BIOCHEMICAL CHARACTERISATION OF DIHYDROLIPOAMIDE DEHYDROGENASE FROM POTATO

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For Mum and Dad

"The natural scientists of the previous age knew less than we do, and believed that they were very close to the goal: we have taken very great steps in its direction and now discover we are still very far away from it."...Georg Christoph Lichtenberg

ABSTRACT

Dihydrolipoamide dehydrogenase (E3), a member of the group of flavin-containing pyridine nucleotide-disulphide oxidoreductases, exists as a homodimer containing 1 flavin adenine dinucleotide (FAD) per subunit and a redox-active disulphide. Dihydrolipoamide dehydrogenase is also an integral component of the 2-oxoacid dehydrogenase complexes and the glycine decarboxylase complex, and is generally believed to be the identical gene product in these multienzyme complexes to those from mammalian sources.

However, there is increasing evidence for the existence of isoforms of E3 from mammalian and bacterial sources. Preliminary immunological analyses of chloroplasts and mitochondria from pea provided the first evidence for the existence of organelle specific isoforms of E3 in plants.

A novel affinity column developed specifically for the one-step purification of E3 from plant sources was successfully used in the purification of E3 from potato tuber mitochondria. Neither pea chloroplastic or mitochondrial E3 would bind effectively to the affinity matrix, but, however, their differing affinities for the column matrix did provide supplementary evidence for the existence of organelle specific forms of E3 from pea. Both potato and pea E3 activities also varied in their sensitivity to salt further confirming the presence of E3 organelle specific isoforms being present in plants.

The affinity purified potato tuber mitochondrial E3 was observed to be composed of two distinct polypeptides α and β with M_r values of 58,000 and 56,000 respectively, which both cross-reacted strongly with anti-E3 IgG raised to the porcine heart enzyme. Further analysis of this E3 involving the use of anion exchange chromatography revealed the presence of three discrete peaks of E3 activity. On SDS-PAGE peak 1 was observed to contain a single band with an M_r value of 58,000, peak 3 a band with an M_r of 56,000, and peak 2 by a combination of the two. All three peaks were observed to cross-react strongly with E3 antiserum, and peaks 1 and 3 to have identical N-terminal sequences. M_r analysis of native E3 preparations on gel

filtration were consistent with the presence of a dimeric organisation for these enzymes. Thus, peak 1 and peak 3 appear to be α_2 and β_2 homodimers respectively, and peak 2 an $\alpha\beta$ heterodimer.

All three isoforms behave as conventional dihydrolipoamide dehydrogenases containing a redox-active disulphide at their active sites, with basic kinetic characterisation providing evidence that they vary in their enzymatic properties, lending weight to the possible conclusion that they interact selectively or exclusively with individual mitochondrial complexes. The three isoforms were also observed to have differing affinities for the inner mitochondrial membrane, providing indirect evidence that the three isoforms appear to be selective. Tissue specific expression of the three isoforms was also observed, with the α_2 isoform observed to be the predominant E3 isoform in potato leaves.

Reconstitution experiments were performed employing bovine heart PDC and OGDC due to the inherent problems involved in the purification of intact multienzyme complexes from plants, which ideally would have been more suitable for this purpose. All three potato E3 isoforms were observed to recognise and bind to the E2/X-PDC and E1/E2-OGDC subcomplexes but with lower affinity than their mammalian counterparts. The three potato isoforms were found to reconstitute approx. 6% of overall PDC activity compared to 35% of reconstituted activity by parent bovine heart E3, but they failed to reconstitute OGDC activity. No direct comparisons could be made between the three isoforms due to the low levels of PDC reconstitution achieved by the plant enzymes.

It is thought that the three peaks of E3 activity present in potato mitochondria may represent complex specific isoforms which may function preferentially or exclusively with one of the three mutienzyme complexes located in plant mitochondria (PDC, OGDC and GDC). To date, it is unknown whether this is a unique situation that occurs only in potato.

