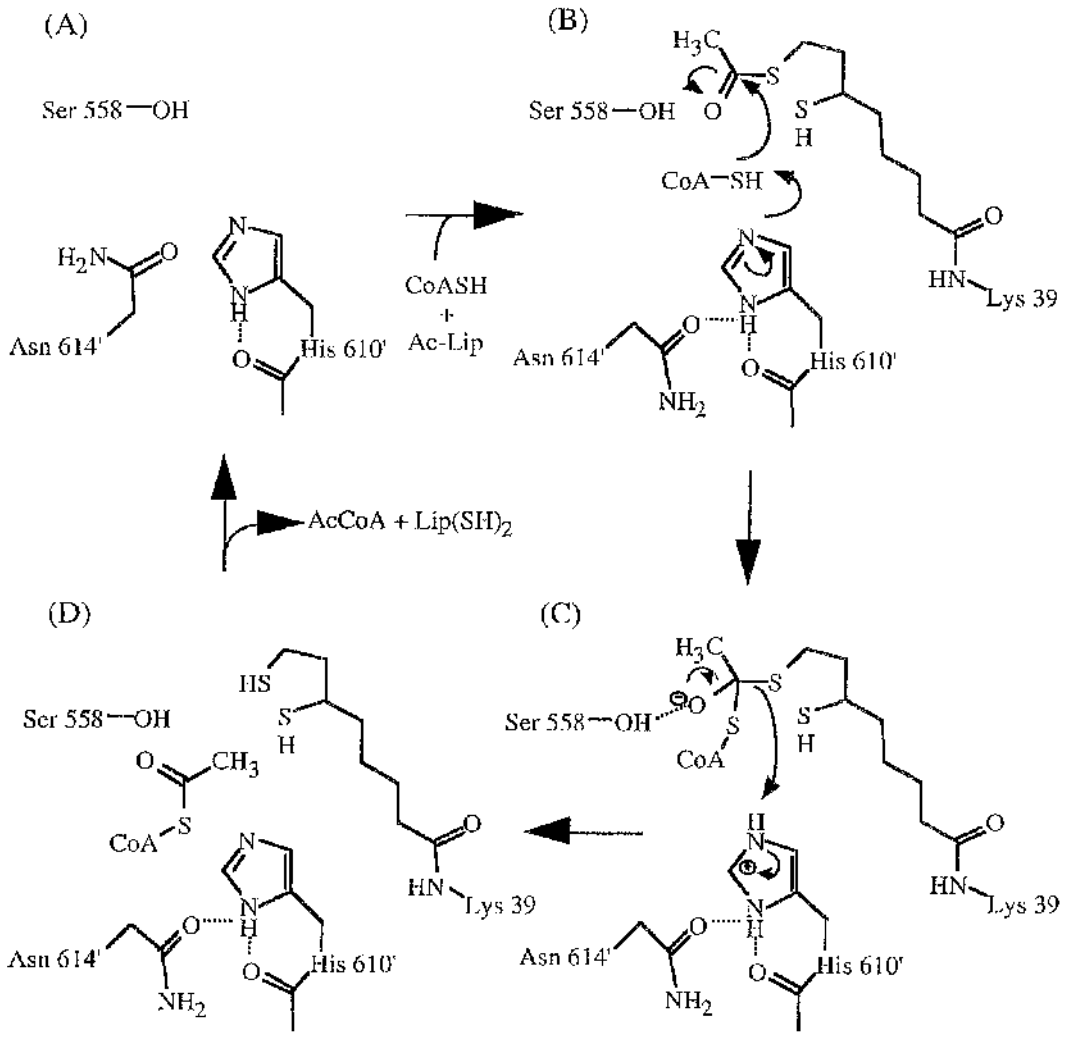

Figure 1.8: Catalytic cycle of the acetyltransferase reaction
Unprimed residues are provided by different subunits in the E2 trimer from the primed residues. (A) In the ground state, the side chain of Asn 614' is engaged in a hydrogen bond with Asp 508 (not shown), thereby blocking the CoA-binding channel at the pantetheine moiety of CoA. (B) During binding of reduced CoA (CoASH), a reorientation of the Asn 614' side chain allows the accommodation of the CoA, and the formation of a new hydrogen bond from the reactive sulphydryl group of CoA. The sulphur atom subsequently attacks the carbonyl carbon of the acetyl moiety of acetyl-dihydrolipoamide. (C) The tetrahedral intermediate is stabilised by a hydrogen bond to Ser 558. Rearrangement of the trigonal planar acetyl carbonyl group results in the simultaneous release of dihydrolipoamide and acetyl-CoA. (D) The cycle is completed by the release of acetyl CoA and dihydrolipoamide from the active site, and the rearrangement of Asn 614'. Reproduced from Hendle *et al.* (1995).

(see Figure 1.9), a feature shared with the biotinyl-lysine swinging arm in the biotinyl domain of pyruvate decarboxylase (Perham, 1996). Additionally, the structure of the catalytic domain of E2 from *Azotobacter vinelandii* PDC has been determined by X-ray crystallography and has been shown to be similar to chloramphenicol acetyltransferase (CAT) (Mattevi *et al.*, 1992a) (see Figure 1.10). CAT (EC 2.3.1.38) catalyses the O-acetylation and subsequent inactivation of chloramphenicol in antibiotic resistant bacteria, using acetyl-CoA as the acyl donor in the inactivation reaction. The C-terminal domain of the PDC E2 enzyme that contains the lipoate acetyltransferase activity also shows remote, but significant sequence similarity with CAT suggesting a resemblance between the mechanisms of E2 and CAT (Guest, 1987).

1.3.3 Dihydrolipoamide Dehydrogenase (E3)

Dihydrolipoamide dehydrogenase is a member of the group of flavoprotein disulphide oxidoreductases (Carothers *et al.*, 1989). It is the third enzymatic component of the three 2-oxoacid dehydrogenase complexes, the distantly related glycine decarboxylase complex (GDC) where it is named L-protein, and the bacterial acetoin dehydrogenase system involved in acetoin utilisation. In all these complexes it is responsible for the NAD^+-dependent reoxidation of the dihydrolipoamide groups which are bound to lysine residues on the acyltransferase component of the 2-oxoacid dehydrogenase complexes or the hydrogen carrier protein (H-protein) of GDC. This allows the acyltransferase component to re-enter the catalytic cycle (Kikuchi and Hiraga, 1982; Carothers *et al.*, 1989; Yeaman, 1989). To date, all E3 enzymes are reported to exist as homodimers with each subunit containing one molecule of non-covalently bound FAD and with a molecular mass of around 50,000 Da. E3 has been isolated from a wide variety of sources and has shown a high degree of sequence conservation, with porcine heart E3 having 96% identity with human E3 and 44% identity with *E. coli*

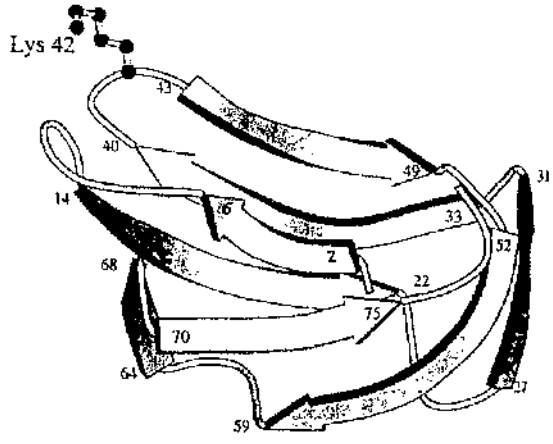

Figure 1.9: Three-dimensional structure of the polypeptide fold of the lipoyl domain of *B. stearothermophilus*

The lipoylation site, Lys 42, is shown on the exposed β-turn containing the lipoyl-lysine residue. Reproduced from Dardel *et al.* (1993).

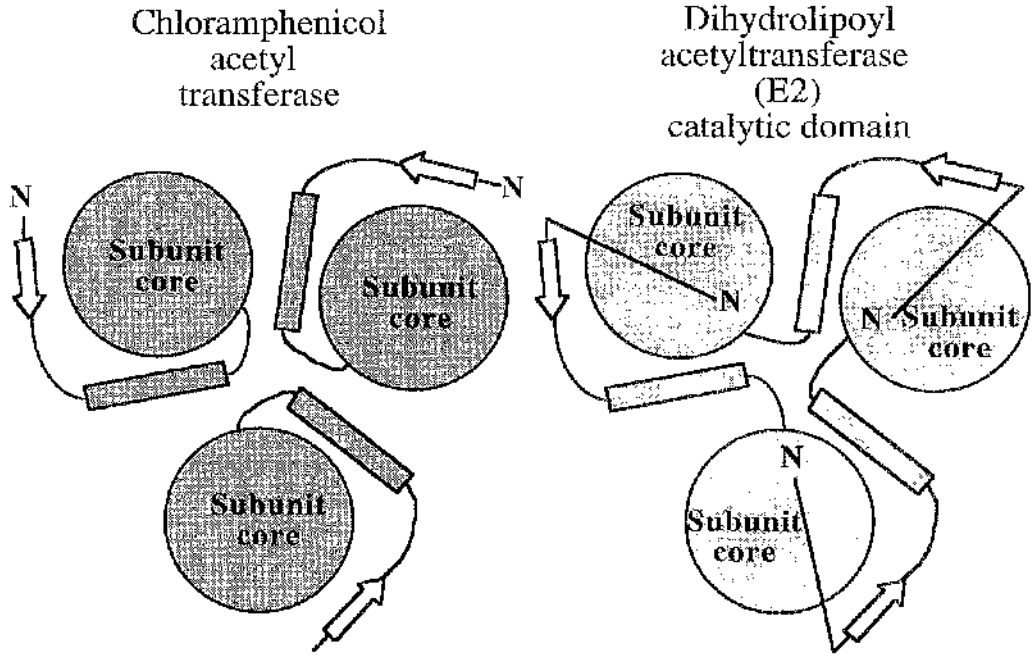

Figure 1.10: Schematic representation of the trimer organisation of the E2 catalytic domains of *A. vinelandii* compared with chloramphenicol acetyltransferase

The N-terminal strand is arranged differently in the two enzymes. Reproduced from Mattevi *et al.* (1992b).

E3.

With the explosion in purification and cloning of E3 in recent years, the idea that a single E3 served the entire family of 2-oxoacid dehydrogenase complexes including GDC within the same species has become increasingly untenable, despite the fact that this enzyme performs the same reaction in all complexes. There is now increasing evidence for the existence of differing isoforms with two immunologically distinct E3s located in rat liver mitochondria, where it has been suggested that the E3 associated with GDC is distinct from that associated with the 2-oxoacid dehydrogenase complexes (Carothers *et al.*, 1987). *Pseudomonas putida* and *P. aeruginosa* are bacteria in which two genetically distinct forms of E3 have been isolated. One form of E3 (LPD-val) is specific for BCDC and is found in the BCDC operon. It is induced in media containing branched-chain amino acids as a carbon source (McCully *et al.*, 1986; Sokatch *et al.*, 1981). This E3 is distinct from the E3 (LPD-glc) found in association with PDC, OGDC and GDC which is induced in glucose synthetic medium (Sokatch and Burns, 1984; Sokatch *et al.*, 1983). A third E3 (LPD-3) has been isolated from *P. putida* (Burns *et al.*, 1989) and appeared able to replace LPD-glc as the component for PDC and OGDC, but was not part of a 2-oxoacid dehydrogenase operon. The precise role of LPD-3 is unknown but it has been suggested that it may be a component of the newly discovered acetoin dehydrogenase complex (Oppermann and Steinbuchel, 1994), and primary sequence comparisons of LPD-3 have demonstrated that it is more closely related to eukaryotic E3. An atypically small E3 subunit associated with GDC has recently been identified in the anaerobic bacterium *Eubacterium acidaminophilum*, and appears to be the first E3 specific to GDC characterised to date (Freudenberg *et al.*, 1989).

While the E3 gene is found either on the PDC operon, or the OGDC operon as in *E. coli* and *A. vinelandii*, recent cloning of the PDC and OGDC operons in *Alcaligenes eutrophus* found genetically distinct E3s for both PDC and OGDC (Hein and

Steinbuchel, 1996). The enzymes are not closely related, but it is unknown whether they are interchangeable. The E3 associated with PDC was found to be a new class of enzyme, containing a lipoyl domain at the N-terminus which was separated from the rest of the enzyme by a flexible hinge region, as in E2 (Hein and Steinbuchel, 1994). Only the E3 component (*aco*L gene product) of the acetoin dehydrogenase enzyme system of the strictly fermentative, gram-positive bacterium *Clostridium magnum* shares this property.

E3 has also been found to exist as an independent enzyme, in the absence of 2-oxoacid dehydrogenase complexes or GDC, in the bloodstream form of the eukaryotic parasitic protozoan *Trypanosoma brucei* (Richarme and Heine, 1986) and also in prokaryotic halophilic and thermophilic archaebacteria (Danson *et al.*, 1987). E3 from *T. brucei* was found to possess an essential catalytic disulphide bond and is closely associated with the plasma membrane. However, it is unknown whether it performs a similar role to a second E3 found in *E. coli*, which is thought to be involved in the transport of maltose and galactose. There were no sequence similarities with either *E. coli* or porcine heart E3 despite the similarities in catalytic function, which may be a result of adaptation by *T. brucei* to high salt conditions (Danson *et al.*, 1986).

In eukaryotes, the evidence of multiple isoforms for E3 associating with specific 2-oxoacid dehydrogenase complexes or GDC is somewhat conflicting. In yeast, a mutation in the *lpd1* gene causes the loss of all PDC and OGDC activity (Dickinson *et al.*, 1986), while immunological data (Matuda and Saheki, 1985) and reconstitutional studies (Sakurai *et al.*, 1970) in mammals all indicated that the same E3 protein is used by PDC, OGDC and BCDC. Evidence for a single copy of E3 in humans comes from the studies of lactic acidosis, which is caused by genetic lesions affecting the production of dihydrolipoamide dehydrogenase. The subsequent symptoms are an accumulation of pyruvate, 2-oxoglutarate and branched-chain amino acids in the serum of those afflicted due to the activities of PDC, OGDC and BCDC being affected

(Robinson *et al.*, 1977). The E3 common to the mammalian 2-oxoacid dehydrogenase complexes is also thought to be common to the glycine decarboxylase complex, although intact complexes have yet to be isolated from mammals. The L-protein gene for GDC from pea leaf mitochondria has been cloned (Bourguignon *et al.*, 1992; Turner *et al.*, 1992) and subsequent comparison with E3 from PDC from the same organelle confirms the presence of a single dihydrolipoamide dehydrogenase complementing both PDC and GDC in pea mitochondria (Bourguignon *et al.*, 1996).

1.3.3.1 Enzymatic Mechanism of E3

E3 reoxidises the reduced lipoamide cofactor on E2 using FAD which in turn becomes re-oxidised with NAD^+ as the final electron acceptor, thus allowing E2 to re-enter the catalytic cycle. The active site of E3 has several essential components. These are a lipoamide-lysine binding region, a redox-active disulphide bridge, hydrogen-bonded histidine and glutamate side chains, an FAD cofactor and an NAD^+ binding site. The reaction has a ping-pong kinetic mechanism, consisting of two half reactions. The enzyme, E, is initially reduced to EH_2, a two electron-reduced form with the electron shared between the flavin and the reactive disulphide in a charge transfer complex. EH_2 is then re-oxidised to E, with the electrons passing from the reduced enzyme to NAD^+ via the flavin. E3 is only able to bind NAD^+ properly and donate the reducing equivalents to the substrate in the EH_2 form. A third oxidation state, EH_4, where both the flavin and disulphide are fully reduced ($FADH_2$ and Cys-SH/Cys-SH) can be formed by the addition of excess NADH.

Through the use of bifunctional arsenoxides (Adamson *et al.*, 1984; Holmes and Stevenson, 1984) and site-directed mutagenesis (Kim and Patel, 1992; Benen *et al.*, 1992a, 1992b), a histidine-side chain has been identified as being directly involved in catalysis (see Figure 1.11). This histidine is found on the opposite subunit to the reactive disulphide and its role is to deprotonate the dihydrolipoyl cofactor on E2 to generate a thiolate anion, activating it for nucleophilic attack on the disulphide group

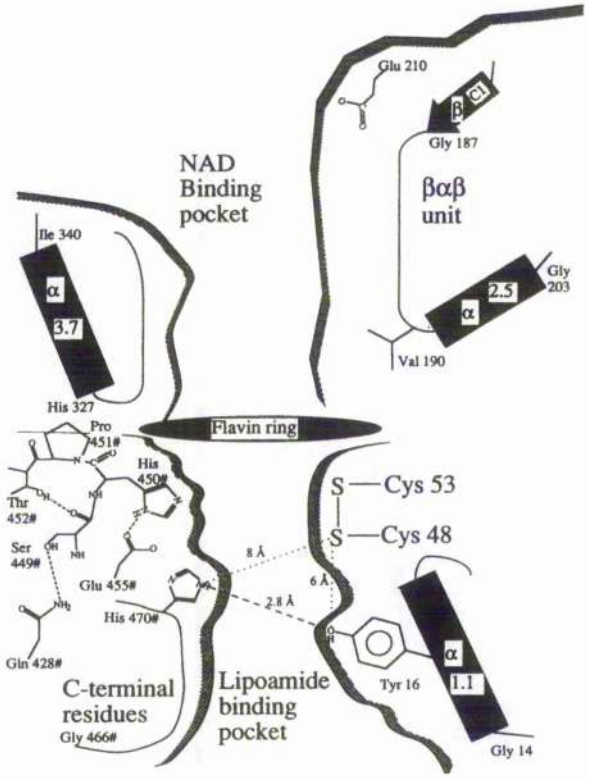

Figure 1.11: A representation of the catalytic centre of *P. fluorescens* dihydrolipoamide dehydrogenase

The figure outlines the active site channel interrupted by the flavin ring, thereby creating two substrate binding sites (residues indicated with # belong to the second subunit). His 450# is crucial for proton abstraction of reduced lipoamide (Williams, 1992; Karplus and Schulz, 1989). Cys 53 and Cys 48 form, in the oxidised form, the redox reactive disulphide. The side chains of Tyr 16 and His 470# are H-bonded to each other and more than 6Å away from the reactive disulphide bridge. However, they exercise a crucial influence on the redox properties of the enzyme. Reproduced from Mattevi *et al.* (1993c).

of the other subunit. Through crystallographic studies, the imidazole ring has been found to be hydrogen-bonded to a conserved glutamate residue and this leads to the increase in pK_a of the histidine that is necessary for the deprotonation of the dihydrolipoyl moiety and stabilisation of the thiolate ion. Other mutagenesis studies have revealed a glutamate side chain important for the stabilisation of the transition state (Benen *et al.*, 1992a, 1992b; Kim and Patel, 1992), C-terminal residues important for the conformational stability (Schulze *et al.*, 1991) and a conserved tyrosine residue located in the N-terminus which is also important in conformational stability. It is thought that a hydrogen bond is formed between this tyrosine and a histidine of the other subunit. Both these decreases in stability lead to over-reduction and inhibition by NADH.

1.3.3.2 Three-Dimensional Structure of E3

A number of dihydrolipoamide dehydrogenases have had their three-dimensional structure determined by X-ray crystallography. These include *Pseudomonas fluorescens* E3 (Mattevi *et al.*, 1993c), *P. putida* E3 (LPD-val) (Mattevi *et al.*, 1992b), yeast E3 (Takenada *et al.*, 1988) and *A. vinelandii* E3 (Schierbeek *et al.*, 1989; Mattevi *et al.*, 1991). These have shown a high similarity to other pyridine nucleotide-disulphide oxidoreductases, such as glutathione reductase (Karplus and Schulz, 1987), mercuric reductase (Schiering *et al.*, 1991), NADH peroxidase (Stehle *et al.*, 1991), tryptanothione reductase (Kuriyan *et al.*, 1991a) and thioredoxin reductase (Kuriyan *et al.*, 1991b), especially around the active site. Despite these similarities around the active site, significant differences in tertiary and quaternary structure were apparent between E3 and the other oxidoreductases, and are probably related to E3 possessing additional regions on the surface of the protein to allow it to function as a component of a multienzyme complex.

The structure of dihydrolipoamide dehydrogenase from *A. vinelandii* is thought to be typical of most E3 enzymes (see Figure 1.12). This enzyme consists of four domains

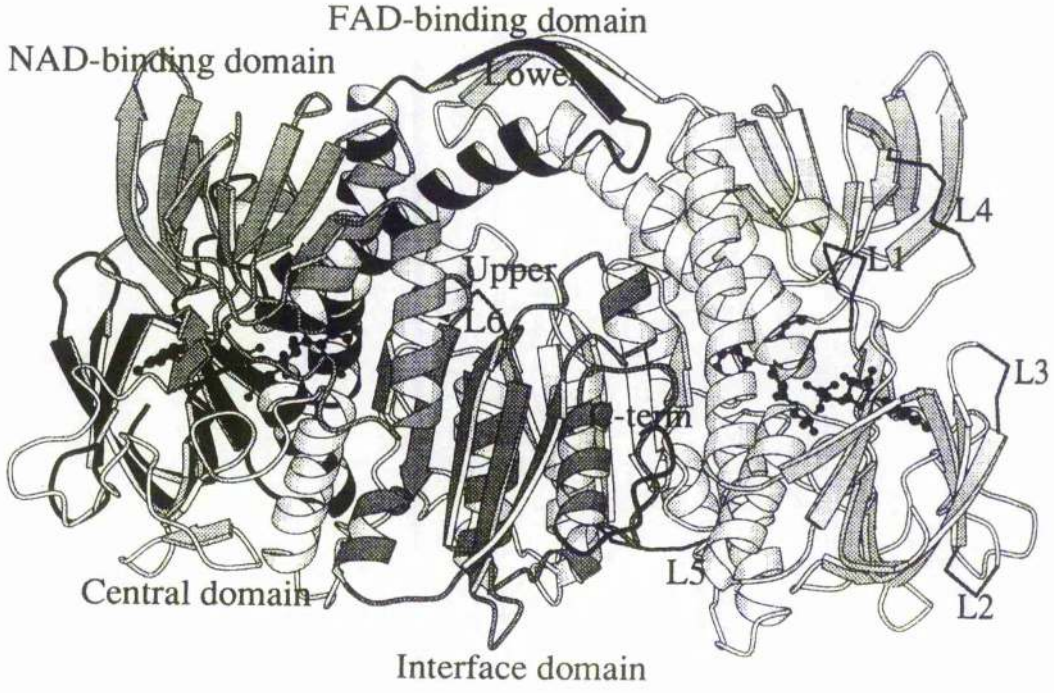

Figure 1.12: Ribbon diagram of *P. fluorescens* dihydrolipoamide dehydrogenase dimer viewed perpendicular to the 2-fold axis

The two subunits can be distinguished by the grey colour of the outer surfaces of the helices and strands of the first subunit. The FAD-binding domain (residues 1 to 150), the NAD^+-binding domain (residues 151 to 280), the central domain (residues 281 to 350) and the interface domain (residues 351 to 472) can be seen in each monomer. In the second subunit, the position of the six previously ill-defined loops are indicated by L1 (residues 37 to 44), L2 (125 to 128), L3 (135 to 137), L4 (158 to 168), L5 (372 to 381) and L6 (389 to 398). The C-terminal tail (466 to 472) of the first monomer is highlighted by the black colour of the ribbon. The black atoms indicate FAD molecules. "Upper" and "Lower" indicate the two regions of the subunit interface, which are separated by a large cavity. Reproduced from Mattevi *et al.* (1993c).

per subunit, with the largest domain, the FAD-binding domain (residues 1-150), interacting with the other three domains. These domains are an NAD^+-binding domain (residues 151-280), a central domain (281-349) and an interface domain (350-466). The two catalytic sites of the E3 dimer contain reactive disulphide groups and are located at the interface between the two subunits of the dimer, with both subunits contributing residues to each active site. When the subunits are in the dimeric form, 16% of the accessible surface becomes buried, involving 95 of the 472 ordered residues, and this produces two major contact areas. The first corresponds to the so-called "lower" region in glutathione reductase (Karplus and Schulz, 1987) and is formed by the loop (residues 61 to 114) which connects two long helices in the FAD domain. The second and larger contact area is formed by the interface domain (residues 350-466) which interacts with the other subunit across the 2-fold axis to form the "upper" interface region. This area contributes the majority of the buried surface and is characterised by the presence of a large number of hydrogen bonds.

1.3.4 Protein/Component X

In addition to the three constituent enzymes, E1, E2 and E3, mammalian and yeast PDC has been demonstrated to contain a tightly associated polypeptide, called protein or component X, with a subunit M_r of 50,000 Da. This polypeptide was initially thought to be a proteolytic fragment of E2, but immunological studies found that antiserum raised to protein X elicited no cross-reaction with E2 or any other component of PDC (De Marcucci and Lindsay, 1985). Protein X contains a lipoate domain and has a structure similar to E2 as deduced from limited proteolysis of bovine heart PDC (Neagle and Lindsay, 1991). Further studies revealed that protein X is tightly bound to the complex and recent evidence suggests there are 12 copies of the polypeptide bound to the complex (Sanderson *et al.*, 1996). It was originally suggested that as protein X is so tightly bound in PDC, it is integrated into the core

complex during the assembly of the E2 core (Rahmatullah *et al.*, 1989). However, evidence exists for a post-assembly interaction in mammals, which is consistent with results from yeast which indicate that the oligomeric E2 is able to form in the absence of protein X (Lawson *et al.*, 1991) leaving the role of protein X unclear.

One possibility is that it could be involved with the binding of the E3 component and facilitating its participation in electron transfer (Gopalakrishnan *et al.*, 1989; Neagle and Lindsay, 1991; Lawson *et al.*, 1991). The lipoyl domain of protein X is capable of becoming reductively acetylated (Jilka *et al.*, 1986; Hodgson *et al.*, 1986) but apparently serves no catalytic function. More recent evidence suggests that protein X has a strong role in binding E3 to the E2 core (McCartney *et al.*, 1997). To date, there is no evidence for the existence of a protein X in PDC from prokaryotes, nor in OGDC or BCDC. However, the sequence of the human and rat E2 gene showed that it lacked the highly conserved E3 and/or E1 binding domain found in all other E2s from OGDC (Nakano *et al.*, 1991; Nakano *et al.*, 1994). Proteolytic analysis revealed that the N-terminus of E1 from bovine heart OGDC was involved in stabilising the interaction of E3 with the assembled complex (Rice *et al.*, 1992). This was thought to be the result of domain shuffling, since sequences are found in the N-terminal region of the E1 enzyme that are related to the lipoyl domain of protein X and its putative E3 binding domain. The E1 component is thought to bind to a sequence in the inner catalytic domain in the eukaryotic OGDC which is yet to be identified.

1.4 Regulation of the Complexes

As the 2-oxoacid dehydrogenase complexes occupy key positions in intermediary metabolism, their activities are subject to strict control. PDC links glycolysis with the TCA cycle and as pyruvate is also used in gluconeogenesis, it is therefore subject to short-term and long-term regulation by nutrient changes. The analogous complexes

from both bacteria and mammals are subject to product inhibition, where the products of the reaction, various acyl-CoAs and NADH, inhibit the multienzyme complexes and this inhibition is reversed competitively by the substrates CoA and NAD^+, respectively. More specifically, the appropriate CoA derivative inhibits the E2 component and NADH inhibits E3 (Koike and Koike, 1976; Yeaman, 1986).

There is no evidence for the phosphorylation of OGDC. Instead the activity state of the complex is governed by a range of allosteric effectors. The complex is predominantly sensitive to the concentration of free Ca^{2+} which decreases the K_m for 2-oxoglutarate (McCormack and Denton, 1979; Denton and McCormack, 1985). In addition, an increase in the ADP:ATP ratio activates the complex by altering the affinity of the E1 enzyme for 2-oxoglutarate, resulting in a decrease in the K_m. This is antagonised by ATP which increases the K_m for 2-oxoglutarate (Lawlis and Roche, 1981). Control of OGDC activity by the energy status of the cell is important, since energy metabolism can be effectively regulated by increasing the flux of carbon atoms around the TCA cycle and away from other biosynthetic pathways when energy demands are high.

1.4.1 Kinases Associated With PDC and BCDC

Besides regulation by product inhibition, mammalian PDC and BCDC are also regulated by reversible phosphorylation of the E1α subunit (Yeaman, 1986, 1989) (see Figure 1.14). This phosphorylation occurs at three specific serine residues on E1α from PDC and at two residues on E1α from BCDC (Cook *et al.*, 1983, 1984; Yeaman *et al.*, 1978). The principal phosphorylation site on both PDC and BCDC has been identified as site 1 (see Figure 1.13). The effect of phosphorylation at this site causes a dramatic reduction in the V_{max}.

PDC Ile-Gly-His-His-**Ser**-Thr-Ser-Asp-Asp-

BCDC Tyr-His-Gly-His-**Ser**-Met-Ser-Asp-Pro-

Figure 1.13: Primary sequence of phosphorylation site one (underlined and bold) on the E1α polypeptides of PDC and BCDC

Site 1 is located at the carboxyl-terminal third of the E1α subunit from PDC and BCDC, and despite both sites having very similar primary structures, their respective kinases are complex specific (Paxton and Harris, 1984). Recent site-directed mutagenesis studies have demonstrated that phosphorylation at the additional sites also causes inactivation, although the rates of inactivation are lower than at site 1 (Korotchkina and Patel, 1995).

In both PDC and BCDC, the complex-specific kinases are bound tightly to the E2 component and copurify with their respective complexes. In bovine PDC, as few as one molecule of kinase is bound to the E2 core, while twenty to thirty molecules of E1 are present. The PDC-specific kinase was originally thought to consist of two non-identical subunits, termed K1, a catalytic subunit, and K2, a regulatory subunit, with M_r values 48,000 and 45,000 respectively (Stepp *et al.*, 1983). However, there is very little evidence for this conclusion as the K1 subunit can bind to the inner lipoyl domain region of E2 and function as a kinase alone. It has been proposed that the subunits form dimers of $[K1]_2$, $[K1K2]_2$ and $[K2]_2$ (for review, see Roche *et al.*, 1996). These dimer states could explain the "hand over hand" mechanism at the surface of the E2 core, which involves the continuous partial dissociation by one subunit followed by the interchange to another lipoyl domain (Liu *et al.*, 1995).

The genes representing the two subunits have been cloned and are very closely related to each other. They have greatest homology with prokaryotic histidine protein kinases, which may reflect the evolutionary origin of mitochondria (Popov *et al.*, 1993, 1994). Both isoforms exhibit kinase activity, although K2 had a lower specific activity

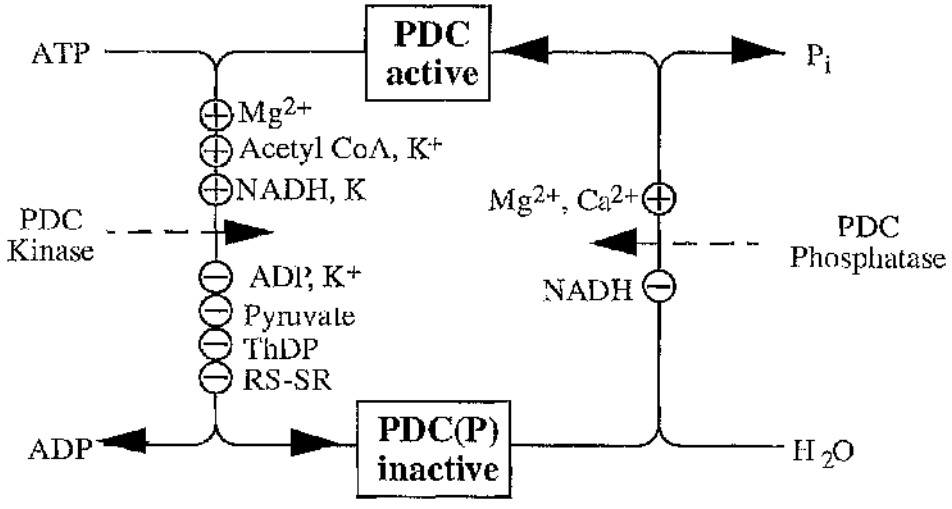

Figure 1.14: Representation of the control of mammalian pyruvate dehydrogenase by the covalent modification and effector molecules

than that of K1 (Stepp *et al.*, 1983). PDC-kinase activity is stimulated by the products of the complex, acetyl CoA and NADH, whereas the substrates CoA and NAD^+ are inhibitory (Pettit *et al.*, 1975). The activity also decreases in the presence of pyruvate and thiamine diphosphate (ThDP) which are thought to bind directly to the active site of the E1 substrate (Pratt and Roche, 1979). Two PDC kinases, termed p45 and p48, have been identified in rat heart using purification, N-terminal sequencing and cDNA cloning and sequencing. Through Northern blot analysis, p45 has been found to be abundant in most rat tissues, while the p48 kinase is only abundant in heart (Popov *et al.*, 1993, 1994). Limited immunological data suggest that the p45 form corresponds to the bovine kidney kinase K1 subunit.

Due to the difficulties involved in purifying BCDC-kinase, little was known about the enzyme (Cook *et al.*, 1985). Recently, however, BCDC-kinase has been purified from rat heart and liver and has been found to be monomeric with a M_r value of 44,000 (Shimomura *et al.*, 1990; Popov *et al.*, 1992). The major regulatory mechanism of the kinase is inhibition by the 2-oxoacid substrates as it has been found to be insensitive to NAD^+:NADH and CoA:acyl CoA ratios (Paxton and Harris, 1984). Cloning and sequencing of rat heart BCDC-kinase has been performed and was found to have little homology with other eukaryotic serine/threonine protein kinases. As with PDC-kinase, the enzyme shows greatest homology with prokaryotic histidine protein kinases (Popov *et al.*, 1992, 1993) .

1.4.2 Phosphatases Associated With PDC and BCDC

The phosphatase responsible for dephosphorylation and subsequent activation of PDC is much more loosely associated with the complex. The enzyme has a M_r value of 150,000 and consists of a catalytic subunit with a M_r value of 50,000 and a larger subunit of M_r 97,000. This larger subunit contains a molecule of FAD, the function of

which remains unknown (Teague *et al.*, 1982; Pratt *et al.*, 1982). PDC-phosphatase has an absolute requirement for Mg^{2+} and is stimulated by Ca^{2+}, which promotes binding of the phosphatase to the E2 core of the complex (Reed and Yeaman, 1987). It is thought that the mode of action of Ca^{2+} is to lower the K_m for Mg^{2+}, as Ca^{2+} ions have no affect on the phosphatase activity at saturating concentrations of Mg^{2+}. The other effectors of phosphatase activity are NADH, which inhibits phosphatase activity and the polyamine, spermine, which stimulates the enzyme by lowering the K_m for Mg^{2+} (Roche *et al.*, 1989) (see Figure 1.14). Acute hormonal Ca^{2+}-mediated control has been demonstrated for PDC from heart (by adrenaline) and liver (by vasopressin) tissue. The activation of PDC is by stimulation of the phosphatase and not by direct allosteric activation (Denton and McCormack, 1985). Insulin is also known to promote the dephosphorylation of PDC, enhancing its activity in adipose tissue, but this effect has been shown to be insensitive to Ca^{2+} concentrations (Marshall *et al.*, 1984).

Knowledge of the BCDC-phosphatase is limited. The native enzyme has been found to have a M_r of 460,000 (Damuni *et al.*, 1984) but the subunit composition has yet to be fully determined. A catalytic subunit with a M_r value of 33,000 has, however, been purified (Damuni and Reed, 1987) but the properties of the remaining subunits have yet to be elucidated

1.5 Plant 2-Oxoacid Dehydrogenase Complexes

While much is known about the mammalian and bacterial 2-oxoacid dehydrogenase complexes, research on their equivalents in plants has been lacking owing to the great difficulty in purifying these complexes and their associated components from plants. This is a consequence of the low abundance of mitochondria per fresh weight of plant material (Randall and Miernyk, 1990). Intact mitochondrial PDC has been purified to

homogeneity only once from a plant source where 1 mg of pure mitochondrial PDC was isolated from 500 kg of broccoli (*Brassica oleracea*) florets (Rubin and Randall, 1977a). However, no SDS-PAGE analysis or definitive subunit composition was provided in this study. Antisera were subsequently raised to broccoli mitochondrial PDC and used to probe other plant extracts.

Research on the plant 2-oxoacid dehydrogenase complexes has so far centred on PDC. While there is considerable overall metabolic similarity between plant and animal cells, there are significant differences in anatomy, physiology and organellar requirements which have led to increasing diversity between these two classes of eukaryotes. Figure 1.15 illustrates the interaction of metabolic pathways found in chloroplasts, peroxisomes and mitochondria of plant cells. A characteristic unique to plants is that they possess two distinct spatially separated forms of PDC, one located in the mitochondrial matrix and the other in the plastid stroma. Each isoform has characteristic structural, catalytic and regulatory properties (Miernyk *et al.*, 1985; Randall *et al.*, 1989). Plant mitochondrial PDC is typical of the complex from eukaryotic sources, where it serves as the primary entry point for carbon into the citric acid cycle. The plastid PDC, however, provides acetyl-CoA and NADH required for fatty acid and isoprenoid biosynthesis. Therefore, the most important mechanism for regulation of plant PDCs is compartmentalisation.

1.5.1 Plant Mitochondrial Pyruvate Dehydrogenase Complexes

Plant mitochondrial PDC has been detected in a wide variety of plant tissues including pea (Randall *et al.*, 1981), cauliflower (Randall *et al.*, 1977), broccoli (Rubin and Randall, 1977a), castor seed endosperm (Rapp *et al.*, 1987), spinach (Rao and Randall, 1980) and potato (Crompton and Laties, 1971). Broccoli mitochondrial PDC was found to possess a sedimentation coefficient of 59.3 S, which was somewhat

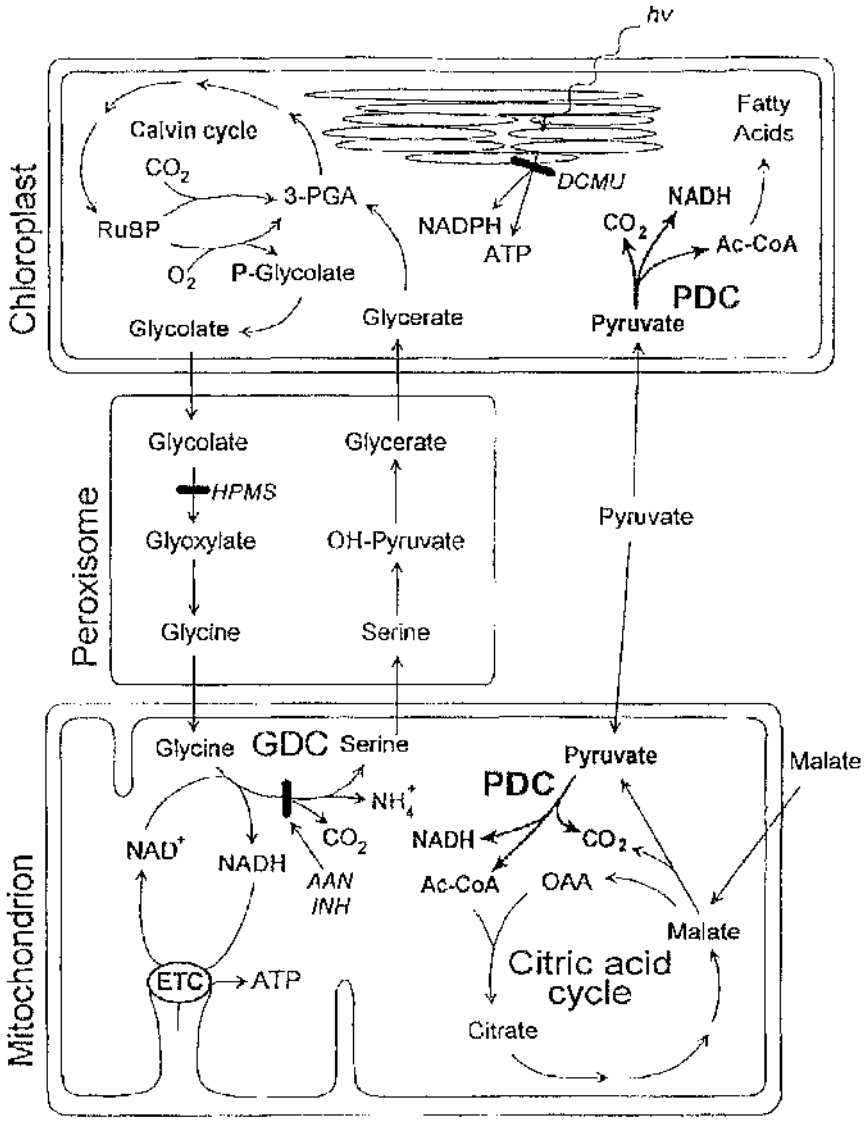

Figure 1.15: Interactions of the photosynthetic, photorespiratory and citric acid cycles

Not all the intermediates are shown. PDC- pyruvate dehydrogenase complex, GDC-glycine decarboxylase complex. Reproduced from Luethy *et al.* (1996).

smaller than the mammalian complex at 70-90 S (Linn *et al.*, 1972). Most plant PDCs have optimal activity at pH 7-8 and substrate and cofactor requirements are typical of the complex from non-plant sources, with broccoli mitochondrial PDC having an absolute requirement for pyruvate, TPP, NAD^+, CoA and Mg^{2+}. Like its mammalian counterpart, broccoli PDC was able to utilise 2-oxobutyrate, giving 10-20% of the rate with pyruvate and also displayed a multisite ping-pong kinetic mechanism as described in section 5.1 (Rubin *et al.*, 1978).

Using antisera raised to broccoli PDC, SDS-PAGE analysis and immunoblotting of pea mitochondrial PDC have revealed subunits of M_r 76,000, 57,000, 53,000, 43,000, and 38,500. The M_r 57,000 subunit is dihydrolipoamide dehydrogenase (E3) as it also cross-reacted with anti-porcine E3 serum (J.A. Miernyk, unpublished observations) and anti-L-protein (from GDC) serum (Turner *et al.*, 1992). The polypeptides of M_r 43,000 and 38,500 correspond to the α and β subunits of pyruvate dehydrogenase (E1), with polyclonal antiserum raised against recombinant *Arabidopsis thaliana* E1β subunit recognising the M_r 38,500 subunit and a monoclonal antibody raised against the maize mitochondrial E1α subunit highlighting the M_r 43,000 subunit (Luethy *et al.*, 1995). This monoclonal antibody recognises the mitochondrial E1α subunit from all plants examined, but does not recognise the E1 subunit of plastid PDC, *E. coli*, or *S. cerevisiae* (Luethy *et al.*, 1995). The polypeptide at M_r 76,000 is thought to be E2 based on the calculated M_r of the E2 deduced amino acid sequence (Guan *et al.*, 1995). The identity of the 53,000 polypeptide is unknown but has always been found to be present in immunoprecipitations with PDC and has been immunopurified using a monoclonal antibody raised against E1α. This polypeptide may be the plant equivalent of protein X, which helps bind E3 to the E2 core in mammalian PDC or PDC-kinase, which has also been proposed to be of this size.

Several clones corresponding to components of plant mitochondrial PDC have recently been isolated and sequenced. These include the E2 component of PDC from *Arabidopsis thaliana* which has shown extensive homology with PDC-E2 from human, yeast and *E. coli* and was found to contain two lipoyl domains, similar to the human complex (Guan *et al.*, 1995). Other clones from *A. thaliana* include the PDC-E1α gene, the open reading frame of which is 1176 bp long and encodes a protein of 389 amino acids. It has 47-51% identity and 63-69% sequence similarity with other eukaryotic E1α sequences (Luethy *et al.*, 1995) and the sequences surrounding the ThDP binding site and phosphorylation sites 1 and 2 are highly conserved. The serine in mammalian E1α is, however, replaced by a threonine at site 2. Another clone from *A. thaliana* is the E1β gene (Luethy *et al.*, 1994), which has an open reading frame of 1230 bp and encodes a protein of 363 amino acids. This has a high sequence identity of 65% with *S. cerevisiae* E1β. Mitochondrial targeting pre-sequences have been found in all these genes.

The only plant E3 to be cloned to date is from pea leaf GDC, in which the E3 component is termed the L-protein (Bourguignon *et al.*, 1992; Turner *et al.*, 1992). The L-protein gene is 1503 bp long which encodes a protein of 501 amino acids, with a calculated M_r of 49,721. This corresponded very closely with the M_r of the L-protein isolated from pea leaves when determined by electrospray mass spectrometry. The amino acid sequence was found to be highly conserved between human, porcine and yeast E3, particularly within the FAD and NAD^+-binding domains. It was also predicted that this protein would share a similar secondary and tertiary structure with other E3s. Subsequently, the L-protein was found to be identical to the E3 derived from PDC in pea leaf mitochondria using various biochemical techniques (Bourguignon *et al.*, 1996).

1.5.2 Regulation of Plant Mitochondrial PDC

PDC in all eukaryotes is an important site of regulation for controlling the entry of carbon into the TCA cycle. This is more so for plants as during photosynthesis energy production is met primarily by the chloroplasts and not by the mitochondria. Greater complexity of subcellular compartmentalisation in plant cells, as compared to mammalian or bacterial cells, allows for the existence of two distinct, spatially-separate types of PDC.

Fine control of plant mitochondrial PDC is achieved by end product inhibition with both NADH and acetyl-CoA, which are competitive with NAD^+ and CoASH, respectively. The complex is more sensitive to the NADH:NAD^+ ratio than to the acetyl-CoA:CoASH ratio, where the K_i for NADH is 5-10 fold lower than the K_m for NAD^+, whereas the K_i for acetyl-CoA is double the K_m for CoASH (Randall *et al.*, 1977; Rubin *et al.*, 1978). In contrast to mammalian PDC, changes in the ATP:ADP ratio over a 20-fold range have little or no effect on the activation state of the plant mitochondrial PDC *in situ* (Budde and Randall, 1988a). PDC in intact mitochondria can be inactivated by ATP added exogenously and inactivation lasts until the ATP is removed or exhausted. Once the ATP is depleted, PDC is rapidly reactivated, and this reactivation occurs in the presence of 20-40 fold less Mg^{2+} than is needed for *in vitro* reactivation (Budde and Randall, 1988b). This concentration of Mg^{2+} is found to be physiologically relevant in inhibiting the kinase.