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ABBREVIATIONS

In addition to the abbreviations recommended (Instrucions to authors, *Biochem. J.* (1992), **281**, 1-19), the following were adopted throughout.

approx.	approximately
BCDC	branched chain 2-oxoacid dehydrogenase complex
BCOADC	branched chain 2-oxoacid dehydrogenase complex
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
cm	centimetres
Da	Daltons
DTT	dithiothreitol
E3	lipoamide dehydrogenase
EDTA	ethylenediaminetetra-aceticacid
EGTA	ethylene glycol-bis(β-aminoethyl ether)
GDC	glycine decarboxylase complex
h	hour
MES	2-(N-morpholino]ethane-sulphonic acid
mg	milligram
min	minute
ml	millilitre
mM	millimolar
MOPS	3-[N-Morpholino] propane-sulphonic acid
M_r	relative molecular mass
MSUD	maple syrup urine disease
NAD^+	nicotinamide adenosine dinucleotide
NADH	nicotinamide adenosine dinucleotide hydride
$NADP^+$	nicotinamide adenosine dinucleotide phosphate

nkat	nanokatal
OGDC	2-oxoglutarate dehydrogenase complex
PAGE	polyacrylamide gel electrophoresis
PBC	primary biliary cirrhosis
PDC	pyruvate dehydrogenase complex
PEG	polyethylene glycol
PMSF	phenylmethylsulphonylfluoride
PVDF	polyvinylidene difluoride
PVP	polyvinylpyrrolidone
SDS	sodium dodecyl sulphate
TCA cycle	tricarboxylic acid cycle
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TES	(N-tris[hydroxymethyl]-methyl-2-aminoethane-sulphonic acid)
TPP	thiamine pyrophosphate
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
Tween 20	polyoxyethylenesorbitan monolaureate
TX-100	Triton X-100
v/v	volume to volume
w/v	weight to volume
X	protein X
μM	micromolar

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 5 : KINETIC CHARACTERISATION OF DIHYDROLIPOAMIDE DEHYDROGENASE FROM POTATO

CHAPTER 6 : RECONSTITUTION STUDIES ON PYRUVATE DEHYDROGENASE COMPLEX AND 2-OXOGLUTARATE DEHYDROGENASE COMPLEX

LIST OF TABLES

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). All members of this group studied to date are homodimers containing one flavin adenine dinucleotide (FAD) per subunit and a redox active disulphide. In eukaryotes and eubacteria, E3 is an integral component of the 2-oxoacid dehydrogenase complexes and the related glycine decarboxylase complex (Motokawa & Kikuchi, 1974). In these multienzyme complexes, the E3 component is responsible for the reoxidation of the lipoamide group and transfers the reducing equivalents onto NAD via its FAD cofactor. Much of the information accumulated on E3 at present has been derived from analyses of these multienzyme complexes from non-plant sources where they are found in high abundance. However, glycine decarboxylase complex (GDC) has been studied fairly extensively from plant sources where it constitutes approx. 30% of the leaf mitochondrial proteins. GDC will be discussed in greater detail at the end of this chapter.

1.2 THE 2-OXOACID DEHYDROGENASE COMPLEXES

The family of 2-oxoacid dehydrogenases comprises three related multienzyme complexes; the pyruvate dehydrogenase complex (PDC), the 2-oxoglutarate dehydrogenase complex (OGDC) and the branched-chain 2-oxoacid dehydrogenase complex (BCDC). These high M_r assemblies (2.5-9.0 x 10^6) have been purified from many diverse organisms and are self assembling (Linn *et al.*, 1972). In eukaryotes these complexes are located in the mitochondrion and are probably loosely associated with the inner face of the inner membrane.

These multienzyme assemblies occupy key positions in intermediary metabolism and catalyse the irreversible oxidative decarboxylation of 2-oxoacids by the coordinated action of multiple copies of three separate enzymes termed E1, E2 and E3 (Fig. 1.1).