1.5.2.1 Plant Mitochondrial PDC-Kinase

All plant mitochondrial PDCs, including pea, spinach, broccoli and cauliflower, have been found to be subject to control by covalent modification via reversible phosphorylation (Rubin and Randall, 1977b; Rao and Randall, 1980; Randall *et al.*, 1981, 1989). The existence of such a mechanism was first identified by demonstrating a Mg^{2+}-dependent inactivation of plant PDC, where loss of activity was observed

when PDC was incubated with Mg-[γ-^{32}P]ATP coupled with the incorporation of the label into a polypeptide of M_r 43,000 (Rubin and Randall, 1977b). This polypeptide also cross-reacted with anti-broccoli PDC, revealing a M_r of identical size to the labelled protein and is slightly larger than the E1α subunit of mammalian PDC at M_r 41,000. As with the mammalian E1α, the residue phosphorylated is serine (Miernyk *et al.*, 1985). Moreover, the additional serine subunits that are phosphorylated on the 43,000 Da subunit appear to have no effect on activity, which is analogous to the mammalian complex (Randall *et al.*, 1989). A synthetic peptide corresponding to a 14 amino acid tryptic fragment containing phosphorylation sites 1 and 2 of bovine PDC was synthesised and antiserum to the peptide prepared (Miernyk and Randall, 1989). Probing pea mitochondrial and plastidic PDC, no cross-reaction was observed with this antiserum, in contrast to porcine, bovine and yeast PDC. This synthetic peptide was also found to act as a substrate for the mammalian PDC-kinase but not for the plant equivalent, suggesting that the phosphorylation sites on the plant PDC are significantly different from those on the mammalian complex.

The mitochondrial PDC-kinase from pea seedlings and from the endosperm of *Ricinus communis* seeds has been studied. Since the kinase is lost from PDC or is inactivated during purification, all studies to date have either used partially purified PDC that retains kinase activity or mitochondrial extracts. Activity of the PDC-kinase has been found to be optimal at pH 7.5, with Mg^{2+}-ATP as the preferred phosphate donor, however, nucleotide specificity was found to be broad.

The K_m for Mg^{2+}-ATP was 2.5-5 μM, about six times the plant PDC-phosphatase, therefore if PDC is not regulated, the complex would always be in an inactivated state (phosphorylated) (Miernyk and Randall, 1987c). Using 8-azido[α-^{32}P]ATP, a polypeptide of M_r 53,000 was labelled and thought to be the plant PDC-kinase. This size is considerably larger than the subunit of the mammalian kinase at M_r 48,000 (Miernyk and Randall, 1987c).

PDC-kinase activity was found to be affected by ADP, pyruvate, acetyl-CoA, NADH, citrate, 2-oxoglutarate and monovalent cations (Miernyk and Randall, 1987c; Schuller and Randall, 1989) (see Figure 1.16). Pyruvate was found to be an uncompetitive inhibitor of the kinase with respect to ATP and is the metabolite with the greatest effect on controlling PDC-kinase and thus adjusting the steady-state level of PDC. Inhibition by ADP is competitive with ATP, similar to the mammalian PDC-kinase. However, in contrast to its mammalian counterpart, K^+ has no effect on this inhibition (Miernyk and Randall, 1987b). Similarly, the plant PDC-kinase enzyme is inhibited by acetyl-CoA and NADH, unlike the mammalian PDC-kinase.

Inhibition by acetyl-CoA was found to be competitive with Mg^{2+}-ATP and inhibition by NADH was non-competitive with respect to Mg^{2+}-ATP. This was unexpected since it was thought that an increase in concentrations of the end products would result in the stimulation of the PDC kinase. Similarly, citrate inhibition of the kinase is also unexpected since increased levels of citrate would indicate a decreased demand for acetyl CoA. It may be possible that there are alternative uses for acetyl CoA and citrate outside the mitochondrion, for example in fatty acid and isoprenoid biosynthesis in chloroplasts (Randall *et al.*, 1989). Monovalent cations, such as NH_4^+ and K^+, stimulated pea leaf PDC-kinase, while Na^+ inhibited the enzyme (Schuller and Randall, 1989). As NH_4^+ is a product of photorespiratory carbon metabolism that occurs within the mitochondria of illuminated leaves (see Figure 1.15), this NH_4^+ stimulation has significant regulatory potential.

1.5.2.2 Plant Mitochondrial PDC-Phosphatase

The PDC-phosphatase from plants has been found to have a requirement for the divalent cation Mg^{2+}, with activation by Mg^{2+}>Mn^{2+}>Co^{2+} having K_m values of 3.8, 1.7 and 1.4 mM respectively (Miernyk and Randall, 1987a). In contrast to the mammalian phosphatase, Ca^{2+} did not activate the pea leaf enzyme, but micromolar levels of Ca^{2+} prevented the Mg^{2+}-dependent dephosphorylation of the plant PDC.

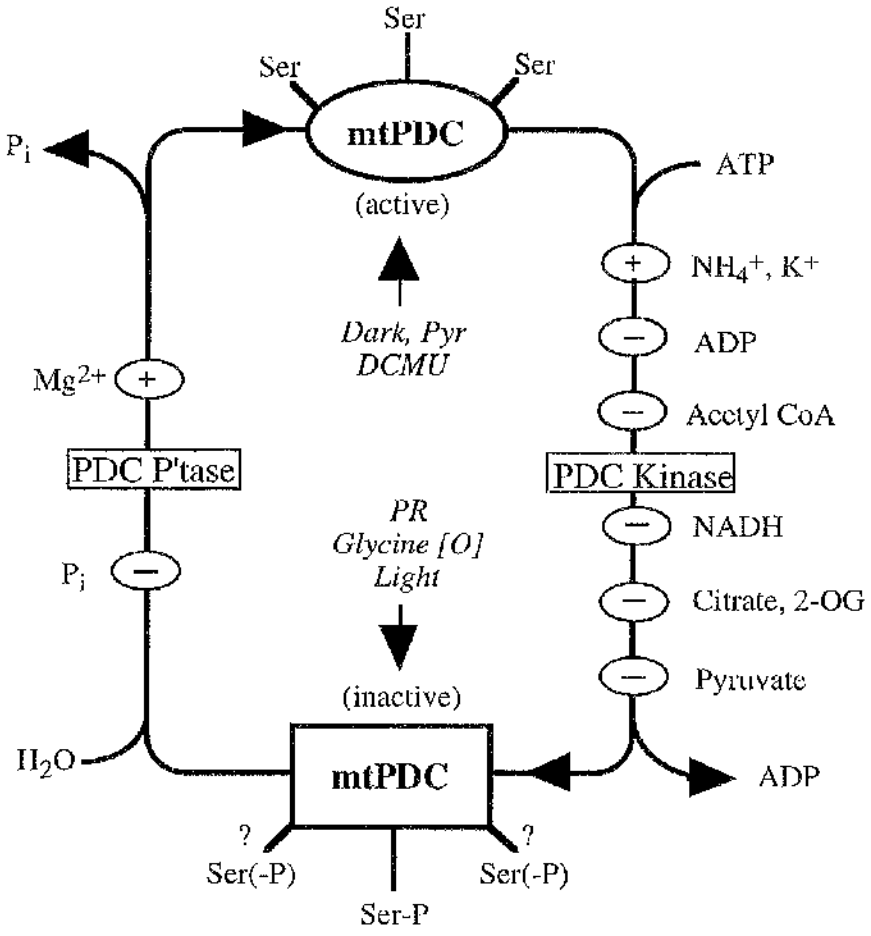

Figure 1.16: Model illustrating the effectors of the reversible phosphorylation of pea leaf mitochondrial PDC

Pyr, pyruvate; DCMU, dichlorophenyl dimethylurea; 2-OG, 2-oxoglutarate; PR, photorespiration; O, oxygen.

1.5.3 Light-Dependent Effects on PDC Activity

Inactivation by phosphorylation has been found to be dependent on light. When pea leaves were illuminated, there was a decrease in steady-state mitochondrial PDC activity to 10-20% of the levels found in leaves adapted to the dark, while activity was retained if the dark conditions were extended. The light-dependent effect was reversed by returning the leaves to the dark and this inactivation could be cycled rapidly by turning the light on and off. Where photosynthesis and/or photorespiration were chemically inhibited, this light-dependent inactivation of PDC did not occur, indicating that both these processes were essential for regulation (Budde and Randall, 1990; Gemmel and Randall, 1992). The conclusion from these studies was that when the mitochondria are able to oxidise substrates other than pyruvate, PDC is quickly inactivated by phosphorylation, conserving carbohydrate when the cell is undergoing photosynthesis (Gemmel and Randall, 1992; Randall *et al.*, 1990). During photosynthesis, the only photorespiratory intermediate metabolised is glycine, which is the preferred substrate for ATP production during illumination. This inactivation is further enhanced by the stimulation of PDC-kinase by NH_4^+ ions, which are produced by glycine oxidation during photosynthesis.

1.5.4 Plastidic Pyruvate Dehydrogenase Complex

Fatty acid biosynthesis occurs in every cell of a plant and is essential to growth. A wide variety of chloroplastic products, including fatty acids and isoprenoids are ultimately derived from acetyl CoA (Camp and Randall, 1985) and since a glycolytic pathway exists in plastids as well as the cytoplasm, a connection was needed between plastidic glycolysis and acetyl-CoA production. Research has revealed a PDC specific to plastids in every plant source investigated, with the exception of the leucoplasts of endosperm from germinating castor oil seeds (Rapp and Randall, 1980). Besides

producing acetyl-CoA for fatty acid biosynthesis, chloroplastic PDC is also the only known source of NADH in chloroplasts (Camp and Randall, 1985).

Plastid PDC catalyses the same overall reaction and has the same kinetic mechanism as the mitochondrial complex. PDC activity has been found to be optimal in the presence of 1 mM pyruvate, 1.5 mM NAD^+, 0.1 mM CoA, 0.1 mM ThDP and 3 mM cysteine-HCl, similar to the optimal conditions for the mitochondrial complex (Qi *et al.*, 1996). Plastid PDC was also found to be sensitive to acetylmethylphosphinate (Golz *et al.*, 1994). However, the chloroplastic complex is structurally different, has higher pH and Mg^{2+} requirements and the Michaelis constant K_m for NAD^+ is approximately half that of its mitochondrial counterpart (Camp and Randall, 1985). This higher Mg^{2+} (10 mM) and alkaline pH (pH 8.0) requirements are in accordance with indirect regulation by the light/dark transitions and may reflect the higher pH and lower NAD^+ concentrations of the plastid environment.

A marked instability of the complex has hampered a definitive estimation of the native M_r of the pea and maize mesophyll chloroplast PDCs (Camp and Randall, 1985; Camp *et al.*, 1988; Treede and Heise, 1986). However, it has been estimated that the M_r of chloroplast PDCs is intermediate in size between that of *Escherichia coli* (M_r 4.3 x 10^6) and bovine mitochondrial (M_r 9-10 x 10^6) PDCs. Subunit analyses of plastid and mitochondrial PDCs by SDS-PAGE and immunoblotting have shown significant differences between the complexes. The only subunit of the plastid complex to be positively identified is the dihydrolipoamide dehydrogenase, with an apparent M_r of 57,000-58,000 (Camp and Randall, 1985). Further immunological analyses by Taylor *et al.* (1992) using antibodies to specific components of bovine heart and yeast PDC were performed on pea mitochondrial and chloroplastic extracts. Using antisera to bovine or yeast E1, no cross-reaction was found in plastid PDC while, in contrast, the pea mitochondrial PDC exhibited a band with a M_r of 41,000 when probed with antiserum to the E1 subunit of yeast PDC. When the different

methods of regulation between the mitochondrial and plastid complexes are taken into account, it is perhaps not surprising that the respective E1 components from these organelles are immunologically distinct polypeptides. A polypeptide of M_r 50,000 from pea chloroplast PDC was found to cross-react with antiserum to E2 from bovine heart E2 and was thought to be the plastid E2. This M_r is substantially smaller than the mammalian E2 enzyme (67,000) but is similar in size to yeast E2. The M_r of the plastid PDC-E2, like E2 from plant mitochondrial PDC, is consistent with the presence of only one lipoyl domain (Taylor *et al.*, 1992). A possible chloroplast E3 protein of M_r 52,000-56,000 was also found in this study when it cross-reacted with antisera to mammalian E3.

1.5.4.1 Regulation of Plastid PDC

(a) Product Inhibition

Similar to the bacterial and mitochondrial PDCs, the plastid complex is sensitive to product inhibition by NADH and acetyl-CoA. NADH is competitive (K_i 10-20 μM) with NAD^+ (K_m 49 μM). Inhibition by acetyl-CoA is competitive with respect to CoASH (K_m 20 μM), and K_i values are also around 20 μM. The plastid complex, like its mitochondrial counterpart, was much more sensitive *in vitro* to the NADH:NAD^+ ratio, with a ratio of 0.2 giving 50% inhibition, than to that of acetyl-CoA to CoA, which required a ratio of 2.2 for 50% inhibition (Camp *et al.*, 1988).

(b) Phosphorylation and Metabolite Effects

Plastid PDCs resemble the bacterial complexes rather than mitochondrial PDCs as they are not regulated by reversible phosphorylation (Randall *et al.*, 1989). The *in vitro* activity of pea chloroplast PDC is insensitive to intermediates of glycolysis, the reductive pentose-phosphate pathway, or amino acid metabolism. Only inorganic phosphate (24% inhibition at 10 mM), oleic acid (57% inhibition by 50 μM), and palmitic acid (36% stimulation by 50 μM) affected pea chloroplast PDC (Camp *et al.*, 1988).

(c) Protein Turnover

Plastid PDC activity showed discrete developmental expression during *Ricinus* endosperm and green tissue maturation. Plastid PDC activity was highest in the developing seeds during the period of maximum storage lipid accumulation, and similarly in leaves, chloroplast PDC activity is maximal during leaf expansion while in a period of rapid lipid synthesis. It was also found that fully mature or senescent tissue lacked detectable plastid PDC activity. This would suggest that on a long term development basis, plastid PDC activity is regulated by protein turnover (Miernyk *et al.*, 1985).

(d) Light Regulation

During photosynthesis the chloroplast stroma becomes more alkaline (pH 8.0-8.3 in the light opposed to pH 7.3-7.5 in the dark) and this pH shift is accompanied by an increase in free Mg^{2+} levels. The pH optimum (pH 8.0) of the plastid PDCs is more alkaline than those of mitochondrial PDCs. The K_m of the pea chloroplast PDC for pyruvate is lowest at pH 8.0 and increases sharply at pH values more acidic or alkaline than pH 8.0 while the values for V_{max} are largely unaffected by changes in pH between 7.0 and 9.0. Plastid PDCs require higher divalent cation concentrations (K_m 1 mM) for maximal *in vitro* activity than do mitochondrial PDCs (K_m 0.4 mM). Thus, the increase in pH and free Mg^{2+} in the plastid stroma in the light would increase the relative activity of PDC (Camp and Randall, 1985; Miernyk *et al.*, 1985; Treede and Heise, 1986; Williams and Randall, 1979). Finally, plastid PDCs are relatively insensitive to inhibition by ATP and NADPH (Reid *et al.*, 1977; Thompson *et al.*, 1977; Camp and Randall, 1985), metabolites which would increase in concentration in illuminated chloroplasts. Overall, then, there is substantial evidence supporting the proposal that chloroplast PDC activity is regulated by the changes in the stroma (i.e. increasing pH and Mg^{2+}) which accompany the light-dark transition and not by reversible phosphorylation.

1.5.4.2 Role of Plastid PDC in Fatty Acid Biosynthesis

Production of acetyl-CoA can occur by at least two pathways. The first employs the plastid PDC, generating acetyl-CoA from the decarboxylation of pyruvate (see Figure 1.17). The second proposed pathway involves the conversion of acetate to acetyl-CoA through the action of acetyl-CoA synthetase (Zeiher and Randall, 1991). Acetate for this pathway is thought to originate in the mitochondria by the action of acetyl-CoA hydrolase (Zeiher and Randall, 1990) on acetyl-CoA produced by the mitochondrial PDC. Plastids that were isolated from developing pea cotyledons were found to contain all of the enzymes required to synthesise acetyl-CoA from triose phosphates (Denyer and Smith, 1988). However, the activity of the acetyl-CoA synthetase was not assessed.

Plastids isolated from the developing embryos of oil seed rape were capable of synthesising fatty acids from ^{14}C-labelled glucose-6-phosphate (G-6-P), dihydroxy-acetone phosphate, pyruvate, malate and acetate (Kang and Rawsthorne, 1994). Several glycolytic enzymes have been found in plastids (Miernyk and Dennis, 1983; Liedvogel and Bauerle, 1986). Glycolysis, however, does not appear to be the only source of pyruvate in plastids as it has been reported that pyruvate is also a by-product of the reaction catalysed by ribulose bisphosphate carboxylase/oxygenase (Rubisco). A further source of pyruvate in plastids may be the decarboxylation of malate (see Figure 1.17), which is able to penetrate the chloroplast envelope via the dicarboxylic acid shuttle system, a process known from C_4 plants (Smith *et al.*, 1992). Pyruvate has been found to be the most effective substrate for fatty acid synthesis in developing embryos of oil seed rape, where rates of ^{14}C incorporation from pyruvate were approximately five fold higher than the rates of incorporation for the label from acetate. Similar results were observed for the leucoplasts of the developing endosperm of castor oil seeds when malate was the added substrate (Miernyk and Dennis, 1983; Smith *et al.*, 1992) and the chloroplasts of mustard cotyledons (Liedvogel and Bauerle, 1986). Acetate, however, has been found to be a better substrate for fatty

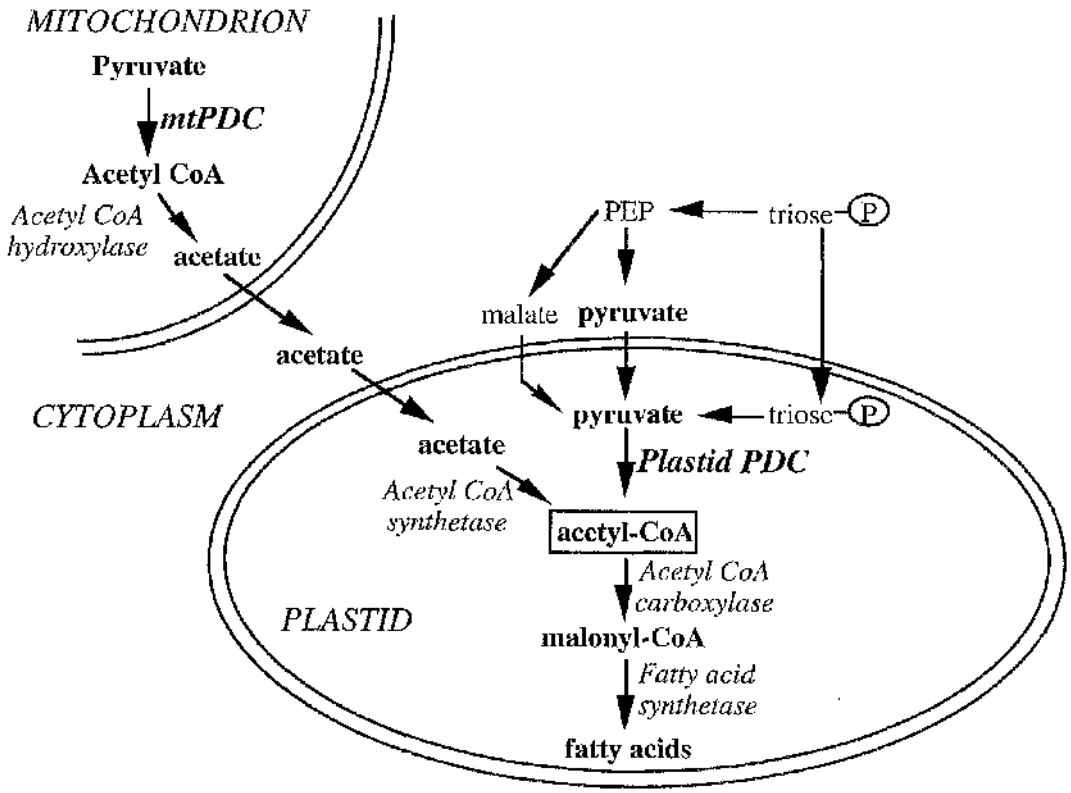

Figure 1.17: Acetyl-CoA formation and *de novo* fatty acid biosynthesis in higher plants

acid synthesis in other instances (Roughan *et al.*, 1979; Springer and Heise, 1989; Masterson *et al.*, 1990). Free acetate is a very small molecule that is able to easily penetrate cellular membranes, including the plastid envelope (Jacobson and Stumpf, 1970). It was reported that incorporation of label from malate into fatty acids occurred at a higher rate than from either pyruvate or acetate. However, malate conversion to fatty acids follows a pathway that includes pyruvate and PDC. Therefore, it appears that the rates of incorporation of the substrate into the fatty acids is highly dependent upon the species and tissue type investigated.

This variation of substrate incorporation into fatty acids between species is probably dependent on the levels of various enzymes present in the plastids of these tissues. For example, castor oil seed endosperm leucoplasts have higher levels of NADP-malic enzyme relative to other plastids (Smith *et al.*, 1992). The levels of acetyl-CoA synthetase in barley chloroplasts are lower than those found in the chloroplasts of spinach leaves (Preiss *et al.*, 1993). It has also been observed that the levels of plastid PDC relative to the mitochondrial PDC are highly dependent on the plant species (Lernmark and Gardestrom, 1994). Mitochondrial PDC levels in pea leaves represent approximately 75-90% of the total cellular PDC (Camp and Randall, 1985; Budde and Randall, 1990), while the chloroplast PDC of barley leaves represents approximately 60% of the total PDC activity (Kromer *et al.*, 1994). In this case, the specific activity of the plastid PDC is comparable to the levels found in pea chloroplasts. However, the specific activity of the mitochondrial complex is approximately 15 times lower. Plastid PDC activity in protoplasts of barley, wheat and spinach represents approximately 85% of the total PDC activity, while the mitochondrial PDC in pea protoplasts was found to represent 85% of the total activity (Lernmark and Gardestrom, 1994).

Plastid PDC, therefore, is a primary source of acetyl-CoA for fatty acid and isoprenoid biosynthesis in many plants, with an additional supply of acetyl-CoA being supplied by a combination of mitochondrial PDC and the carnitine:acetyl-CoA cycle

which would transport the mitochondrially produced acetyl-CoA to the plastids (Wood *et al.*, 1992).

1.5.5 Plant 2-Oxoglutarate Dehydrogenase Complex (OGDC)

Compared to plant mitochondrial PDC, plant OGDC has not been studied as extensively due to the high levels of NADH-oxidase activity, which makes detection of OGDC-catalysed reactions difficult (Poulson and Wedding, 1970). Coupled with this, purification techniques commonly used for the isolation of the mammalian complexes resulted in the partial or complete dissociation of the E3 component from plant OGDC. As a result, to observe a detectable level of intact OGDC activity, the introduction of mammalian E3 is usually required (Poulson and Wedding, 1970). The reconstituted complexes of plant OGDC displays very similar properties to the mammalian and bacterial counterparts where it was found to have an absolute requirement for 2-oxoglutarate, CoA, ThDP, exogenous E3 and consequently NAD^+. Cauliflower OGDC has been reported to be activated by AMP, increasing the maximal velocity by approx. 3-fold and decreasing the K_m for 2-oxoglutarate by approx. 10-fold. It has been suggested that this results in the tighter binding of Mg^{2+}-ThDP to the E1 component (Wedding and Black, 1971a, 1971b; Craig and Wedding, 1980). This complex also has an absolute requirement for divalent cations in common with all other 2-oxoacid dehydrogenase complexes and utilises Ca^{2+}, Mg^{2+} and Sr^{2+} with a strong preference for Mg^{2+}. The extent of AMP-induced stimulation of OGDC activity is equal irrespective of which divalent cation is introduced (Wedding and Black, 1971b; Craig and Wedding, 1980). Both PDC and OGDC utilise CoA resulting in competition for the available substrate between the two complexes. Pyruvate has been found to exhibit an inhibitory effect on plant OGDC activity and it is proposed that this is as a consequence of PDC and OGDC interaction via a limited size of CoA pool in the mitochondrial matrix (Dry and Wiskich, 1985, 1987).

To date, OGDC has only been found in mitochondria with the only other organelle examined being chloroplasts, where OGDC was not found. The first extensive screening for OGDC in organelles using enzymatic and immunological techniques was performed by A. Carmichael (Ph.D. thesis, Glasgow University, 1994), where OGDC could not be detected in peroxisomal or plastid compartments. However, the lack of data or research in this area means that the presence of OGDC in other plant organelles cannot be ruled out at present.

1.5.6 Plant Branched-Chain 2-Oxoacid Dehydrogenase Complex (BCDC)

BCDC activity has been detected only in the peroxisomes of mung bean hypocotyls (Gerbling and Gerhardt, 1988, 1989). The reaction mechanism involving the release of CO_2 from branched chain 2-oxoacids was found to be a NAD^+ and CoA-dependent process, with the concomitant formation of NADH and the acyl-CoA. The cofactor requirements, product analysis and observed stoichiometries are consistent with a BCDC-catalysed reaction. Gerbling and Gerhardt (1989) proposed pathways for the peroxisomal degradation of 2-oxoisocaproate, 2-oxoisovalerate and 2-oxo-3-methylvalerate through HPLC analysis of the intermediate CoAs. Their results suggested that branched-chain amino acids are oxidatively decarboxylated by a BCDC-like activity. The degradation of amino acids is a basic metabolic function of higher plant peroxisomes. After formation of the appropriate acyl-CoA from branched-chain 2-oxoacids, intermediates are further metabolised by β-oxidation to acetyl CoA.

The absence of BCDC activity in mitochondria and plastids of mung bean hypocotyls has been reported (Gerbling and Gerhardt, 1988) and it has been suggested that plant BCDC is located exclusively in peroxisomes. However, BCDC activity was recorded

in solubilised pea and potato mitochondrial fractions concentrated by high speed centrifugation (A. Carmichael, Ph.D. thesis, Glasgow University, 1994). Since BCDC activity produces CoA derivatives which are able to enter the TCA cycle, then it would be advantageous to have a BCDC located in the mitochondrion. One possible explanation for the lack of detection of BCDC in the past may be due to the complex being inactivated by a phosphorylation mechanism, as in mammalian tissues, BCDC is present largely in its inactive phosphorylated state. Another possibility is that BCDC activity or synthesis is governed by a developmental programme and, therefore, may have been developmentally suppressed in the tissue screened. Surprisingly, in this study, no BCDC activity was observed in peroxisomes, contrary to what was previously reported in mung bean.

1.6 Glycine Decarboxylase Complex (GDC)

The glycine decarboxylase complex (GDC) or the glycine cleavage system (EC 2.1.2.10) is a distant relation of the family of 2-oxoacid dehydrogenase complexes. It catalyses the reversible oxidation of glycine in a multistep process to produce N^5, N^{10}-methylenetetrahydrofolate, CO_2 and NH_4^+ (see Figure 1.18).

$$\text{Glycine} + NAD^+ + THF \longrightarrow \text{methylene-THF} + CO_2 + NH_3 + NADH$$

Figure 1.18: Reaction sequence performed by GDC

This multienzyme system has been purified from a variety of sources including mammals (Klein and Sagers, 1966), bacteria (Kikuchi and Hiraga, 1982) and plants (Walker and Oliver, 1986). It is located in the mitochondrial compartment and exists as a loosely associated complex consisting of four enzymes, P, H, T and L proteins (termed P_1, P_2, P_3 and P_4 respectively in bacterial systems). In mammalian tissue, GDC converts glycine derived from protein catabolism into one-carbon fragments that

are used in the anabolic functions of mitochondria, and serine that can enter three-carbon metabolism and glycolysis (Oliver, 1994).

Because of its involvement in photorespiration, GDC is extremely important to plant cells and this is reflected in the levels of GDC found in leaves, where it has been reported that GDC may represent 30-50% of the total mitochondrial matrix protein. This is in contrast to mammalian sources where it constitutes only a small fraction of the total mitochondrial matrix protein (Kikuchi, 1973; Oliver *et al.*, 1990). During photosynthesis, glycolate is produced in chloroplasts and is transported to peroxisomes where it is oxidised to glyoxylate, which is then transaminated to glycine (see Figure 1.15). Glycine, in turn, migrates to leaf mitochondria where it is subsequently decarboxylated by GDC to CO_2, NH_4^+ and N^5,N^{10}-methylene-tetrahydrofolate with the reduction of NAD^+ to NADH. The latter compound reacts with a second molecule of glycine to form serine in a reaction catalysed by serine hydroxymethyltransferase (SHMT). Serine then returns to the peroxisomes where it is converted to glycerate for re-entry to the chloroplast. Ribulose 1,5-bisphosphate is regenerated from the glycine via glycerate-3-phosphate.

Thus, photorespiration involves the co-operation of three separate plant organelles (see Figure 1.15). Very low levels of glycine metabolism are observed in green leaf tissue in the dark, but upon illumination there is rapid synthesis of GDC, largely regulated at the transcriptional level (Kim and Oliver, 1990; Bourguignon *et al.*, 1988). In C_3 plants, the flux of carbon through the photorespiratory pathway is approximately equal to the flux through the photosynthetic C_3 cycle, on a molar basis and so it represents the major metabolic pathway in mitochondria in leaves. The conversion of glycine to serine in green leaf mitochondria is currently considered to be the major source of CO_2 released during photorespiration (Douce *et al.*, 1994) and is about 25% of the amount that is fixed by ribulose bisphosphate carboxylase/oxygenase (Rubisco). Therefore, this pathway decreases the amount of

carbon that is available to support plant growth, while the rate of NH_4^+ release is almost ten times higher than the rate of primary NH_4^+ fixation. Thus, NH_4^+ must be efficiently recycled to prevent the loss of nitrogen (Oliver, 1994) and although the NADH produced is available to the mitochondrial electron transport chain, the photorespiratory cycle results in a net energy consumption.

The P protein (P_1) is a pyridoxal phosphate-dependent enzyme which binds and catalyses the decarboxylation of glycine in concert with the smaller acid and heat stable H protein (P_2) (see Figure 1.19). The resulting aminomethyl moiety is covalently bound to the lipoic cofactor of the H protein. The T protein (P_3) then catalyses the release of ammonia from the methylamine intermediate which is bound to the H protein. Tetrahydrofolate serves as an acceptor for the remaining one-carbon unit of glycine and forms N^5, N^{10}-methylenetetrahydrofolate. The lipoic acid of the H-carrier protein (H protein) is left in a reduced state and its subsequent reoxidation catalysed by the L protein (P_4), a flavin containing dihydrolipoamide dehydrogenase, with the formation of NADH. The lipoic acid cofactor is attached to the H protein via an amide linkage to the ε-amide group of a lysine residue, which is, in effect, equivalent to a free lipoyl domain which acts as a substrate for the P, T and L proteins. The activity of the L protein is analogous to the electron transfer reaction in the decarboxylation of the 2-oxoacid dehydrogenase complexes.

Owing to the ease of dissociation of the complex, characterisation of GDC has been limited to either whole mitochondria or a crude protein extract (Sarojini and Oliver, 1983). The high protein concentration in the mitochondrial matrix has been found to promote the formation of this loosely associated complex with an apparent subunit ratio of 2 P dimers, 27 H protein monomers, 9 T protein monomers and 1 L protein dimer (Oliver *et al.*, 1990).

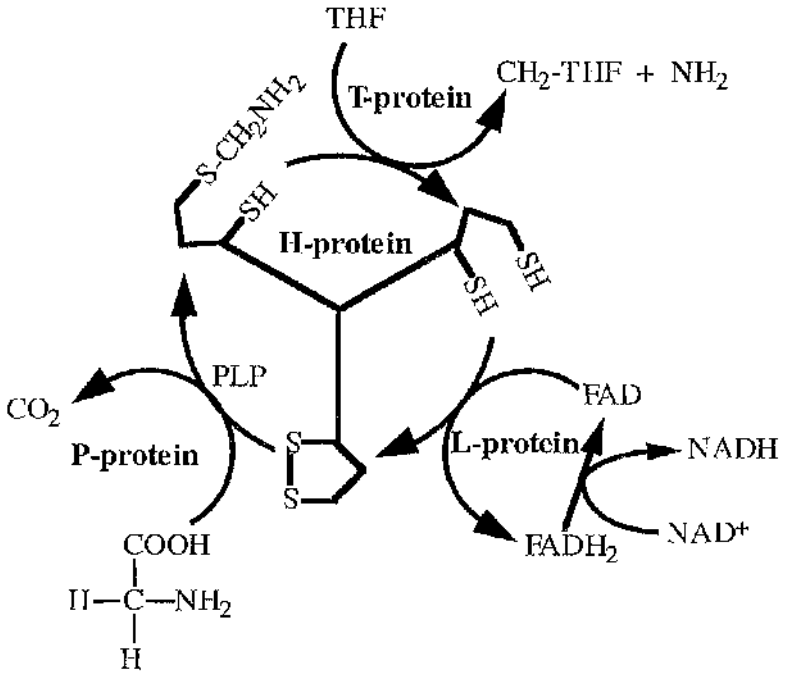

Figure 1.19: Reaction mechanism of glycine decarboxylase complex

The reaction catalysed by GDC requires all four component proteins of the multienzyme complex. The lipoic acid cofactor on the H protein carries the reaction intermediates between the reactive sites of the P, T and L proteins. Reproduced from Oliver (1994).

The crystal structure of the H-protein with the oxidised and reduced lipoyl group has been determined by Pares *et al.* (1995). It was found that when the H-protein was in the oxidised state, the lipoyl arm was located on the surface of the polypeptide. However, when the prosthetic group was loaded with methylamine, the covalently modified methylamine-lipoamide group was found to be tucked in a cleft at the surface of the protein where it is stabilised by hydrogen bonds and hydrophobic contacts. Thus, the group is totally protected and not free to move in aqueous solution. This protective mechanism is also thought to be found in the E2 polypeptide of the 2-oxoacid dehydrogenase complexes.

cDNA clones encoding all four proteins of GDC (P, H, T and L) of pea mitochondria have now been isolated and characterised (Kim and Oliver, 1990; Macherel *et al.*, 1990; Turner *et al.*, 1992; Bourguignon *et al.*, 1992, 1993). All four proteins were found to be encoded by unique nuclear genes which are translated on cytosolic ribosomes and imported into the mitochondrial matrix. With the exception of the L protein, the expression of the genes encoding the proteins of GDC occurs specifically in mature leaf tissue. In addition, upon illumination of the leaves, the mRNA levels of the P, T and H proteins increase several fold, with the mRNA of the dihydrolipoamide dehydrogenase (L protein) accumulating to identical levels in all tissues (Kim *et al.*, 1991; Bourguignon *et al.*, 1992; Turner *et al.*, 1992). This latter result can be attributed to the fact that dihydrolipoamide dehydrogenase is also associated with the pyruvate dehydrogenase complex (PDC) and the 2-oxoglutarate dehydrogenase complex (OGDC) as well as GDC (Bourguignon *et al.*, 1996). There is also evidence for spatial regulation in the expression of GDC activity. In spinach, GDC is abundant in mitochondria from photosynthetic tissues, whereas in the mitochondria of the roots, stalks and leaf veins, it is expressed at much lower levels (Gardestrom *et al.*, 1980).

GDC is subject to feedback inhibition by the products of the reaction, NADH and serine (Bourguignon *et al.*, 1988). However, this is prevented by unique substrate

transporters found in plant mitochondria. NADH is shuttled out of the mitochondrion by an oxaloacetate/malate transporter in conjunction with malate dehydrogenase activity in the matrix and cytosol (Oliver and Walker, 1984), while glycine/serine exchange proteins in the inner mitochondrial membrane prevent inhibition by serine.

1.7 Aims of Thesis

Much is known about mammalian and bacterial 2-oxoacid dehydrogenase complexes, but very little is known about their plant equivalents. Evidence exists for complex-specific isoforms of E3 in prokaryotes, while there is some evidence for isoforms of mammalian E3. Knowledge on plants, however, has been lacking, with the added complication of the existence of organelle-specific forms of the pyruvate dehydrogenase complex. The primary aim of this work was to isolate the chloroplast-specific dihydrolipoamide dehydrogenase and to provide conclusive evidence for its unique identity in comparison to the mitochondrial E3. Previous immunological analysis indicated that in plant tissue there was a possibility of organelle specific isoforms of dihydrolipoamide dehydrogenase in pea (Taylor *et* al., 1992).

Further aims were to provide evidence for the existence of the chloroplastic dihydrolipoamide dehydrogenase in other species of plants and to isolate and sequence a cDNA encoding the chloroplastic enzyme.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Plants

Thirum-treated pea seeds (*Pisum sativum L.*, cv. Little Marvel) were purchased from Sharpes International Seeds Ltd. (Sleaford, Linconshire); barley seeds (*Hordeum vulgare* cv. Hart) from Plant Breeding International (Maris Lane, Trumpington, Cambridge, UK); oilseed rape seeds (*Brassica napus*) were kindly donated by Dr. Steve Rawsthorne (John Innes Centre, Norwich) and spinach leaves (*Spinacea oleracea*) were obtained from Safeway, Byres Rd, Glasgow.

2.1.2 Chemicals and Equipment

The following reagents were purchased from Sigma Chemical Co., Poole, Dorset, UK:

NAD^+, NADH, $NADP^+$, NADPH, sorbitol, bovine serum albumin (BSA), isoascorbic acid, polyvinylpyrrolidine (PVP-40), copper sulphate, phenylmethylsulphonyl fluoride (PMSF), benzamidine-HCl, Coomassie Brilliant Blue type R250, Pyronin Y, bromophenol blue, amido black, Tween 20, 3-(N-Morpholino) propane-sulphonic acid (MOPS), polyvinylpyrrolidine (PVP), N-tris[hydroxymethyl]-methyl-2-aminoethane-sulfonic acid (TES), Percoll, thioctic acid amide, thiamine diphosphate (ThDP), formaldehyde, 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), silver nitrate, ammonium hydroxide, Ponceau S, ampicillin, kanamycin, glutathione (reduced), isoamyl alcohol, HPLC grade distilled water, ethidium bromide, 3-[cyclohexylamino]-1-propanesulphonic acid (CAPS), 2-[N-cyclohexylamino]ethane-sulphonic acid (CHES), 2-[N-morpholino]ethanesulphonic acid (MES), 3-(N-morpholino) propane-sulphonic acid (MOPS), Freund's complete

and incomplete adjuvants, broad range protein standards and N-tris-[hydroxymethyl]methyl-glycine;N-[2-hydroxy-1,1-bis(hydroxylmethyl)ethyl]-glycine (Tricine).

The following were obtained from Fisons, Loughborough, UK:
Polyethylene glycol (PEG) 6000 grade, Triton X-100, calcium chloride, potassium phosphate, sodium carbonate, potassium tartrate, ethylenediaminetetra-acetic acid (EDTA), sucrose, Triton X-100, methanol, ethanol, chloroform, isopropanol, acetic acid, magnesium chloride, Folin and Ciocalteu phenol reagent, acrylamide, N,N'-methylenebisacrylamide, ammonium persulphate, citric acid, glycine and sodium chloride.

Ultra pure acrylamide used for protein sequencing was from Fluka, Derbyshire, UK. DTT was obtained from Boehringer Mannheim GmbH, Germany. Toluene was from RP Normapur, Rhone-Poulenc Ltd, Manchester, UK. Heptane was from Synchemica, Chadwell Heath, Essex, UK. Hybond-C nitrocellulose membrane, ECL chemicals, ECL secondary antibody and Hyperfilm-ECL were from Amersham, Amersham Place, Little Chalfont, Buckinghamshire, UK. Piperazine diacrylamide (PDA) crosslinker was from Biorad, Richmond, CA, USA.

X-Omat S film was from Kodak Ltd., Dallimore Rd, Manchester, UK. Non-immune donkey serum was supplied by the Scottish Antibody Production Unit (SAPU), Lanarkshire, Scotland. Plast-X autoradiography cassettes were from Anthony Monk Ltd., Sutton-in-Ashfield, UK.

Centricon microconcentrators were from Amicon Ltd., Stonehouse, Gloucestershire, UK. Non-fat milk and Saran Wrap were obtained from local shops. All other chemicals were of Analar grade from BDH Chemicals Ltd., Poole, U.K., or were of the highest grade commercially available. The Taq DNA polymerase, assay buffer,

$MgCl_2$, DNA Ligase, Bam HI and Eco RI were obtained from Promega (Southampton, UK).

Low melting point agarose and standard agarose were from Appligene, Pinetree Centre, Durham Road, Birtley, Chester-le-Street, Co. Durham, UK. Sodium borohydride was from Aldrich chemicals, The Old Brickyard, New Road, Gillingham, Dorset, UK. Tris-HCl was from Gibco, Life Technologies Limited, Trident House, Renfrew Road, Paisley, Scotland. Bacto-tryptone, bacto-yeast extract and agar were from Merck Lab Supplies, 21 Polmadie Avenue, Glasgow, Scotland. VCS-M13 Helper phage was from Stratagene, 140 Cambridge Science Park, Milton Road, Cambridge, UK. DNA molecular weight marker V was from Boehringer Mannheim, Bell Lane, Lewes, East Sussex, UK.

FPLC columns and gel chromatography materials were purchased from Pharmacia Ltd., Milton Keynes, UK. Nitrocellulose filters (0.22 μm) were from Millipore S.A., Molsheim, France. Immobilon PVDF membrane for protein sequencing was from BioRad, Richmond, CA, USA.

2.2 Methods

2.2.1 Growth of Plants

Pea seeds (*Pisum sativum L.*, cv. Little Marvel) were cultivated in moist vermiculite in a growth chamber with a 12 h photoperiod (22°C light / 18°C dark) at a photon flux rate of 200 μmol m^{-2} s^{-1}. In order to avoid the development of an extensive lateral root system the pea roots were harvested after 4 days. Leaf tissue was collected 12 days after planting when the primary leaves were fully expanded.

Barley seeds (*Hordeum vulgare* cv. Hart) were grown in moist vermiculite at 200 μmol $m^{-2}s^{-1}$ in a growth chamber with a 12 h photoperiod (22°C light / 18°C dark) and watered using Hoagland's reagent. Leaf tissue was harvested after 10 days. Oil seed rape was grown in similar conditions to barley seeds, with leaf tissue being harvested after 14 days.

2.2.2 Organelle Isolation

Organellar extracts from pea leaf and root tissue were prepared by the general method of Bonner (1967) modified by Douce *et al.* (1972).

Pea leaf and root tissue (25g per homogenisation) were ground in a Polytron homogeniser (System Tecknik, Ruschlikon, Switzerland) for four short bursts at setting five to limit organelle damage in the presence of 3-4 volumes of ice-cold grinding medium containing 300 mM sorbitol, 50 mM MOPS, pH 7.5, 1 mM EDTA, 0.1% (w/v) BSA (essentially fatty acid free) and 0.5% (w/v) polyvinylpyrrolidine 40 (PVP-40). Iso-ascorbic acid (10 mM), benzamidine (1 mM) and phenylmethylsulphonyl fluoride (PMSF, 1 mM) were added on the day of preparation.

The homogenate was filtered through 4 layers of pre-wetted muslin, then a sandwich of 8 layers of pre-wetted muslin and cotton wool. At this stage, the filtrate was treated by one of the following protocols, depending on the organelle to be isolated:

(A) Mitochondrial Isolation

Mitochondria were purified essentially according to Douce *et al.* (1972) with the following modifications.

The crude extract isolated by the above procedure was centrifuged at 1,500g for 15 min at 4°C to pellet starch granules and debris and the resulting supernatant centrifuged at 3,000g for 10 min to pellet chloroplasts. The supernatant was centrifuged at 10,000g in order to pellet the mitochondria. These were resuspended in wash buffer containing 300 mM sorbitol, 40 mM potassium phosphate, pH 7.2, 1 mM EDTA and 0.1% (w/v) BSA.

The mitochondrial fraction was then layered onto a self-generating Percoll gradient (28% (v/v) Percoll, 300 mM sucrose, 10 mM potassium phosphate, pH 7.2, 1 mM EDTA and 0.1% BSA) and centrifuged at 40,000g for 30 min as described by Neuburger *et al.* (1982). The mitochondria formed a broad band near the bottom of the gradient while chloroplasts and broken organelles were unable to enter the gradient.

Mitochondria were removed, diluted 10 fold in wash buffer minus BSA (as previously described but without the BSA) and centrifuged at 10,000g for 20 min. Pelleted mitochondria were rinsed in wash buffer minus BSA, re-pelleted as described before and then resuspended in wash buffer minus BSA containing 0.1% (v/v) Triton X-100. The solubilised mitochondrial fraction was heat-treated at 65°C for 60 min since E3 has previously been shown to be stable at temperatures up to 80°C (Van Muiswinkel-Voetberg *et al.*, 1973; Van Berkel *et al.*, 1991), centrifuged at 10,000g for 20 min and the supernatant fluid dialysed overnight in dialysis buffer (see 2.2.3).

(B) Chloroplast Isolation From Leaves

Chloroplasts were isolated from the crude extract prepared by the above procedure, employing modifications of the method described by Murphy and Leech (1977; 1978).

The crude extract was centrifuged at 200g for 3 min at 4°C to pellet starch granules and debris and the supernatant centrifuged at 3020g for 3 min to pellet the chloroplasts. Chloroplasts were gently resuspended in wash buffer containing 330 mM sorbitol, 50 mM MOPS, pH 7.8, 2 mM EDTA and 0.15% (w/v) BSA using a paintbrush and further purified by isopycnic centrifugation on Percoll gradients following the procedure of Mourioux and Douce (1981).

The resuspended chloroplasts were layered onto a pre-formed Percoll gradient (containing 50% (v/v) Percoll in wash buffer as described previously and centrifuged at 10,000g for 100 min), before centrifugation at 5000g for 10 min. Intact chloroplasts, which formed a broad band near the bottom of the gradient, were removed and diluted 10 fold in wash buffer minus BSA.