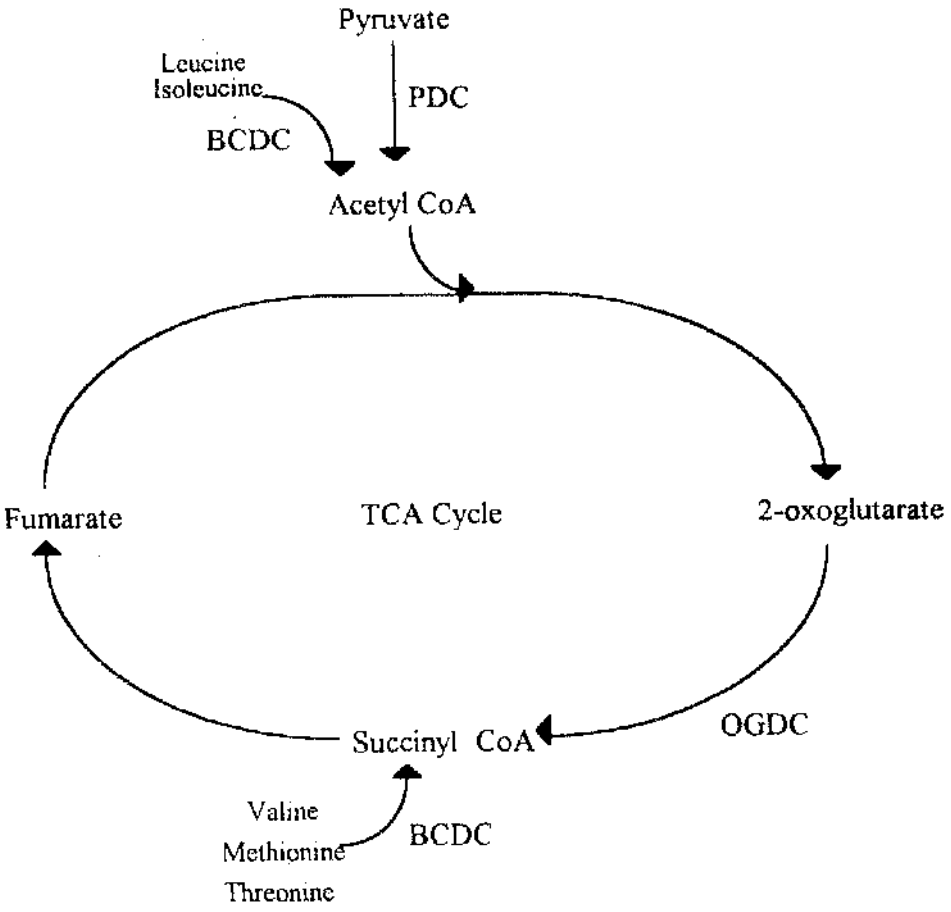


Figure 1.1: Partial representation of the tricarboxylic acid cycle illustrating the positions of the 2-oxoacid dehydrogenase complexes in central metabolism.

E1 is a 2-oxoacid dehydrogenase which requires thiamine pyrophosphate (TPP) as a catalytic cofactor. E2 is a dihydrolipoamide acyltransferase with a covalently attached lipoic acid prosthetic group and E3 is 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). PDC is responsible for the oxidative decarboxylation of pyruvate to acetyl CoA and NADH with the release of CO_2, an irreversible step linking glycolysis with the tricarboxylic acid cycle (TCA). PDC therefore regulates the entry of two carbon units (as acetyl CoA) into the TCA cycle where it is either oxidised to generate energy, or alternatively used as a precursor for various biosynthetic pathways. OGDC is a regulatory enzyme of the TCA cycle itself where it controls carbon flux in the latter stages of the cycle. OGDC converts 2-oxoglutarate to succinyl CoA which in turn is involved in the production of porphyrins, and amino acids in mammals. BCDC catalyses the second step in the catabolism of valine, leucine and isoleucine. It oxidatively decarboxylates the 2-oxoacids produced by the transamination of these compounds. BCDC is also capable of metabolising 2 oxobutyrate, an intermediate formed during the breakdown of threonine and methionine (Yeaman, 1986).