The chloroplast fraction was removed from the gradient, diluted 10 fold with wash buffer minus BSA and pelleted by centrifugation at 3000g for 10 min at 4°C. The pellet was rinsed in wash buffer minus BSA and re-centrifuged after resuspension. Chloroplast pellets were resuspended in a minimal volume of wash buffer minus BSA containing 0.2% (v/v) Triton X-100 and the suspension incubated at 65°C for 60 min. The heat-treated solution was centrifuged at 40,000g for 30 min and the resulting supernatant dialysed overnight in dialysis buffer (see 2.2.3).

(C) Whole Organellar Extraction From Pea Leaves

To ensure maximal yields of dihydrolipoamide dehydrogenase from both mitochondria and chloroplasts the following procedure was devised. The crude extract prepared above was centrifuged at 20,000g for 20 min to pellet the organelles. Organelles were resuspended in 50 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid (HEPES), pH 6.8 buffer containing 0.2% (v/v) Triton X-100 and incubated at 65°C for 60 min.

After the incubation the heat-treated solution was centrifuged at 40,000g for 30 min at 4°C to pellet broken membranes and precipitated proteins. The resultant solution, designated the whole organellar extract, was dialysed overnight in dialysis buffer (see 2.2.3).

2.2.3 Dialysis Of Plant Extracts

Extracts of either plant mitochondria, chloroplasts or total organelle were dialysed overnight into 100 vol of 25 mM HEPES, pH 6.8 containing 1 mM dithiothreitol (DTT), 1 mM benzamidine and 1 mM PMSF in preparation for chromatographic analysis. Following dialysis, the extract was concentrated by placing the dialysis tubing in polyethylene glycol (PEG) 6000 grade for several hours at 4° C.

2.2.4 Chromatographic Analysis of Plant Extracts

Organellar extracts from plant tissue, which had been dialysed overnight, were loaded onto a Pharmacia anion-exchange column (HR 5/5 or 10/10) using a fast protein liquid chromatography (FPLC) system, pre-equilibrated in 10 mM potassium phosphate containing 25 mM HEPES, pH 6.8, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and 1 mM mercaptoethanol. Bound proteins were eluted at 0.5 ml min^{-1} for the HR 5/5 column or 1.5 ml min^{-1} for the HR 10/10 column using a linear 10-400 mM potassium phosphate gradient and collected in 1 ml fractions for the HR 5/5 column or 1.5 ml fractions for the HR 10/10 column. Dihydrolipoamide dehydrogenase activity was determined in each fraction using the assay described in section 2.2.7.

2.2.5 Preparation Of Dihydrolipoamide

Dihydrolipoamide was prepared according to Reed *et al.* (1958). Lipoamide (1g) (thioctic acid) was dissolved in 80% (v/v) methanol at 4°C. 1g of sodium borohydride was dissolved in 5 ml distilled water at 4°C and then added to the lipoamide solution. The solution was stirred at room temperature for approximately 45 min until it became clear. The pH of the solution was then lowered to 2.0 with 0.25M HCl and extracted with 100 ml of chloroform. The lower layer containing the dihydrolipoamide was removed and dried under N_2 and the resulting powder was redissolved in 150 ml toluene/heptane (2.5:1) by stirring on a hot plate. This solution was then dried under N_2 until a minimal volume of liquid remained and the dihydrolipoamide allowed to crystallise. The dihydrolipoamide was stored at -20°C and a 100 mM stock solution (dissolved in ethanol) was used for enzyme assays.

2.2.6 DTNB Assay for Thiol Groups

In order to determine the yield of dihydrolipoamide derived from lipoamide by the method of Reed *et al.* (1958), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) was employed according to Ellman (1959). DTNB reacts with reduced thiol groups which are present on DHL but not lipoic acid. A 10 mM solution of DTNB dissolved in 100 mM potassium phosphate, pH 7.0 was prepared in addition to 100 µM solutions of DHL and standards mercaptoethanol and dithiothreitol (DTT). The 100 µM DTNB was added to the 100 µM samples in the following volumes:

	Blank (ml)	Sample (ml)
100 mM KP_i, pH 8.0	2	2
dH_2O	8	5
Sample	-	3

5 ml of 100 µM DTNB solution was added to 0.75 ml of the blank solution and 0.75 ml the sample solution. This was left for 3 min at room temperature, the absorbance of the resulting yellow product measured at 412 nm and the concentration of thiol groups calculated according to the equation:

$C_o = \frac{AD}{\varepsilon}$ where C_o- Concentration

A - Absorbance at 412 nm

D - Dilution factor

ε - 13,600M^{-1} cm^{-1}

One molecule of DNTB reacts with each thiol group on a molecule of DHL or DTT. As DHL and DTT both have two thiol groups per molecule, the concentration of thiol groups found will be twice the molar concentration of that found in mercaptoethanol.

2.2.7 Enzymatic Assay for Dihydrolipoamide Dehydrogenase (EC 1.8.1.4)

Assays were performed according to Jackman *et al.* (1990). The assay medium, which was pre-warmed to 30°C prior to assaying, contained 3 mM NAD^+, 1 mM $MgCl_2$, 0.2 mM TPP, 3 mM dihydrolipoamide (for preparation see 2.2.5) and 50 mM potassium phosphate buffer, pH 7.6 and the reaction initiated by the addition of enzyme. The activity was followed by monitoring NADH formation at 340 nm using a Shimadzu UV-2101PC spectrophotometer. All enzymatic data presented in subsequent chapters of this thesis represent an average of duplicate determinations differing by less than 10% unless otherwise stated (the extinction coefficient for NADH is 6.22 mM^{-1} cm^{-1}).

2.2.8 Concentration of Protein Samples

Gradient fractions from the Mono Q anion exchange column and other samples were concentrated using Amicon Centricon-30 disposable microconcentrators (Amicon Ltd., Upper Mill, Stonehouse, Gloucestershire, UK) and rinsed with 20 mM potassium phosphate buffer, pH 7.0. These were also used to prepare samples for electrospray mass spectrometry (see 2.2.19).

2.2.9 Determination of Protein Concentrations

(A) Micro BCA Assay

For measuring protein amounts in the range of 0.5-15 µg, a BCA Protein Assay Reagent kit (Pierce, Post Office Box 117, Rockford, Illinois 61105, USA) was employed. The following solutions were supplied with the kit:

Microreagent A (MA): sodium carbonate, sodium bicarbonate and sodium tartate in 0.2N NaOH.

Microreagent B (MB): 4% (v/v) bicinchoninic acid (BCA) in H_2O.

Microreagent C (MC): 4% (v/v) cupric sulphate, pentahydrate in H_2O.

Working Reagent: 2 parts of MC mixed with 48 parts of MB and then 50 parts of MA added.

The working reagent was stable for 1 day. A standard curve was constructed in the range of 0.5-15 µg using the 2 mg/ml BSA standard supplied with the kit and both the standards and unknowns were made up to a final volume of 1 ml. Working reagent (1 ml) was added to each standard and unknown, and incubated at 60°C for 60 min. Once the incubation was complete the samples were left to cool to room temperature and then their absorbance read at 562 nm using the 0 µg samples to zero the spectrophotometer.

(B) Lowry Procedure

Protein amounts in the range of 10-150 µg were determined using the method of Lowry *et al.* (1951) modified according to Markwell *et al* (1976).

The following solutions were prepared:

Solution A: 25% (w/v) sodium carbonate, 0.4% (w/v) sodium hydroxide, 0.16% (w/v) potassium tartrate and 1% (w/v) SDS.

Solution B: 4% (w/v) copper sulphate.

Solution C: 1% (v/v) of solution B added to solution A.

Solution D: 50% (v/v) Folin and Ciocalteu reagent diluted with distilled water.

Using a 1 mg/ml solution of BSA, a standard curve in the range of 10-150 µg of protein was constructed. The final volume was made up to 1 ml with distilled water and 3 ml of solution C then added. The tubes were vortexed and left to stand at room temperature for 15 min. Solution D (0.3 ml) was added and the absorbance was measured after a further 30 min at 660 nm. The procedure was repeated for samples whose concentrations were unknown.

2.2.10 Preparation of Protein Samples for SDS/Polyacrylamide Gel Electrophoresis

An equal volume of Laemmli sample buffer containing 2% (w/v) sucrose, 0.001% (w/v) Pyronin Y and 62.5 mM Tris-HCl, pH 6.8 was added to samples of protein for electrophoresis with 10 mM DTT. These were boiled for 2 min and then loaded onto individual lanes of an SDS/polyacrylamide gel.

2.2.11 Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were analysed using the discontinuous buffer system according to the method of Laemmli (1970). Proteins were resolved on either 7% or 15% (w/v) polyacrylamide gel slabs (pH 8.8, 16 x 19.5 x 0.15 cm), with a 5% (w/v) stacking gel (pH 6.8).

The following solutions were prepared for making SDS-polyacrylamide gels:

Acrylamide stock: 29.2% (w/v) acrylamide

0.8% (w/v) N,N'-methylenebisacrylamide.

Resolving gel buffer: 0.75M Tris-HCl (pH 8.8)

0.2% (w/v) SDS.

Stacking gel buffer: 0.17M Tris-HCl (pH 6.8)

0.14% (w/v) SDS.

Solution of 10% (w/v) ammonium persulphate.

For a 7% resolving gel, 8.5 ml acrylamide stock, 18 ml resolving gel buffer, 0.36 ml 10% (w/v) ammonium persulphate and 9.1 ml distilled water were mixed and degassed before the addition of 36 µl TEMED (NNN'N'-tetramethylethylenediamine) to initiate polymerisation. This mixture was then poured into the casting apparatus and a layer of isopropanol was poured on top of the resolving gel to ensure an even surface. This was removed once the gel had set by washing with distilled water and the residual water removed.

For a 15% resolving gel 17.5 ml acrylamide stock, 18 ml resolving gel buffer, 0.36 ml 10% (w/v) ammonium persulphate and 0.14 ml distilled water were mixed and degassed before the addition of 36 µl TEMED. This mixture was then poured into the casting apparatus and allowed to set.

A 5% stacking gel was prepared with 1.8 ml acrylamide stock, 8.2 ml stacking gel buffer, 0.12 ml 10% (w/v) ammonium persulphate and 1.9 ml distilled water. This solution was degassed, polymerisation initiated with 12 µl TEMED and poured into the casting apparatus on top of the resolving gel, a comb inserted and the gel allowed to set. Gel plates were wrapped in damp tissue and cling film and stored at 4°C until use.

Gels were subjected to electrophoresis at a constant current in the range of 8 mA to 65 mA in buffer containing 1 mM glycine, 0.1% (w/v) SDS, 25 mM Tris-HCl, pH 8.3 using a vertical gel electrophoresis apparatus, until the Pyronin Y tracker dye reached the end of the gel (approx. 2.5 h at 65 mA and 16 h at 8 mA).

2.2.12 Silver Staining of Proteins

This technique was used to stain 0.1-2 µg of protein when analysed by gel electrophoresis and was carried out according to Wray *et al.* (1981). After electrophoresis, the proteins were fixed by incubating the gel in 50% (v/v) methanol at room temperature for at least 1 h. After this, the gel was placed in staining solution containing 0.8% (w/v) silver nitrate, 0.21 M ammonium hydroxide and 0.08% (w/v) NaOH for 20 min. The gel was rinsed several times with distilled water for 40 min, then the developing solution, containing 0.24 mM citric acid and 0.02% (v/v) formaldehyde (prepared immediately before use) added. Once the protein bands had developed the gel was rinsed with distilled water several times, then placed in stopping solution containing 10% (v/v) acetic acid and 20% (v/v) methanol.

2.2.13 Coomassie Staining of Proteins

This technique was used to detect proteins in the range of 2-10 µg when analysed by gel electrophoresis. After electrophoresis, gels were stained in solution containing 0.04% (w/v) Coomassie Brilliant Blue R250, 10% (v/v) acetic acid and 25% (v/v) methanol for 1 h at room temperature with gentle agitation. Unbound stain was then removed by washing the gel in destain solution containing 10% (v/v) acetic acid and 20% (v/v) methanol. This solution was changed regularly until the excess stain was removed (1-2 h).

2.2.14 Determination of M_r Values by SDS-PAGE

Determination of the subunit M_r values of proteins was performed by comparing their mobility to proteins from molecular mass standards. These were phosphorylase b (M_r 94,000), albumin (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 31,000), soybean trypsin inhibitor (M_r 20,100) and lysozyme (M_r 14,000).

The relative mobility, R_f, was calculated for each protein by dividing the distance migrated by the protein by the distance migrated by the dye front. A plot of R_f versus log M_r of the standards yielded a calibration curve from which the subunit molecular mass of the unknown species was determined.

2.2.15 Preparation of Antisera

Purified denatured pea mitochondrial and chloroplastic dihydrolipoamide dehydrogenases were prepared by electroelution. Samples were subjected to electrophoresis on a 7% (w/v) SDS-polyacrylamide gel which was poured with a

comb containing a well of 11.5 x 1.0 cm. The gel was stained for 30 min with stain solution containing 0.1% (w/v) Coomassie Brilliant Blue type R250, 50% (v/v) methanol and 10% (v/v) acetic acid. The gel was then rinsed with destain, containing 10% (v/v) methanol and 20% (v/v) acetic acid, for 1h to remove any unbound stain and the protein bands of interest excised from the gel.

The gel pieces were placed in an S+S Biotrap Filtration and Separation apparatus (Schleicher & Schuell, Postfach 4, D-3354 Dassel, Germany) and subjected to electrophoresis overnight at 100V in electrode buffer containing 25 mM Tris, 192 mM glycine and 0.025% (w/v) SDS, and the solution in the trapping chamber removed. This solution was concentrated and rinsed with 20 mM potassium phosphate buffer, pH 7.0 using Amicon Centricon-30 microconcentrators (see 2.2.8).

Antiserum was prepared by dissolving approx. 0.2-1.0 mg of the desired antigen with 0.5 ml of 0.9% (w/v) NaCl mixed with 0.5 ml of Freund's complete adjuvant and injected subcutaneously into rabbits at various sites in the neck, back and thighs. A booster dose consisting of 0.1-0.5 mg of antigen in 0.5 ml of 0.9% (w/v) NaCl and 0.5 ml of Freund's incomplete adjuvant was administered 4 weeks later. The rabbits received a further booster 2 weeks prior to bleeding.

Antiserum was collected by bleeding the rabbit from an ear vein. The blood was allowed to clot overnight at 4°C and the antiserum removed using a Pasteur pipette. The antiserum was centrifuged at 700g for 5 min to remove any remaining red blood cells and stored in 1 ml aliquots at -80°C. Further collections of antisera were made monthly, two weeks after additional booster injections, using the procedure described above.

2.2.16 Western Blotting Using ^{125}I-Protein A

Protein samples were subjected to electrophoresis on an SDS-acrylamide gel and then transferred electrophoretically onto Hybond-C nitrocellulose membrane in the presence of 192 mM glycine, 0.02% (w/v) SDS, 20% (v/v) methanol and 25 mM Tris-HCl, pH 8.3 as described by Towbin *et al.* (1979) and Batteiger *et al.* (1982). The procedure was carried out in a BioRad Trans-Blot cell at 40 mA overnight.

To ensure that the proteins had transferred to the nitrocellulose membrane, the membrane was stained briefly with Ponceau S solution to visualise the transferred proteins and after visualisation, the membrane was rinsed with distilled water to remove the Ponceau S stain.

Excess binding sites were blocked by incubating the membrane for 1 h in wash buffer containing 0.15 M NaCl, 0.5% (v/v) Tween 20 and 20 mM Tris-HCl, pH 7.2. At this stage, the membrane was incubated with the desired antibody (diluted 1:100 with fresh wash buffer containing 5% (v/v) heat inactivated donkey serum) for 90 min and after incubation, rinsed 4 times with wash buffer over a period of 1 h to remove unbound antibody.

^{125}I-protein A (3×10^6 c.p.m.) was added to the membrane with 50 ml of wash buffer and incubated for 1 h before being rinsed with wash buffer 4 times over 1 h as before. The membrane was allowed to dry in air, placed in a cassette with X-Omat S film and stored at -80°C for 1-7 days to develop. After the cassette was removed from the freezer and allowed to reach room temperature, the film was removed and developed.

2.2.17 Western Blotting Using Enhanced Chemiluminescence (ECL)

Proteins were transferred onto nitrocellulose membrane overnight as described above except in the presence of 190 mM glycine, 20% (v/v) methanol and 25 mM Tris-HCl, pH 8.3.

Once transferred, the membrane was incubated for 4 h at 37°C in blocking solution containing 15 mM NaCl, 5% (w/v) non-fat milk, 5% (v/v) heat inactivated donkey serum, 0.2% (v/v) Tween 20 and 20 mM Tris-HCl, pH 7.2. The membrane was rinsed briefly with distilled water, then incubated overnight at 4°C in primary antibody solution containing 1% (w/v) non-fat milk, 1% (v/v) normal donkey serum, 0.1% (v/v) Tween 20, 20 mM Tris-HCl, pH 7.2 and a 1 in 5000 to 10,000 dilution of primary antibody.

After the incubation, the membrane was rinsed for 4 x 30 min in wash solution containing 15 mM NaCl, 1% (w/v) non-fat milk, 1% (v/v) heat inactivated donkey serum and 20 mM Tris-HCl, pH 7.2 and then incubated for 2 h in 150 mM NaCl, 1% (w/v) non-fat milk, 1% (w/v) heat inactivated donkey serum, 20 mM Tris-HCl, pH 7.2 and a 1:1000 dilution of anti-rabbit horseradish peroxidase-linked antibody (from donkey). The membrane was then rinsed for 3 x 30 min with wash solution and finally for 30 min with 150 mM NaCl and 20 mM Tris-HCl, pH 7.2.

Detection of bound antibody was performed by incubating the membrane in detection reagents A and B from Amersham for 1 min (0.125 ml cm^{-2} of membrane), removing excess solution from the membrane and covering with Saran Wrap. Hyperfilm-ECL was exposed to the membrane for between 2 s and 5 min depending on the strength of chemiluminescence.

2.2.18 N-Terminal Protein Sequencing

Samples for N-terminal sequencing were subjected to electrophoresis on a 7% (w/v) SDS-acrylamide gel which was made as follows:

Acrylamide Solution: 30% (w/v) BDH Electran molecular biology grade 1 acrylamide
0.8% (w/v) piperazine diacrylamide (PDA) crosslinker
This solution was filtered through Whatman filter paper.

Lower Tris Buffer: 380 mM Tris-HCl, pH 8.8
0.1% (w/v) SDS

Upper Tris Buffer: 125 mM Tris-HCl, pH 6.8
0.1% (w/v) SDS

Electrophoresis Buffer: 25 mM Tris-HCl pH 8.0
1 mM glycine
0.1% (w/v) SDS

A 7% (w/v) SDS-acrylamide resolving gel solution containing 16.8 ml acrylamide solution, 18 ml lower Tris buffer, 540 µl of 10% (w/v) ammonium persulphate and 37.2 ml distilled water was de-gassed and 72 µl of NNN'N'-tetramethylethylene-diamine (TEMED) added before the solution was poured into a casting apparatus. A layer of isopropanol was poured on top of the resolving gel to ensure an even surface. Once the gel had set, the isopropanol was removed by washing with distilled water, and residual water removed from the top of the gel.

A 5% (w/v) stacking gel was prepared with 5.9 ml acrylamide stock, 9.0 ml lower Tris buffer, 270 µl 10% (w/v) ammonium persulphate and 21.1 ml distilled water. This solution was degassed, polymerisation initiated with 36 µl TEMED, poured into the casting apparatus on top of the resolving gel, a comb inserted and the gel allowed to set.

5-10 µg of protein was heated at 100°C for 2 min with sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 2.5% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol. After heating, bromophenol blue was added to a final concentration of 0.002% (w/v).

The gel was subjected to electrophoresis, prior to loading the samples, at 25 mA using Upper Tris buffer containing 50 µM reduced glutathione in the top reservoir, and with electrophoresis buffer in the bottom reservoir. After 30 min, the buffers in the top and bottom reservoirs were removed and fresh electrophoresis buffer added to both. The samples were loaded and the gel subjected to a current of 40-65 mA.

Prior to transferring the proteins, a Problott membrane (Applied Biosystems, 850 Lincoln Drive, Foster City, California 94404) was wetted with methanol for a few seconds and placed in electroblotting buffer containing 10 mM 3-[cyclohexylamino]-1-propanesulphonic acid (CAPS), pH 11.0 and 10% (v/v) methanol. Once the bromophenol blue dye had reached the bottom of the gel, the proteins in the gel were transferred overnight to the Problott membrane in electroblotting buffer.

After transferring the proteins, the Problott was rinsed with distilled water and stained with 0.1% (w/v) Amido Black in 40% (v/v) methanol and 1% (v/v) acetic acid for 1 min. The Problott was then destained by washing extensively in distilled water. Bands of interest were then excised and allowed to air-dry.

The Problott membranes containing the proteins of interest were analysed at the Protein Sequencing Facility, University of Aberdeen on one of two Applied Biosystems model 477A automated sequencing instruments, each equipped with on-line PTH analysis and utilising either (a) gas-phase delivery with the manufacturer's standard 50 min program or (b) pulsed-liquid delivery using a 'Fastblot' program of 27 min duration which was designed 'in-house'.

2.2.19 Determination of Molecular Mass Using Electrospray Mass Spectrometry (ESMS)

Samples for mass spectrometry were prepared in HPLC grade distilled water by centrifuging in Amicon Centricon-30 microconcentrators and rinsing with one hundred volumes of HPLC grade water. The samples were concentrated to 1-2 mg/ml to obtain 1.6 nmol in 40 µl (for a 100 kDa protein).

Mass spectrometry was performed on a VG Platform quadruple mass spectrometer (2-3000 amu range) fitted with a pneumatically assisted electrospray (ionspray) source and controlled via the VG Mass-Lynx software (VG Biotech. Ltd., Altrincham, Cheshire, UK). Carrier solvent (1:1 (v/v) acetonitrile/water infusion) was controlled at 10 ml min^{-1} using a Harvard syringe Pump (Harvard Apparatus, South Natic, MA, USA). Capillary voltages were between 2.8 and 3.2 kV, extraction cone voltages 20-30 V, and the focusing cone voltage offset by +10 V. The source temperature was set at 65°C, the nebulising gas flow at 10 l h^{-1}, and the drying gas flow at 250 l h^{-1}. Lens stack voltages were adjusted to give maximum ion currents. The M_r range of 45,000-55,000 which contained >95% of the signal intensity for both purified E3 enzymes was scanned with a sweep time of 5 s. The instrument was calibrated over this M_r range with horse heart myoglobin.

Samples for analysis were diluted with an equal volume of 4% (v/v) formic acid in acetonitrile and 10-20 µl aliquots injected directly into the carrier stream. The MaxEnt deconvolution procedure (Ferridge *et al.*, 1992) was applied for quantitative analysis of the raw data using 1.0 Da peak width and 1 Da/channel resolution.

2.2.20 Phenol and Chloroform Extraction of DNA

To remove contaminating proteins from DNA samples, an equal volume of phenol (equilibrated to pH 8.0 with Tris) was added to the DNA sample. The solution was vortexed for several seconds before centrifuging at high speed on a microfuge for 10 min. The aqueous (upper) phase was removed and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) added. The solution was again vortexed and centrifuged, and the aqueous (upper) layer removed. An equal volume of chloroform:isoamyl alcohol (24:1) was added, vortexed and centrifuged. The aqueous phase was removed and the DNA ethanol precipitated (see 2.2.21).

2.2.21 Ethanol Precipitation of DNA

Precipitation of DNA was carried out for concentration purposes to remove contamination. The volume of the DNA sample was increased to approximately 200-400 µl to allow ease of handling. One tenth volume of 3 M sodium acetate was added followed by two volumes of ice-cold 100% (v/v) ethanol and this incubated at -80°C for 30 min. The solution was centrifuged at 17,500 g for 15 min (for large fragments of DNA) or 30 min (for small fragments of DNA) to pellet the DNA. The pellet was washed with 70% (v/v) ethanol and recentrifuged for either 15 min or 30 min, the supernatant removed and the pellet allowed to air-dry. The final DNA pellet was resuspended in either TE buffer, pH 8.0 (containing 10 mM Tris, pH 8.0 and 1 mM EDTA) or sterile distilled water.

The concentration of DNA in the samples was calculated by measuring the absorbance at 260 nm.

2.2.22 Isolation of mRNA

Isolation of mRNA was performed using the Mini Message Maker from R&D Systems Europe Ltd (4-10 The Quadrant, Barton Lane, Abingdon, OX14 3YS, UK) as follows.

(A) Preparation of Plant Tissue

Pea leaves were grown and harvested as described in section 2.2.1 and 130 mg of tissue weighed. This was transferred to a pre-chilled mortar and pestle and liquid nitrogen added. The leaves were ground to a very fine powder and 1.5 ml of pre-shaken lysis buffer (supplied with the kit) added to the ground tissue. The buffer was allowed to gradually thaw out and once it began to melt, it was agitated with the pestle until the homogenate was fully liquid.

The liquid was transferred to a microcentrifuge tube and spun for 3 min at full speed. The supernatant was removed, transferred to a fresh microcentrifuge tube and genomic DNA sheared by passing the solution four times through a 25 gauge needle attached to a syringe. This was recentrifuged at high speed for 3 min before transferring the supernatant to a fresh microcentrifuge tube and the volume made up to 1.5 ml using lysis buffer.

(B) Hybridization Reaction

The Oligo dT-latex beads (supplied with the kit) were resuspended by gently rotating for 3 min, 50 µl added to the microcentrifuge tube containing the total RNA solution and the tube inverted 10 times prior to leaving the tube to incubate at room temperature for 10 min. The mixture was centrifuged at full speed for 5 min in a microcentrifuge and the supernatant removed and discarded. The pellet was resuspended in 350 µl lysis buffer, the mixture transferred to a spin column and the column centrifuged at full speed for 2 min in a microcentrifuge.

(C) Washing

400 µl of wash buffer (supplied with the kit) was added to the spin column and the beads gently resuspended by pipetting up and down. The column was recentrifuged at full speed for 1 min in a microcentrifuge and the eluate discarded. The addition of wash buffer, resuspension and centrifugation was repeated, with the last centrifugation step for 2 min.

(D) Elution

The spin column insert was transferred to a microcentrifuge tube supplied with the kit and 50-100 µl of elution buffer (supplied with the kit), pre-heated to 70°C, added. The beads were resuspended by pipetting up and down ten times, and the column centrifuged at full speed for 1 min in a microcentrifuge. For maximum mRNA yield, the beads were resuspended again in another 50-100 µl of hot elution buffer and the column centrifuged for 1 min. The eluate contained mRNA.

2.2.23 First-Strand cDNA Synthesis

First-strand cDNA synthesis was performed using the First-Strand cDNA Synthesis Kit from Pharmacia Biotech (23 Grosvenor Rd, St. Albans, Herts. AL1 3AW) as follows.

To the sample containing the mRNA, RNAse-free water was added to bring the volume to 20 µl. The RNA solution was heated at 65°C for 10 min, then placed on ice. 11 µl of the Bulk First-Strand cDNA Reaction Mix, 1 µl DTT solution and 1 µl of Not I-d$(T)_{18}$ primer were added to a sterile microcentrifuge tube followed by the heat-denatured mRNA. The mixture was pipetted up and down several times prior to incubation at 37°C for 1 h. The completed first-strand cDNA reaction product was

incubated at 90°C for 5 min to denature the mRNA-cDNA duplex and inactivate the reverse transcriptase, then chilled on ice prior to PCR.

2.2.24 Polymerase Chain Reaction (PCR)

For PCR, the following amounts and volumes were used:
100 ng-1 µg of template DNA, 5 µg Primer 1, 5 µg Primer 2, 2.5 units Taq DNA Polymerase, 1x Assay buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0, 1% (v/v) Triton X-100), 2.5 mM $MgCl_2$, 0.2 mM each of dATP, dGTP, dTTP and dCTP and sterile water to a final volume of 50 µl.
A layer of mineral oil was applied to the surface prior to the PCR reaction.

PCR reactions were carried out in a Techne PHC-3 Thermal Cycler and the conditions used were:

Denaturation Step	94°C	3 min	1 cycle
Step Cycle	94°C	30 sec	35 cycles
	50°C	1 min	
	65°C	1 min	
Extension Cycle	72°C	10 min	
Soak Cycle	4°C	5 min to overnight	

2.2.25 Acrylamide Electrophoresis of DNA

Vertical acrylamide gels were used for visualising DNA fragments in the range of 20 bp to 500 bp. 10% (w/v) gels were poured by the following method.

A 30% (w/v) stock solution of acrylamide/bisacrylamide was prepared by dissolving 29 g of acrylamide and 1g of bisacrylamide in distilled H_2O to a final volume of 100 ml and filtered. For a single gel, 8.25 ml of 30% (w/v) stock solution was mixed with 2.5 ml 10x TBE (0.5 M Tris-HCl, pH 8.0, 0.4 M boric acid, 10 mM EDTA), 14.25 ml distilled H_2O and 313 µl 10% (w/v) ammonium persulphate. The solution was degassed prior to the addition of 31.3 µl TEMED to initiate polymerisation. The solution was poured into a casting apparatus (16 x 19.5 x 0.1 cm), a comb inserted between the plates and the gel allowed to set.

Electrophoresis was carried out in TBE (1x) at a constant voltage of 60 V for approx. 3 h prior to visualising the DNA with ethidium bromide.

2.2.26 Agarose Gel Electrophoresis of DNA

Agarose gels were made to a final concentration of 1.5% (w/v) in 1x TBE (see 2.2.25). Agarose solution was heated in a microwave for several minutes to dissolve the agarose, prior to pouring into a horizontal slab gel apparatus. TBE (1x) was used as electrode buffer.

Low melting point agarose gels were made to a final concentration of 1% (w/v) in 1x TAE (stock solution was 50x and contained 2.25 M Tris-HCl, pH 8.0, 6% (v/v) glacial acetic acid, 50 mM EDTA) and poured as described above for the TBE gels. TAE (1x) was used as electrode buffer.

2.2.27 Visualising DNA Using Ethidium Bromide

Gels were soaked in a solution of 0.5 μg ml^{-1} ethidium bromide for 15-30 min, then rinsed with distilled water for a further 15-30 min prior to visualisation under UV.

2.2.28 Digestion of dsDNA With Restriction Enzymes

Digestion of restriction sites on either the PCR products or the plasmid pBKS II+ were performed with the required amount of DNA, 0.1 vol Bam HI (10 units μl^{-1}), 0.1 vol Eco RI (10 units μl^{-1}), 0.1 vol restriction enzyme buffer and sterile distilled water at 37°C for up to 60 min.

2.2.29 Ligation of DNA

Ligation of the PCR products into the plasmid pBKS II+ was performed using 0.1 vol DNA ligase, 0.1 vol 10x ligase buffer, the digested PCR products and plasmid, and sterile distilled water overnight at 16°C.

2.2.30 Growth of *E. coli* in Luria-Bertani (LB) Medium

E. coli cells (XL1-Blue) were grown overnight at 37°C in LB medium, which contained 10 g l^{-1} bacto-tryptone, 5 g l^{-1} bacto-yeast extract and 10 g l^{-1} NaCl. The pH was brought to 7.0 with NaOH and sterilised by autoclaving. Agar plates were made to a final concentration of 1.5% (w/v).

2.2.31 Transformation of *E. coli* Cells Using $CaCl_2$

A sample of stock cells was grown overnight at 37°C. These cells were subcultured by taking 200 µl and growing in 5 ml fresh LB medium. After several hours 1 ml of the cells per transformation were pelleted by centrifugation at high speed for 30 s in a microcentrifuge. The cells were resuspended in 500 µl sterile 50 mM $CaCl_2$ and incubated on ice for 30 min. The cells were pelleted again and resuspended in 300 µl 50 mM $CaCl_2$ and incubated on ice for 10 min. DNA (100 ng to 1 µg) was added to the cells and incubated on ice for 20 min prior to heat-shock at 42°C for 90 s. The cells were placed on ice, 1 ml of LB added and the cells grown for approx. 1 h at 37°C. The cells were then pelleted and resuspended in 100 µl LB. Resuspended cells were spread on LB plates containing ampicillin (50 µg ml^{-1}) with 3.4 µmol IPTG (isopropyl β-D-thiogalactopyranoside) and 2.0 µmol X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) spread previously. The plates were then placed in an incubator at 37°C and the cells grown overnight.

2.2.32 Isolation of Double-Stranded Plasmid DNA

Double-stranded plasmid DNA was isolated from *E. coli* cells as follows (the volumes are for a 50 ml culture).

Solution 1: 50 mM glucose
25 mM Tris-HCl, pH 8.0
10 mM EDTA

Solution 2: 0.2 M NaOH
1% (w/v) SDS

Solution 3: 3 M potassium acetate
12% (v/v) acetic acid

Solution 4: 5 M LiCl
50 mM Tris-HCl, pH 8.0

A culture of cells was grown overnight at 37°C in LB containing ampicillin (50 µg ml^{-1}) and pelleted by centrifugation at 4500 g for 10 min. The pellet was resuspended in

2.5 ml solution 1 plus 2.5 ml solution 2 and 1.9 ml solution 3 added. The cells were centrifuged at 14,000 g for 15 min at 4°C and the resulting supernatant retained.

Isopropanol (6.9 ml) was added to the supernatant and the mixture incubated at room temperature for 15 min. The DNA and RNA were pelleted by centrifugation at 14,000 g for 15 min at 4°C and the pellet resuspended in 0.5 ml TE, pH 7.8. Solution 4 (0.5 ml) was added and the solution incubated on ice for 10 min. RNA was pelleted by centrifugation at 14,000 g for 15 min at 4°C then the DNA precipitated from the supernatant by the addition of 2 vol 100% (v/v) ethanol and incubated on ice for 15 min.

The DNA was pelleted by centrifugation at 14,000 g for 15 min at 4°C and the resulting pellet resuspended in 125 µl TE buffer, pH 8.0. DNAse-free RNAse (300 µg) was added to the resuspended DNA and incubated at 37°C for 60 min. DNA was then extracted with phenol and chloroform (see 2.2.20) followed by ethanol precipitation (see 2.2.21). The final pellet was resuspended in 50 µl TE buffer, pH 8.0.

2.2.33 Isolation of Single-Stranded Plasmid DNA

Colonies were grown overnight in 5 ml LB medium containing ampicillin (50 µg ml^{-1}) a subculture of 200 µl taken and grown in 5 ml of fresh LB containing ampicillin at 37°C for 2-3 h. To 500 µl, 5 µl VCS-M13 helper phage was added prior to incubation at room temperature for 15 min. This culture was then placed in 5 ml LB containing ampicillin and grown for 5 h before 2 ml was added to 50 ml LB containing ampicillin (150 µg ml^{-1}) and kanamycin (75 µg ml^{-1}) and grown overnight at 37°C.

The cells grown overnight were pelleted by centrifugation at 4500 g for 15 min. To the supernatant, 1/9th vol of 40% (w/v) polyethylene glycol 6000 (PEG) and 1/9th vol of 5 M sodium acetate (pH 7.0) were added and incubated at 4°C. After 15-30 min the mixture was centrifuged at 14,000 g for 10 min and the supernatant discarded. The pellet was resuspended in 200 µl TE buffer, pH 8.0, the DNA extracted with phenol and chloroform (see 2.2.20) and precipitated with ethanol (see 2.2.21). The final pellet was resuspended in 50 µl TE buffer, pH 8.0.

2.2.34 Single-Stranded DNA Sequencing

Single-stranded DNA sequencing was performed using the Sequenase v2.0 DNA Sequencing Kit from Amersham Life Science (Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK). This was performed as follows.

To 7 µl single-stranded DNA isolated using the procedure described in section 2.2.33, 2 µl reaction buffer and 1 µl -40 primer were added. The primer was annealed to the template DNA by heating at 65°C for 2 min before cooling slowly to <35°C over 25 min. The mixture was centrifuged briefly and placed on ice. While the annealing mixture was cooling, 2.5 µl of each dGTP Termination Mixture (G, A, T and C) was placed in microcentrifuge tubes and kept at room temperature.

The Labelling Mix was diluted 15-fold in sterile H_2O, the Sequenase Polymerase diluted 8-fold in ice-cold Enzyme Dilution Buffer and the four Termination Mixture tubes pre-warmed to 37°C.

To the 10 µl ice-cold annealed DNA mixture, 1 µl 0.1 M DTT, 2 µl diluted Labelling Mix, 0.5 µl [^{35}S] dATP and 2 µl diluted Sequenase Polymerase were added and the mixture incubated at room temperature for 4 min. The termination reaction was

performed by transferring 3.5 µl labelling reaction to each termination tube (G, A, T and C) and the incubation continued at 37°C for 5 min. The reaction was stopped by the addition of 4 µl Stop Solution. The reactions were then either loaded onto a sequencing gel or stored at -20°C overnight for loading the next day. Prior to loading the samples onto the gel, they were heated at 75°C for 2 min and 5 µl loaded in each lane.

Sequencing gels were prepared by dissolving 84 g of urea in 20 ml 10x TBE (see 2.2.25), 40 ml 29% (w/v) acrylamide/1% (w/v) bisacrylamide (see 2.2.25) and distilled water to a final volume of 200 ml. This was filtered and degassed. 40 ml was removed and 200 µl TEMED added to the 40 ml. This solution was used to seal the bottom of the sequencing plates (BioRad Sequi-Gen® Sequencing Cell). Once the acrylamide had polymerised, 160 µl TEMED was added to the remaining 160 ml acrylamide solution and this poured between the plates, placing a comb at the top to form wells for loading. This was allowed to polymerise before the electrophoresis apparatus was set up with 1x TBE as the electrode buffer. The gel was pre-warmed by running at 100 W for 60-90 min and 3 µl sequencing reactions then loaded onto the gel.

The gel was again run at 100 W until the second dye in the Stop Solution added to the sequencing reactions was two-thirds of the way down the gel. The gel was then placed in 10% (w/v) methanol and 10% (w/v) acetic acid for 15-20 min to rinse the urea from the gel, and the gel placed on a piece of large Whatmann filter paper and dried at 80°C. The dried gel was placed in a cassette with sequencing film in direct contact with the gel. The film was developed 2-3 days later.

Chapter 3

Isolation and Purification of a Dihydrolipoamide Dehydrogenase from Pea Leaf Chloroplasts

3.1 Introduction

Dihydrolipoamide dehydrogenase (E3), as stated previously, is an integral component of the 2-oxoacid dehydrogenase complexes and the glycine decarboxylase complex in eukaryotes and eubacteria. It catalyses the NAD^+-dependent reoxidation of dihydrolipoamide, which is bound to the acyltransferase (E2) component of the 2-oxoacid dehydrogenase complexes (Yeaman, 1989), or the hydrogen-carrier protein (H-protein) component of the glycine decarboxylase complex . It is a member of the group of flavin-containing pyridine nucleotide-disulphide oxidoreductases (Carothers *et al.*, 1989). Other members of this group are glutathione reductase, pantetheine-4'4"-diphosphate reductase, thioredoxin reductase, bis-γ-glutamyl cystine reductase, trypanothione reductase and mercuric reductase (Shames *et al.*, 1986; Fox & Walsh, 1983; Pigiet & Conley, 1977; Sundquist & Fahey, 1988; Swerdlow & Setlow, 1983). These enzymes are homodimers that contain one flavin adenine dinucleotide (FAD) molecule per subunit and a redox-active disulphide. They are resistant to heat and proteolysis while they are sensitive to some divalent cations, such as Hg^{2+} and Cu^{2+} and some arsenical derivatives (Holmgren, 1980; Knowles, 1985; Williams, 1976).

The three dimensional structure of a number of prokaryotic E3s have been determined by X-ray diffraction. Crystallographic studies of *Azotobacter vinelandii* (Mattevi *et al.*, 1991) and *Pseudomonas putida* (Mattevi *et al.*, 1992b) have revealed a dimeric organisation of identical subunits (M_r 50,000), with a catalytic centre at the interface between the two subunits. The flavin ring, which separates the binding sites of the NAD^+ and the dihydrolipoamide, has an adjacent disulphide bridge forming the redox centre that is involved in the electron transfer reaction between the substrates, dihydrolipoamide and NAD^+.

Dihydrolipoamide dehydrogenase has been characterised from a wide variety of prokaryotic and eukaryotic sources, with the most extensively studied from *E. coli*

and porcine heart. The cloning and sequencing of dihydrolipoamide dehydrogenase from many species has allowed extensive comparisons to be made (Stephens *et al.*, 1983; Otulakowski and Robinson, 1987). There are considerable similarities between E3s from *E. coli*, yeast, pig and human, in particular in the catalytic and structural domains (Carothers *et al.*, 1989). In general, it has been found that a common E3 is shared by PDC, OGDC, BCDC and GDC. However, in two species of *Pseudomonas*, two distinct E3s (LPD-val and LPD-glc) are expressed from separate genes (LPD-val and LPD-glc). The enzymes display complex specificity, with LPD-glc associating with PDC, OGDC and GDC, and LPD-val associating with BCDC (Sokatch and Burns, 1984). There is now also increasing evidence for the existence of isoenzymes of E3 in some species.

Dihydrolipoamide dehydrogenase has been isolated from the bloodstream form of *Trypanosoma brucei* (Danson *et al.*, 1987; Lohrer *et al.*, 1990) despite the absence of functional mitochondria and therefore, the multienzyme complexes with which E3 is normally associated. This E3 is also located specifically in the plasma membrane and may be involved in the shuttling of sugars across the membrane (Richarme and Heine, 1986), similar to a form of the enzyme in *E. coli*, which aids the transport of maltose and galactose. The exact physiological function of this enzyme is still unknown, although kinetic analysis and chemical modification studies indicate that it could be a true dihydrolipoamide dehydrogenase. However, E3 can be found in the mitochondrion in the insect procyclic form of *T. brucei* and is probably associated with the pyruvate dehydrogenase and 2-oxoglutarate complexes found there (Danson *et al.*, 1987; Jackman *et al.*, 1990; Cook *et al.*, 1990). Therefore, it appears that this species of E3 exists both independently and associated with the 2-oxoacid complexes. Moreover, both forms of E3 are encoded by the same gene and there is little if any difference between them (Else *et al.*, 1994).

The possibility of multiple isoforms of E3 in mammalian cells remains in dispute. Immunological observations indicate that mammalian cells may express multiple forms of E3. Carothers *et al.* (1987) identified two immunologically distinct dihydrolipoamide dehydrogenases in rat liver mitochondria. Antisera were raised to E3 from highly purified PDC and from commercially available porcine heart E3. As the commercial purification procedure does not involve isolation of a specific 2-oxoacid complex or of GDC, it may be possible that there is co-purification of potential isoenzymes. The resulting antiserum precipitated rat liver mitochondrial E3 whereas anti-PDC E3 serum failed to inactivate rat liver and pig heart E3 completely. In addition, anti-PDC E3 had no effect on GDC activity whereas anti-pig heart E3 inhibited glycine oxidation. Immunological investigations, therefore, suggest that rat liver mitochondria contain two forms of E3, one of which may be the L-protein involved in the decarboxylation of glycine.

In contrast to its mammalian counterpart, there is very little known about plant dihydrolipoamide dehydrogenase. Compared to mammalian and bacterial systems, there is greater difficulty in purifying the 2-oxoacid complexes and their associated E3 components from plant tissue, owing to the low abundance of mitochondria per unit of fresh weight of plant material (Randall *et al.*, 1990). Mitochondrial PDC has been purified to homogeneity only once, where 500 kg of broccoli (*Brassica oleracea*) florets yielded 1 mg of pure mitochondrial PDC (Rubin and Randall, 1977a). Despite this isolation, no analysis by SDS-PAGE or definitive subunit composition was provided. In addition to the difficulty in purifying plant mitochondria, phenols and endogenous proteases are present in plant tissue at high levels which can cause problems in the purification of intact multienzyme complexes and, generally, other plant proteins.

In plants, PDC is the most extensively studied of the 2-oxoacid dehydrogenase complexes. This complex is unusual in that there is substantial evidence for two

spatially distinct types, with one located in the mitochondrial matrix and the other located in the plastid stroma. Similar to its mammalian counterpart, plant mitochondrial PDC acts as the primary entry point of carbon into the tricarboxylic acid cycle. The subunit and cofactor requirements of this complex are typical of the complex from non-plant sources. Plastid PDC, however, differs in structural, catalytic and regulatory properties and is thought to provide acetyl CoA and NADH for fatty acid and isoprenoid biosynthesis (Camp and Randall, 1985; Randall *et al.*, 1989). Plant OGDC, in comparison, is a component enzyme of the TCA cycle itself and has only been identified in corn and soybean mitochondria where it displays approx. 20% of the activity of mitochondrial PDC (Cho *et al.*, 1988).

Previously, it was reported that BCDC is absent from plant mitochondrial and plastids and is instead located in peroxisomes (Gerbling and Gerhardt, 1988). However, recent research indicates that BCDC may also be present in plant mitochondria. Very low levels of BCDC activity were detected, albeit inconsistently, in pea leaf and root mitochondria (A. Carmichael, Ph.D. thesis, Glasgow University, 1994). Like PDC, GDC has been extensively studied, having been purified from a number of plant sources where it is located in the mitochondrial matrix and constitutes approx. one third of the soluble matrix protein (Walker and Oliver, 1986; Bourguignon *et al.*, 1988; Oliver *et al.*, 1990). In plants, GDC plays an important role in photorespiration where it catalyses the decarboxylation of glycine.

Recent investigations indicate that the mitochondrial E3 (L-protein) is shared by the 2-oxoacid dehydrogenase complexes and GDC (Bourguignon *et al.*, 1996). Thus, in plant mitochondria, there is a need of only one dihydrolipoamide dehydrogenase. However, with the substantial evidence of a plastid-specific PDC, the question of whether this complex shares the mitochondrial E3 or possesses a distinct plastid-specific dihydrolipoamide dehydrogenase has to be addressed. With this in mind, the

detection, isolation and characterisation of this plastid-specific enzyme from pea leaves was undertaken.

3.2 Results and Discussion

3.2.1 Isolation of Dihydrolipoamide Dehydrogenases from Organellar Extracts

Indications that two distinct organelle-specific dihydrolipoamide dehydrogenases were present in pea leaves arose from previous immunological comparison of highly purified pea leaf/root mitochondrial and chloroplast preparations which detected a separate cross-reacting species in plastids, with a subunit mass of 52 kDa, in addition to the known mitochondrial isoform. With this in mind, extraction of pea leaves was performed using a modification of the procedure described by Douce *et al.*(1972) (see 2.2.2).

Pea leaves were homogenised in grinding medium (see 2.2.2), filtered, then centrifuged at 20,000g for 20 min to pellet all organellar material. The pellets were resuspended in a minimal volume of 50 mM HEPES, pH 6.8 containing 0.2% (v/v) Triton X-100 to release the stromal and matrix proteins from the organelles. The use of detergent has previously been found to be more effective than other methods, such as freeze-thaw cycling, in solubilising organellar membranes and releasing membrane-bound proteins (R. Fullerton, PhD thesis, Glasgow University, 1995). As mentioned previously, dihydrolipoamide dehydrogenases from a variety of sources have been shown to be resistant to heat. Therefore, heat treatment of the organellar extracts was performed as a simple purification step. As can be seen in Table 3.1, this leads to a purification of approx. 10 fold from the previous stage, with the precipitation of proteins and organellar membranes that are sensitive to temperatures up to 65°C,

Stage of Purification	Volume (ml)	Protein Conc. (mg ml^{-1})	Total protein (mg)	Enzyme activity (units ml^{-1})	Total enzyme units	Percent recovery	Specific activity (units mg^{-1})	Purification factor
Total leaf extract	5223	6.8	35,516	0.67	3,499	100	0.099	1
Crude organellar extract	210	37.7	7,917	-	-	-	-	-
As above after heat treatment	169	4.7	795	14.7	2,487	71	3.13	31.8
Mono-Q purified Mitochondrial E3	0.27	13.8	3.2	3,421	920	28	295	2,394
Mono-Q purified Chloroplastic E3	0.20	5.4	1.08	335	67		62	

Table 3.1: Purification of pea mitochondrial and chloroplastic dihydrolipoamide dehydrogenase enzymes from total pea leaf extract

Percent recovery and purification factor refers to the combined dihydrolipoamide dehydrogenase activities. The above table refers to a 2kg preparation of pea leaves, with subsequent resolution on two runs on a MonoQ HR 10/10 column.

without significant loss of dihydrolipoamide dehydrogenase activity. Another advantage of heat treatment is the removal of heat-sensitive proteolytic enzymes present in the extracts. The supernatant, referred to as the whole organellar extract, was dialysed overnight into 25 mM HEPES, pH 6.8 (see 2.2.3), in preparation for resolution on an ion-exchange column. This procedure was also performed on pea roots.

3.2.2 Anion-Exchange Chromatography of Pea Leaf and Pea Root Organellar Extracts

Mitochondrial dihydrolipoamide dehydrogenase has previously been isolated from pea leaves by Bourguignon *et al.* (1992) and Turner *et al.* (1992). The authors utilised anion-exchange chromatography to further purify the enzyme from a partially-purified extract to near-homogeneity. Thus, the whole organellar extract from pea leaves, which had been dialysed overnight, was loaded onto a Pharmacia anion-exchange column (HR 5/5) using a fast protein liquid chromatography (FPLC) system, pre-equilibrated in 10 mM potassium phosphate containing 25 mM HEPES, pH 6.8, 1 mM EGTA and 1 mM mercaptoethanol (see 2.2.4). Bound proteins were eluted using a linear 10-400 mM potassium phosphate gradient and collected in 1 ml fractions. Dihydrolipoamide dehydrogenase activity was then determined in each fraction.

Figure 3.1 demonstrates the activity profile obtained from a whole organellar extract of pea leaves. Two peaks of dihydrolipoamide dehydrogenase activity can be seen, with the first major peak eluting at 130-150 mM potassium phosphate. The second minor peak was found to elute at 180-210 mM potassium phosphate, and corresponds to approx. 10-15% of total activity. When the three fractions with highest activity in each peak were pooled, concentrated, exchanged into 20 mM potassium phosphate, pH 7.0 (see 2.2.8), and subjected to electrophoresis on a 7% SDS-acrylamide gel (see

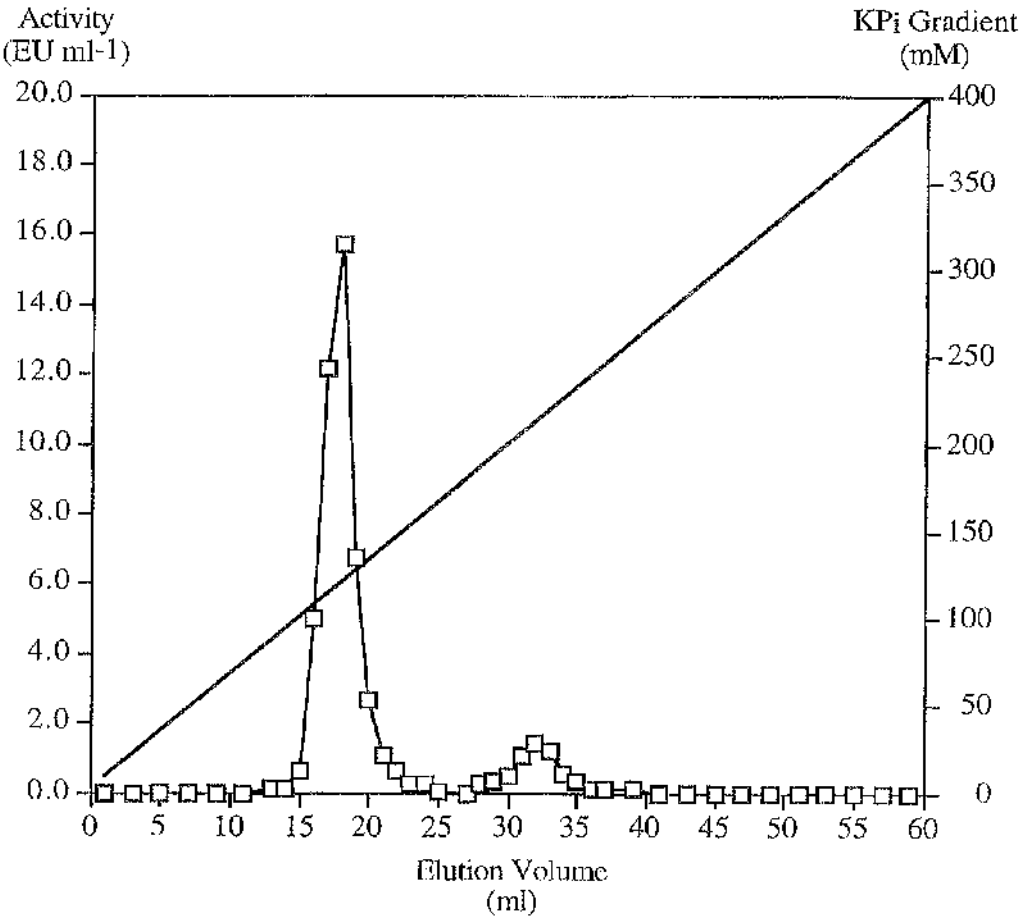

Figure 3.1: Resolution of pea leaf dihydrolipoamide dehydrogenase enzymes by anion exchange chromatography

Organellar extracts of pea leaves were solubilised in 50 mM HEPES buffer, pH 6.8 containing 0.2% (v/v) Triton X-100 and heat treated prior to resolution on a Mono Q HR 5/5 ion-exchange column using a Pharmacia FPLC System. Bound proteins were eluted at a flow rate of 0.5 ml min^{-1} with a potassium phosphate gradient (solid line, 10-400 mM) and 1.0 ml fractions collected for analysis. Dihydrolipoamide dehydrogenase activity (□), expressed in EU ml^{-1} (µmol NADH produced ml^{-1} min^{-1}), was determined in duplicate according to the method described in Materials and Methods, section 2.2.7.

2.2.11), a band of approx. 56 kDa can be resolved from the first major peak of activity (see Figure 3.2). The second minor peak of activity, however, reveals a band of approx. 52 kDa, noticeably lower than that obtained in the first peak. Thus, two dihydrolipoamide dehydrogenase activities were detected in pea leaves which apparently had different subunit molecular masses. Table 3.1 shows the purification regime for both enzymes, indicating that total dihydrolipoamide dehydrogenase activity can be recovered in a high yield in a three-step purification involving: (i) initial detergent release of the two enzymes from a crude organellar pellet; (ii) a thermal denaturation step to remove heat-sensitive polypeptides by precipitation and (iii) final resolution by Mono Q ion exchange chromatography.

Most of the dihydrolipoamide dehydrogenase activity (80-95%) was removed from the supernatant fluid by pelleting the crude organelle fraction. However, it proved impossible to obtain reliable estimates of dihydrolipoamide dehydrogenase activity in the detergent-solubilised organellar extract owing to the high background absorbance from photosynthetic pigments. Heat treatment was extremely effective in removing both the contaminating pigments and approx. 90% of protein with no appreciable loss of dihydrolipoamide dehydrogenase activity. After Mono Q separation of the two isoenzymes, the extent of purification of total dihydrolipoamide dehydrogenase activity is approx. 2400-fold with overall recoveries in the 25-30% range. It should be noted that recoveries from the Mono Q step were routinely 70-100%. However, it was necessary to pool only the peak fraction(s) to ensure a high degree of purity of the dihydrolipoamide dehydrogenase samples. Some additional losses were also incurred in the subsequent concentration steps.

A similar activity profile was obtained when extracts of pea roots were eluted from the Mono Q anion exchange column, where the first major peak of activity also eluted at 130-150 mM potassium phosphate. However, the second activity, eluting at 180-210 mM, was lower than the second peak observed in pea leaf extracts, corresponding to

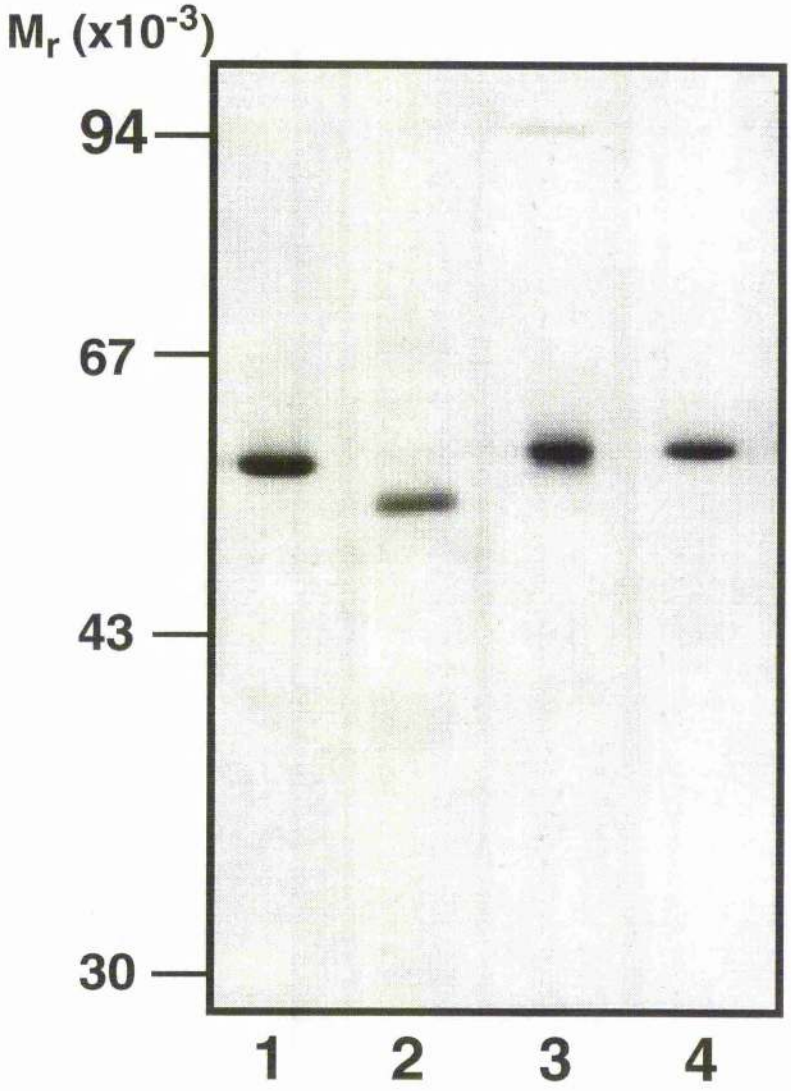

Figure 3.2: Analysis by SDS-PAGE of Mono Q-purified pea leaf dihydrolipoamide dehydrogenase isoenzymes

The two distinct dihydrolipoamide dehydrogenases purified from total leaf extract and finally resolved by Mono Q ion-exchange chromatography, as shown in Table 3.1, were subjected to electrophoresis on 7% (w/v) SDS gels and visualised by silver staining as described in Materials and Methods, section 2.2.12.

Lane 1, Mono Q purified dihydrolipoamide dehydrogenase (peak 1, 2 μg); lane 2, Mono Q purified dihydrolipoamide dehydrogenase (peak 2, 1 μg); lane 3, porcine E3 (2 μg) and yeast (*Candida utilis*) E3 (2 μg). M_r standards were resolved on an adjacent lane.

approx. 1-2% of total activity (see Figure 3.3).

3.2.3 Isolation of Dihydrolipoamide Dehydrogenases from Pea Leaf Chloroplasts and Mitochondria

The possibility that the two dihydrolipoamide dehydrogenase activities found in leaves represented specific mitochondrial and chloroplastic dihydrolipoamide dehydrogenase isoforms was investigated using similar detergent extracts prepared from Percoll-purified pea leaf mitochondria and chloroplasts. When an extract of pea leaf mitochondria was eluted from the Mono Q column and the fractions assayed for dihydrolipoamide dehydrogenase activity only one peak of activity is found, eluting at 130-150 mM potassium phosphate (Figure 3.4). This is at an identical position to the first peaks in the pea leaf and root whole organellar extract Mono Q profiles. In contrast, analysis of purified chloroplastic extracts by FPLC Mono Q ion exchange chromatography provided evidence for the exclusive association of the second, minor peak of dihydrolipoamide dehydrogenase activity in the whole organellar extracts with the plastids in pea leaves (see Figure 3.5).

Low levels of contamination of chloroplast-specific (peak 2) activity with the mitochondrial isoform (peak 1) could also be detected on occasions but were absent in the best chloroplast preparations. The low proportion of dihydrolipoamide dehydrogenase activity in chloroplasts (10-15%) is in line with previous estimates of the relative activities of PDC in these two compartments (Miernyk *et al.*, 1985; Lernmark & Gardestrom 1994).

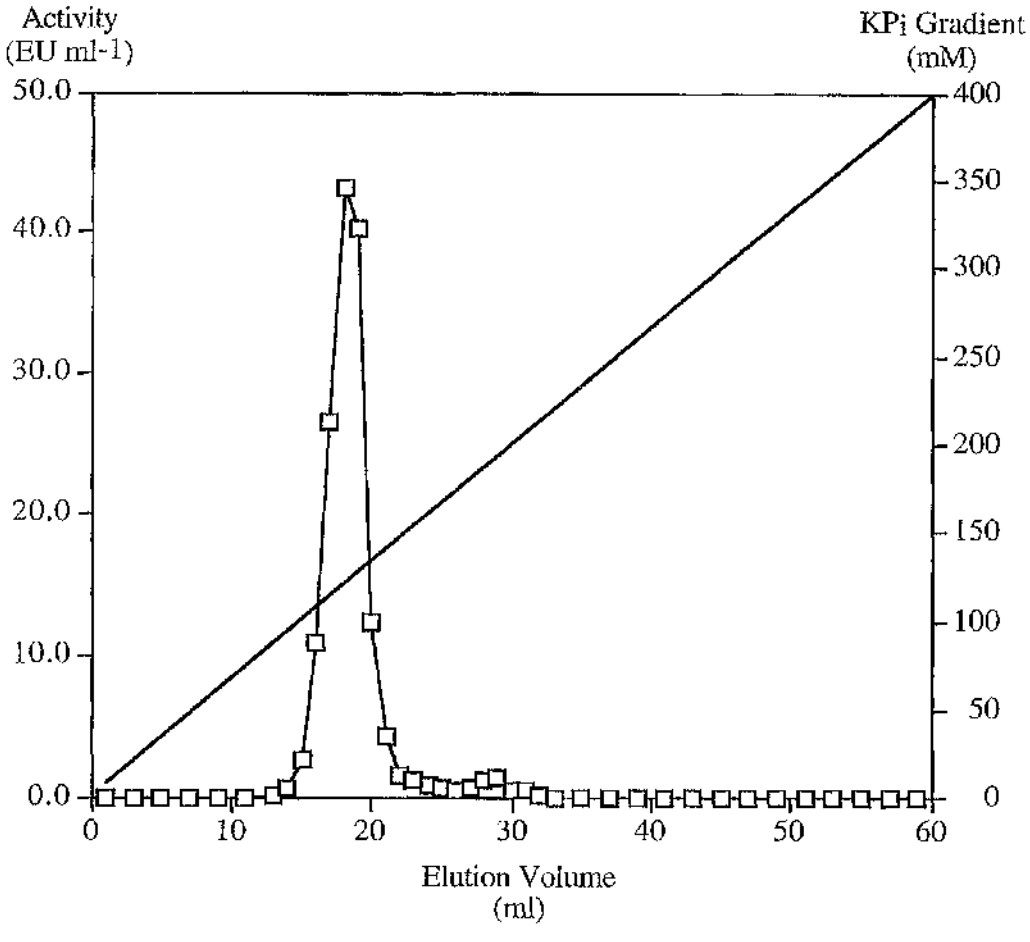

Figure 3.3: Resolution of pea root dihydrolipoamide dehydrogenase enzymes by anion exchange chromatography

Organellar extracts of pea roots (150g) were solubilised in 50 mM HEPES buffer, pH 6.8 containing 0.1% (v/v) Triton X-100 and heat treated prior to resolution on a Mono Q HR 5/5 ion-exchange column using a Pharmacia FPLC System. Bound proteins were eluted at a flow rate of 0.5 ml min^{-1} with a potassium phosphate gradient (solid line, 10-400 mM) and 1.0 ml fractions collected for analysis. Dihydrolipoamide dehydrogenase activity (□), expressed in EU ml^{-1} (μmol NADH produced ml^{-1} min^{-1}), was determined in duplicate according to the method described in Materials and Methods, section 2.2.7.

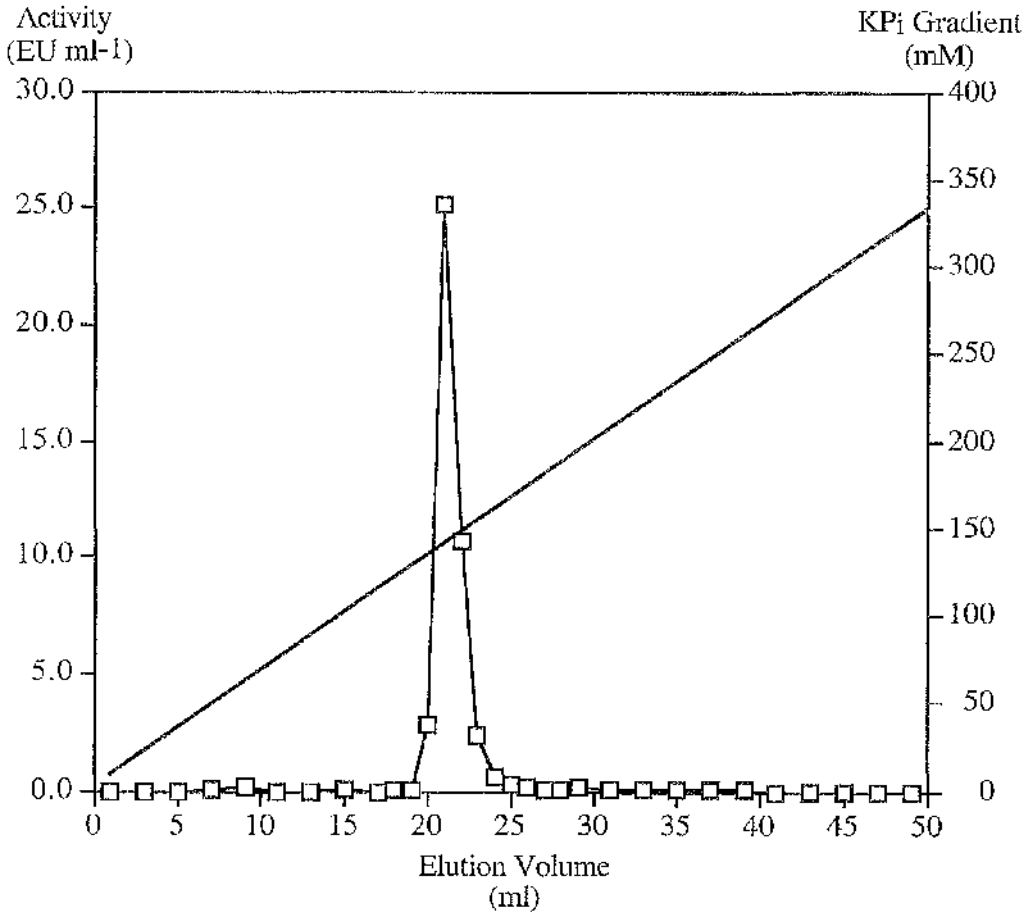

Figure 3.4: Resolution of pea leaf mitochondrial dihydrolipoamide dehydrogenase by anion exchange chromatography

Pea leaf mitochondrial extracts were solubilised in wash buffer (minus BSA) containing 0.1% (v/v) Triton X-100 and heat treated prior to resolution on a Mono Q HR 5/5 ion-exchange column using a Pharmacia FPLC System. Bound proteins were eluted at a flow rate of 0.5 ml min^{-1} with a potassium phosphate gradient (solid line, 10-400 mM) and 1.0 ml fractions collected for analysis. Dihydrolipoamide dehydrogenase activity (□), expressed in EU ml^{-1} (µmol NADH produced ml^{-1} min^{-1}), was determined in duplicate according to the method described in Materials and Methods, section 2.2.7.

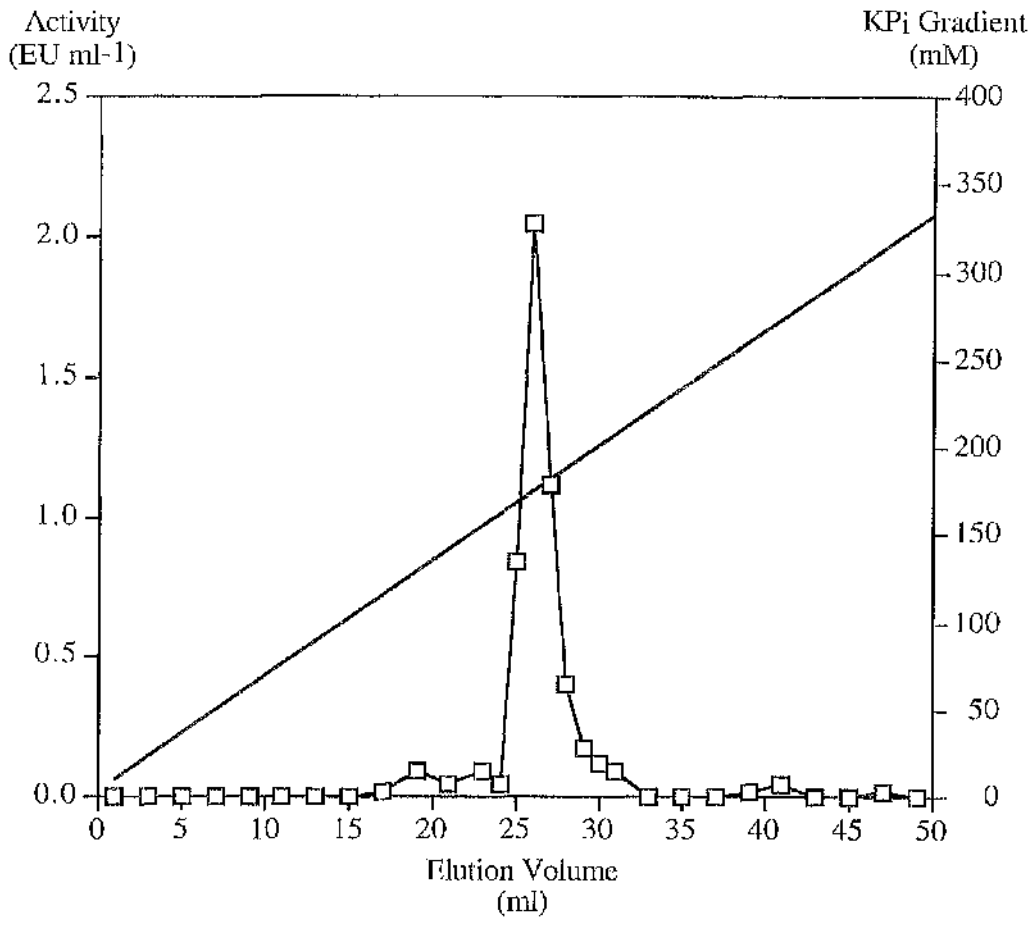

Figure 3.5: Resolution of pea leaf chloroplastic dihydrolipoamide dehydrogenase by anion exchange chromatography

Pea leaf chloroplastic extracts were solubilised in wash buffer (minus BSA) containing 0.2% (v/v) Triton X-100 and heat treated prior to resolution on a Mono Q HR 5/5 ion-exchange column using a Pharmacia FPLC System. Bound proteins were eluted at a flow rate of 0.5 ml min^{-1} with a potassium phosphate gradient (solid line, 10-400 mM) and 1.0 ml fractions collected for analysis. Dihydrolipoamide dehydrogenase activity (□), expressed in EU ml^{-1} (μmol NADH produced ml^{-1} min^{-1}), was determined in duplicate according to the method described in Materials and Methods, section 2.2.7.

When these peak fractions were subjected to electrophoresis with the peak fractions from the whole organellar extracts from pea leaves and pea roots, it was observed that the fractions corresponding to the first peak were identical in mass to the pea leaf mitochondrial dihydrolipoamide dehydrogenase as determined by SDS-PAGE (Figure 3.6). The fractions corresponding to the second peak, however, were identical in mass to the polypeptide obtained from pea chloroplasts i.e. 52 kDa, which again, displayed a lower M_r value than the mitochondrial band. Thus, a polypeptide with a lower molecular mass than mitochondrial E3 that possessed dihydrolipoamide dehydrogenase activity could be detected in pea chloroplasts. Once the precise location of these two distinct activities had been established, it was unnecessary to resort to isolation of Percoll-purified chloroplasts or mitochondria, resulting in routinely higher yields of both enzymes.

3.2.4 Immunological Detection of Dihydrolipoamide Dehydrogenase Isolated from Pea Leaves and Roots

The cross reactivities of the mitochondrial and chloroplastic dihydrolipoamide dehydrogenase using antisera to pea leaf mitochondrial dihydrolipoamide dehydrogenase and pea leaf chloroplastic dihydrolipoamide dehydrogenase was investigated in Figures 3.7 and 3.8. Pea root whole organellar peaks 1 and 2, pea leaf whole organellar peaks 1 and 2, pea leaf mitochondrial and pea leaf chloroplastic dihydrolipoamide dehydrogenase were subjected to SDS-PAGE electrophoresis (Figure 3.6), transferred to nitrocellulose membrane, and blotted with either anti-pea leaf mitochondrial E3 serum or anti-pea leaf chloroplastic dihydrolipoamide dehydrogenase serum. Figure 3.7 reveals that the anti-mitochondrial E3 IgG reacted only with the purified enzyme from mitochondrial sources. This antiserum was also unable to cross-react with either the porcine E3 or the yeast E3, suggesting that it is highly specific for plant mitochondrial dihydrolipoamide dehydrogenases. The anti-

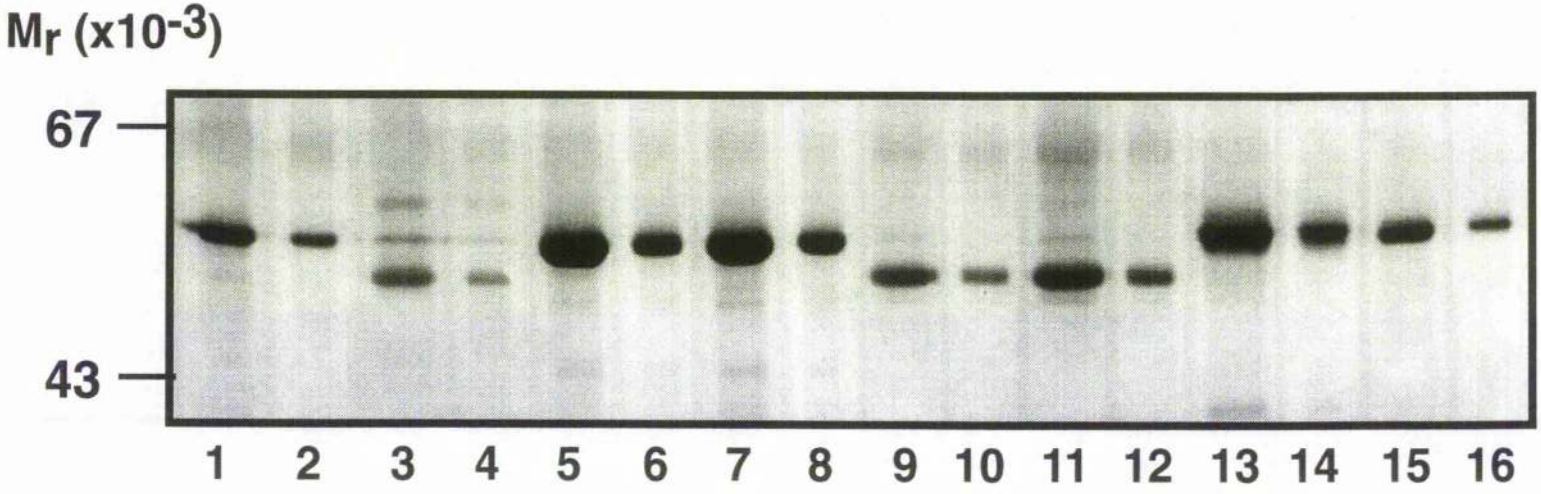

Figure 3.6: Analysis by SDS-PAGE of Mono Q-purified pea leaf and pea root crude organelle, pea mitochondrial and chloroplastic dihydrolipoamide dehydrogenases

Purified dihydrolipoamide dehydrogenases from crude leaf and root extracts, and Percoll-purified pea leaf mitochondria and chloroplasts, were subjected to electrophoresis on 7% (w/v) SDS/polyacrylamide gels prior to detection by silver staining as described in the Materials and Methods, section 2.2.12.

Lanes 1-4, purified E3 peak 1 fraction (lane 1, 1 μg; lane 2, 0.25 μg) and peak 2 fractions (lane 3, 1 μg; lane 4, 0.25 μg) from Mono Q-resolved enzyme derived from crude pea root extracts (see Figure 3.3). Lanes 5-8, purified E3 from pea leaf mitochondria (lane 5, 2 μg; lane 6, 1 μg)(see Figure 3.4) and peak 1 fractions (lane 7, 2 μg; lane 8, 1 μg) from crude pea leaf extracts (see Figure 3.1). Lanes 9-12, purified E3 from Percoll-purified chloroplasts (lane 9, 1 μg; lane 10, 0.25 μg)(see Figure 3.5) and peak 2 fractions (lane 11, 1 μg; lane 12, 0.25 μg) from crude pea leaf extracts (see Figure 3.1). Lanes 13 and 14, porcine E3 (lane 13, 2 μg; lane 14, 0.5 μg). Lanes 15 and 16, yeast (*Candida utilis*) E3 (lane 15, 1 μg; lane 16, 0.25 μg). M_r standards were run on adjacent lanes.

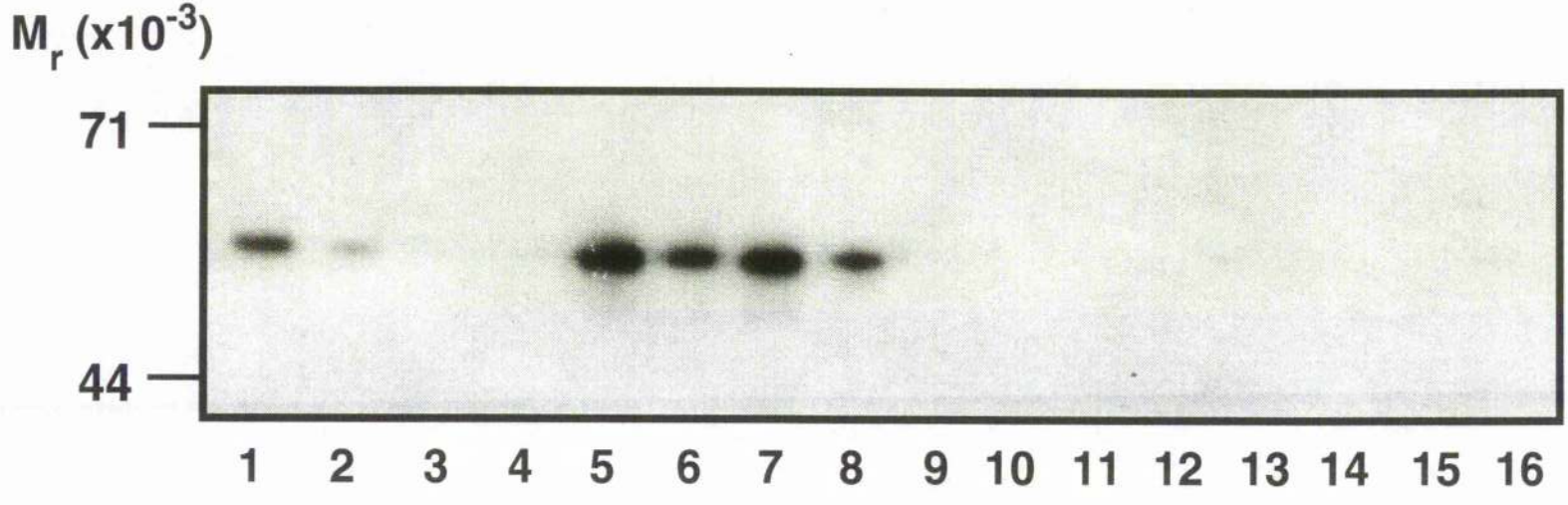

Figure 3.7: Lack of cross-reactivity of pea plastid dihydrolipoamide dehydrogenase with antibodies raised to the mitochondrial enzyme

Purified dihydrolipoamide dehydrogenases from crude leaf and root extracts, and Percoll-purified pea leaf mitochondria and chloroplasts, were subjected to electrophoresis on 7% (w/v) SDS/polyacrylamide gels and were transferred to nitrocellulose prior to detection by anti-pea leaf mitochondrial E3 serum as described in the Materials and Methods, section 2.2.16.

Lanes 1-4, purified E3 peak 1 fraction (lane 1, 0.5 μg; lane 2, 0.1 μg) and peak 2 fractions (lane 3, 0.5 μg; lane 4, 0.1 μg) from Mono Q-resolved enzyme derived from crude pea root extracts (see Figure 3.3). Lanes 5-8, purified E3 from pea leaf mitochondria (lane 5, 1 μg; lane 6, 0.5 μg)(see Figure 3.4) and peak 1 fractions (lane 7, 1 μg; lane 8, 0.5 μg) from crude pea leaf extracts (see Figure 3.1). Lanes 9-12, purified E3 from Percoll-purified chloroplasts (lane 9, 0.5 μg; lane 10, 0.1 μg)(see Figure 3.5) and peak 2 fractions (lane 11, 0.5 μg; lane 12, 0.1 μg) from crude pea leaf extracts (see Figure 3.1). Lanes 13 and 14, porcine E3 (lane 13, 1 μg; lane 14, 0.25 μg). Lanes 15 and 16, yeast (*Candida utilis*) E3 (lane 15, 0.5 μg; lane 16, 0.1 μg). M_r standards were run on adjacent lanes.

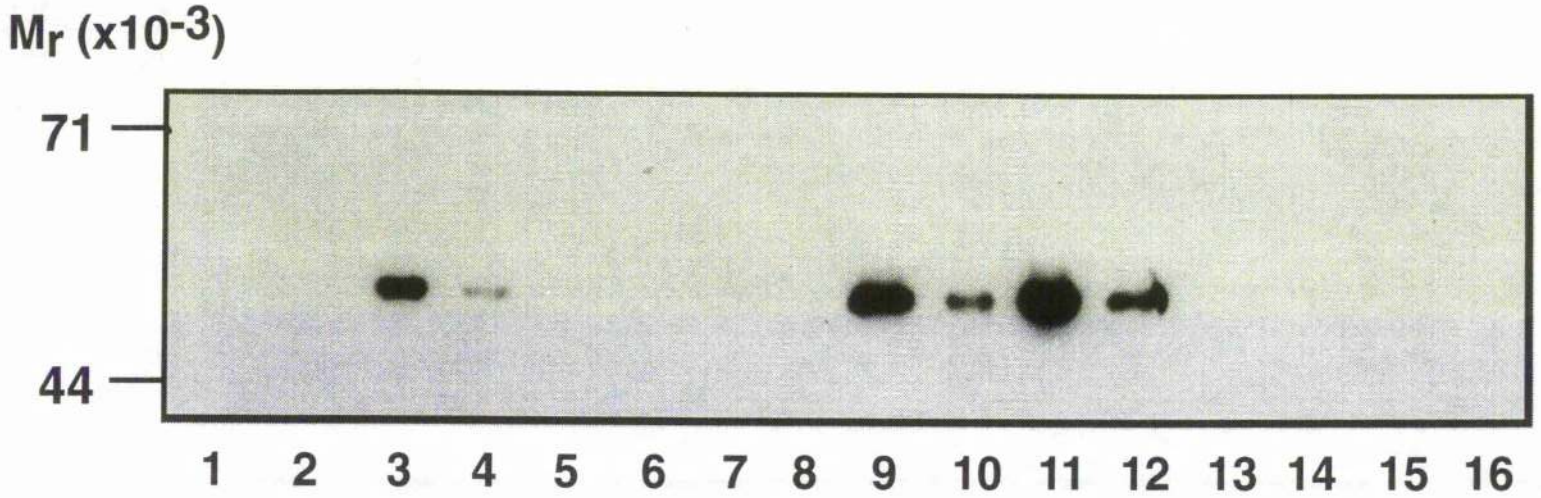

Figure 3.8: Lack of cross-reactivity of pea mitochondrial dihydrolipoamide dehydrogenase with antibodies raised to the chloroplastic enzyme

Purified dihydrolipoamide dehydrogenases from crude leaf and root extracts, and Percoll-purified pea leaf mitochondria and chloroplasts, were subjected to electrophoresis on 7% (w/v) SDS/polyacrylamide gels and were transferred to nitrocellulose prior to detection by anti-pea leaf chloroplastic dihydrolipoamide dehydrogenase serum as described in the Materials and Methods, section 2.2.16.
Lanes 1-4, purified E3 peak 1 fraction (lane 1, 0.5 μg; lane 2, 0.1 μg) and peak 2 fractions (lane 3, 0.5 μg; lane 4, 0.1 μg) from Mono Q-resolved enzyme derived from crude pea root extracts (see Figure 3.3). Lanes 5-8, purified E3 from pea leaf mitochondria (lane 5, 1 μg; lane 6, 0.5 μg)(see Figure 3.4) and peak 1 fractions (lane 7, 1 μg; lane 8, 0.5 μg) from crude pea leaf extracts (see Figure 3.1). Lanes 9-12, purified E3 from Percoll-purified chloroplasts (lane 9, 0.5 μg; lane 10, 0.1 μg)(see Figure 3.5) and peak 2 fractions (lane 11, 0.5 μg; lane 12, 0.1 μg) from crude pea leaf extracts (see Figure 3.1). Lanes 13 and 14, porcine E3 (lane 13, 1 μg; lane 14, 0.25 μg). Lanes 15 and 16, yeast (*Candida utilis*) E3 (lane 15, 0.5 μg; lane 16, 0.1 μg). M_r standards were run on adjacent lanes.

chloroplastic dihydrolipoamide dehydrogenase serum, however, failed to react with the mitochondrial E3s and recognised only dihydrolipoamide dehydrogenases from plastidic sources (Figure 3.8). Again, no reaction was observed with yeast or porcine E3. These results suggest a large degree of sequence difference as seen by a lack of cross-reaction between polyclonal antisera made to denatured pea leaf mitochondrial dihydrolipoamide dehydrogenase and the chloroplastic isoenzyme, and vice versa. When the peak activities obtained from pea root whole organellar extracts were probed with antiserum to the pea leaf mitochondrial enzyme, a band of approx. 56 kDa was observed in the first major peak of activity. However, in the second minor peak of activity, the antisera to the chloroplastic dihydrolipoamide dehydrogenase revealed a polypeptide of 52 kDa, identical in size to the dihydrolipoamide dehydrogenase from pea leaf chloroplasts. Therefore, it appears that this second peak of activity is also a dihydrolipoamide dehydrogenase, distinct from the mitochondrial isoform, and represents a plastid-specific dihydrolipoamide dehydrogenase isoform, which is probably expressed as the identical gene product in chloroplasts and plastids from non-photosynthetic tissues.

Previous immunological studies on related families of proteins from different species e.g. lysozyme or haemoglobins have shown that 50-60% sequence identity at the amino acid level is required before a significant degree of cross-reactivity is observed, indicating that the degree of similarity between the chloroplastic and mitochondrial isoenzymes might be less than 50% (Prager and Wilson, 1971).

The data presented here provide chromatographic and immunological evidence for a distinctive dihydrolipoamide dehydrogenase found only in pea leaf chloroplasts and plastids from non-photosynthetic tissues.

Chapter 4

Comparison of Biochemical Properties of Pea Leaf Mitochondrial and Chloroplastic Dihydrolipoamide Dehydrogenases

4.1 Introduction

In chapter three, the isolation of a dihydrolipoamide dehydrogenase associated with pea leaf chloroplasts was performed and an apparent difference in antigenic properties using antibodies raised to both the pea leaf mitochondrial and the chloroplastic isoforms detected. In order to further differentiate between the chloroplastic and the mitochondrial dihydrolipoamide dehydrogenase, characterisation of the biochemical properties of both enzymes was performed.

4.2 Results and Discussion

4.2.1 Analysis of the Molecular Mass of Chloroplastic and Mitochondrial Dihydrolipoamide Dehydrogenase by Electrospray Mass Spectrometry (ESMS)

Electrospray mass spectrometry is based on an idea proposed by Dole (1968) which involves passing the solution of molecules to be analysed through a needle that is kept at high electrical potential. The solution is dispersed at the end of the needle into a fine mist of small, highly charged droplets containing protein molecules. The small droplets rapidly evaporate and by a process of either field desorption or residual solvent evaporation, protonated molecules are released into the gas phase. Once the protein ions are in the gas phase, they are leaked into the vacuum of a mass spectrometer, where they are separated and detected according to their mass/charge ratio (Figure 4.1).

When subjected to SDS-polyacrylamide electrophoresis, the chloroplastic enzyme has an apparent molecular mass of approx. 52 kDa and the mitochondrial E3 has an apparent molecular mass of approx. 56 kDa. To obtain the precise molecular

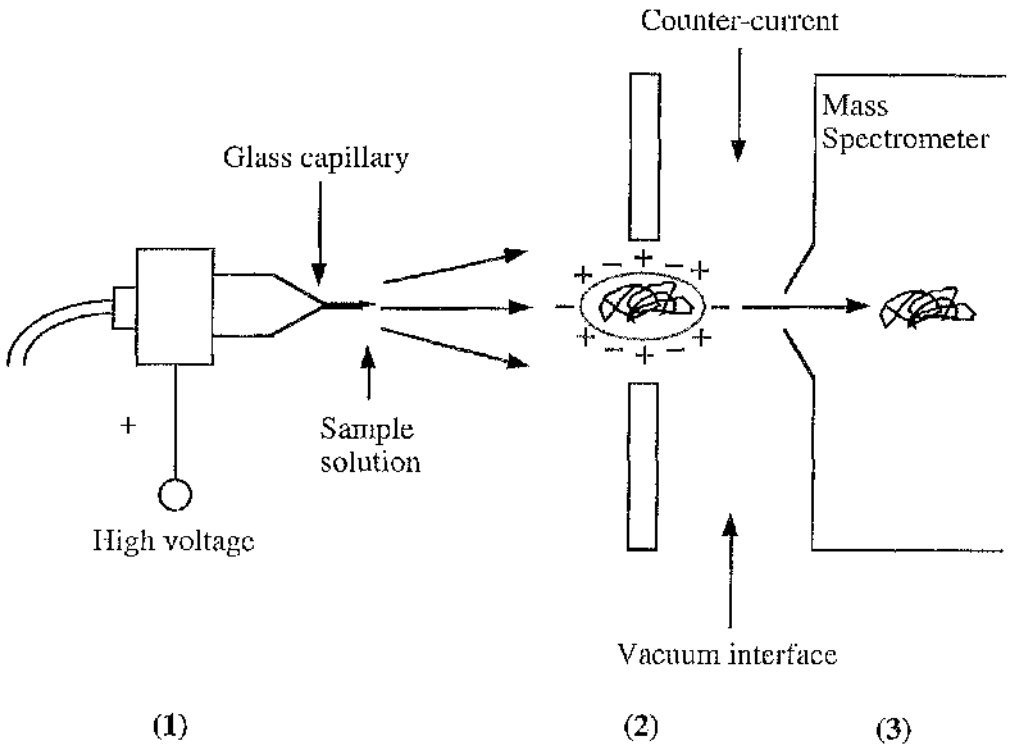

Figure 4.1: Illustrative diagram of electrospray mass spectrometry. Step 1 involves the formation of small, highly charged droplets by electrostatic dispersion of a solution under the influence of a high electric field. Step 2 involves desorption of protein ions from the droplets into the gas phase (assisted by a countercurrent of hot N_2 gas). Mass analysis of the ions is performed in a mass spectrometer in step 3.

mass of chloroplastic and mitochondrial dihydrolipoamide dehydrogenase, electrospray mass spectrometry was employed (see 2.2.19).