Deficiencies in the activities of these multienzyme complexes can cause various forms of metabolic acidosis, for example, 'Maple Syrup Urine Disease' (MSUD), caused by a deficiency in BCDC (Chuang & Nin 1981). This is an autosomal recessive inborn error of metabolism involving a dysfunction of the E1 and E2 subunits of BCDC. This condition 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 Billiary 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 PBS 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). Approx. 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 and Bradford *et al.*, 1987). Deficiencies in OGDC activity have also been recently implicated in Alzheimers disease. Recent studies of Alzheimer patients revealed both defective OGDC activity and abnormality of the E2 component, whereas all other mitochondrial proteins were normal including PDC.

1.2.1 STRUCTURE AND SUBUNIT COMPOSITION OF THE 2-OXOACID DEHYDROGENASE COMPLEXES

As mentioned earlier the 2-oxoacid dehydrogenase complexes are large multimeric enzyme assemblies with M_r values ranging between 2.5-9.0 x 10^6 (Linn *et al.*, 1972) and are composed of three separate enzyme components termed E1, E2 and E3. The E1 component is complex specific and is a pyruvate dehydrogenase (EC 1.2.4.1) in PDC, a 2-oxoglutarate dehydrogenase (EC 1.2.4.2) in OGDC and in BCDC (EC 1.2.4,4) the equivalent enzyme is capable of interacting with leucine, isoleucine and valine and also methionine and threonine. Likewise E2 is also specific for its complex. E2 from PDC is an acetyltransferase (EC 2.3.1.12) whereas it functions as a succinyltranferase (EC 2.3.1.61) in OGDC and an acyltransferase in BCDC (no EC number) where it has a broad substrate specificity and is capable of transferring a range of acyl groups. In contrast, E3 is a dihydrolipoamide dehydrogenase (EC 1.8.1.4), which is believed to be common to all of the 2-oxoacid dehydrogenase complexes and is also highly conserved across species.

In general the structure of these complexes is such that a number of E2 polypeptides form a symmetrical core around which are arranged multiple copies of E1 and E3 enzymes which bind non-covalently (Reed, 1974). The exact arrangement of

the E2 polypeptides is complex and species specific with the existence of two distinct forms of E2 core, the cube and pentagonal dodecahedron. OGDC and BCDC have 24 identical subunits of E2 arranged with octahedral (432) symmetry as does PDC from Gram negative bacteria such as *E. coli* and *A. vinelandii* (Oliver & Reed, 1982; Perham *et al.*, 1987). In contrast, the pentagonal dodecahdron is composed of 60 E2 subunits arranged with icosahedral (532) symmetry and is associated with PDC from Gram positive sources such as *B. subtilis*, yeast such as *S. cerevisiae* and also from mammalian, avian and fungal sources (Lowe *et al.*, 1983; Keha *et al.*, 1982; for review see Perham, 1991). Therefore there are two structural types of PDC, one associated with Gram negative bacteria and the other with Gram positive bacteria and mammalian species. The former, like that found in *E. coli* (Fig. 1.2), has octahedral symmetry where 12 E1 dimers bind along the twelve edges of the E2 core and 6 E3 dimers bind along the six faces of the cube. The second structural type of PDC is that which can be located in mammalian mitochondria. Here 60 subunits of E2 form an icosahedral core, 20-30 E1 tetramers ($\alpha_2\beta_2$) bind to the thirty edges and 6 E3 dimers to the twelve faces of the pentagonal dodecahedron (Reed *et al.*, 1975; Barerra *et al.*, 1972). In mammals, both PDC and BCDC E1 components are composed of two non-identical subunits (termed α and β) whereas the E1 component of OGDC is a homodimer (Koike & Koike, 1976).