Upon analysis of the mass spectrometry profile for the chloroplastic E3 (Figure 4.2), it can be seen that this enzyme has a molecular mass of 52,614 ± 9.89 Da, which compares favourably with the apparent molecular mass as determined by SDS-PAGE (52,000 ± 1000 Da). The mitochondrial E3 (Figure 4.3), however, has a subunit molecular mass of 49,760 ± 1.49 Da, which is considerably lower than the apparent molecular mass (56,000 Da) determined by SDS-PAGE. The molecular basis for the anomalous behaviour of mitochondrial dihydrolipoamide dehydrogenase is not understood but clearly it is a property not shared by the chloroplastic enzyme. Interestingly, the M_r of the mitochondrial enzyme is close (within 30 mass units) to the predicted value from the corresponding cDNA sequence and relative molecular mass obtained by ESMS as isolated by Bourguignon *et al.* (1992). This small difference is likely to reflect the presence of conserved substitutions as different varieties of pea were employed in these two studies.

The possibility that the chloroplastic dihydrolipoamide dehydrogenase could be a proteolytic fragment of the apparently larger mitochondrial enzyme was discounted by the above results since the chloroplastic isoform is actually larger than the mitochondrial enzyme.

This discrepancy in molecular mass is also observed upon analysis of the M_r values of the subunits comprising the three distinct mitochondrial dihydrolipoamide dehydrogenases from potato tuber (personal communication, S. Khan, Glasgow University) which have higher apparent molecular masses as estimated by SDS-PAGE acrylamide gel electrophoresis than the actual molecular mass obtained by electrospray mass spectrometry.

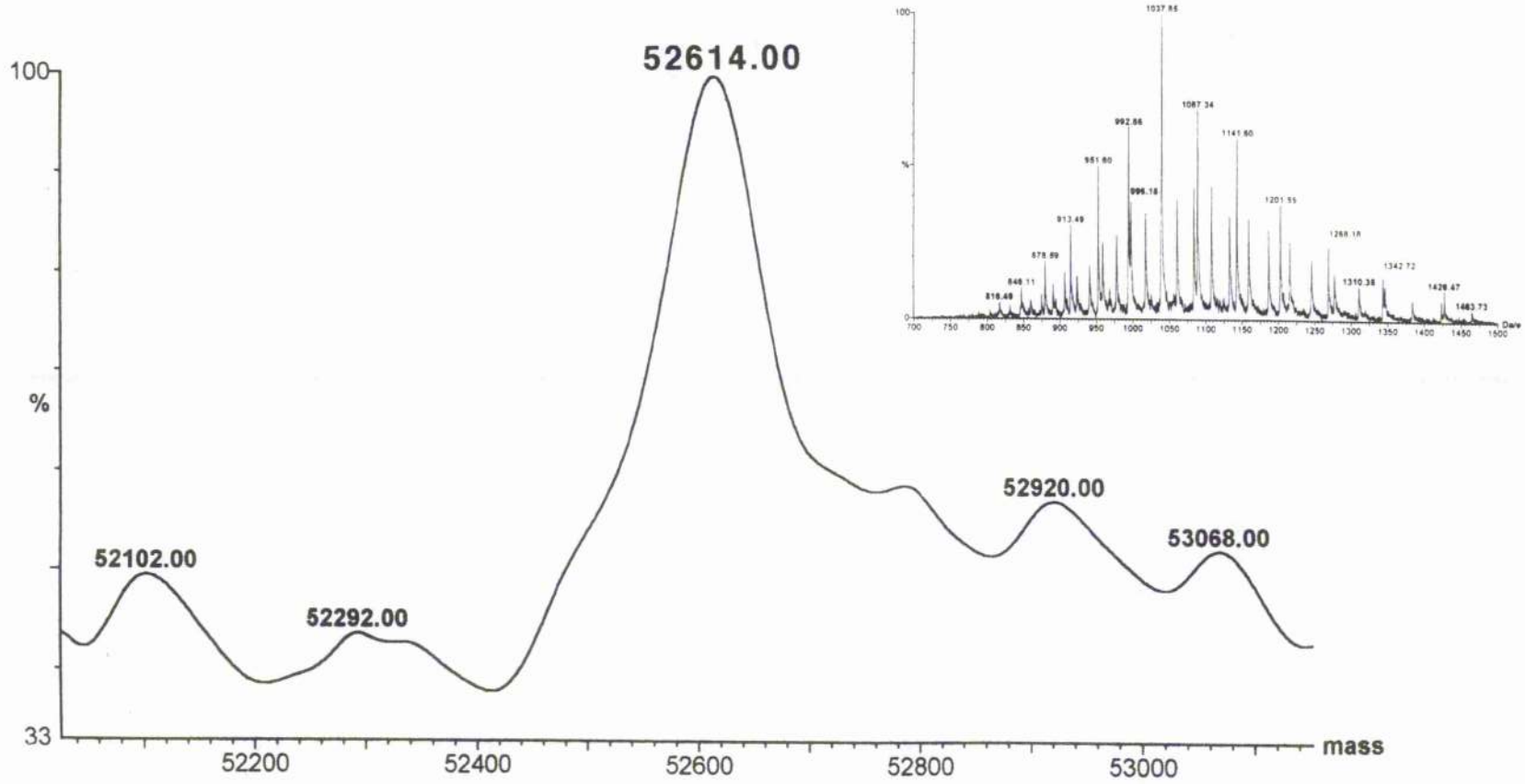

Figure 4.2: Computer deconvolution of the series of mass-to-charge ratio peaks to molecular mass peaks for chloroplastic dihydrolipoamide dehydrogenase

The chloroplastic enzyme corresponding to the major peak at 52,614, with minor contaminating peaks before and after the major peak. The inset contains a spectrum of mass-to-charge ratio peaks of chloroplastic dihydrolipoamide dehydrogenase.

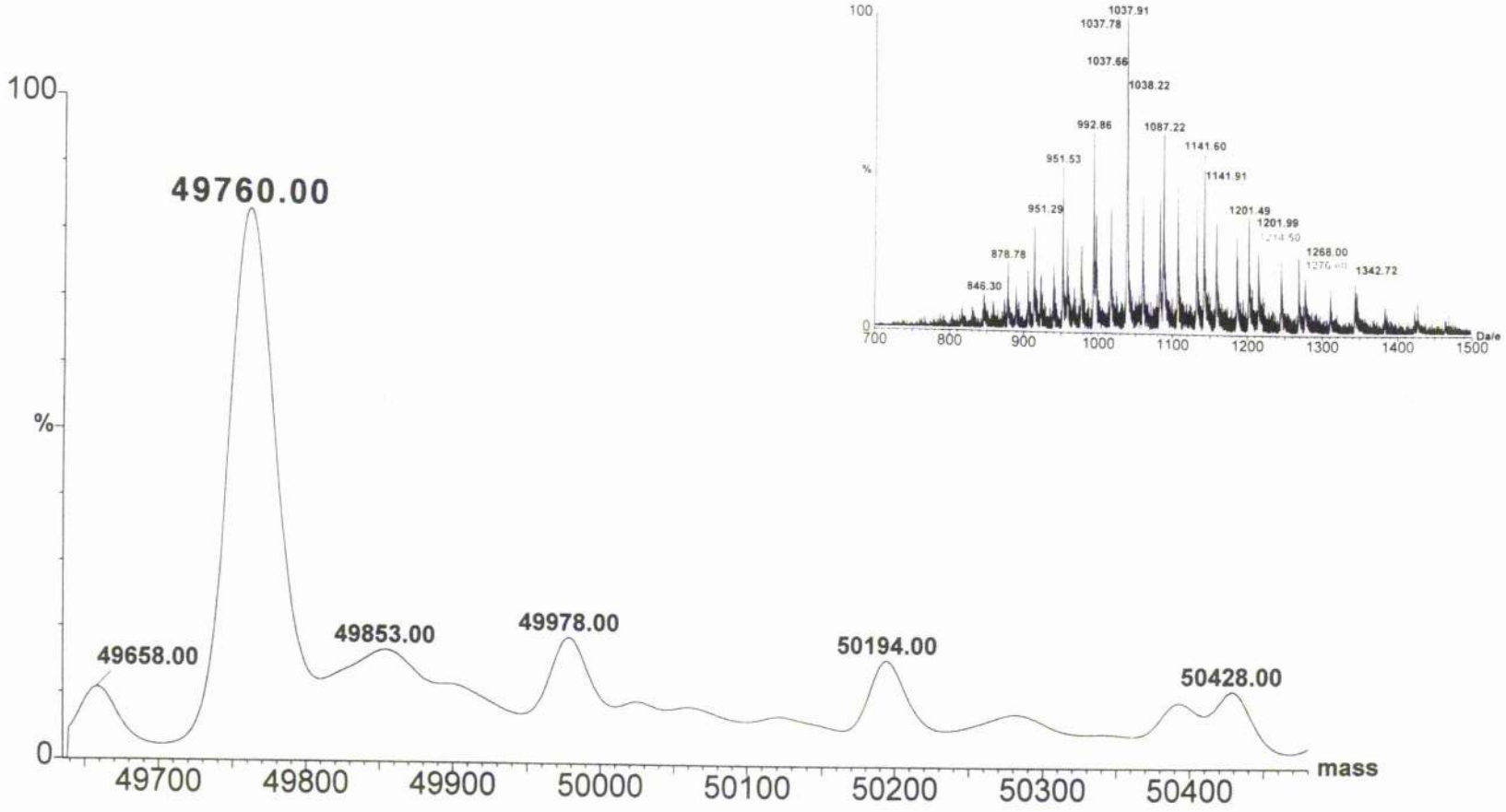

Figure 4.3: Computer deconvolution of the series of mass-to-charge ratio peaks to molecular mass peaks for mitochondrial dihydrolipoamide dehydrogenase

The mitochondrial enzyme corresponding to the major peak at 49,760, with very minor contaminating peaks before and after the major peak. The inset contains a spectrum of mass-to-charge ratio peaks of mitochondrial dihydrolipoamide dehydrogenase.

4.2.2 Determination of Native Molecular Mass by Gel Filtration

All mitochondrial dihydrolipoamide dehydrogenases to date have been found to exist as homodimers, with the catalytic sites shared between the two subunits. Pea leaf mitochondrial E3, or L-protein in GDC, has also been shown to be a dimer of identical subunits (Bourguignon *et al.*, 1992). However, whether this applies to the chloroplastic dihydrolipoamide dehydrogenase is unknown, since this form of the enzyme has not been isolated or studied until now and may be of different evolutionary origin. To determine the subunit composition of the plastidic enzyme, the native molecular mass was determined using gel filtration.

Initially, standard molecular mass marker proteins (Blue Dextran, M_r 2,000,000; β-amylase, M_r 200,000; alcohol dehydrogenase, M_r 150,000; bovine serum albumin, M_r 66,000; carbonic anhydrase, M_r 29,000) were resolved on a Superdex 200 gel filtration column from Pharmacia Biotech, connected to a fast protein liquid chromatography (FPLC) system. The markers were eluted from the column at 1.0 ml min^{-1} using running buffer containing 20 mM potassium phosphate, pH 7.0 / 20 mM NaCl, and the absorbance at 280 nm recorded. The elution volume for Blue Dextran represented the void volume of the column and the elution volume for the remaining markers determined using the same method. The ratio of elution volume to the void volume (V_e/V_o) was then plotted against the log of their M_r values permitting the construction of a standard curve (Figure 4.4).

Chloroplastic dihydrolipoamide dehydrogenase was isolated from pea leaves by anion exchange chromatography and passed through the gel filtration column in an identical manner to the standard markers. The eluted protein was collected in 1.0 ml fractions and dihydrolipoamide dehydrogenase activity was determined in each fraction (see Figure 4.5). Using the standard curve obtained in Figure 4.4, a native molecular mass of 120,000 Da was determined. Comparing this to the subunit molecular mass

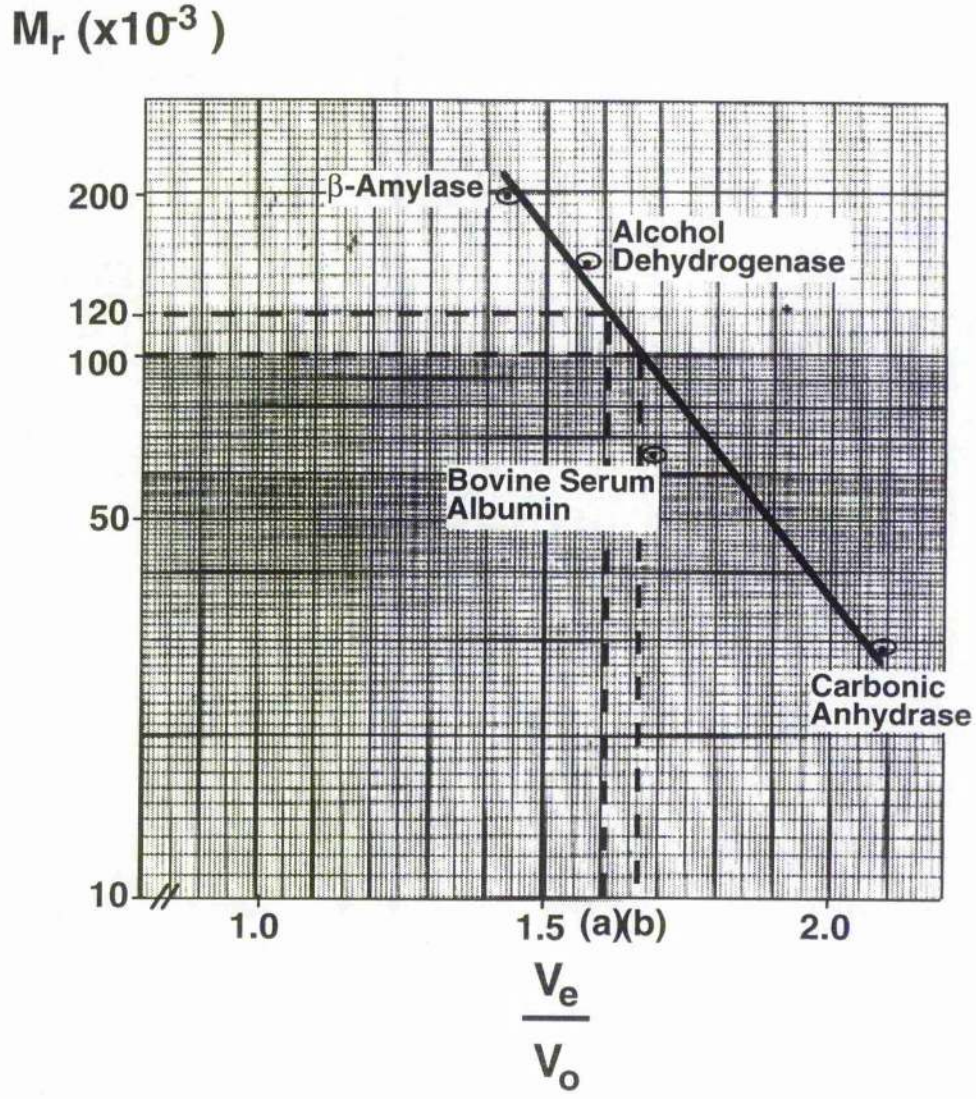

Figure 4.4: Calibration curve for the molecular mass determination of pea leaf chloroplastic and mitochondrial dihydrolipoamide dehydrogenases by gel filtration

The void volume of the Superdex 200 gel filtration column was calculated by determining the elution volume of Blue Dextran. The elution volumes of each of the molecular mass markers were divided by the void volume and plotted against their molecular mass. The V_e/V_o for the pea leaf chloroplastic (a) and mitochondrial (b) dihydrolipoamide dehydrogenases were determined and their molecular masses calculated using the standard curve.

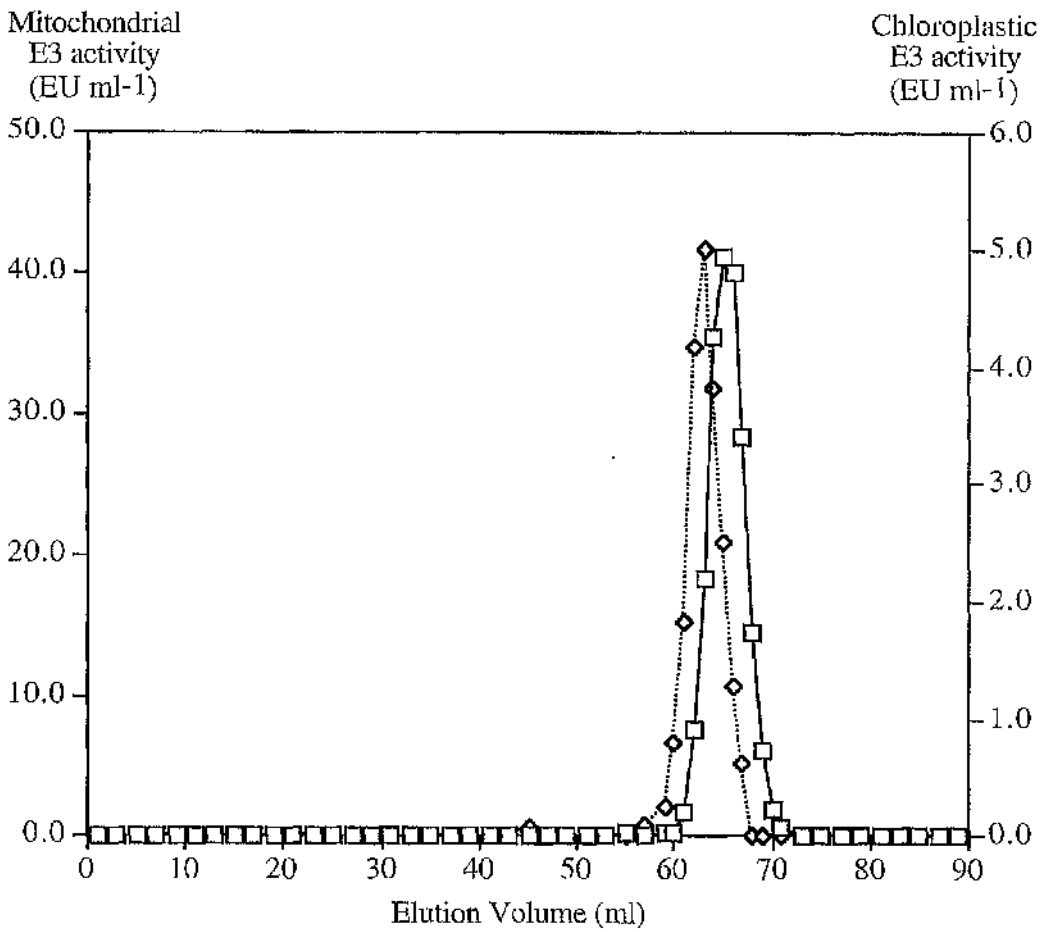

Figure 4.5: Elution profile of pea leaf mitochondrial and chloroplastic dihydrolipoamide dehydrogenases determined by gel filtration

Pea leaf mitochondrial (□) and chloroplastic (◇) dihydrolipoamide dehydrogenases were isolated and loaded separately onto a Superdex 200 gel filtration column and eluted at 1.0 ml min^{-1}. Eluted protein was collected in 1.0 ml fractions and dihydrolipoamide dehydrogenase activity determined (see 2.2.7 for method).

calculated by SDS-PAGE (see section 3.2.2) and ESMS (see section 4.2.1), this corresponds closely to twice the mass of individual E3 polypeptide chains. Therefore, the chloroplastic enzyme appears to exist as a dimer in its native state.

The native molecular mass of the pea leaf mitochondrial dihydrolipoamide dehydrogenase was also calculated, and this was found to be 100,000 Da (see Figure 4.5). This agrees with published data indicating that the mitochondrial isoenzyme exists as a homodimer (Bourguignon *et al.*, 1992).

4.3.3 N-terminal Analysis of Chloroplastic and Mitochondrial Dihydrolipoamide Dehydrogenases

N-terminal sequencing is a powerful and useful technique for elucidating the identity of an unknown protein. The technique utilises the automated Edman degradation reaction to remove one amino acid, sequentially from the N-terminus of the peptide or protein. The terminal amino group of the peptide, in an uncharged state, forms a phenylthiocarbamoyl derivative after reacting with phenyl isothiocyanate. Then, under mildly acidic conditions, a cyclic derivative is liberated, and the peptide, shortened by one amino acid is left intact (see Figure 4.6). The cyclic compound formed is a phenylthiohydantoin (PTH) amino acid, and is then identified using high-pressure liquid chromatography (HPLC).

N-terminal sequencing was performed on dihydrolipoamide dehydrogenase from the following sources:
pea root mitochondria, pea leaf mitochondria, pea leaf crude organellar extract peak 1 (corresponding to mitochondrial E3) and pea leaf crude organellar extract peak 2 (corresponding to chloroplastic E3).

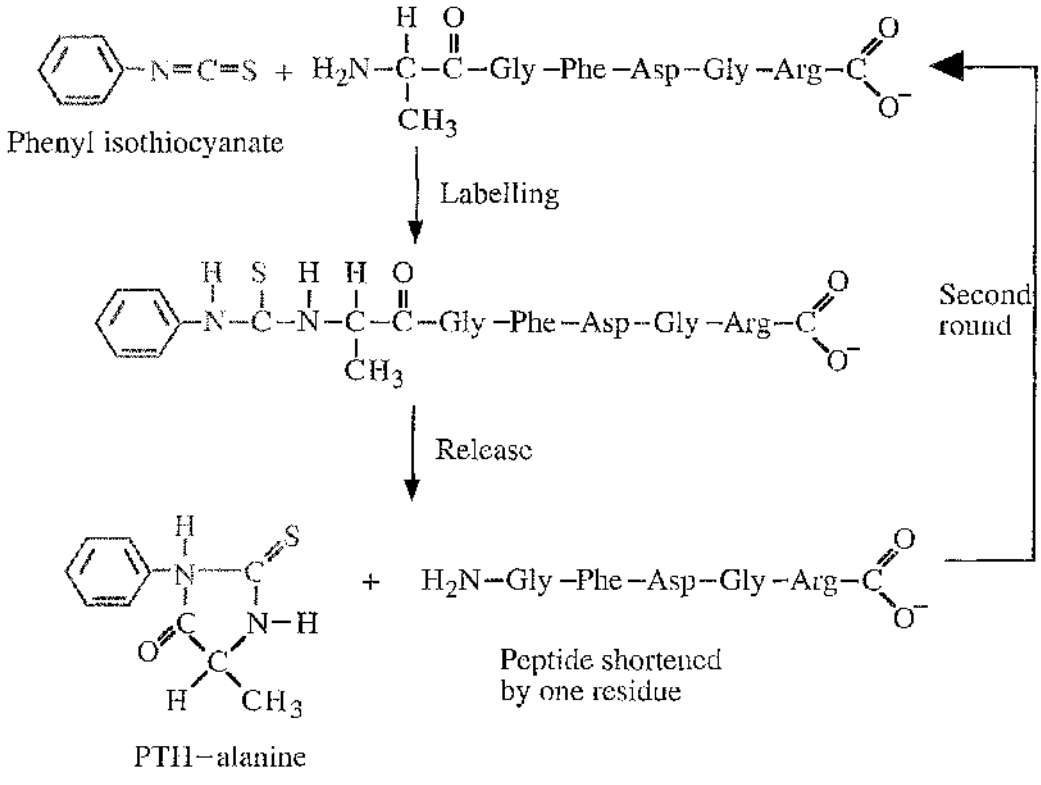

Figure 4.6: The Edman degradation reactions

Phenyl isothiocyanate covalently binds the N-terminal amino acid (alanine in this example). The labelled amino-terminal residue (PTH-alanine in the first round) is released and identified using HPLC.

The sequences obtained for the above samples are displayed in Figure 4.7. The N-terminal sequence determined for the purified peak 2 (chloroplastic dihydrolipoamide dehydrogenase) reveals a distinct sequence from that obtained for the purified pea leaf peak 1 (mitochondrial E3) enzyme, with only 10-20% homology in this region. Moreover, pea root mitochondrial E3, pea leaf mitochondrial E3 and pea leaf peak 1 E3 sequences are identical indicating that they are the same polypeptides and this confirms the proposition that the pea leaf whole organellar peak 1 and the pea leaf mitochondrial E3 are the same enzymes. In addition these sequences, are identical to the N-terminal sequence as predicted from the cDNA sequence obtained by Bourguignon *et al.* (1992) and Turner *et al.* (1992).

On comparing the entire mitochondrial dihydrolipoamide dehydrogenase primary sequence (Bourguignon *et al.*, 1992; Turner *et al.*, 1992), it was observed that the N-terminal sequence of the chloroplastic isoenzyme did not correspond to any part of the internal amino acid sequence. This is consistent with the ESMS data which verifies that the chloroplastic dihydrolipoamide dehydrogenase cannot be a proteolytic fragment of mitochondrial E3 and is a unique isoform, probably encoded by a separate gene.

When the chloroplastic N-terminal sequence was entered into the National Centre for Biotechnological Information (NCBI) database sequence database, the closest matches obtained were glutathione reductases, with several dihydrolipoamide dehydrogenase from non-plant and non-mammalian sources also displaying some sequence similarity (see Figure 4.8). Thus, chloroplastic dihydrolipoamide dehydrogenase appears to have closest homology not with mitochondrial E3s but with glutathione reductases over this limited region of the N-terminal sequence.

This could be explained by the fact that the mitochondrial dihydrolipoamide dehydrogenase is a member of the flavoprotein disulphide oxidoreductase family,

Mono Q Purified Dihydrolipoamide Dehydrogenase (Peak 2, chloroplastic):

Ala-Ala-Arg-Ser-Asp-Asn-Gly-Ser-Ser-Thr-Gly-Gly-Ser-Phe-Asp-Tyr-Asp-Leu-X-Ile-Ile-Gly-Ala-X-X-Gly

Mono Q Purified Dihydrolipoamide Dehydrogenase (Peak 1, mitochondrial):

Ala-Ser-Gly-Ser-Asp-Glu-Asn-Asp-Val-Val-Ile-Ile-Gly-Gly-Gly-Pro-Gly-Gly-Tyr-Val-Ala-Ala-Ile-Lys-Ala-Ala-Gln-Leu-Gly-Phe

Pea Leaf Mitochondrial E3:

Ala-Ser-Gly-Ser-Asp-Glu-Asn-Asp-Val-Val-Ile-Ile-Gly-Gly-Gly-Pro-Gly-Gly-Tyr-Val-Ala-Ala-Ile-Lys-Ala-Ala-Gln-Leu-Gly-Phe

Pea Root Mitochondrial E3:

Ala-Ser-Gly-Ser-Asp-Glu-Asn-Asp-Val-Val-Ile-Ile-Gly-Gly-Gly-Pro-Gly-Gly-Tyr-Val-Ala-Ala-Ile-Lys-Ala-Ala-Gln-Leu-Gly-Phe

Figure 4.7: N-terminal sequence comparison of pea dihydrolipoamide dehydrogenase from different tissues determined by automated Edman degradation

Identical residues between pea leaf organellar peak 2 and peak 1 are underlined. For sequencing protocol see section 2.2.18 in Materials and Methods.

(1) [1]A A R S D N G S S T G G S F D Y D L X I I G A X X G [26]

(2) [18]G G S Y D Y D L F V I G A V S G [33]

(3) [74]A S T D N G A E S D R H Y D F D L F T I G A G S G [98]

(4) [60]A E S Q N G A D P A R Q Y D F D L F T I G A G S G [84]

(5) [70]A E S Q N G A D P A R Q Y D F D L F T I G A G S G [94]

(6) [1]S K S E E F D Y D L F T I G A G S G [18]

(7) [66]A E S S N G A D A P R H Y D F D L F T I G A G S G [90]

(8) [4]F D Y D L V I I G A [13]

(9) [2]T F D Y D L F V I G A G S G [15]

(10) [16]G E E Q G K V Y D F D L F I I G A G S G [35]

(11) [15]F D Y D L F T I G A [24]

(12) [2]S F D F D L F V I G A G S G [15]

(13) [5]F D Y D L V I I G A G V G [17]

(14) [9]F D Y D L V I I G A G V G [21]

(15) [239]R S D L S Q S S G G N F N F D L [254]

(16) [12]G S S S G G S R E Y K V V M L G A [28]

(17) [256]T D N M S I T G E S F D Y [268]
[277]Y D L N F I A A [284]

(18) [10]S Y D Y D L I I I G G G S G [23]

(19) [113]R N D L G S P S G [121]
[258]T G G G V D Y S F E V I G [270]

(20) [113]R N D L G S P S G [121]
[258]T G G G V D Y S F E V I G [270]

(21) [157]R T D V S L N D E G N Y D Y D Y I I I G S [177]

(22) [12]G S A S G G S R E Y K V V M L G A [28]

(23) [12]G S A S G G S R E Y K V V M L G A [28]

(24) [8]T G G S F S Y E [15]

(25) [97]A R A N H G G N S G G A F D [110]

(26) [949]R S D Q G F P T S G Y F D Y [962]

(27) [909]G G G P G G S F D Y D [919]

(28) [41]R A F N G K N A N G S Y D Y G L [56]

Figure 4.8: Sequence alignment of pea chloroplastic dihydrolipoamide dehydrogenase with sequences obtained from the National Centre for Biotechnological Information (NCBI) database

The N-terminal sequence of the pea chloroplastic dihydrolipoamide dehydrogenase was compared to sequences held in the NCBI nr database using the blastp program. Amino acid residues underlined in the sequences obtained are identical to the equivalent residues in the chloroplastic N-terminal sequence, while letters in bold are conservative substitutions. The aligned sequences are:

(1) Pea leaf chloroplastic dihydrolipoamide dehydrogenase
(2) Glutathione reductase, cytosolic from rice (accession P48642)
(3) Glutathione reductase, chloroplast precursor from *Arabidopsis thaliana* (accession P42770)
(4) Glutathione reductase, chloroplast precursor from *Pisum sativum* (accession P27456)
(5) Glutathione reductase, from *Pisum sativum* (accession 100043)
(6) Glutathione reductase, from *Pinus sylvestris* (accession 1326449)
(7) Glutathione reductase, precursor from *Nicotinia tobacum* (accession P80461)
(8) Soluble hydrogenase, 50 kDa subunit from *Anabaena cylindrica* (accession P17874)
(9) Glutathione reductase, from *Anabaena Sp.* (accession P48638)
(10) Glutathione reductase, *Pisum sativum* (accession 1370285)
(11) Glutathione reductase isozyme 2H, from *Picea rubens* (accession 765183)
(12) Glutathione reductase, from *Pseudomonas aeruginosa* (accession P23189)
(13) Dihydrolipoamide dehydrogenase, from *Synechocystis PCC6803* (accession 1321941)
(14) Dihydrolipoamide dehydrogenase, from *Synechocystis sp.* (accession 1651828)
(15) CooC, from *Escherichia coli* (accession 488736)
(16) rin, from *Mus musculus* (accession 1655999)
(17) Hypothetical protein, from *Synechocystis sp.* (accession 1652555)
(18) Thioredoxin reductase, from *Homo sapiens* (accession 1184537)
(19) Alcohol dehydrogenase, from *Alligator mississippiensis* (accession 479932)
(20) Alcohol dehydrogenase Major, from *Alligator mississippiensis* (accession P80222)
(21) Mercuric reductase, from *Bacillus sp.* (strain RC607) (accession P16171)
(22) rin, from *Homo sapiens* (accession 1656003)
(23) rin (ric-related gene expressed in neurons) from *Homo sapiens* (accession 1702926)
(24) T-cell receptor beta chain VDJ region (clone 1), from *Mus sp.* BALB/C LN cells (accession 456925)
(25) Immunoglobulin heavy chain variable region, from *Homo sapiens* (accession 392754)
(26) D2045.1, from *Caenorhabditis elegans* (accession 1066914)
(27) Hypothetical protein, from *Synechocystis sp.* (accession 1652555)
(28) Lysozyme C, from *Equus asinus* (accession P11375)

which includes glutathione reductase and mercury(II) reductase. The highest similarity between these enzymes can be found in the disulphide active site and the FAD binding domains (Carothers *et al.*, 1989). As this group of oxidoreductases are thought to be derived from a common evolutionary ancestor, then the chloroplastic dihydrolipoamide dehydrogenase may have diverged from the common ancestor prior to the divergence of the mitochondrial E3, the glutathione reductase and the mercury(II) reductase from their common ancestor (see Figure 4.9a). Another possibility is that the mitochondrial enzyme may have diverged initially from a common ancestor, leaving the ancestor of the glutathione and mercury(II) reductases and the chloroplastic dihydrolipoamide dehydrogenase, followed by the divergence of the chloroplastic enzyme from this ancestor (see Figure 4.9b). Since chloroplasts are thought to be derived from gram -ve bacteria and mitochondria from gram +ve bacteria, then the first scheme, as shown in Figure 4.9a, is more plausible, since the divergence of the chloroplastic and mitochondrial dihydrolipoamide dehydrogenases would have to have occurred at a very early evolutionary stage.

One item of interest was, due to the presence of unknown residues (X) the subunit of a soluble hydrogenase from *Anabaena cylindrica* (Ewart *et al.*, 1990) was not the closest match despite the known residues matching a 10 amino acid section (see Figure 4.9). This region may represent a conserved motif in dihydrolipoamide dehydrogenases from non-mitochondrial sources.

Thus, the chloroplastic dihydrolipoamide dehydrogenase has been shown to possess a higher subunit molecular mass and distinct N-terminal sequence from the mitochondrial isoform. However, similar to the mitochondrial E3, the chloroplastic isoform has been shown to exist as dimer. These results support the existence of a distinct dihydrolipoamide dehydrogenase found in pea leaf chloroplasts.

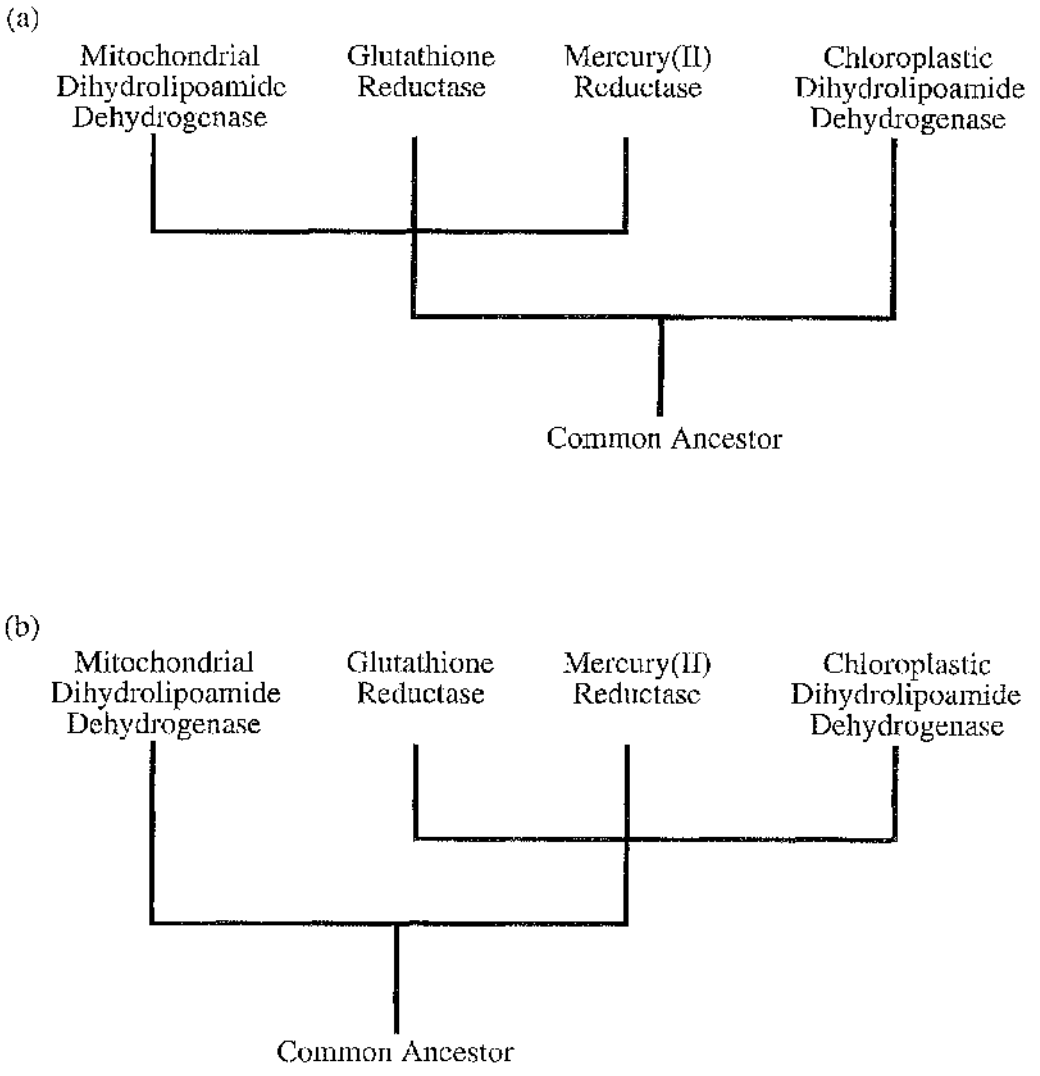

Figure 4.9: Proposed schemes for the evolutionary divergence of chloroplastic dihydrolipoamide dehydrogenase from mitochondrial dihydrolipoamide dehydrogenase, glutathione reductase and mercury(II) reductase

Chapter 5

Kinetic Analysis of Mitochondrial and Chloroplastic Dihydrolipoamide Dehydrogenases

5.1 Introduction

The reaction sequence of the 2-oxoacid dehydrogenase complexes from mammalian sources has previously been shown to proceed via a three site ping-pong mechanism first described by Cleland (Cleland, 1973; Tsai *et al.*, 1973; Hamada *et al.*, 1975; Boyer & Odessey, 1991). The mechanism involves the reaction of the enzyme with the initial substrate to produce a covalently modified enzyme, with the release of a product. This covalently-modified enzyme then reacts with a second substrate to produce a second product, derived from the covalent modification of the enzyme from the first substrate and the second substrate. Therefore, one or more products are released before all the substrates are bound. 2-oxoacid dehydrogenase complexes catalyse the reactions at distinct active sites on each of their individual enzyme components, E1, E2 and E3. The reaction sequence can be illustrated diagrammatically (see Figure 5.1).

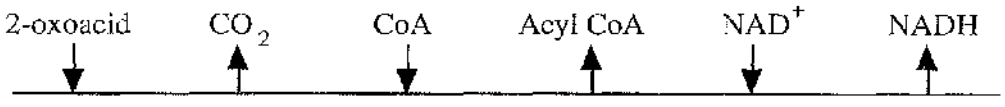

Figure 5.1: Illustration of the ping-pong reaction sequence of the 2-oxoacid dehydrogenase complex family

The ping-pong mechanism for these complexes can be confirmed by analysing the initial velocity patterns of one substrate, A, at varying concentrations of a second substrate, B, which will result in a series of parallel lines on a double reciprocal plot (see Figure 5.2).

Kinetic characterisation of the plant complexes has focused primarily on plant mitochondrial PDC. This complex, along with the chloroplastic PDC, from pea, broccoli and cauliflower has been shown to exhibit a three site ping-pong reaction mechanism (Randall *et al.*, 1977; Thompson *et al.*, 1977a & 1977b; Miernyk &

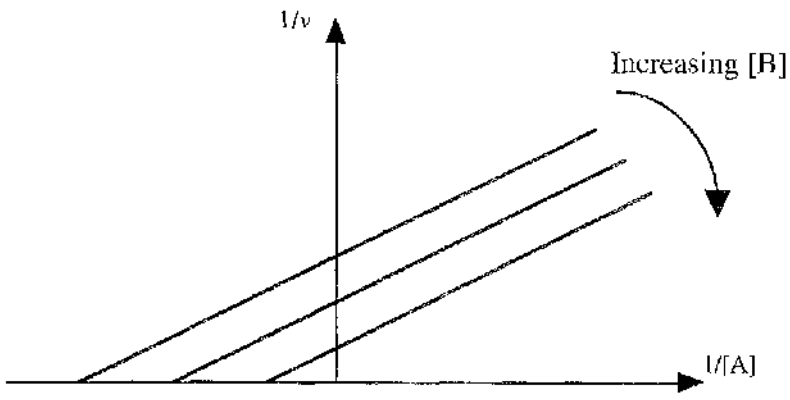

Figure 5.2: Example of a double reciprocal plot derived from a complex undergoing a three site ping-pong mechanism

Plot of $1/v$ versus 1/[A] at several concentrations of B, where A and B are substrates of a multiple substrate reaction.

Randall, 1987b). Both the mitochondrial and chloroplastic complexes, in common with their mammalian and microbial counterparts, are inhibited by the products of the overall reaction, namely NADH and acetyl CoA. NADH and acetyl CoA inhibit their respective substrates, NAD^+ and CoA respectively, competitively for all plant PDCs studied to date, while inhibition by NADH or acetyl CoA versus pyruvate is uncompetitive. This type of behaviour reflects the distinct active sites of the multienzyme complex and correlates with the inhibition patterns predicted by a ping-pong mechanism. Typical K_m and K_i values for the product inhibitors of various plant mitochondrial and chloroplastic PDCs are given in Table 5.1. The K_i value for NADH of the mitochondrial and plastidic complexes is lower than the K_m value for NAD^+ demonstrating that these complexes are very sensitive to the NAD^+:NADH ratio in common with prokaryotic and other eukaryotic PDCs. Thus, these complexes are subject to negative feedback by the products of the overall reaction. It is thought that the E3 component of the complexes is responsible for the sensitivity to the NAD^+:NADH ratio.

Plant Source	K_m(Pyr) μM	K_m (NAD^+) μM	K_m (CoA) μM	K_i (NADH) μM	K_i (Acetyl CoA) μM
Mitochondrial PDC					
Pea Leaf	57	122	4	18	10
Broccoli floret	250	110	6	13	19
Cauliflower floret	207	125	7	34	13
Plastidic PDC					
Pea leaf	120	36	10	9	16
Castor seed endosperm	62	130	6	27	23
Maize shoot	120	16	4	12	18

Table 5.1: Michaelis constants for a range of mitochondrial and plastidic PDCs from a variety of plant tissue

Pea leaf mitochondrial PDC from Miernyk and Randall (1987b); broccoli floret mitochondrial PDC from Rubin and Randall (1977a); cauliflower floret mitochondrial PDC from Randall *et al.* (1977); pea leaf plastidic PDC from Camp *et al.* (1988); castor seed endosperm plastidic PDC from Thompson *et al.* (1977a) and maize shoot plastidic PDC from Cho *et al.* (1988).

Kinetic characterisation of dihydrolipoamide dehydrogenase has concentrated on E3 from non-plant sources such as porcine heart and *E. coli*. E3 has been found to exist as a homodimer, containing one molecule of FAD per subunit, a redox-active disulphide and a histidine residue in each catalytic site. E3 catalyses the oxidation of dihydrolipoamide to lipoamide with the concomitant reduction of NAD^+ to NADH (see Figure 5.3).

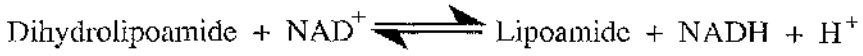

Figure 5.3: Reaction performed by dihydrolipoamide dehydrogenases

The binding sites of NAD^+ and dihydrolipoamide are located on opposite faces of the flavin in E3, with the redox active dithiol situated on the same side as the dihydrolipoamide binding site. A histidine side chain was reported to be located in front of the reactive thiol groups and is thought to be responsible for removing a proton from dihydrolipoamide (Matthews *et al.*, 1977). The role of E3 in the 2-oxoacid dehydrogenase complexes and the glycine decarboxylase complex (GDC) is to re-oxidise the dihydrolipoamide groups bound to the lysine residues present on the acyltransferase component (E2 of the 2-oxoacid complexes and the H-protein in GDC) of the complexes. This, in turn, is coupled to the reduction of NAD^+ to NADH, which is then utilised in the respiratory chain. The reaction proceeds via a ping-pong mechanism as described previously for the 2-oxoacid dehydrogenase complexes. The electrons from the dihydrolipoamide follow a complex path to the dithiol groups (see Figure 1.11) and then via the flavin to reach NAD^+ with the concomitant production of NADH (Massey *et al.*, 1960; Reed, 1973; Williams, 1992; Ghisla & Massey, 1989).

Previously, in chapters three and four, the isolation, purification and partial characterisation of a chloroplast-specific dihydrolipoamide dehydrogenase from pea leaf, distinct from the mitochondrial enzyme was described. The chloroplast enzyme has been shown to exhibit distinctive molecular characteristics, including its profile

from Mono Q anion exchange chromatography, subunit and native molecular mass determined by SDS-acrylamide gel electrophoresis, electrospray mass spectrometry and gel filtration analysis, and N-terminal sequence. The enzyme is thought to be present in the chloroplast stroma where NAD^+ and NADH levels are lower and $NADP^+$ and NADPH levels are higher than in the mitochondria. Therefore, it is probable that the mitochondrial and chloroplastic E3 isoforms differ in their kinetic properties, reflecting their differences in evolutionary origin and *in vivo* environment. With this in mind, characterisation of the kinetic parameters of both the mitochondrial and chloroplastic E3s was performed.

5.2 Results and Discussion

5.2.1 Kinetic Constants for the Substrates of the Chloroplastic and Mitochondrial Dihydrolipoamide Dehydrogenase-Catalysed Reactions

Similar to the 2-oxoacid dehydrogenase complexes, E3 is inhibited by the products of the overall reaction, i.e. NADH and lipoamide, and this occurs via a ping-pong mechanism as described previously. The Michaelis-Menten model is representative for the kinetic properties of dihydrolipoamide dehydrogenase enzymes. Competitive inhibition or uncompetitive inhibition can be revealed by constructing Lineweaver-Burke plots and from these plots, the kinetic constants K_m and K_i can be deduced (see Figure 5.4).

The K_m for NAD^+ was calculated for each isoform by varying the concentration of NAD^+, while maintaining a fixed, saturating concentration of dihydrolipoamide (DHL). The K_i for NADH was calculated by assaying the enzymes in increasing concentrations of NAD^+ at 0 μM, 100 μM and 200 μM NADH. The K_m for DHL was

calculated by varying the concentration of DHL while maintaining a saturating concentration of NAD^+.

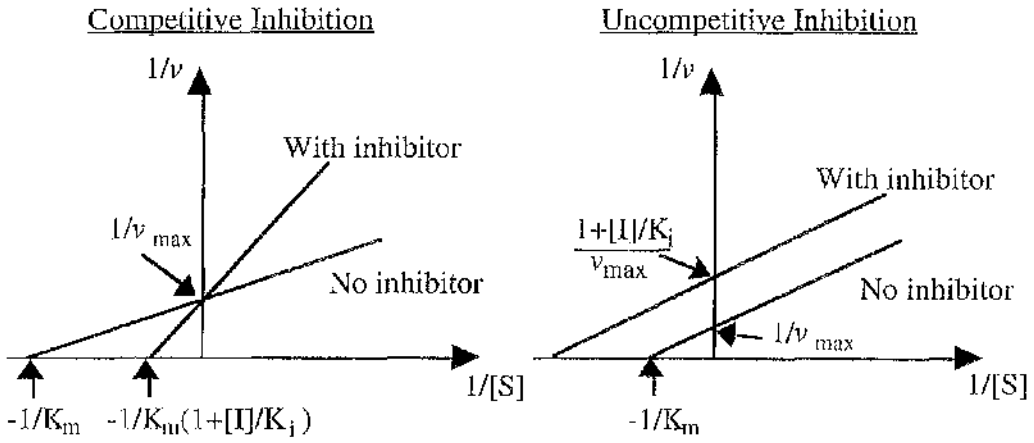

Figure 5.4: Lineweaver-Burke plots for competitive and uncompetitive inhibition in the presence and absence of the inhibitor

The enzymes were prepared as described in Materials and Methods, section 2.2.2 to 2.2.4, and all E3 assays were performed in triplicate at pH 7.6. The apparent Michaelis constants, K_m for NAD^+, K_i for NADH and K_m for DHL, were derived from Lineweaver-Burke plots (see Figure 5.5 to Figure 5.8) and are presented in Table 5.2 and Table 5.3 with the K_m for NAD^+ and the K_i for NADH for potato tuber mitochondrial E3 α_2, $\alpha\beta$, β_2 isoforms, porcine heart E3, bovine mucosal E3 and yeast E3 (R. Miller, Ph.D. Thesis, Glasgow University) for comparison. Best fit lines were calculated by regression analysis using a least squares method (Curve Fit, Linear function, CA Cricket Graph III).

Since NAD^+ and NADH levels in the chloroplast stroma are lower than levels of $NADP^+$ and NADPH, it was proposed that the chloroplastic dihydrolipoamide dehydrogenase may be able to utilise $NADP^+$ as a substrate. Therefore, both the pea leaf mitochondrial and chloroplastic E3s were investigated for $NADP^+$-linked activity. NADPH was found not to be produced in the presence of $NADP^+$ and DHL, and therefore it appears that neither the chloroplastic or mitochondrial E3 are able to utilise

$NADP^+$. $NADP^+$ and NADPH were then investigated to find whether they exerted inhibitory effects on NADH production from NAD^+. $NADP^+$, as expected, was not found to inhibit NADH production. However, at 0.5 mM NADPH inhibited both the mitochondrial and chloroplastic enzymes. This effect was similar for both the isoenzymes and was thus thought not to be physiologically relevant.

Both the K_m for NAD^+ and K_i for NADH values for the pea leaf chloroplastic dihydrolipoamide dehydrogenase were found to be lower than the values for the mitochondrial enzyme (Table 5.2). The K_m was more than 5-fold lower for the chloroplastic enzyme than the mitochondrial enzyme and the K_i 3-fold lower. Comparing these to the potato, bovine intestinal mucosal, porcine heart and yeast E3 values, the pea leaf chloroplastic enzyme has both the lowest K_m and K_i. As reported earlier, the chloroplastic enzyme is unable to utilise $NADP^+$, and only catalyses an NAD^+-linked reaction although this cofactor is present at lower concentrations in the chloroplast stroma than in the mitochondrial matrix. The low K_m and K_i reflect these low levels of substrate and product giving rise to considerable differences in the kinetic constants between the pea leaf mitochondrial and chloroplastic dihydrolipoamide dehydrogenases.

Differences between the K_m for DHL, like the K_m for NAD^+, were found in the chloroplastic and mitochondrial enzymes. However, the K_m of the chloroplastic enzyme was 3-fold higher (as opposed to lower) at 2 mM compared to a K_m of 0.77 mM for the mitochondrial enzyme (see Table 5.3).

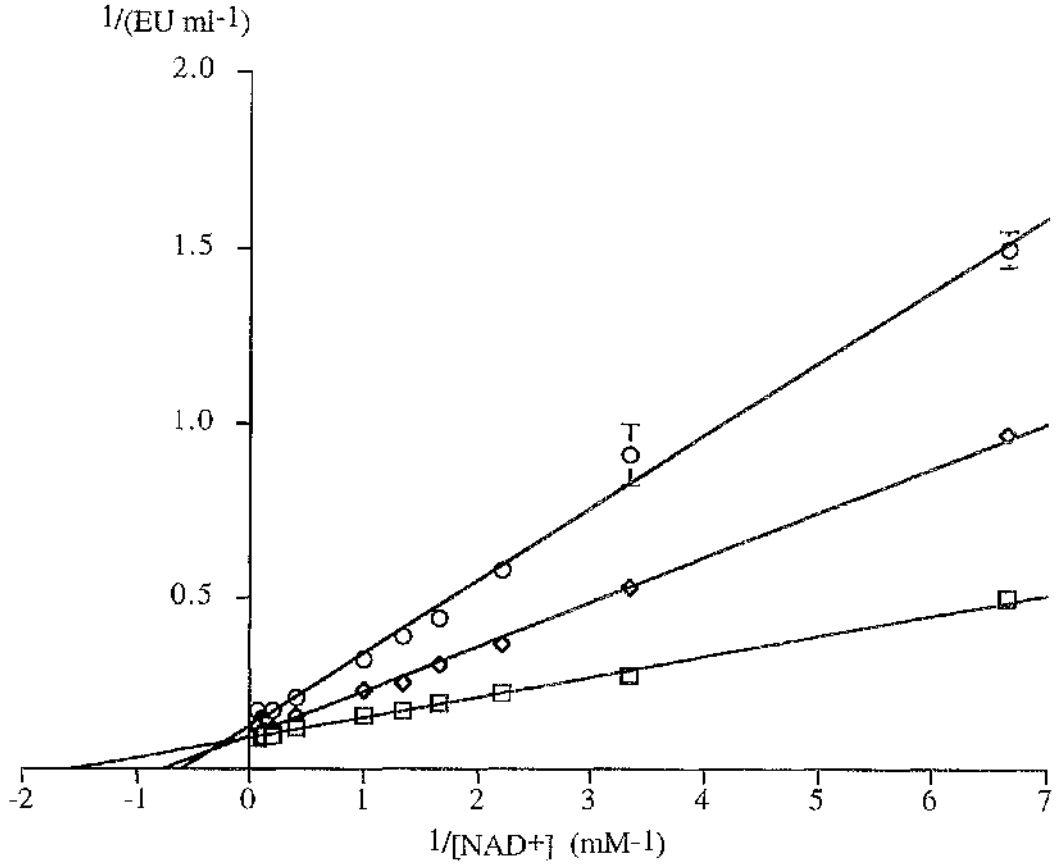

Figure 5.5: Double reciprocal plot of velocity of a pea mitochondrial dihydrolipoamide dehydrogenase-catalysed reaction

E3 activity, expressed as EU ml^{-1} (µmol NADH produced ml^{-1} min^{-1}), was determined as described in Materials and Methods section 2.2.7, except increasing concentrations of NAD^+ were used: 0 µM (□), 100 µM (◇) and 200 µM NADH (○). Best fit lines were calculated by regression analysis using a least squares method (Curve Fit command, Linear function in CA Cricket Graph III).

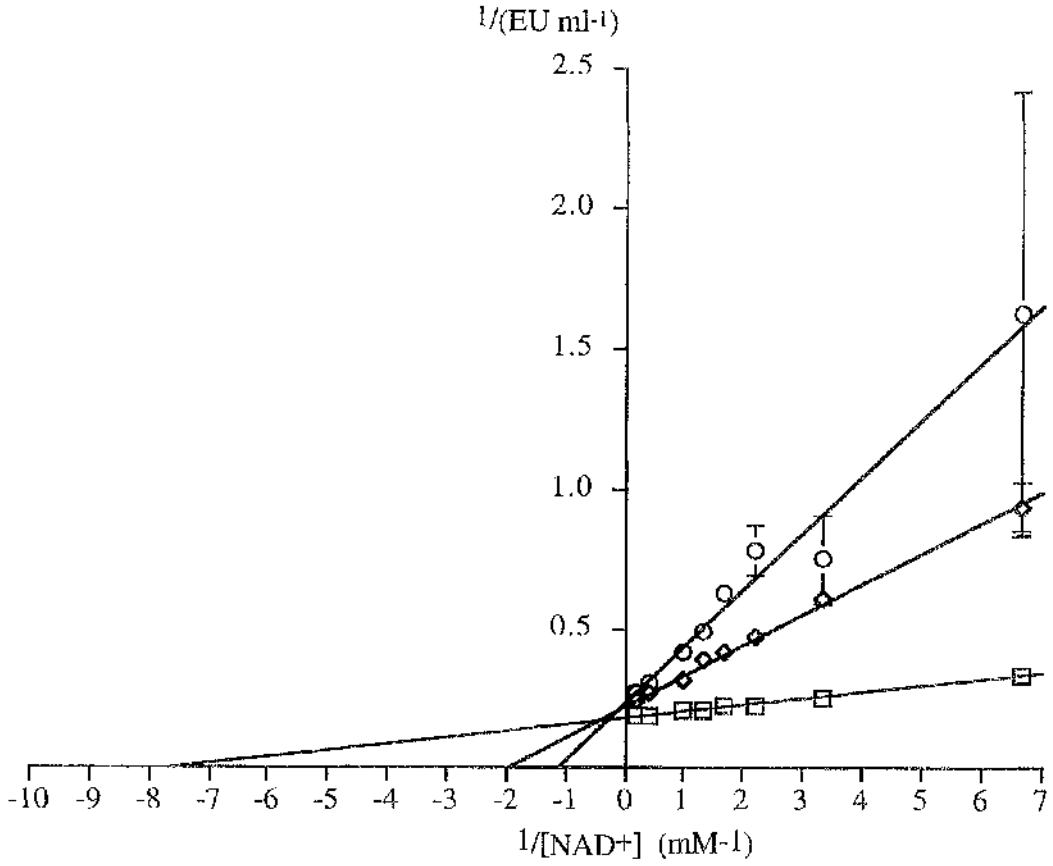

Figure 5.6: Double reciprocal plot of velocity of a pea chloroplastic dihydrolipoamide dehydrogenase-catalysed reaction

E3 activity, expressed as EU ml^{-1} (µmol NADH produced ml^{-1} min^{-1}), was determined as described in Materials and Methods section 2.2.7, except increasing concentrations of NAD^- were used: 0 µM (□), 100 µM (◇) and 200 µM NADH (○). Best fit lines were calculated by regression analysis using a least squares method (Curve Fit command, Linear function in CA Cricket Graph III).

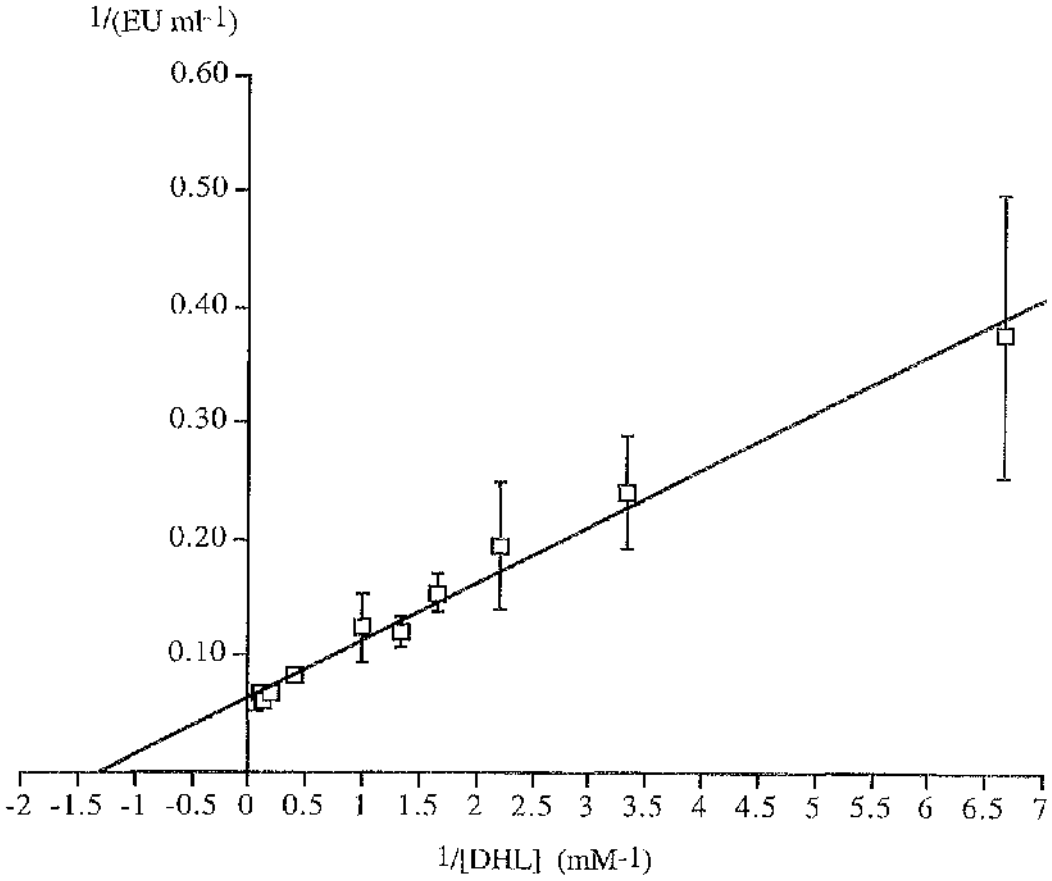

Figure 5.7: Double reciprocal plot of velocity of a pea mitochondrial dihydrolipoamide dehydrogenase-catalysed reaction versus DHL

E3 activity, expressed as EU ml^{-1} (µmol NADH produced ml^{-1} min^{-1}), was determined as described in Materials and Methods section 2.2.7, except increasing concentrations of DHL were used at a fixed concentration of 3 mM NAD^+. Best fit lines were calculated by regression analysis using a least squares method (Curve Fit command, Linear function in CA Cricket Graph III).

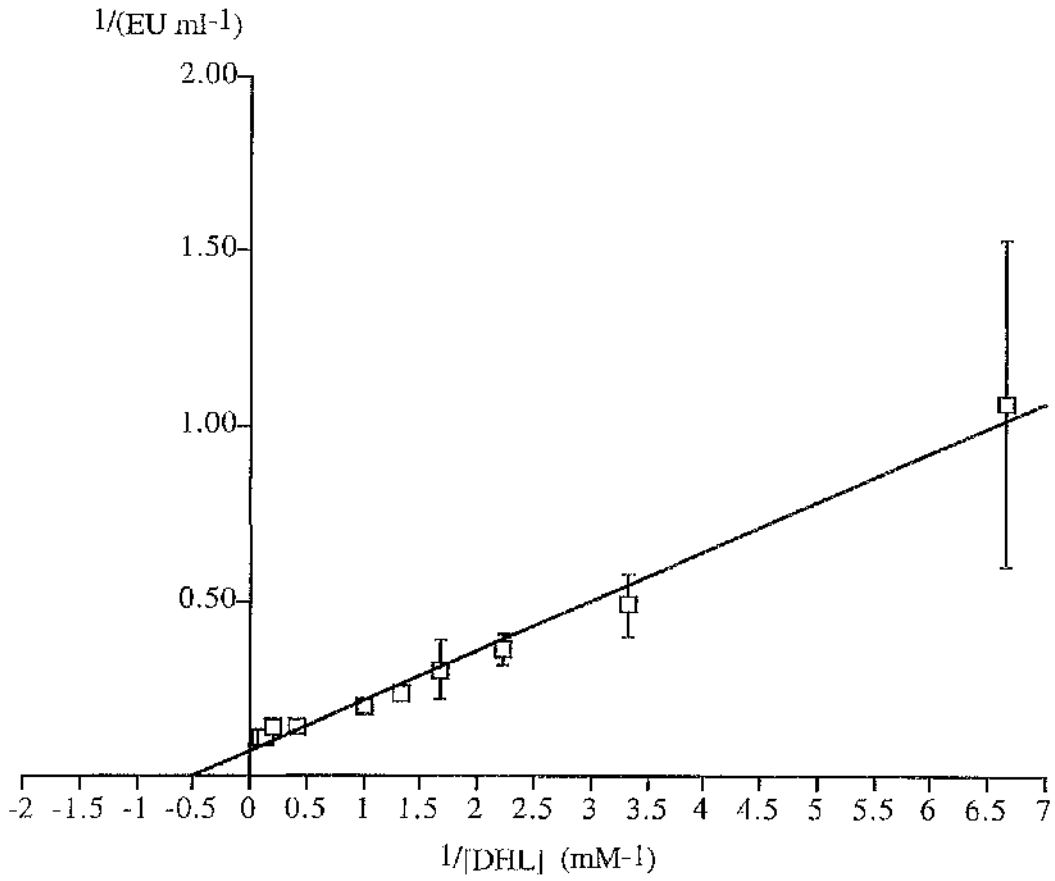

Figure 5.8: Double reciprocal plot of velocity of a pea chloroplastic dihydrolipoamide dehydrogenase-catalysed reaction versus DHL

E3 activity, expressed as EU ml^{-1} (µmol NADH produced ml^{-1} min^{-1}), was determined as described in Materials and Methods section 2.2.7, except increasing concentrations of DHL were used at a fixed concentration of 3 mM NAD^+. Best fit lines were calculated by regression analysis using a least squares method (Curve Fit command, Linear function in CA Cricket Graph III).

	K_m (NAD^+) μM	K_i (NADH) μM
Pea Mitochondrial E3	667 ± 43	114 ± 4.1
Pea Chloroplastic E3	130 ± 12	35 ± 3.1
Potato tuber E3 α_2 isoform	461 ± 28	105 ± 6.3
Potato tuber E3 $\alpha\beta$ isoform	547 ± 26	143 ± 6.8
Potato tuber E3 β_2 isoform	491 ± 46	128 ± 4.6
Porcine heart E3	227 ± 15	97 ± 6.2
Bovine intestinal mucosal E3	178 ± 9	68 ± 3.3
Yeast E3	358 ± 13	83 ± 6.3

Table 5.2: Michaelis constants for pea leaf mitochondrial and chloroplastic dihydrolipoamide dehydrogenase catalysed reactions

K_m and K_i values for NAD^+ were calculated from Lineweaver-Burke plots for pea mitochondrial and chloroplastic E3, potato tuber E3 α_2, $\alpha\beta$ and β_2 isoforms, porcine heart, bovine and yeast E3 as described in section 5.2.1.

	K_m (DHL) mM
Pea Leaf Mitochondrial E3	0.77 ± 0.16
Pea Leaf Chloroplastic E3	2.0 ± 0.33

Table 5.3: Michaelis constants for pea leaf mitochondrial and chloroplastic dihydrolipoamide dehydrogenase catalysed reactions

K_m values for DHL were calculated from Lineweaver-Burke plots for pea mitochondrial and chloroplastic E3 as described in section 5.2.1.

5.2.2 Specific Activities of Chloroplastic and Mitochondrial Dihydrolipoamide Dehydrogenases

The specific activities were calculated for the pea leaf mitochondrial and chloroplastic enzymes and for porcine heart, bovine intestinal mucosal and yeast E3 for controls. Samples of dihydrolipoamide dehydrogenase were assayed for E3 activity using the method described in section 2.2.7 and the protein concentration estimated using the BCA method described in section 2.2.9. For the pea leaf mitochondrial isoform, the specific activity was found to be most similar to the porcine E3, at 296 ± 41 μmol NADH produced min^{-1} mg^{-1} (see Table 5.4). This value is 20-fold higher than the specific activity reported by Bourguignon *et al.* (1996), who calculated a specific activity of 15.8 μmol NADH produced min^{-1} mg^{-1}. A similar discrepancy was found for the porcine heart E3. The reason for these disagreement of values is unknown but may be due to the differences in methods of assaying or calculation of specific activities by Bourguignon *et al.* (1996).

The specific activity for the chloroplastic enzyme was calculated to be 98.3 ± 4.9 μmol NADH produced min^{-1} mg^{-1}, approx. 3-fold lower than pea mitochondrial E3. Interestingly, this activity was found to possess the lowest specific activity of the dihydrolipoamide dehydrogenases assayed, with only the yeast E3 possessing a similar value.

5.2.3 Optimal pH for Pea Mitochondrial and Chloroplastic Dihydrolipoamide Dehydrogenase Activity

Previously, mitochondrial E3 from rat liver mitochondria (Reed, 1973) and from human liver (Ide *et al.*, 1967) were found to exhibit a maximal activity at approximately pH 8.0. This pH value is largely thought to be determined by the

	Specific Activity (μmol NADH produced min^{-1} mg^{-1})
Pea Leaf Mitochondrial E3	296 ± 41
Pea Leaf Chloroplastic E3	98.3 ± 4.9
Porcine heart E3	324 ± 39
Bovine Intestinal Mucosal E3	254 ± 48
Yeast E3 *(Candida utilis)*	120 ± 9.3

Table 5.4: Specific activities calculated for dihydrolipoamide dehydrogenases from various sources

E3 activity was determined as described in Materials and Methods section 2.2.7 and protein concentration determined using the BCA method as described in Materials and Methods section 2.2.9.

cysteine groups involved in catalysis. Previous results suggest that the chloroplastic dihydrolipoamide dehydrogenase has distinctive catalytic properties and this could be reflected in the amino acid residues involved in catalysis, thus possibly giving rise to a different activity profile as a function of pH.

To determine the pH at which maximal activity is observed in the chloroplastic E3, the mitochondrial and chloroplastic enzymes were assayed at various pHs (see section 2.2.7 for procedure). However, to enable effective buffering at pH values outside the buffering range of potassium phosphate, this was substituted with the following buffers:

2-[N-morpholino]ethanesulphonic acid (MES) for pH 5.5-6.5

3-[N-morpholino] propanesulphonic acid (MOPS) for pH 6.5-7.5

N-tris[hydroxymethyl]methylglycine;N-[2-Hydroxy-1,1-bis(hydroxylmethyl)ethyl]-glycine (Tricine) for pH 7.5-9.0

2-[N-cyclohexylamino]ethanesulphonic acid (CHES) for pH 9.0-10.0 and

3-[cyclohexylamino]-1-propanesulphonic acid (CAPS) for pH 10.0-11.0.

The relationship between pH and activity of both mitochondrial and chloroplastic E3s was found to be typical of a biological catalyst, forming a bell shaped curve. The maximal activity for the mitochondrial enzyme was observed at pH 8.0 (see Figure 5.9), which is in agreement with the published results for the rat liver and human liver mitochondrial E3. The pH profile for the chloroplastic dihydrolipoamide dehydrogenase is similar, with maximal activity occurring also at pH 8.0.

However, differences can be observed at the extremes of the pH activity curve. At pH 5.5 the mitochondrial enzyme is inactive while the chloroplastic enzyme appears to retain approx. 10% of activity. This is repeated at pH 10.0 and 10.5 where the chloroplastic enzyme appears more active than the mitochondrial isoform and at pH 11.0 is still active when the mitochondrial has lost all activity. To ensure this was not

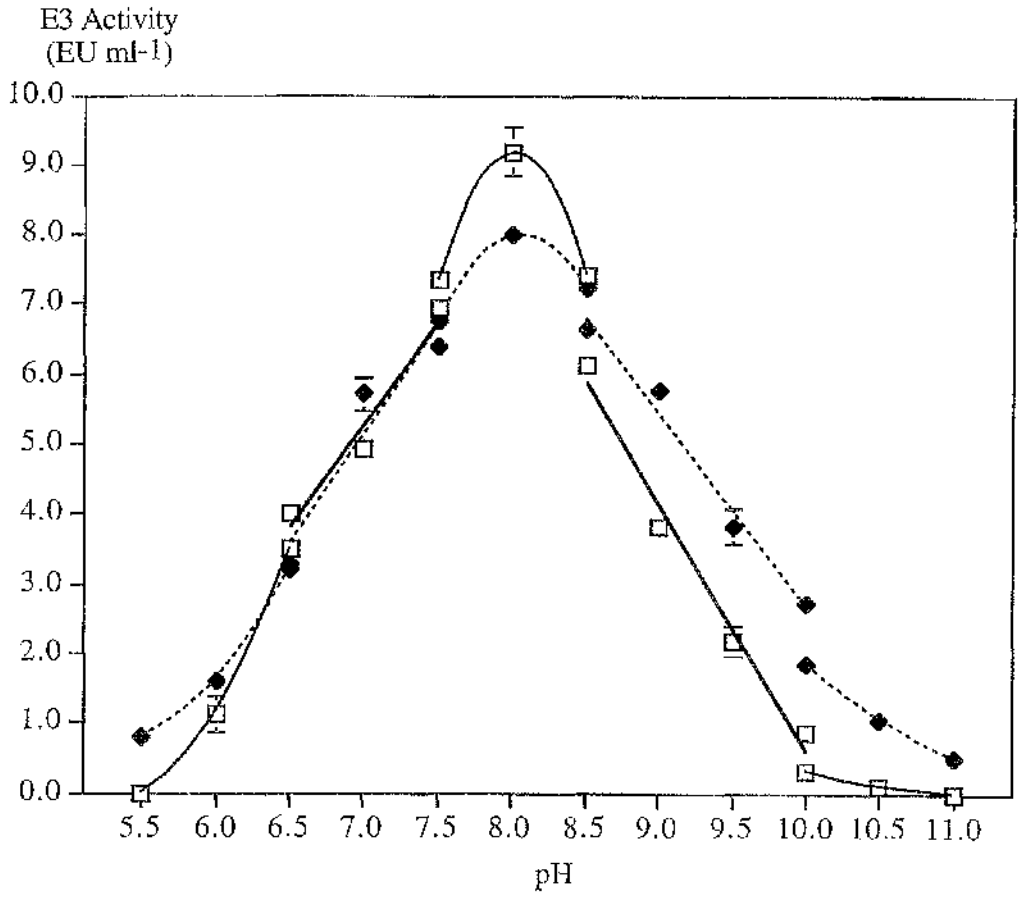

Figure 5.9: Effect of pH on the pea leaf mitochondrial and chloroplastic dihydrolipoamide dehydrogenase

Mitochondrial (—□—) and chloroplastic (--◆--) dihydrolipoamide dehydrogenase activity, expressed as EU ml^{-1} (μmol NADH produced ml^{-1} min^{-1}), was determined as described in Materials and Methods section 2.2.7 with the following buffers used to substitute potassium phosphate at the appropriate pH: MES (pH 5.5-6.5), MOPS (pH 6.5-7.5), Tricine (pH 7.5-9.0), CHES (pH 9.0-10.0) and CAPS (pH 10.0-11.0).

an artefact of experimental procedure, this was repeated at pHs 5.5-6.5 and pHs 10.0-11.0 in quadruplicate and the results, with standard deviation calculated, are presented in Table 5.5.

These results confirm the differing responses to variations in pH reported above between the mitochondrial and chloroplastic enzymes. While at pH 5.5, more than 10% of chloroplastic activity is present, less than 4% of mitochondrial activity is measurable. This pattern is repeated at pH 11.0, while at pH 10.0 the chloroplastic enzyme is more than twice as active at 39% than the mitochondrial E3 at 16.4%. Thus, the chloroplastic dihydrolipoamide dehydrogenase retains more activity at the extremes of the pH activity curve than the mitochondrial enzyme. These results indicate the presence of catalytic and/or structural differences.

5.2.4 Inhibition of Pea Mitochondrial and Chloroplastic Dihydrolipoamide Dehydrogenase Activity Using ρ-Aminophenyldichloroarsene Oxide (APA)

To date, all lipoamide dehydrogenases utilise a pair of cysteine amino acids in catalysis (see Figure 1.11). Chemical modification of these cysteine groups with the tervalent arsenical reagent ρ-aminophenyldichloroarsene oxide (APA) is indicative of the involvement in catalysis of a reversibly reducible disulphide bond. APA possesses a dichloro group bound to an arsenide atom (see Figure 5.10) which irreversibly binds with the catalytic cysteine groups, which must be in a reduced state and in close proximity to each other.

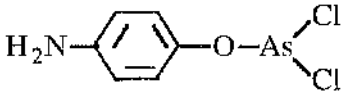

Figure 5.10: Chemical structure of ρ-aminophenyldichloroarsene oxide

	pH 5.5	pH 6.0	pH 6.5		pH 10.0	pH 10.5	pH 11.0
Mitochondrial E3	3.3 (0.87)	23.4 (1.88)	35.6 (2.88)		16.4 (1.02)	7.2 (0.91)	3.6 (0.66)
Chloroplastic E3	13.3 (0.86)	21.4 (1.27)	34.9 (1.34)		39.0 (1.77)	23.1 (1.47)	14.8 (1.80)

Table 5.5: Residual activity at extremes of pH of pea leaf mitochondrial and chloroplastic dihydrolipoamide dehydrogenase

E3 activity was determined in quadruplicate as described in Materials and Methods section 2.2.7 with the following buffers used to substitute potassium phosphate at the appropriate pH: MES (pH 5.5-6.5) and CAPS (pH 10.0-11.0) and compared to activity at pH 7.6 (standard deviations from the mean is given in brackets).

Thus, APA will specifically inhibit the redox active disulphide group at the active site of dihydrolipoamide dehydrogenases in the presence of their substrate, dihydrolipoamide and product NADH. This reaction is diagnostic for E3 and related enzymes with similar catalytic mechanisms (Danson *et al.*, 1984; Loving *et al.*, 1992).

Pea leaf mitochondrial and chloroplastic E3s were prepared as described in Materials and Methods, section 2.2.2 to 2.2.4. APA was prepared by dissolving in 95% ethanol to final concentrations of 2.5 mM and 250 μM. NADH was dissolved in 50 mM potassium phosphate buffer, pH 7.6 to a final concentration of 250 μM. The pea leaf mitochondrial and chloroplastic dihydrolipoamide dehydrogenase were incubated over a time course of 30 min on ice with the following reagents added and assayed after incubation as described in section 2.2.7 :

no NADH or APA	50 μM NADH only
500 μM APA only	50 μM APA only
500 μM APA and 50 μM NADH	50 μM APA and 50 μM NADH

A concentration of 50 μM of NADH was selected since this was found to have a minimal effect on activity but allowed NADH to reduce the putative cysteine groups thus enabling APA to irreversibly bind and inhibit E3 activity.

Both mitochondrial and chloroplastic E3s were irreversibly inhibited only in the presence of APA and NADH (see Figures 5.11 and 5.12). At 50 μM APA, it can be observed that the activity is irreversibly inhibited more slowly in both isoforms than at 500 μM APA, as expected. Moreover, an unexpected difference between the mitochondrial and chloroplastic isoforms was observed where the mitochondrial E3 activity was irreversibly lost after 3 min in the presence of 500 μM APA and after 15 min in the presence of 50 μM APA. The chloroplastic isoform, however, lost activity after approximately 8 min in the presence of 500 μM APA and only lost all activity

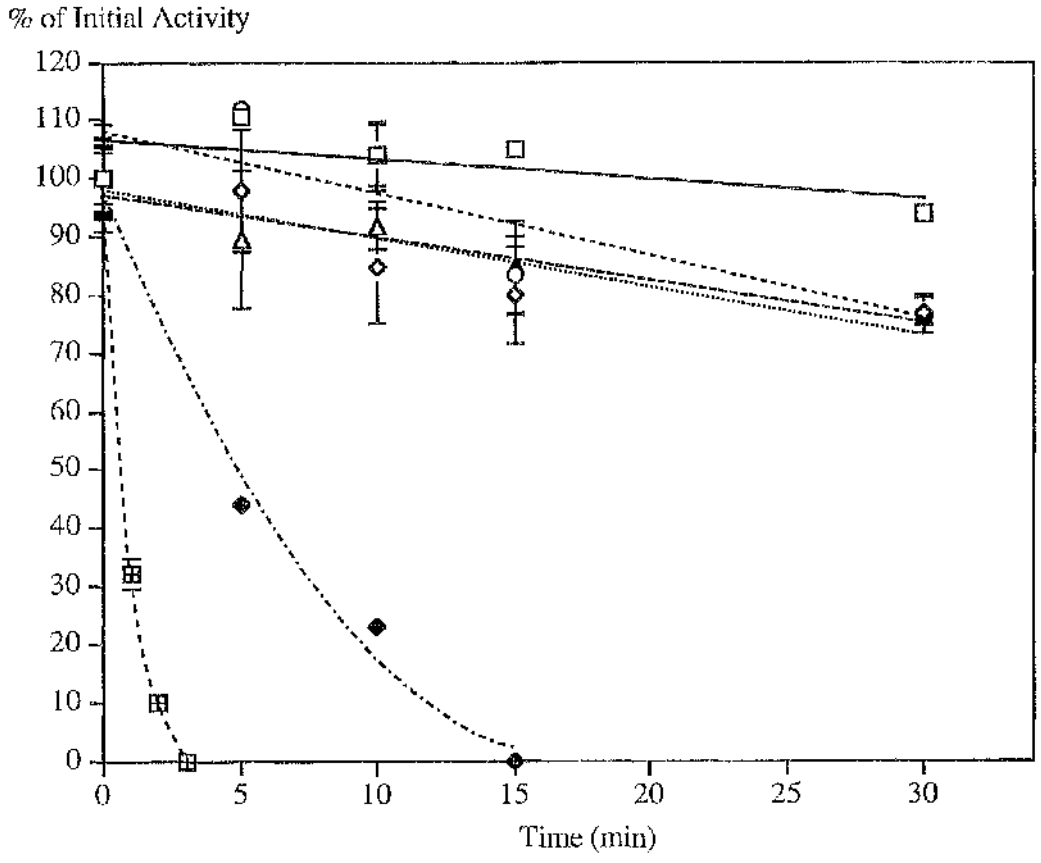

Figure 5.11: Chemical modification of pea leaf mitochondrial dihydrolipoamide dehydrogenase with APA

Mitochondrial E3 activity after incubation with no NADH or APA (□), 50 μM NADH only (◇), 500 μM APA only (○), 50 μM APA only (Δ), 500 μM APA with 50 μM NADH (⊞) and 50 μM APA with 50 μM NADH (◈).

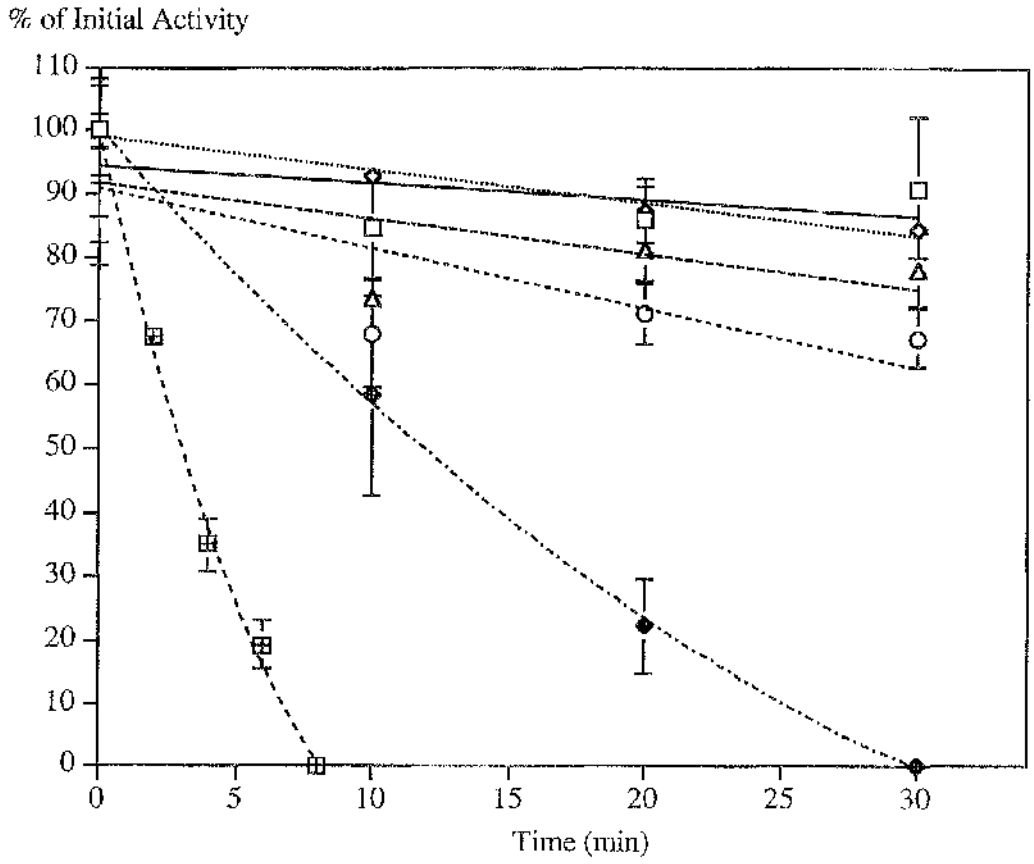

Figure 5.12: Chemical modification of pea leaf chloroplastic dihydrolipoamide dehydrogenase with APA

Chloroplastic E3 activity after incubation with no NADH or APA (□), 50 μM NADH only (◇), 500 μM APA only (○), 50 μM APA only (△), 500 μM APA with 50 μM NADH (⊞) and 50 μM APA with 50 μM NADH (◈).

after 30 min in the presence of 50 μM APA. This could reflect a difference in the accessibility of the active site by APA, where the chloroplastic E3 site is less accessible than the site of the mitochondrial enzyme, resulting in lower inactivation rates.

Chapter 6

Structure and Stability of Pea Mitochondrial and Chloroplastic Dihydrolipoamide Dehydrogenases

6.1 Introduction

The initial data from the isolation of chloroplastic dihydrolipoamide dehydrogenase from pea leaves indicated that its structural and enzymatic properties were significantly different from the mitochondrial enzyme, enabling it to bind to the Mono Q anion exchange column more tightly and causing it to be unrecognised by anti-pea mitochondrial E3 sera. Furthermore, kinetic analyses revealed a 5-fold lower K_m for NAD^+, a 3-fold lower K_i for NADH and a 3-fold higher K_m for DHL. These data suggest that the chloroplastic enzyme differs in primary structure and thus in secondary and tertiary structure. Therefore, to investigate their protein-chemical properties in more detail, a comparison of the stability of the enzymes to denaturation using chemical and physical techniques was employed. This was made simpler by the fact that the chloroplastic and mitochondrial dihydrolipoamide dehydrogenases are enzymatically active, enabling observation of protein secondary and/or tertiary unfolding by measuring any decrease in enzymatic activity. The techniques employed involved denaturation with guanidine hydrochloride (GdnHCl), the effects of high ionic strength and elevated temperatures to compare any differences in their susceptibility to denaturing agents.

6.2 Guanidine Hydrochloride (GdnHCl) Treatment of Chloroplastic and Mitochondrial E3s

For many years, guanidine hydrochloride (GdnHCl) has been used as a tool for investigating secondary and tertiary structure in individual proteins and multi-enzyme complexes. GdnHCl exerts a chaotropic effect on protein structure, thought to affect the hydration shell surrounding proteins, enabling researchers, for example, to selectively separate individual components of a multienzyme complex without destroying the tertiary structures of the individual components (West *et al.*, 1995). In

the following work, GdnHCl has been used to detect differences in secondary and tertiary structure between the chloroplastic and mitochondrial E3. This consisted of three types of experiments:

1. Inactivation and unfolding of each enzyme in the presence of guanidine hydrochloride- this investigated the ability of the enzymes to maintain structure and hence, activity, in the presence of increasing concentrations of GdnHCl.
2. Inactivation of each enzyme in the presence of sodium chloride- this investigated the ability of the enzymes to maintain their structure/activity in the presence of increasing ionic strength. Besides revealing any differences between the isoenzymes, it also shows that the effects of GdnHCl are not due only to salt effects.
3. Denaturation and renaturation of each enzyme in the presence of increasing concentrations of guanidine hydrochloride- this investigated the ability of the enzymes to reassemble after treatment with and then removal of GdnHCl.

6.2 Results and Discussion

6.2.1 Inhibition of Chloroplastic and Mitochondrial E3s using GdnHCl

Initially, pea leaf chloroplastic and mitochondrial dihydrolipoamide dehydrogenases were incubated in E3 assay solution (minus DHL) (see 2.2.7 in Materials and Methods) containing increasing concentrations of guanidine hydrochloride (0 to 2.0 M). These were incubated for 15 min at 25°C before beginning assaying by the addition of 3 mM DHL.

Figure 6.1 reveals that the activity of the mitochondrial E3 is completely absent after incubation with 0.5 M GdnHCl. However, the chloroplastic enzyme retains approx. 60% of activity at this concentration and only when the concentration reaches 1.75-2.0M does the activity fall to zero.

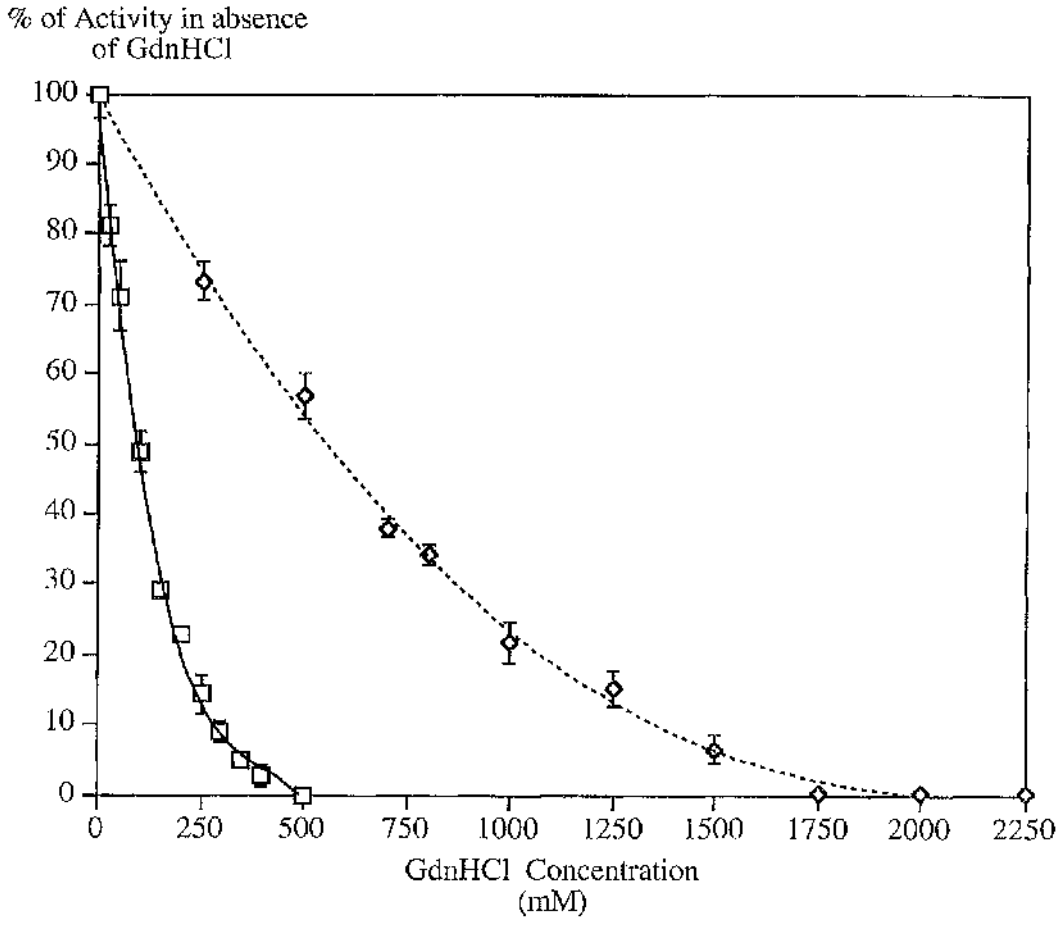

Figure 6.1: Pea leaf mitochondrial and chloroplastic dihydrolipoamide dehydrogenase activity in the presence of increasing concentrations of GdnHCl

Mitochondrial (□) and chloroplastic (◇) dihydrolipoamide dehydrogenase activity, expressed as % of activity in 0 M GdnHCl was determined as described in Materials and Methods section 2.2.7, except in the presence of increasing concentrations of GdnHCl. Best fit lines were calculated by regression analysis using a least squares method (Curve fit function in CA Cricket Graph III).

6.2.2 Inhibition of Chloroplastic and Mitochondrial E3s using NaCl

To ensure the effects exerted by GdnHCl are not solely caused by interference of substrate or co-factor binding at high ionic strength, this experiment was repeated, replacing GdnHCl with NaCl (0 to 4.5 M). In this instance, the mitochondrial activity was not completely lost until approx. 3.0 M NaCl (see Figure 6.2). A similar result was observed with the chloroplastic E3. However, at 3.0 M NaCl approximately 25% of activity was still present, and even at 4.5 M NaCl 20% of activity was retained.

6.2.3 Renaturation of Chloroplastic and Mitochondrial E3s using GdnHCl

The previous experiments were designed to compare loss of activity promoted by GdnHCl or NaCl which may be caused by reversible or permanent disruption of protein structure or interference with substrate or cofactor binding. However, to investigate irreversible denaturation of protein structure, the chloroplastic and mitochondrial enzymes were initially incubated in increasing concentrations of GdnHCl (0 to 3.0 M) at 25°C. After 15 min, the enzyme was added to E3 assay solution (minus DHL) to dilute the GdnHCl to a maximum of 25 mM (when initial concentration was 3.0 M) and the enzyme allowed to renature by incubating at 25°C for a further 15 min. Assays were initiated by the addition of 3 mM DHL (see section 2.2.7 for protocol).