The situation with mammalian PDC is more complicated since it also contains an additional component, termed protein or component X, with a subunit M_r of approx. 50,000 (De Marcucci & Lindsay, 1985). This protein copurifies with and is tightly bound to E2 and was thought to be a proteolytic fragment of E2 for many years. Through extensive immunological, peptide mapping and proteolytic digestion studies, it has since been identified as a distinct polypeptide in its own right (De Marcucci *et al.*, 1986). Both protein X and the E2 component of PDC from *S. cerevisiae* have been cloned and sequenced revealing that these two enzymes are separate gene products with protein X exhibiting close homology (approx. 45-50%) with E2 over its N-terminal region (Niu *et al.*, 1988; Behal *et al.*, 1989). Protein X contains one lipoyl domain at

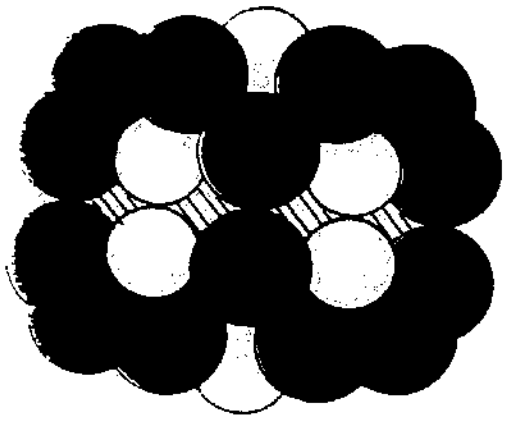


Figure. 1.2: Model of the native *Escherichiacoli* PDC. Figure is reproduced from Stryer (3rd Edition, p381). The periperal subunits (●) and E3 (O) are non-covalently bound to the central multimeric E2 core (⊗).

its N-terminus which participates in the acetylation reactions of the complex (Hodgson *et al.*, 1986; Neagle *et al.*, 1989; Rahmatullah *et al.*, 1989), but is not required for full catalytic activity of the complex. A truncated 35,000 M_r fragment of protein X from which the lipoyl domain has been removed is sufficient to sustain the activity of the complex but reduces its affinity for E3 (Lawson *et al.*, 1991; Neagle & Lindsay, 1991). Current evidence suggests that protein X plays primarily a structural role in the binding of E3 to the E2 core assembly. It was demonstrated initially that the cleavage of protein X by protease arg C was protected by the presence of exogenously added E3 (Gopalakrishman *et al.*, 1989). Additional evidence implicating protein X in the binding of E3 to the E2 core assembly of PDC was provided by Powers-Greenwood *et al.*, (1989). Protein X was removed from the isolated E2/X subcomplex of PDC and the purified E2 enzyme used in reconstitution experiments with E1 and E3. The results showed that the E2 enzyme in the absence of protein X was incapable of maintaining the overall reaction sequence and exhibited reduced affinity for binding the E3 enzyme. In addition to this, a putative E3 binding domain has been located on protein X of *S. cerevisiae* (Lawson *et al.*, 1991). However, since E2 also contains putative E3 binding domains, it is still unclear why PDC has evolved a specific polypeptide, protein X, required for high-affinity binding of E3 to the E2 core assembly. To date, OGDC and BCDC have not been found to contain protein X . It has been reported, however, that sequences related to protein X are located in the N-terminal region of the 2-oxoglutarate dehydrogenase (E1) of OGDC isolated from bovine heart (Rice *et al.*, 1992).

1.2.2 REACTION CATALYSED BY THE 2-OXOACID DEHYDROGENASE COMPLEXES

The decarboxylation and dehydrogenation of 2-oxoacids is catalysed by the 2-oxoacid dehydrogenase complexes in a sequential and coordinated multistep process involving the activities of multiple copies of three separate enzymes to generate CO_2 and the corresponding acyl CoA (Reed, 1974; Yeaman, 1989) (Fig. 1.3). The overall