Figure 6.3 reveals that the chloroplastic dihydrolipoamide dehydrogenase is more resistant to irreversible denaturation than the mitochondrial isoform, although both enzymes can recover 100% of their activities following pre-incubation in 1.0 - 1.3 M, a concentration range in which the mitochondrial isoform has no detectable activity. Interestingly, both E3 isoforms are irreversibly unfolded over a similar range

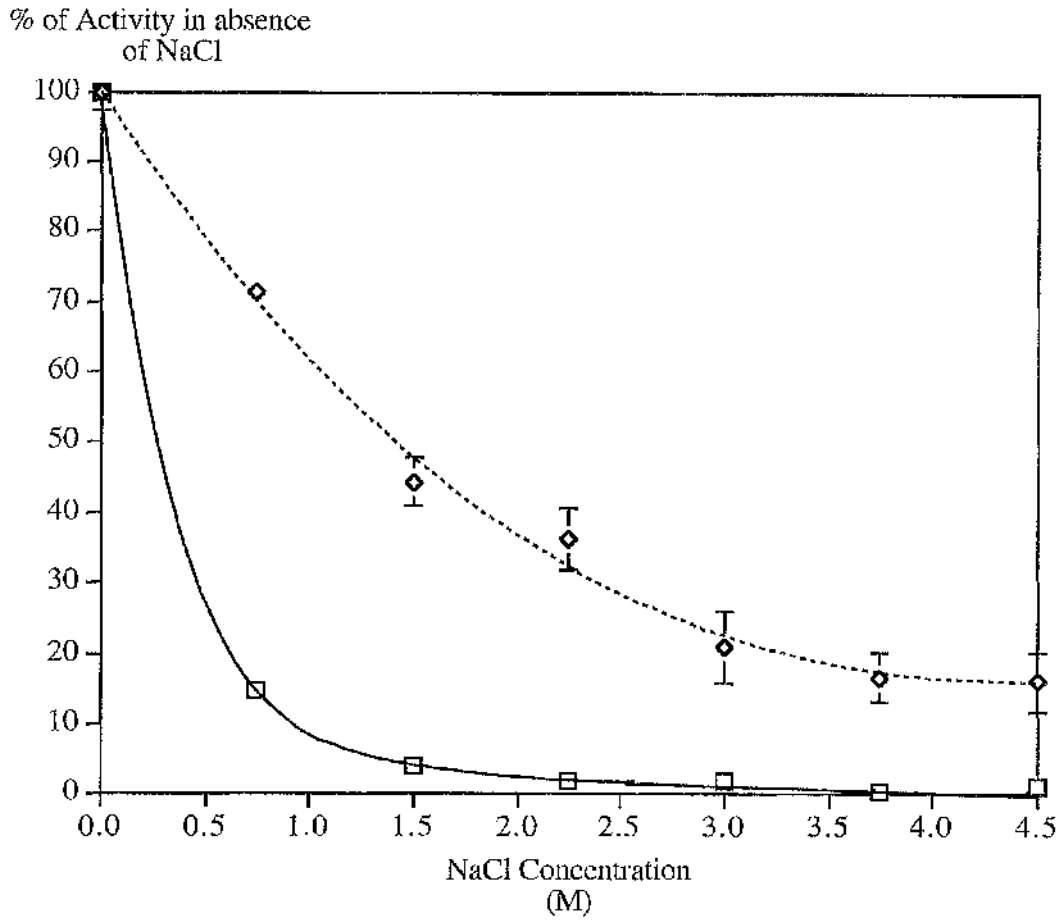

Figure 6.2: Pea leaf mitochondrial and chloroplastic dihydrolipoamide dehydrogenase activity in the presence of increasing concentrations of NaCl

Mitochondrial (□) and chloroplastic (◇) dihydrolipoamide dehydrogenase activity, expressed as % of activity in 0 M NaCl was determined as described in Materials and Methods section 2.2.7, except in the presence of increasing concentrations of NaCl. Best fit lines were calculated by regression analysis using a least squares method (Curve fit function in CA Cricket Graph III).

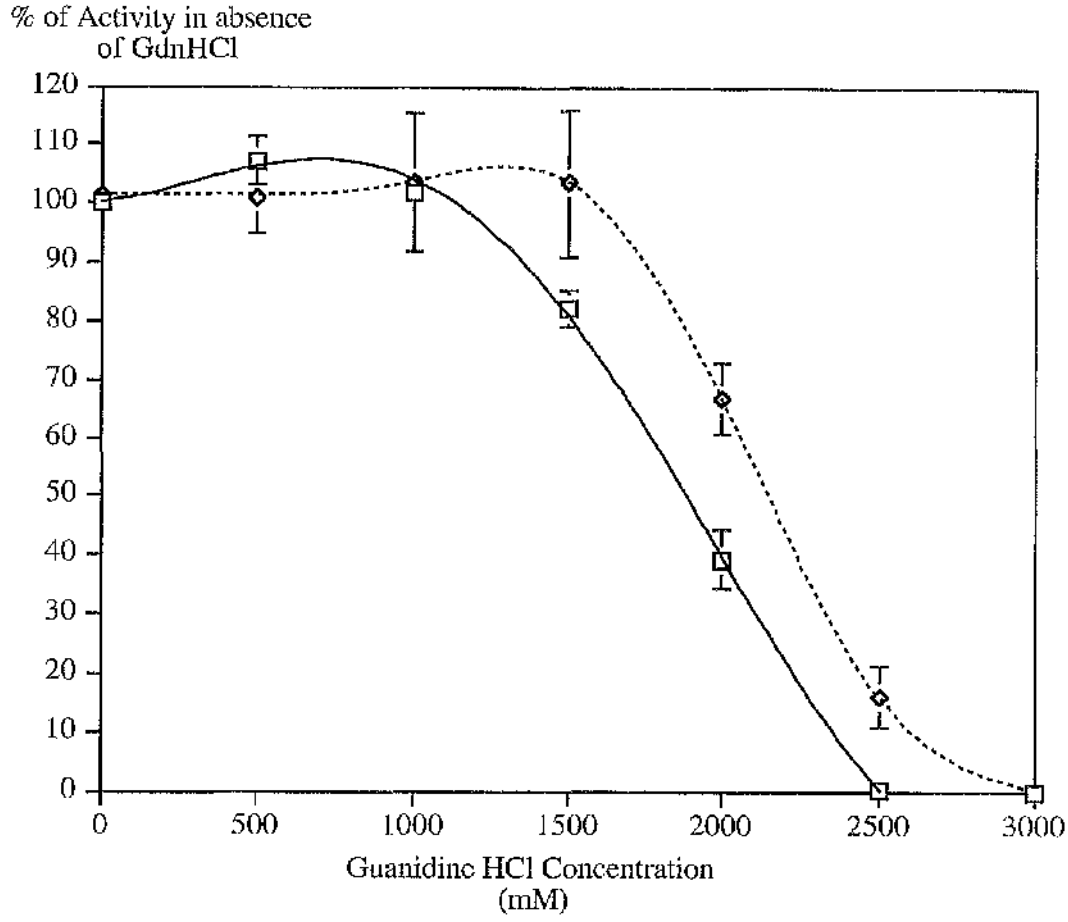

Figure 6.3: Pea leaf mitochondrial and chloroplastic dihydrolipoamide dehydrogenase activity after incubation with, then removal of increasing concentrations of GdnHCl

Mitochondrial (□) and chloroplastic (◇) dihydrolipoamide dehydrogenase activity, expressed as % of activity in 0 M GdnHCl was determined as described in Materials and Methods section 2.2.7, except assaying was performed after incubation and dilution of increasing concentrations of GdnHCl. Best fit lines were calculated by regression analysis using a least squares method (Curve fit function in CA Cricket Graph III).

of GdnHCl concentrations (1.5 - 3.0 M), where the mitochondrial enzyme is markedly more sensitive to the presence of GdnHCl in the final assay mix. These results suggest that the chloroplastic E3 structure and/or activity is more resistant to reversible inhibition than the mitochondrial isoform, where GdnHCl either interferes with the binding or catalysis of the substrate or perturbs the overall structure of the mitochondrial dihydrolipoamide dehydrogenase. Thus, a significant difference in secondary and/or tertiary structure and stability exists between the chloroplastic and mitochondrial E3s.

6.2.4 Gel Filtration Analysis of Chloroplastic and Mitochondrial E3s in the Presence of GdnHCl

From these results, several theories were postulated. While the mitochondrial enzyme separated into its constituent subunits at 0.5 M, the chloroplast enzyme was resistant to this dissociation and remained present as dimers until 2.0 M GdnHCl, thus affecting only mitochondrial activity.

Another possibility was that both enzymes were present as dimers at 0.5 M GdnHCl but only the activity of the mitochondrial enzyme was disrupted by GdnHCl, through either reversible alteration of the protein structure or interference of substrate binding, thus preventing catalysis.

To determine if dissociation of the mitochondrial and/or chloroplastic dihydrolipoamide dehydrogenases was occurring and thus affecting mitochondrial activity only, pea leaf mitochondrial and chloroplastic dihydrolipoamide dehydrogenase were passed through a Superdex 200 gel filtration column in the presence of 1.0M GdnHCl and the native molecular mass of the protein and activity peaks calculated using molecular mass standards.

Owing to the denaturing effects on the multisubunit proteins used as molecular mass standards, these were passed through the column in the absence of GdnHCl. Therefore, an assumption had to be made where any change in molecular mass of the chloroplastic or mitochondrial enzymes was not due to GdnHCl altering the resolution of the gel filtration column but to dissociation of the dihydrolipoamide dehydrogenase dimers into monomers.

Previously, it was reported that the pea leaf mitochondrial E3 has a native molecular mass of 100 kDa (see Figure 4.4) and the chloroplastic isoenzyme a native molecular mass of 120 kDa, when calculated by gel filtration. This revealed that both enzymes exist as dimers. When the mitochondrial and chloroplastic enzymes were passed through the Superdex 200 gel filtration column separately in the presence of 1.0 M GdnHCl, similar molecular masses were obtained (see Figures 6.4 and 6.5), indicating that under these conditions, both enzymes are still present as dimers. This discounts the first proposition, where the loss of activity in the mitochondrial dihydrolipoamide dehydrogenase is attributed to the dissociation of the dimers into monomers. Therefore, GdnHCl must be exerting an effect through either interference of substrate binding or through a general and reversible disruption of protein structure of the mitochondrial enzyme while the chloroplastic enzyme is able to resist these effects to a greater degree.

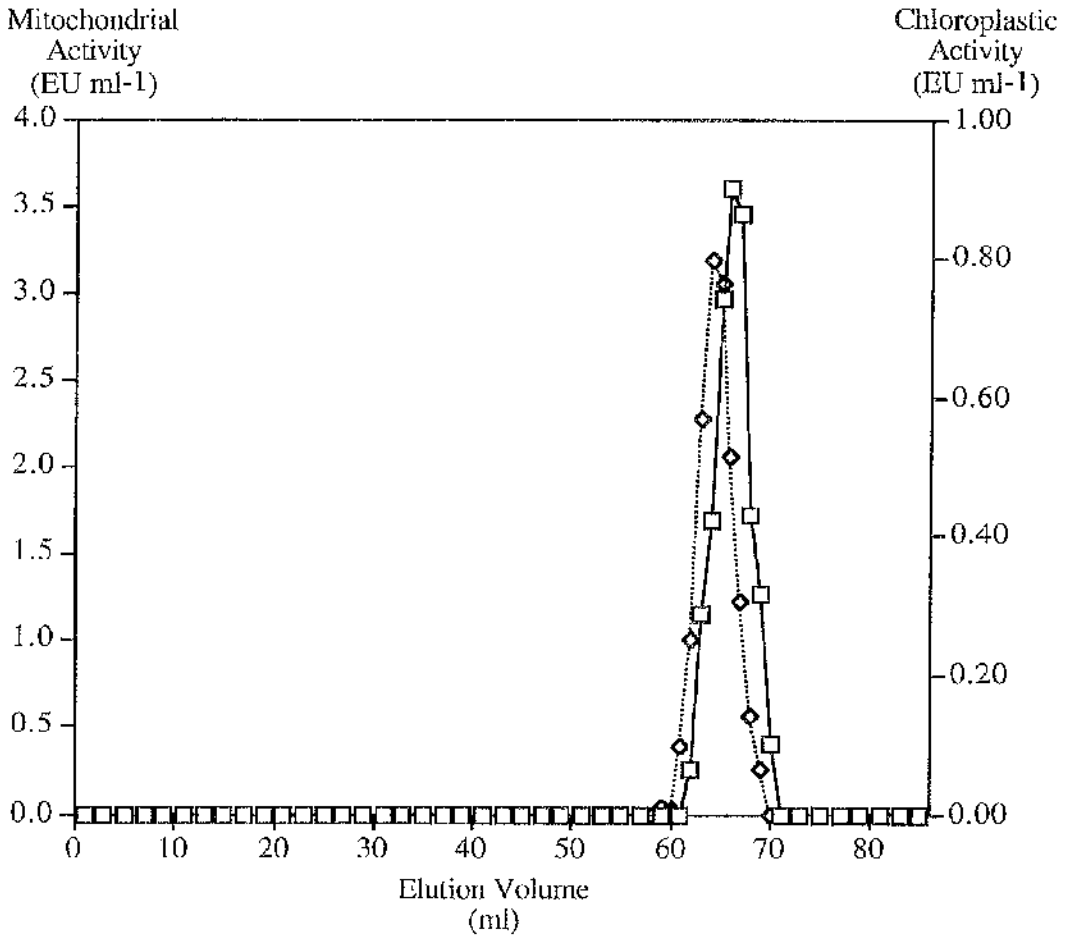

Figure 6.4: Activity profile obtained from the gel filtration of pea leaf mitochondrial and chloroplastic dihydrolipoamide dehydrogenases in the presence of GdnHCl

Mitochondrial (□) and chloroplastic (◇) dihydrolipoamide dehydrogenase was eluted from a Superdex 200 gel filtration column at 1.0 ml min^{-1} using 20 mM KP_i, pH 7.0 + 20 mM NaCl containing 1.0 M GdnHCl as buffer and the eluted protein collected in 1 ml fractions. E3 activity, expressed as EU ml^{-1} (µmol NADH produced ml^{-1} min^{-1}) was determined in each fraction as described in Materials and Methods section 2.2.7.

6.3 Heat Denaturation of Chloroplastic and Mitochondrial Dihydrolipoamide Dehydrogenases

Dihydrolipoamide dehydrogenases from porcine heart and *Azobacter vinelandii* have previously been shown to be stable at high temperature (Van Muiswinkel-Voetberg *et al.*, 1973; Van Berkel *et al.*, 1991). This heat stability is exploited here in the purification of both pea leaf mitochondrial and chloroplastic enzymes where extracts are heat treated for 20 min to 1 h at 65°C (see 2.2.2). To further characterise the differences between the chloroplastic and mitochondrial isoenzymes, measurement of the extent of loss of enzymatic activity and hence, denaturation of protein structure, with increasing temperature and also at a specified chosen temperature was performed.

Initially, in order to determine the optimum temperature for revealing differences in heat stability, pea leaf chloroplastic and mitochondrial dihydrolipoamide dehydrogenase were incubated for 15 min at increasing temperatures from 50°C to 95°C prior to assaying for activity (see Figure 6.5). This revealed that the chloroplastic enzyme retained practically all of its activity up to and including 80°C, while the mitochondrial E3 had lost approx. 30% of its activity after a 15 min incubation at this temperature. When the temperature is increased past 80°C both enzymes lose activity until 90°C, where no activity can be detected in either enzyme.

Once this had been performed, a temperature of 80°C was selected since a difference in activities at this temperature between the chloroplastic and mitochondrial enzymes could be detected. Again the enzymes were incubated, this time at 80°C and assayed over time. (see Figure 6.6). As expected, the chloroplastic E3 was shown to be significantly more resistant to denaturation by heat at 80°C, whereas the mitochondrial enzyme lost all enzymatic activity after 180 min incubation at this temperature.

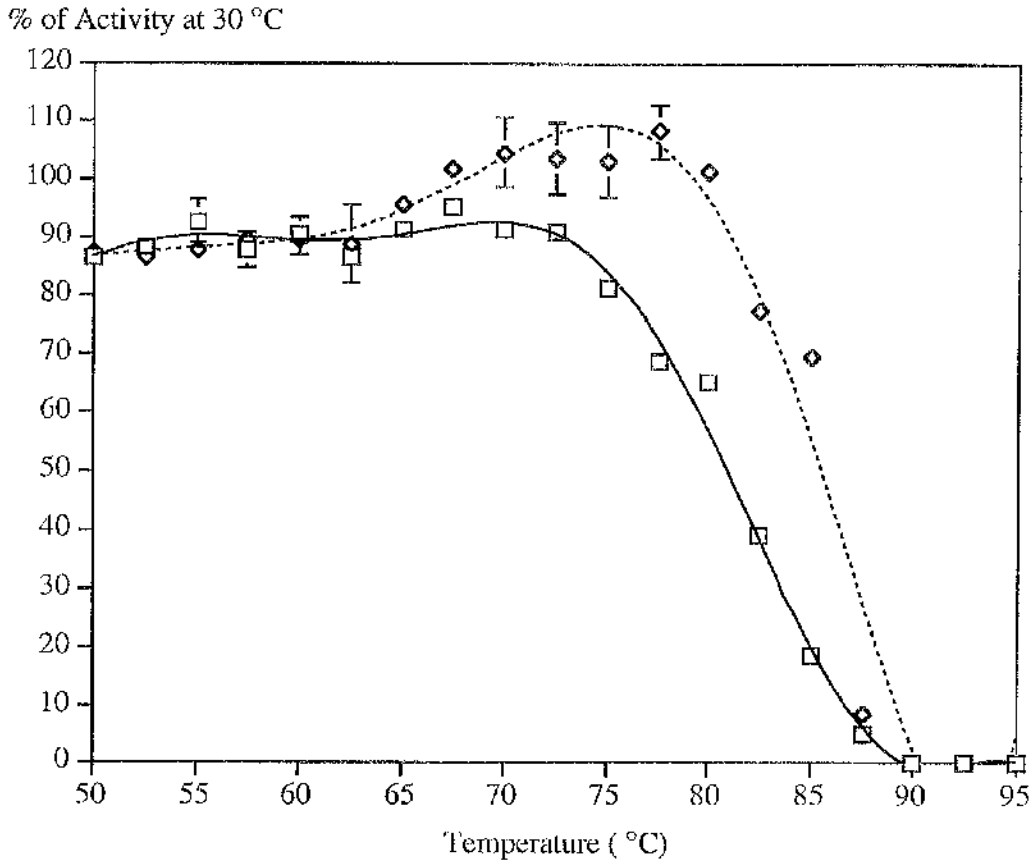

Figure 6.5: Pea leaf mitochondrial and chloroplastic dihydrolipoamide dehydrogenase activity after incubation at 50°C to 95°C

Pea leaf mitochondrial (□) and chloroplastic (◇) dihydrolipoamide dehydrogenases were initially incubated at temperatures from 50°C to 95°C for 15 min. E3 activity, expressed as % of activity at 30°C was then determined at each temperature step as described in Materials and Methods section 2.2.7. Best fit lines were calculated by regression analysis using a least squares method (Curve fit function in CA Cricket Graph III).

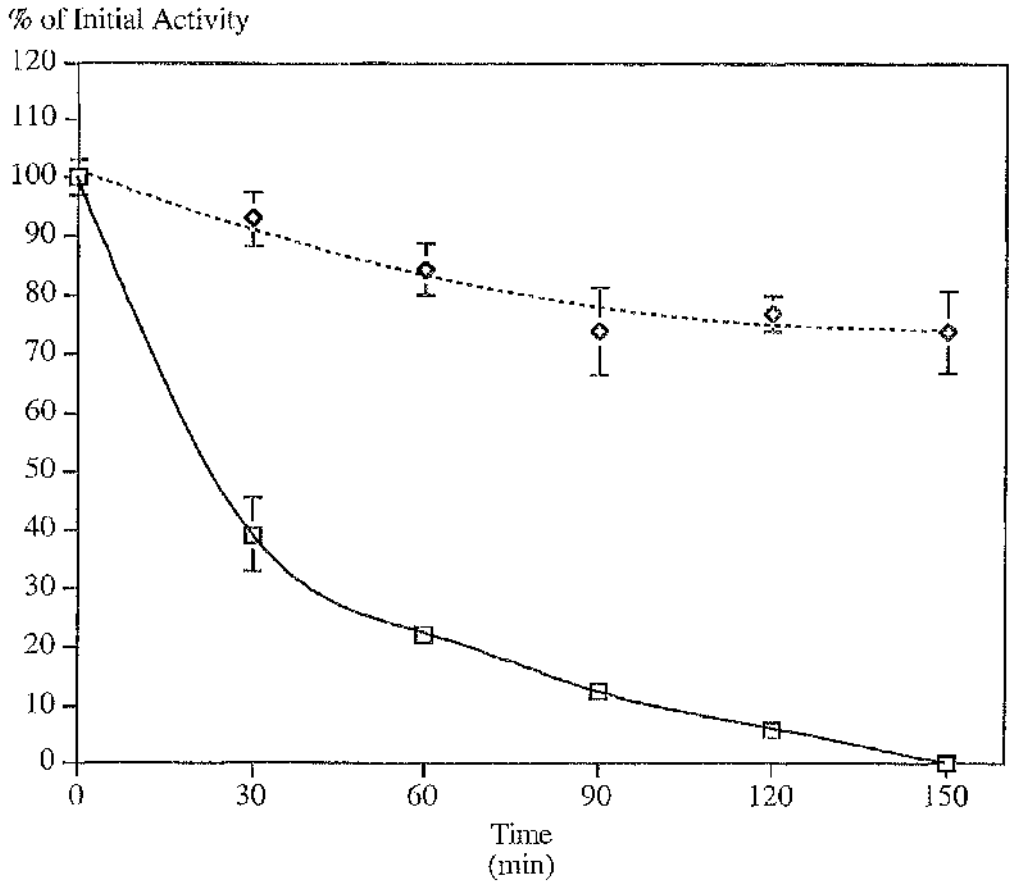

Figure 6.6: The effect of temperature on the activity of pea leaf mitochondrial and chloroplastic dihydrolipoamide dehydrogenases

Pea leaf mitochondrial (□) and chloroplastic (◇) dihydrolipoamide dehydrogenase was incubated at 80°C and samples assayed at the time intervals indicated on the graph as described in Materials and Methods section 2.2.7. Best fit lines were calculated by regression analysis using a least squares method (Curve fit function in CA Cricket Graph III).

As observed for the GdnHCl inhibition and renaturation experiments discussed previously, differences in structural stability between the chloroplastic and mitochondrial enzymes can also be detected using high temperatures to disrupt secondary and tertiary structure. These structural differences are also reflected in their kinetic properties which are consistent with the requirements of the plastid enzyme to operate in the chloroplast stroma. Levels of NAD^- are thought to be significantly lower in the chloroplast stroma than in the mitochondrial matrix, leading to the chloroplastic enzyme having a lower K_m to allow the reduction of NAD^+ to NADH to proceed. Thus, the properties of the chloroplastic enzyme reflect its suitability to operate under the conditions prevailing in the plastid stroma.

These protocols could provide an improved procedure for the purification of the chloroplastic dihydrolipoamide dehydrogenase from the mitochondrial isoform, particularly with the treatment of a crude extract with elevated temperatures to inactivate the mitochondrial enzyme but not the chloroplastic isoform.

Chapter

7

Chromatographic and Immunological Detection of Mitochondrial and Chloroplastic Dihydrolipoamide Dehydrogenases from Other Plant Species

7.1 Introduction

Chapters three and four were concerned with the identification of a dihydrolipoamide dehydrogenase unique to pea plastids which was distinct from the mitochondrial isoform, while chapters 5 and 6 examined the kinetic and physical properties of the chloroplastic enzyme as compared to the mitochondrial enzyme. In this chapter the possibility was investigated that a similar plastid-specific dihydrolipoamide dehydrogenase could be detected in other plant species using chromatographic and immunological investigations on leaves from barley, spinach and oil seed rape.

7.2 Results and Discussion

7.2.1 Ion-exchange Chromatography of Organellar Extracts of Barley Leaves

An organellar extract of barley leaves was prepared as described in Materials and Methods, section 2.2.2c, loaded onto a Pharmacia Mono Q anion exchange column and the bound proteins eluted using a potassium phosphate gradient. Dihydrolipoamide dehydrogenase activity was determined in each fraction. The resulting activity profile revealed four peaks of dihydrolipoamide dehydrogenase activity (see Figure 7.1). The first three peaks of activity, eluting at approx. 50 mM to 140 mM potassium phosphate, were similar to the profile observed in potato mitochondria, where three isoforms of mitochondrial E3 could be resolved (R. Fullerton, Ph.D. thesis, Glasgow University, 1995). The fourth peak, not observed in potato tuber mitochondria owing to the low levels of plastidic dihydrolipoamide dehydrogenase in non-photosynthetic tissues, eluted from the column at approx. 180 mM potassium phosphate, a similar position in the salt gradient to the elution of the pea leaf chloroplastic E3.

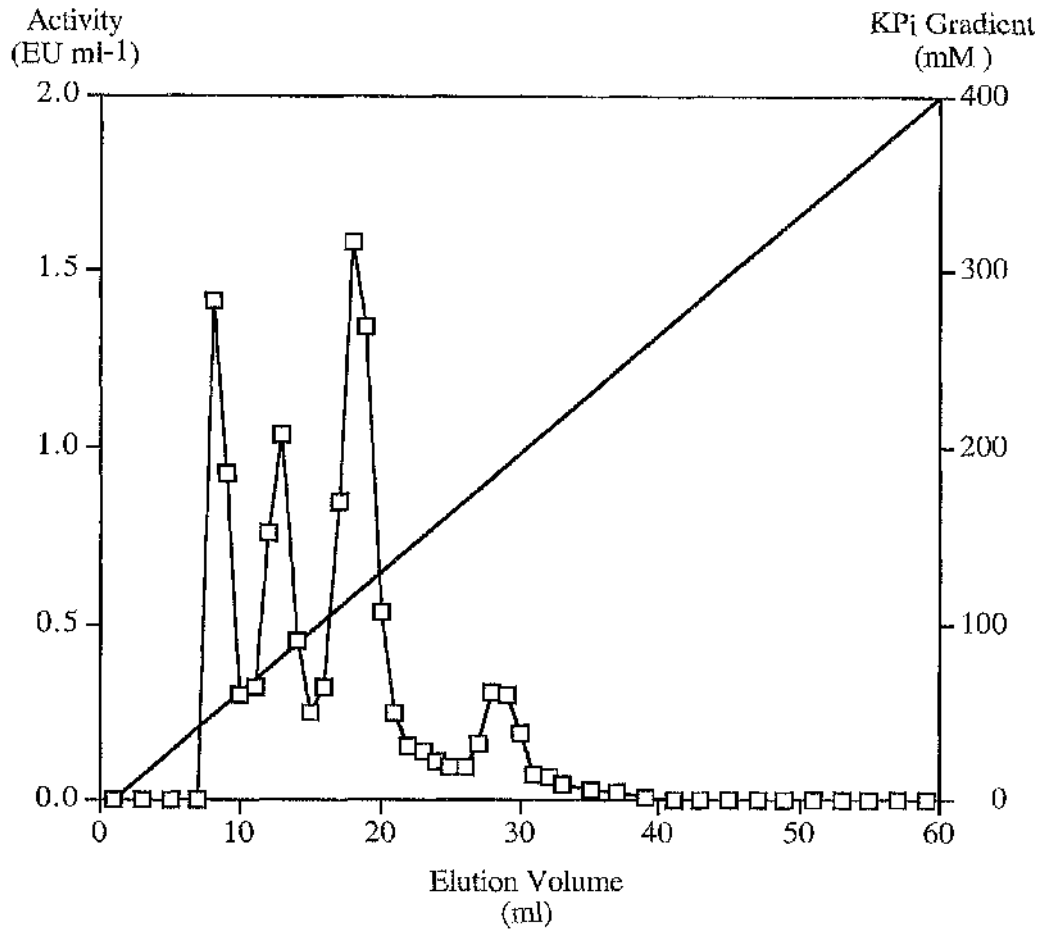

Figure 7.1: Resolution of an organellar extract of barley leaves by anion exchange chromatography

Organellar extracts of barley leaves (100g) were solubilised in 25 mM HEPES, pH 6.8 containing 0.2% (v/v) Triton X-100 and heat treated prior to resolution on a Mono Q HR 5/5 ion-exchange column on a Pharmacia FPLC System. Bound proteins were eluted at a flow rate of 1.5 ml min^{-1} with a potassium phosphate gradient (solid line, 10-400 mM) and 1.5 ml fractions collected for analysis. Dihydrolipoamide dehydrogenase activity (□), expressed in EU ml^{-1} (µmol NADH produced ml^{-1} min^{-1}), was determined in duplicate according to the method described in Materials and Methods, section 2.2.7.

These four peaks of activity were concentrated separately, resolved on a 7% (w/v) SDS-acrylamide gel and silver-stained. The first peak resolved as a single polypeptide with a molecular mass of approx. 58 kDa (see Figure 7.2). In contrast, the second peak contained two subunits with molecular masses of 58 kDa and 56 kDa. The third peak of activity again contained only one polypeptide, with a mass identical to the smaller subunit in peak two. Moreover, the molecular mass of the fourth peak was found to be smaller than the subunit masses of the first three peaks at approx. 52 kDa. By analogy with potato mitochondrial isoforms, the first three peaks appear to be a combination of two subunits of 58 kDa and 56 kDa, while the fourth peak has a mass of 52 kDa. It was therefore deduced that peaks one to three may be associated with mitochondria and the fourth peak was chloroplastic in origin, by comparison with the potato mitochondrial and pea chloroplastic isoforms detected previously.

7.2.2 Immunological Detection of Chloroplastic and Mitochondrial Isoforms of Dihydrolipoamide Dehydrogenase in Barley Leaves

A chloroplastic location for the fourth peak of E3 activity was confirmed when antisera specific to pea leaf mitochondrial and chloroplastic E3s were used to probe nitrocellulose blots of the four peaks from barley leaves seen in the elution profile in Figure 7.1 (see Figure 7.3). The anti-mitochondrial E3 serum highlighted only the first three peaks, at approx. 58 kDa and 56 kDa, and not the fourth peak of dihydrolipoamide dehydrogenase activity at 52 kDa, whereas the converse result was obtained with anti-chloroplastic dihydrolipoamide dehydrogenase serum (see Figure 7.3b). In this instance, only the fourth peak of activity was detected with no bands present in the lanes containing the first three peaks of E3 activity. Therefore, a peak of dihydrolipoamide dehydrogenase is present in barley which does not cross-react with antiserum to pea leaf mitochondrial dihydrolipoamide dehydrogenase but is recognised by antiserum directed against the pea chloroplastic isoform.

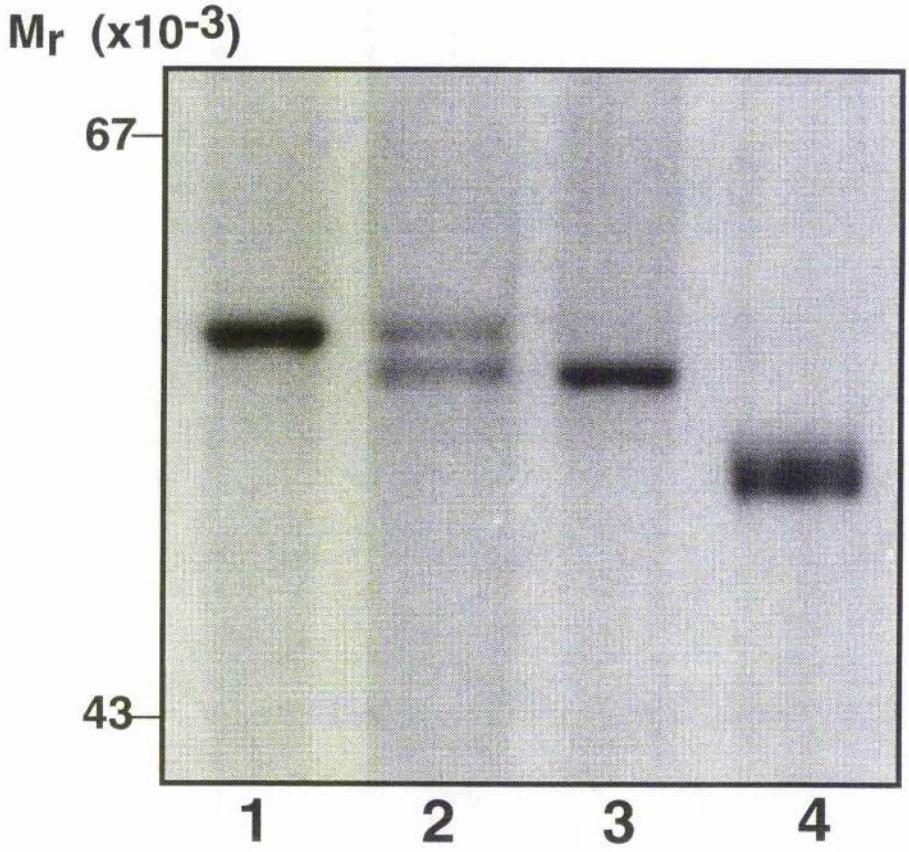

Figure 7.2: Analysis by SDS-PAGE of Mono Q-purified peaks of dihydrolipoamide dehydrogenase activity from organellar extracts of barley

Purified dihydrolipoamide dehydrogenase activities from organellar extracts of barley were subjected to electrophoresis on a 7% (w/v) SDS/polyacrylamide gel prior to detection by silver staining as described in the Materials and Methods, section 2.2.12. Lanes 1, 2, 3 and 4 contain 2 μg of peaks 1, 2, 3 and 4, respectively, from the organellar extraction of barley leaves as depicted in Figure 7.1. M_r standards were run on adjacent lanes.

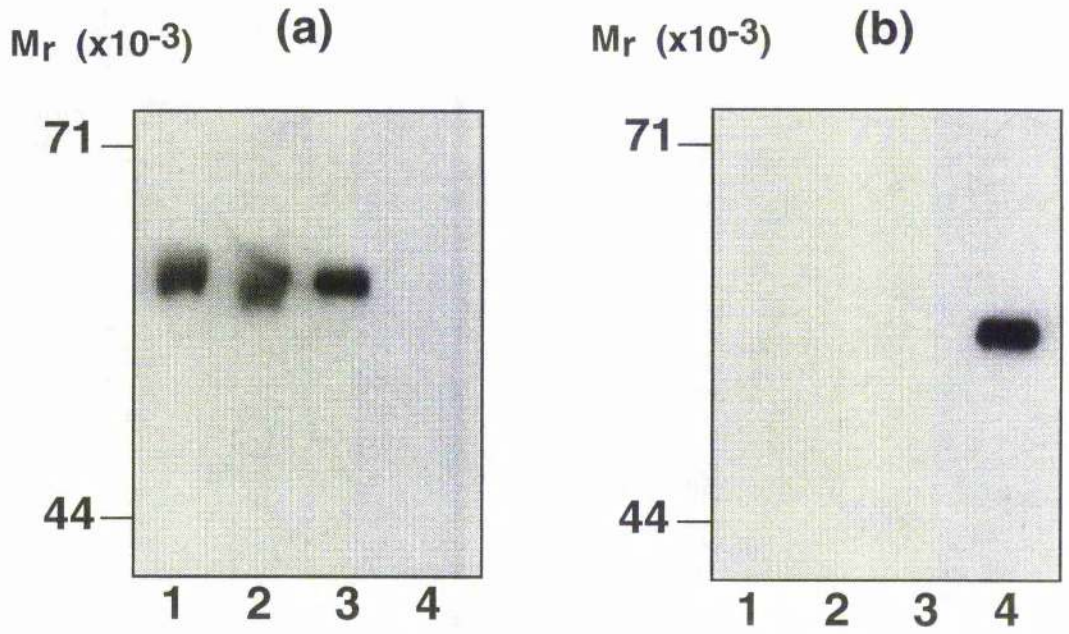

Figure 7.3: Cross-reactivity of Mono Q elution peaks of dihydrolipoamide dehydrogenase from organellar extracts of barley with antibodies raised to either pea mitochondrial E3 or its chloroplastic isoform

Purified dihydrolipoamide dehydrogenase activities from organellar extracts of barley were subjected to electrophoresis on 7% (w/v) SDS/polyacrylamide gels and transferred to nitrocellulose prior to detection by either anti-(pea leaf) mitochondrial E3 serum (a) or anti-(pea leaf) chloroplastic dihydrolipoamide dehydrogenase serum (b) as described in the Materials and Methods, section 2.2.17.

Lanes 1, 2, 3 and 4 (panels a and b) contain 0.2 μg of peaks 1, 2, 3 and 4 of E3 activity, respectively, from the organellar extraction of barley leaves. M_r standards were run on adjacent lanes.

Interestingly, barley leaves, similar to potato tubers, are the only other tissue so far found to display multiple isoforms of mitochondrial dihydrolipoamide dehydrogenase composed of the varying combinations of two closely related subunits, α and β.

7.2.3 Ion-Exchange Chromatography of Organellar Extracts of Spinach Leaves

As described previously for barley, organellar extracts were made from spinach leaves, dialysed and loaded onto a Mono Q ion-exchange column. Bound proteins were eluted using a potassium phosphate gradient and dihydrolipoamide dehydrogenase activity determined. As can be seen in Figure 7.4, only one major peak of activity appears to be resolved in spinach organellar extracts, at approx. 100 mM potassium phosphate. However, at approx. 160 mM, a very small secondary peak could be detected.

These peaks (fractions 29-31 for the first peak and fractions 51-53 for the second peak) were concentrated separately and resolved on a 7% (w/v) SDS-acrylamide gel prior to silver-staining. In this instance, the first major peak resolved as two polypeptides, with a prominent band of approx. 56 kDa and a smaller band of approx. 52 kDa (see Figure 7.5). The second, very minor peak was found to possess predominantly the lower band of 52 kDa, with again, a polypeptide of 56 kDa present in small quantities. The origins of the 52 kDa polypeptides were unclear at this stage and subsequent immunoblotting analysis was performed to determine whether they were mitochondrial, a proteolytic fragment of the 56 kDa polypeptide, chloroplastic or a contaminating peptide.

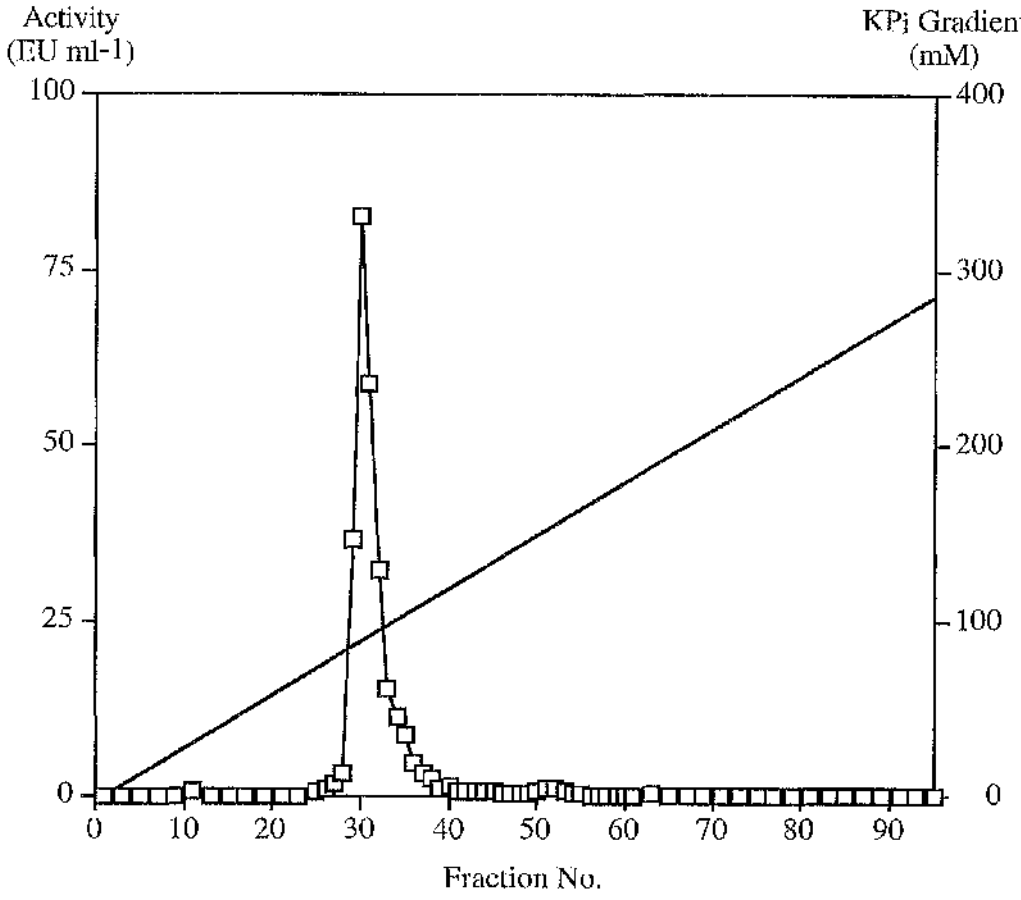

Figure 7.4: Resolution of an organellar extract of spinach leaves by anion exchange chromatography

Organellar extracts of spinach leaves (100g) were solubilised in 25 mM HEPES, pH 6.8 containing 0.2% (v/v) Triton X-100 and heat treated prior to resolution on a Mono Q HR 10/10 ion-exchange column on a Pharmacia FPLC System. Bound proteins were eluted at a flow rate of 1.5 ml min^{-1} with a potassium phosphate gradient (solid line, 10-400 mM) and 1.5 ml fractions collected for analysis. Dihydrolipoamide dehydrogenase activity (□), expressed in EU ml^{-1} (µmol NADH produced ml^{-1} min^{-1}), was determined in duplicate according to the method described in Materials and Methods, section 2.2.7.

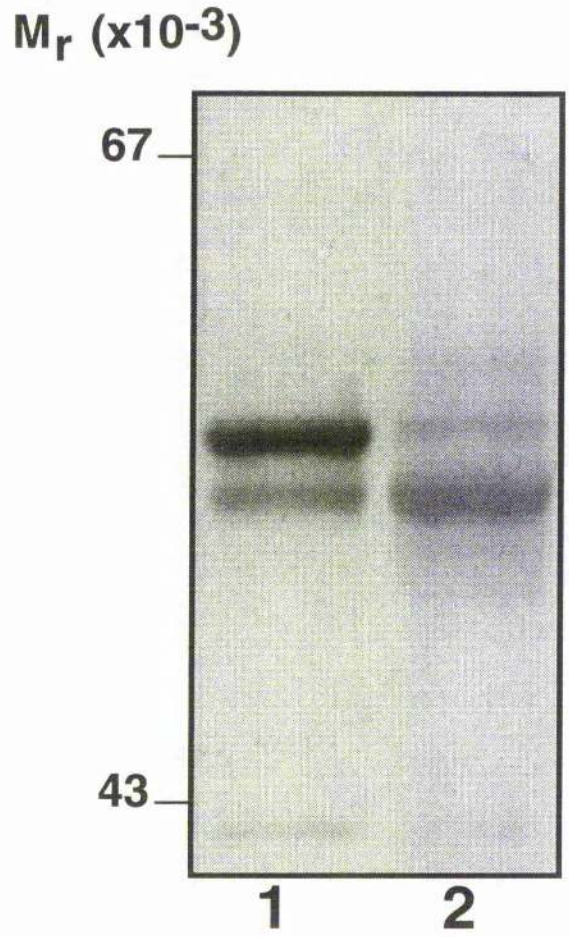

Figure 7.5: Analysis by SDS-PAGE of Mono Q-purified peaks of dihydrolipoamide dehydrogenase activity from organellar extracts of spinach

Purified dihydrolipoamide dehydrogenase activities from organellar extracts of spinach were subjected to electrophoresis on a 7% (w/v) SDS/polyacrylamide gel prior to detection by silver staining as described in the Materials and Methods, section 2.2.12.

Lanes 1 and 2 contain 2 μg of peaks 1 and 2, respectively, from the organellar extraction of spinach leaves as depicted in Figure 7.4. M_r standards were run on adjacent lanes.

7.2.4 Immunological Detection of Chloroplastic and Mitochondrial Isoforms of Dihydrolipoamide Dehydrogenase in Spinach Leaves

The peaks were transferred to nitrocellulose and probed with either anti-(pea leaf) mitochondrial E3 serum or anti-(pea leaf) chloroplastic dihydrolipoamide dehydrogenase serum. The anti-mitochondrial E3 serum highlighted two polypeptides in the first peak of activity, the major of approx. size 56 kDa and a minor of approx. 52 kDa, and in the second activity peak, a faint band also at 56 kDa. This reaction probably stemmed from contamination of the second activity peak by the mitochondrial isoform. The anti-chloroplastic dihydrolipoamide dehydrogenase serum, however, highlighted only one band in the second peak of activity, at approx. 52 kDa (see Figure 7.6). Moreover, this antiserum did not react with the 52 kDa band present in the first activity peak, leading to the conclusion that this band is either a proteolytic fragment of the polypeptide of 56 kDa, a smaller subunit of the spinach mitochondrial E3 dimer, or an alternative E3 polypeptide, distinct from, but related to, the E3 polypeptide at 56 kDa.

It therefore appears that the anti-mitochondrial E3 serum has recognised two bands, while antibodies to the chloroplastic dihydrolipoamide dehydrogenase were unable to recognise either of the polypeptides present in the first activity peak. This antiserum, however, cross-reacted specifically with a similar sized polypeptide of 52 kDa in the second, minor peak of activity. As a result of this lack of reactivity by the anti-mitochondrial E3 serum to the polypeptide of 52 kDa in the second, minor activity peak, it is proposed that this peak contains a plastid-specific dihydrolipoamide dehydrogenase, eluting from the anion exchange column at a similar concentration of potassium phosphate to the pea chloroplastic enzyme.

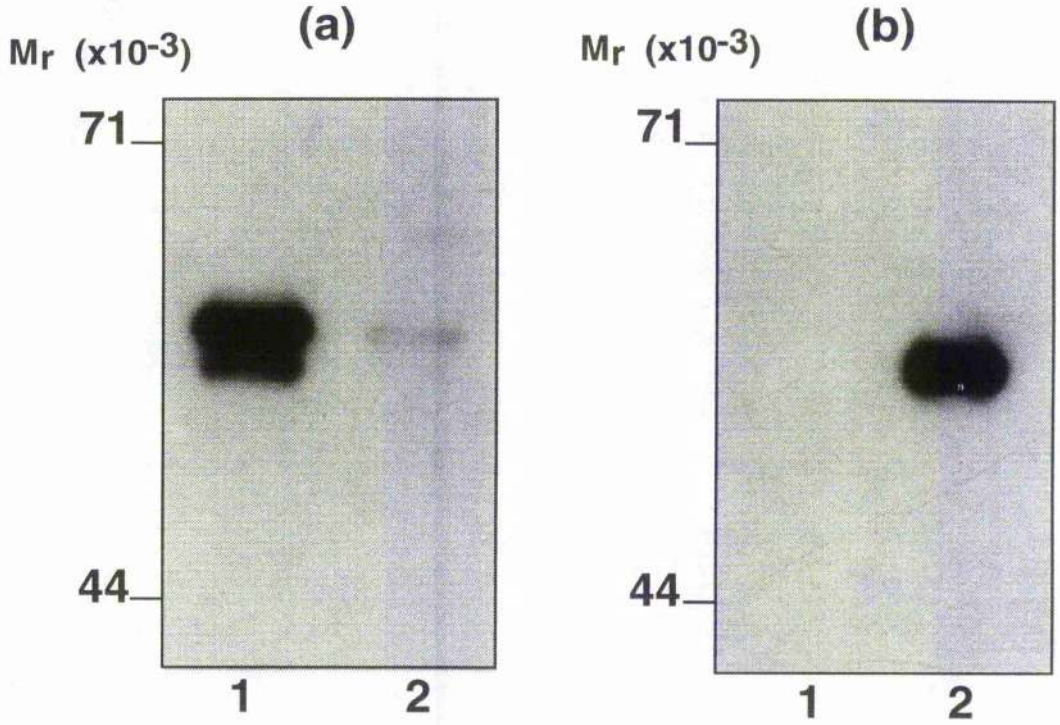

Figure 7.6: Cross-reactivity of Mono Q elution peaks of dihydrolipoamide dehydrogenase from organellar extracts of spinach with antibodies raised to either pea mitochondrial E3 or its chloroplastic isoform

Purified dihydrolipoamide dehydrogenase activities from organellar extracts of spinach were subjected to electrophoresis on 7% (w/v) SDS/polyacrylamide gels and transferred to nitrocellulose prior to detection by either anti-pea leaf mitochondrial E3 serum (a) or anti-pea chloroplastic dihydrolipoamide dehydrogenase serum (b) as described in the Materials and Methods, section 2.2.17.

Lanes 1 and 2 (panel a and b) contain 0.2 μg of peaks 1 and 2, respectively, from the organellar extraction of spinach leaves. M_r standards were run on adjacent lanes.

7.2.5 Ion-exchange Chromatography of Organellar Extracts of Oil Seed Rape Leaves

Similar organellar extracts were made from the leaves of oil seed rape, dialysed and loaded onto a Mono Q ion-exchange column. Bound proteins were eluted again with a potassium phosphate gradient and dihydrolipoamide dehydrogenase activity determined in the collected fractions. As can be seen in Figure 7.7, the resolution of individual dihydrolipoamide dehydrogenase activities was poor in this case, with the resulting recovery of a broad asymmetric peak with a leading shoulder. This suggests the possible presence of two isoforms of dihydrolipoamide dehydrogenase possessing similar elution profiles and therefore, overlapping activity peaks.

To test if the broad peak of E3 activity represented two overlapping peaks, fractions 35-44 and fractions 45-65 were concentrated separately and resolved on a 7% (w/v) SDS-acrylamide gel prior to silver-staining. In this instance, the first set of pooled fractions resolved as a single polypeptide with a molecular mass of approx. 56 kDa (see Figure 7.8). The second "peak" was found to possess two bands, with one of mass 56 kDa and a lower band with a mass of approx. 52 kDa. Thus, it was thought that this may mirror the situation in peas, where the first "peak" eluting from the Mono Q column is mitochondrial in origin, while the second represents the chloroplastic enzyme, with contamination of the second activity "peak" with the mitochondrial isoform.

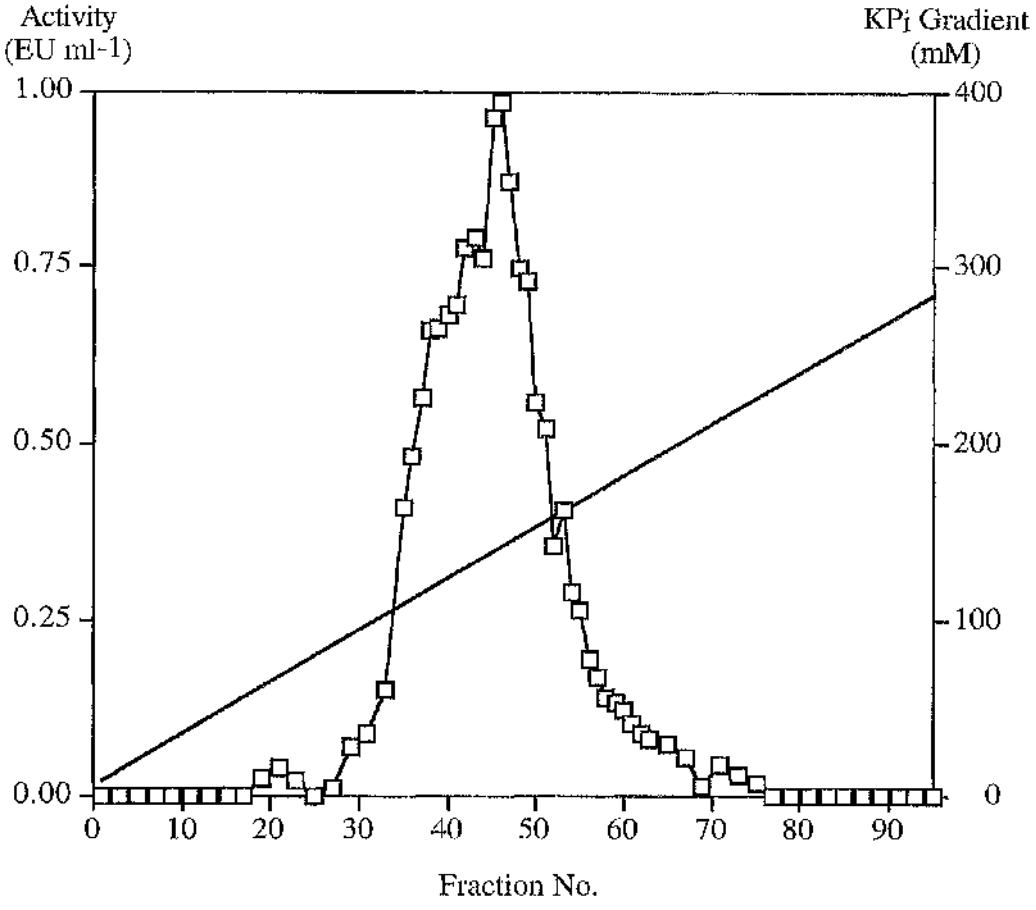

Figure 7.7: Resolution of an organellar extract of oil seed rape leaves by anion exchange chromatography

Organellar extracts of oil seed rape leaves (100g) were solubilised in 25 mM HEPES, pH 6.8 containing 0.2% (v/v) Triton X-100 and heat treated prior to resolution on a Mono Q HR 10/10 ion-exchange column on a Pharmacia FPLC System. Bound proteins were eluted at a flow rate of 1.5 ml min^{-1} with a potassium phosphate gradient (solid line, 10-400 mM) and 1.5 ml fractions collected for analysis. Dihydrolipoamide dehydrogenase activity (□), expressed in EU ml^{-1} (µmol NADH produced ml^{-1} min^{-1}), was determined in duplicate according to the method described in Materials and Methods, section 2.2.7.

Figure 7.8: Analysis by SDS-PAGE of Mono Q-purified peaks of dihydrolipoamide dehydrogenase activity from organellar extracts of oil seed rape

Purified dihydrolipoamide dehydrogenase activities from organellar extracts of oil seed rape were subjected to electrophoresis on a 7% (w/v) SDS/polyacrylamide gel prior to detection by silver staining as described in the Materials and Methods, section 2.2.12.

Lanes 1 and 2 contain 2 µg of peaks 1 and 2, respectively, from the organellar extraction of oil seed rape leaves as depicted in Figure 7.7. M_r standards were run on adjacent lanes.

7.2.6 Immunological Detection of Chloroplastic and Mitochondrial Isoforms of Dihydrolipoamide Dehydrogenase in Oil Seed Rape Leaves

This was further substantiated when the pooled and concentrated fractions were transferred to nitrocellulose and probed with either anti-pea mitochondrial or chloroplastic E3 serum. In this case, anti-mitochondrial E3 antibodies weakly highlighted a single polypeptide of approx. 56 kDa in the first set of pooled fractions, and strongly highlighted an identical-sized peptide in the second pool. The anti-chloroplastic dihydrolipoamide dehydrogenase serum strongly highlighted a band of approx. 52 kDa in the second "peak" of activity and weakly highlighted a similar sized band in the first activity "peak" (see Figure 7.9).

It was therefore proposed that the mitochondrial and chloroplastic dihydrolipoamide dehydrogenase isoforms were present in both sets of pooled samples whose fractions were arbitrarily selected, reinforcing the proposition that there were two closely spaced peaks of E3 activity eluting from the anion exchange column. Thus, the anti-chloroplastic enzyme antiserum reacted with a band of approx. 52 kDa in both "peaks" that were selected. However, due to the contamination of the second peak, it was impossible to determine whether this polypeptide possessed E3 activity.

In this chapter, evidence has been presented using enzymatic and immunological criteria for the possible existence of a chloroplast-specific dihydrolipoamide dehydrogenase in barley, spinach and oil seed rape leaves, all possessing a molecular mass of around 52 kDa. The mitochondrial isoforms, however, displayed more variability in molecular masses with M_r values in the 54-58 kDa range.

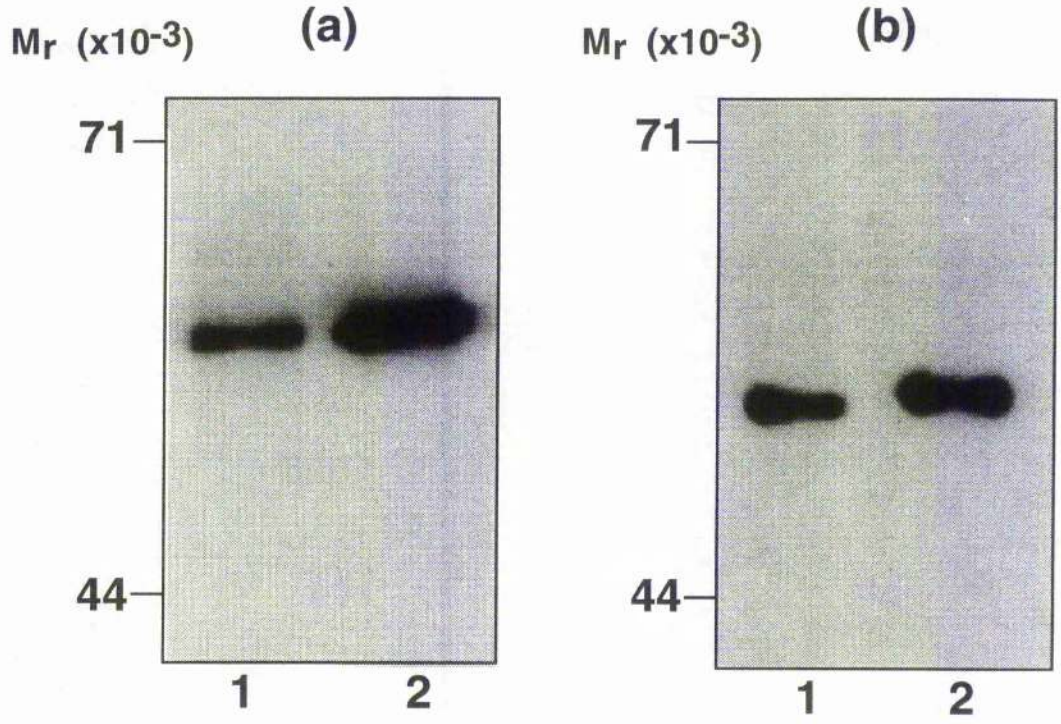

Figure 7.9: Cross-reactivity of Mono Q elution peaks of dihydrolipoamide dehydrogenase from organellar extracts of oil seed rape with antibodies raised to either pea mitochondrial E3 or its chloroplastic isoform

Purified dihydrolipoamide dehydrogenase activities from organellar extracts of oil seed rape were subjected to electrophoresis on 7% (w/v) SDS/polyacrylamide gels and transferred to nitrocellulose prior to detection by either anti-pea leaf mitochondrial E3 serum (a) or anti-pea chloroplastic dihydrolipoamide dehydrogenase serum (b) as described in the Materials and Methods, section 2.2.17.

Lanes 1 and 2 (panel a and b) contain 0.2 μg of peaks 1 and 2, respectively, from the organellar extraction of spinach leaves. M_r standards were run on adjacent lanes.

The possible existence of subunits of mitochondrial E3 with different molecular masses has also been demonstrated in barley, with three isoforms arising from the various combinations of two subunits of differing sizes, closely mirroring the situation in potato leaves and tubers. In spinach leaves the situation was less clear, with two peptides eluting from the anion exchange column as a single peak of activity with a similar elution profile to the pea mitochondrial enzyme. The apparent major peptide was of similar size to the pea mitochondrial E3, while the smaller peptide was of similar size to the chloroplastic enzyme (revealed by immunoblotting). These polypeptides may be subunits of a heterodimeric E3 as found in barley leaves and potato tubers, although this is unlikely as there appears to be a lack of formation of multiple isoforms formed by mixed binding of the individual subunits in all possible combinations e.g. homodimers as well as heterodimers. However, both polypeptides could possess similar net charges, preventing the separation of the mixture of heterodimers and homodimers by Mono Q ion exchange chromatography.

A second plausible explanation could be that the smaller peptide is a proteolytic fragment of the larger protein. This would imply that the spinach mitochondrial E3 is more susceptible to proteolysis than the pea isoform and other dihydrolipoamide dehydrogenases, which are generally known for their resistance to proteolysis. Another possibility could be that both bands represent subunits of two distinct but related E3 homodimers, with one possibly associating with GDC and the other with OGDC. Further investigation is necessary to provide a definitive answer to these alternatives.

In conclusion, the presence of an invariant 52 kDa polypeptide species in the leaves of barley, spinach and oil seed rape was revealed by anti-chloroplastic dihydrolipoamide dehydrogenase serum but not with antiserum raised to the mitochondrial isoform. However, in oil seed rape, it was impossible to determine whether the polypeptide possessed dihydrolipoamide dehydrogenase activity. The specificity of the antibody

response to the different isoenzymes present in different compartments within the same cell highlighted the distinctive nature of these two enzymes, indicating that they have less than 50% identity at the amino acid level which is in agreement with the limited protein sequence information obtained.

In addition, anti-chloroplastic and anti-mitochondrial sera have been shown to be sensitive and specific probes for revealing organelle-specific dihydrolipoamide dehydrogenases and could be used for studies on a wide variety of plant species.

Chapter 8

Attempted Isolation of the Gene Encoding the Chloroplastic Dihydrolipoamide Dehydrogenase from Pea Leaves

8.1 Introduction

The previous chapters have dealt with the isolation and characterisation of the chloroplastic dihydrolipoamide dehydrogenase, in order to provide evidence for its distinctiveness from the mitochondrial isoform. However, to provide definitive evidence of the uniqueness of the enzyme and to elucidate its role in plant metabolism, it would be highly advantageous to clone and sequence the cDNA corresponding to the chloroplastic E3. The only dihydrolipoamide dehydrogenase to be cloned and sequenced from plant sources is that of pea mitochondrial PDC, or the L-protein from GDC (Bourguignon *et al.*, 1992; Turner *et al.*, 1992). The cDNA encoded a 501 amino acid polypeptide including a 31 amino acid presequence. The mature protein was predicted to consist of 470 amino acids, giving a protein of 49,721 Da (or 50,441 Da if the FAD cofactor is included). The open reading frame reported by Turner *et al.* (1992) was 28 residues longer than that obtained by Bourguignon and co-workers. This was found to result from a sequencing error where the inclusion of an extra nucleotide at position 1535 resulted in a C-terminal extension to the open reading frame of 28 residues. Re-sequencing confirmed that the extra nucleotide was an error, that the cDNA sequences obtained by the two groups were identical and were derived from the same gene. However, to date, no component of the chloroplast-specific pyruvate dehydrogenase complex has been cloned, and since this work represents the first isolation of a chloroplast-specific dihydrolipoamide dehydrogenase, probably associated with a chloroplast-specific PDC, isolation of the cDNA encoding the protein would be very useful.

To obtain the cDNA sequence, a strategy was adopted using PCR amplification, with the initial amplification of a larger segment at the 5'-end using two smaller primers. This enlarged cDNA sequence, corresponding to the N-terminal region of the mature polypeptide, would then be used either to directly probe a pea leaf cDNA library or be

used to design a specific 5' primer for the amplification of the complete cDNA sequence, with an oligo-dT primer for the 3' primer.

8.2 Results and Discussion

8.2.1 Synthesis of Degenerate Oligonucleotide Primers

Using the N-terminal sequence obtained for the pea leaf chloroplastic dihydrolipoamide dehydrogenase (see 4.3.3), degenerate oligonucleotide probes containing either a Bam HI or an Eco RI restriction site were obtained from DNA Synthesis Unit, University of Glasgow (see Figure 8.1). These areas of N-terminal sequence were selected for their lowest degeneracy compared to the remaining sequences: primer 1 had a degeneracy of 256 and primer 2, a degeneracy of 512. The primers were prepared by ethanol precipitation according to the method described in section 2.2.21.

8.2.2 mRNA Isolation and cDNA Synthesis

mRNA was isolated from pea leaves using the Mini Message Maker from R&D Systems (see Materials and Methods, section 2.2.22) and then first strand cDNA synthesised using a First-strand cDNA Synthesis Kit from Pharmacia Biotech (see 2.2.23).

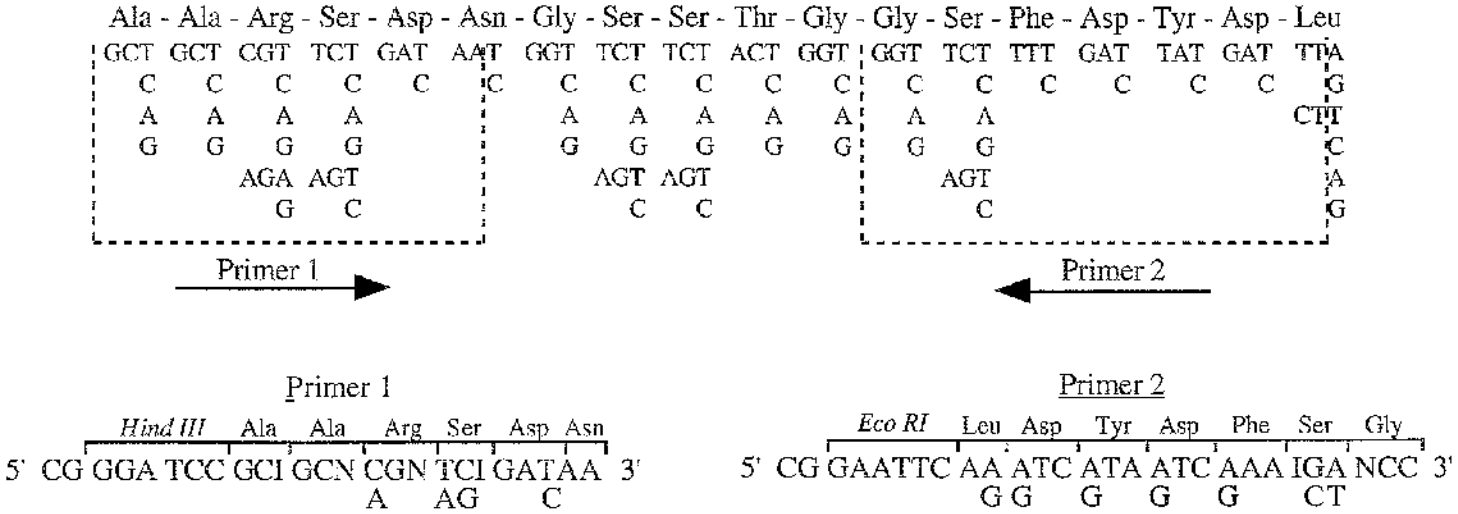

Figure 8.1: N-terminal sequence and corresponding degenerate oligonucleotide probes

The N-terminal region of the chloroplastic dihydrolipoamide dehydrogenase was found by sequencing (see 4.3.3), and the degenerate codons deduced for the sequence. The design of the primers were based on their low degeneracy, compared to the remaining sequence. N denotes A, G, T or C and I denotes the base inosine.

8.2.3 PCR Amplification of First Strand cDNA

PCR was performed on the first strand cDNA using the above degenerate primers (see 2.2.24 for PCR conditions) and electrophoresis of samples performed on a 10% (w/v) acrylamide vertical gel (see 2.2.25). The gel was subsequently stained using ethidium bromide to visualise any DNA oligonucleotides present (see 2.2.27).

As can be observed in Figure 8.2, a PCR product of approx. 70 bp, as calculated using the base pair standards, can be observed. This was close to the size of fragment expected from the N-terminal protein sequence.

8.2.4 Cloning of PCR Products

Isolation of this product was carried out by pooling and subsequent phenol:chloroform extraction (see 2.2.20), followed by ethanol precipitation, on several PCR reactions (see 2.2.21). The resulting purified DNA was treated with Bam HI and Eco RI to digest the restriction sites on the primer sequences that were incorporated into the DNA products (see 2.2.28). Once the digestion was completed, the resulting digested PCR product was subjected to electrophoresis, again on a 10% (w/v) acrylamide gel and the DNA was visualised by staining with ethidium bromide. A product at approx. 65-70 bp was observed, similar to that shown in Figure 8.2.

In order to assist purification of the fragment at approx. 70 bp, the band was excised from the acrylamide gel, placed into an elongated well of a 1% (w/v) low melting point high purity agarose gel (see 2.2.26) and subjected to electrophoresis until the selected fragment of DNA had entered the agarose gel (approx. 15 to 30 min). The

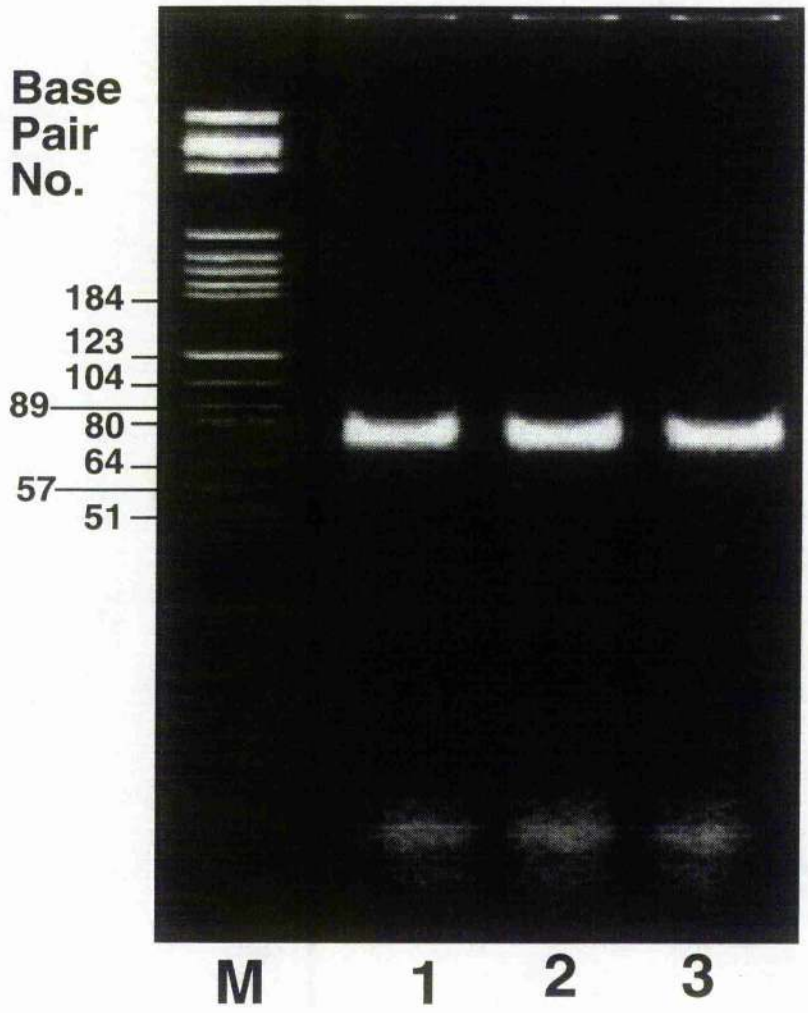

Figure 8.2: Electrophoretic analysis of the PCR reaction products obtained using primers 1 and 2

Identical samples of the PCR reaction products (lanes 1-3) were subjected to electrophoresis on a 10% (w/v) acrylamide vertical gel. The gel was then stained using ethidium bromide and visualised under UV light. Base pair marker ladders were run on adjacent lanes.

fragment was visualised under UV without the necessity of further staining due to the presence of ethidium bromide in the DNA fragment.

This DNA band was then excised and purified from the agarose gel, and ligated into plasmid pBluescript KS II+ (see Figure 8.3) using the method described in section 2.2.29. This plasmid had previously been digested with Bam HI and Eco RI (see 2.2.28) prior to ligation, and *E. coli* cells transformed with the resulting ligation mixture using $CaCl_2$ (see 2.2.31). Transformed cells were selected for their resistance to the antibiotic ampicillin (see 2.2.30) and plasmids containing inserts identified by the production of white colonies in the presence of IPTG and X-gal. Numerous white colonies were identified and removed from the agar plates for further analysis.

8.2.5 Analysis of Cloned PCR Products

The selected colonies were grown in LB medium and double-stranded plasmid DNA isolated from the cells using the method described in section 2.2.32. Restriction digestions using Bam HI and Eco RI were performed on the isolated samples of plasmids, the products subjected to electrophoresis on a 10% (w/v) acrylamide gel, and those containing a fragment of size of approx. 70 bp were identified.

8.2.6 Single-Stranded DNA Sequencing of PCR Products

These colonies were again grown in LB medium and in this instance, single stranded DNA isolated using the method described in the protocol for the Sequenase v2.0 DNA Sequencing Kit from Amersham (see 2.2.34). Once the single stranded DNA had been isolated, sequencing of these samples was performed using the Sequenase v2.0 DNA Sequencing Kit and the sequence obtained shown in Figure 8.4.

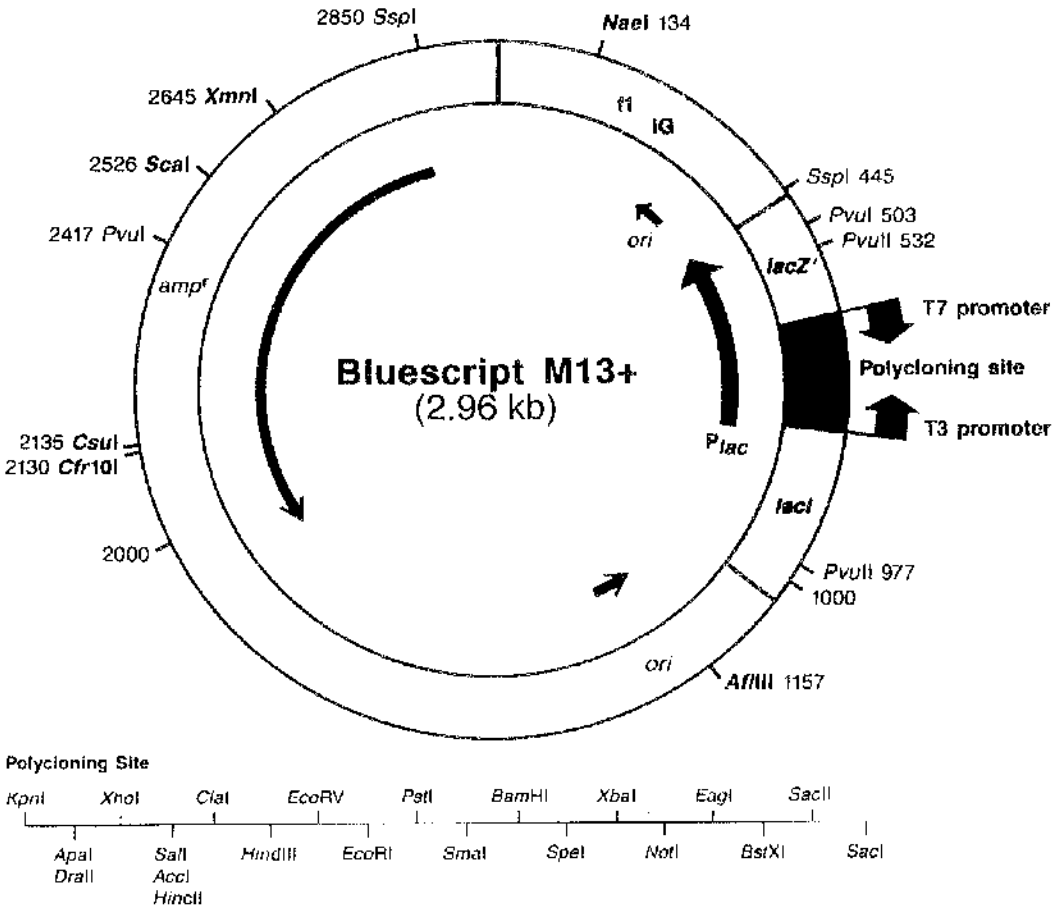

Figure 8.3: Sequence of plasmid Bluescript KS II M13+

Bluescript M13+ is a vector that contains the bacteriophage M13 origin of DNA replication inserted in opposite orientation into a vector derived from a pUC plasmid that contains bacteriophage T3 and T7 promoters. This vector can thus be used to generate single-stranded DNA *in vivo*, or RNA *in vitro*, that is complementary to either of the two strands of foreign DNA inserted into the multiple cloning site (Short *et al.*, 1988).

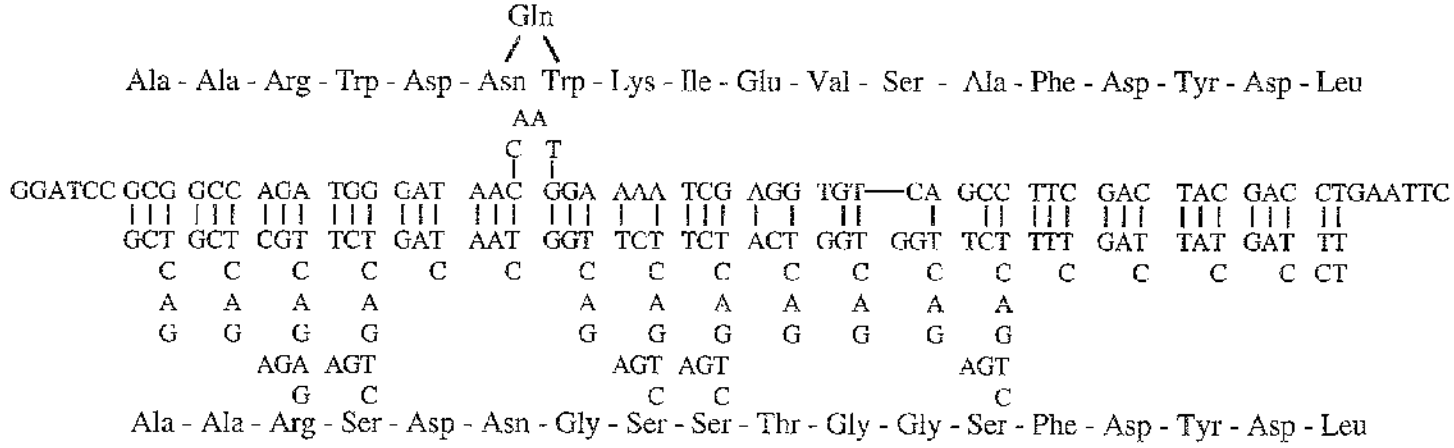

Figure 8.4: Comparison of the amplified cDNA sequence with the sequence predicted from the N-terminal sequence

Single stranded DNA containing the cloned PCR products of approx. 65-70 bp were sequenced using the Sequenase v2.0 DNA Sequencing Kit (top). The degenerate nucleotide sequence obtained from the N-terminal sequence is also shown for comparison (bottom sequence).

The sequence shown was typical of many of the PCR products analysed. Comparing this to the expected sequence it can be observed that the PCR product has limited similarity to it, except over the primer sequences. To try to resolve this discrepancy, the series of experiments were repeated several times, including the initial isolation of the mRNA and the subsequent synthesis of cDNA. PCR amplification using genomic DNA as a template was also performed several times. Despite this, an identical sequence was always obtained (see Figure 8.4).

These results were puzzling as the primers were binding to a similar-sized sequence with quite high specificity, but upon amplification and sequencing the internal sequence contained many differences to the expected sequences. The possibility of non-specific binding seems remote as the PCR product was a clean band of approximately the correct base pair number. The most obvious possibility appears to be that the primers may have been binding to and amplifying either a pseudogene or a related member of the family of pyridine disulphide oxidoreductases. A search in the NCBI **nr** database using the **blastn** program with the DNA sequence produced a match of 73% from base number 10 to 55 with the cosmid TO7C12 from *Caenorhabditis elegans*. This appeared to be a non-specific match with no physiological relevance. Thus, the reason for this difference in sequence obtained could not be determined at this stage.

One further approach for the isolation of the chloroplastic gene could be using slightly different primers from those used here. This, however, would be difficult as the sequences to the primers used were chosen for their lowest degeneracy, which at 256 to 512, were not low in absolute terms. Another approach would be to screen a cDNA expression library from pea leaves using the antisera described in chapters 3 and 7. A common problem of expressing eukaryotic proteins in prokaryotic systems is that the resulting polypeptide is expressed as a fusion protein with β-galactosidase which can cause partial or full denaturation. This, however, would be advantageous with the

anti-mitochondrial and anti-chloroplastic E3 sera since they have been raised to the denatured and not the native proteins. A third approach may be amplification of a full length cDNA using the N-terminal sequence obtained as the 5' primer above with an oligo-dT primer for the 3' end. This would obviously need to rely on the obtained N-terminal sequence binding to the required sequence with a few mismatches. This approach has been attempted but was found, with the limited conditions tried, to yield no amplified product.

Chapter 9

Discussion and Future Research

9.1 Detection and Purification of a Distinct Dihydrolipoamide Dehydrogenase from Pea Leaf Chloroplasts

The possible existence of a chloroplast-specific dihydrolipoamide dehydrogenase in pea leaves was first reported by Taylor *et al.* (1992). Immunological analysis revealed a polypeptide of approx. 52 kDa in chloroplasts probed with antisera raised to the E3 subunit of mammalian PDC, differing in size to the mitochondrial enzyme. Kinetic data also reveal the possible existence of a pyruvate dehydrogenase complex exclusive to plant plastids with differences in the Michaelis constant for NAD^+, pH optima and several other kinetic parameters (Camp and Randall, 1985). The difficulties in the isolation of intact pyruvate dehydrogenase complexes along with the heat stability, resistance to proteolysis and the ease and sensitivity of assaying the E3 component from a variety of sources, made E3 an attractive candidate to screen for.

Isolation of the potato and pea mitochondrial dihydrolipoamide dehydrogenase had previously been performed by anion exchange chromatography where mitochondrial extracts were eluted using a linear gradient of potassium phosphate. Using a similar approach, extracts of pea leaves were eluted from the anion exchange column and the E3 activity profile carefully monitored for the presence of more than one peak of activity. Two peaks were obtained of which the first, eluting at 130-150 mM potassium phosphate, contained 85-90% of overall activity and the second, which eluted at the higher concentration of 180-210 mM potassium phosphate, corresponded to 10-15% of overall activity. The first and second peak were subsequently shown to be present in separate compartments, being associated exclusively with highly purified mitochondria and chloroplasts, respectively.

The levels of activity of the mitochondrial and chloroplastic E3s found here in pea leaves were similar to the distribution of mitochondrial PDC (87%) and chloroplastic PDC (13%) found by Lernmark and Gardestrom (1994).

The distinctive nature of the two organelle-specific E3s was confirmed by immunological criteria, as antibodies to either failed to elicit a detectable cross-reaction with its sister E3.

9.2 Comparison of Biochemical Properties of Pea Leaf Mitochondrial and Chloroplastic Dihydrolipoamide Dehydrogenases

The possibility that the chloroplastic dihydrolipoamide dehydrogenase was either a proteolytic fragment or derived from the same gene as the mitochondrial isoform via gene-splicing was investigated. N-terminal sequencing was performed on both the mitochondrial and the chloroplastic dihydrolipoamide dehydrogenase isolated from pea leaves. This revealed a sequence for the chloroplastic enzyme which differed significantly from the mitochondrial N-terminal sequence. Moreover, the chloroplastic sequence did not correspond to any part of the internal protein sequence of the mitochondrial E3 as predicted from the cDNA sequence obtained by Bourguignon *et al.* (1992) and Turner *et al.* (1992).

Further biochemical analysis determined the precise subunit molecular mass using electrospray mass spectrometry where it was found that the chloroplastic enzyme possessed a molecular mass which agreed closely with the apparent molecular mass determined by SDS-PAGE. The mitochondrial E3, however, had a precise molecular mass which was considerably lower than the apparent molecular mass determined by SDS-PAGE, making this enzyme the smaller of the two isoforms. The molecular

basis for the anomalous behaviour of mitochondrial dihydrolipoamide dehydrogenase is not understood but clearly it is a property not shared by the chloroplastic enzyme. Both this and the N-terminal sequencing data discount the possibility that the chloroplastic enzyme is a proteolytic fragment of the mitochondrial E3.

A common feature of the chloroplastic and mitochondrial dihydrolipoamide dehydrogenases was that they were both dimers as determined by gel filtration and electrospray mass spectrometry. This is a property shared by all members of the flavoprotein family of pyridine disulphide oxidoreductases studied to date.

9.3 Kinetic Analysis of Mitochondrial and Chloroplastic Dihydrolipoamide Dehydrogenases

Basic kinetic analysis was performed to determine whether the pea leaf chloroplastic and mitochondrial dihydrolipoamide dehydrogenases differed in their biochemical properties. Significant differences in the K_m for NAD^+ and DHL and in the K_i for NADH were observed, reflecting their differences in organellar origin. The chloroplastic dihydrolipoamide dehydrogenase was found to possess a 5-fold lower K_m for NAD^+ and 3-fold lower K_i for NADH, reflecting the lower NAD^+ and NADH levels found in the chloroplastic environment. This is in agreement with the Michaelis constants found for pea chloroplastic PDC, where the K_m for NAD^+ is around half that of mitochondrial PDC (Camp and Randall, 1985). A difference in the K_m for DHL was also found while the specific activity for the chloroplastic enzyme was, again, found to be significantly lower than the mitochondrial enzyme.

Treatment of the chloroplastic and mitochondrial E3s with p-aminophenyl-dichloroarsene oxide (APA) not only confirmed that both the mitochondrial and chloroplastic isoenzymes utilise a redox active thiol disulphide group at their active

sites, but also displayed a difference in the time taken for the enzymes to become totally inactivated by APA. The inactivation time for the chloroplastic E3 was 3-fold longer than the mitochondrial enzyme, revealing a possible difference in accessibility and reactivity of the active site to APA.

9.4 Structure and Stability of Pea Mitochondrial and Chloroplastic Dihydrolipoamide Dehydrogenases

The stability of dihydrolipoamide dehydrogenase from many sources to denaturation by heat has previously been documented (Van Muiswinkel-Voetberg *et al.*, 1973; Van Berkel *et al.*, 1991). To determine whether any difference between the chloroplastic and mitochondrial enzymes existed, for further evidence of their differing origins, inactivation of activity by both chemical agents and heat was performed on these enzymes. Major differences using guanidine hydrochloride and sodium chloride were found, with the chloroplastic E3 proving more resistant to loss of activity. The possibility that it was caused by differences in subunit interaction, that is, the mitochondrial enzyme was dissociating into its component subunits at lower concentrations than the chloroplastic dihydrolipoamide dehydrogenase was not supported by gel filtration analysis in the presence of guanidine hydrochloride.

A smaller but still statistically significant difference in the resistance to loss of activity at high temperatures was found, with the chloroplastic dihydrolipoamide dehydrogenase again more resistant to thermal denaturation.

The low specific activity of the chloroplastic E3 and increased resistance to heat suggests the presence of a more rigid, stable structure by analogy with the general organisation of heat-stable enzymes from a variety of thermophilic bacteria, where a

network of salt bridges provides a compact, rigid structure which is resistant to denaturation but is also incompatible with high turnover rates.

9.5 Chromatographic and Immunological Detection of Mitochondrial and Chloroplastic Dihydrolipoamide Dehydrogenases from Other Plant Species

Once evidence for the individuality of plastidic dihydrolipoamide dehydrogenase, compared with the mitochondrial isoform, had been obtained, the possibility that the enzyme may be present in other plants was investigated using the chromatographic procedure initially used to isolate the pea chloroplastic enzyme, and employing the polyclonal antisera raised to both the pea mitochondrial and pea chloroplastic dihydrolipoamide dehydrogenases as specific probes. This revealed the presence of polypeptides in barley, spinach and oil seed rape, at approximately the same molecular mass as the pea chloroplastic enzyme, all of which reacted exclusively with the anti-pea chloroplastic E3 sera. The presence of mitochondrial E3, as expected, could also be observed with the use of the anti-pea mitochondrial E3 antisera. Moreover, there was no cross-reaction between the polypeptides highlighted by the appropriate organelle-specific anti-pea E3 antibodies. Thus, both these antisera appear to be highly specific for their respective antigens, although previous studies had identified the possible presence of both chloroplastic and mitochondrial E3s, using antisera raised to the mammalian mitochondrial E3, in pea leaves (Taylor *et al.*, 1992) and to broccoli mitochondrial PDC (Camp and Randall, 1985).

Previous immunological studies on related families of proteins from different species e.g. lysozyme or haemoglobins have shown that 50-60% sequence identity at the amino acid level is required before a significant degree of cross-reactivity is observed, indicating that the degree of similarity between the chloroplastic and mitochondrial

isoenzymes might be less than 50% (Prager and Wilson, 1971). This is consistent with limited N-terminal amino acid sequence data where there is only 10-20% identity over the first 22 amino acids.

Unlike the relative levels of mitochondrial and chloroplastic PDC and E3 found in pea leaves, the levels of dihydrolipoamide dehydrogenase from barley and spinach leaves were found to be markedly different from those of PDC found in the same tissue, as reported by Lernmark and Gardestrom (1994). In this study, of the total PDC activity in barley and spinach leaves, 15% of activity was mitochondrial, while the remaining 85% was chloroplastic. These discrepancies may be due to difficulties in purifying the intact complexes, where the mitochondrial complex may be more susceptible to proteolysis, dissociation of the mitochondrial complexes in particular or mitochondrial PDC present in an inactive state due to phosphorylation, compared to the chloroplastic PDC.

9.6 Attempted Isolation of the Gene Encoding the Chloroplastic Dihydrolipoamide Dehydrogenase from Pea Leaves

The isolation of the cDNA sequence corresponding to the chloroplastic dihydrolipoamide dehydrogenase would represent the first full sequence of a probable component of the plastidic PDC. This was attempted but proved to be unsuccessful with the selected procedure. A sequence was continually obtained which, outside of the areas corresponding to the primers used, bore only limited resemblance to the expected sequence. Possible reasons for this were that the primers were binding to and amplifying pseudogenes or related members of the pyridine disulphide oxidoreductase family. Non-specific binding may be ruled out since the PCR product obtained was a single strong band of very similar size to that expected. Owing to time

constraints, it was not possible to investigate these discrepancies further. However, the availability of the isoform-specific antisera presents new opportunities for an immunological screening approach to the isolation of the corresponding cDNA.

9.7 Future Research

The data presented in this thesis represents the first isolation and characterisation of a probable component of the PDC from chloroplasts. However, despite the now ample immunological and kinetic evidence for this complex reported elsewhere, no evidence has been presented here that conclusively proves that the dihydrolipoamide dehydrogenase is a component of this complex. Recent access to the chloroplastic E2 clone should permit overexpression of this distinct dihydrolipoamide acetyltransferase in *E. coli* or other heterologous hosts, enabling analysis of any specific physical and functional association with the chloroplastic E3 when compared to the mitochondrial E3, thereby providing definitive evidence for its role as an integral component of plastidic PDC (Soll and Lindsay, unpublished observations).

The role for this complex is in providing acetyl CoA and NADH for fatty acid synthesis, but the question of whether PDC is the major regulatory step in controlling flux into the acetyl CoA remains to be answered, especially as other pathways are available for producing acetyl CoA, e.g. acetyl-CoA synthetase. As the components of the chloroplast-specific complex are most likely encoded by separate genes, the isolation of a cDNA clone that encodes the E3 component or any of the other constituent enzymes would be very useful in elucidating the role of plastidic PDC in plant cellular metabolism.

The E2 and E3 cDNA clones could also be useful for probing libraries of other plants and organisms in order to identify the presence of a counterpart in other species.

Using antisense technology, it is intended to produce transgenic plants, e.g. oil seed rape, in conjunction with Dr Steve Rawsthorne, John Innes Centre. These plants would have the rate of production of plastidic PDC selectively lowered by preventing the production of the E2 and/or E3 component, which, like the mitochondrial counterpart, are assumed to be essential to the functioning of the complex as a whole. This, in turn, could lead to transgenic plants that are able to produce fatty acids at elevated levels by either inserting multiple copies of the genes encoding the chloroplastic PDC component enzymes, or by selective mutation of the genes to produce an unregulated complex with a higher product turnover. Indeed, the plastidic complex is likely to be expressed at basal levels in all plant tissues which have an ability to promote lipid biosynthesis and may be involved in other hitherto unrecognised metabolic functions.

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