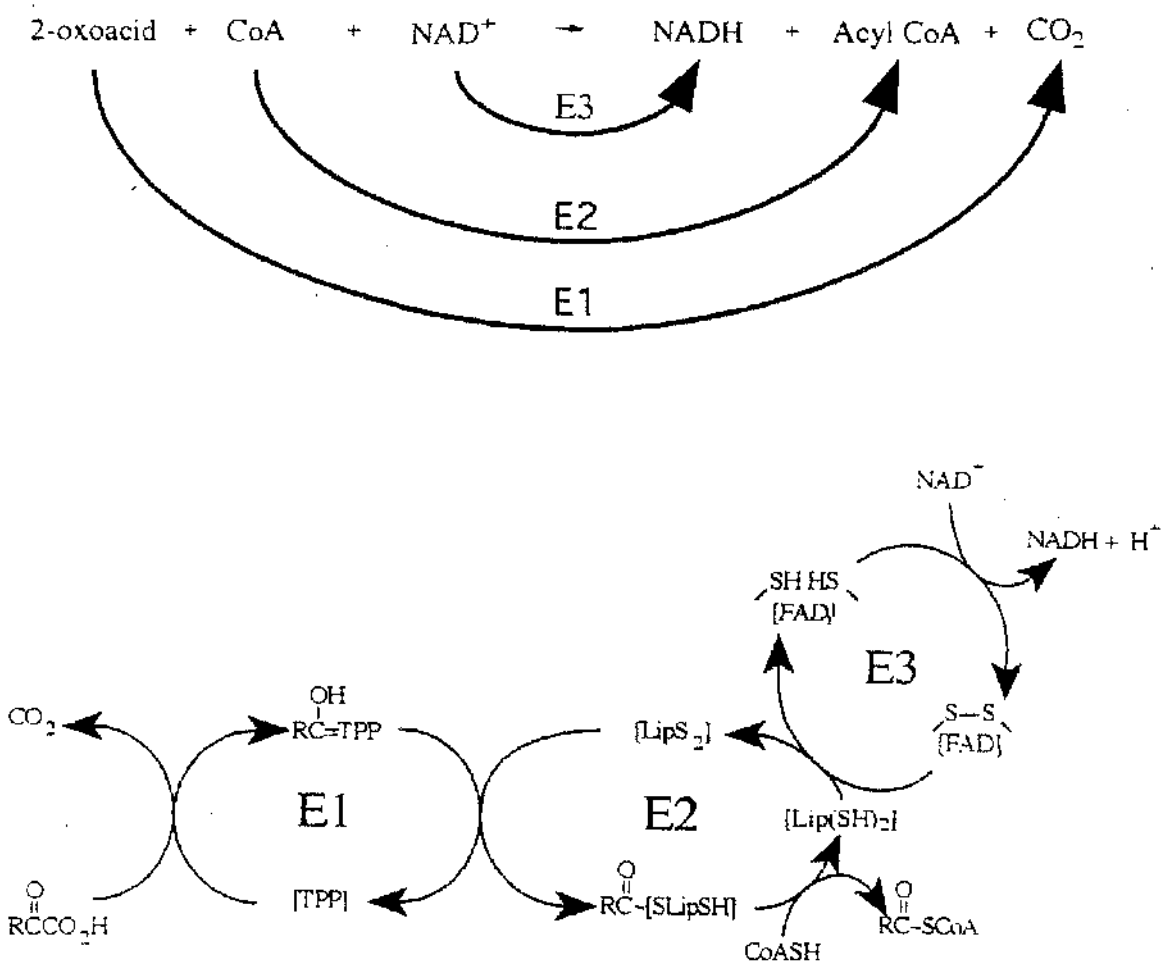


Figure 1.3: Schematic representation of the reaction mechanism of the 2-oxoacid dehydrogenase complexes highlighting the specific reactions catalysed by the component enzymes.

reaction catalysed by the 2-oxoacid dehydrogenase complexes can be summarised as follows:

$$RCO\text{-}COOH + CoASH + NAD^+ \quad RCOSCoA + CO_2 + NADH + H^+$$

The substate-specific E1 enzyme (2-oxoacid dehydrogenase), which requires thiamine pyrophosphate (TPP) as an essential cofactor, catalyses the decarboxylation of the appropiate 2-oxoacid with the formation of an 2-(1-hydroxyethylidene)-TPP intermediate and a molecule of CO_2. E1 is also responsible for the reductive acylation of the lipoic acid moiety which is covalently attached to E2 (dihydrolipoamide acyltransferase) via a specific lysine residue (Bleile *et al.*, 1979). E2 transfers the acyl group to the CoA acceptor leaving the the dithiolane ring of the lipoic acid in the reduced state. The lipoamide group is reoxidised by E3 (dihydrolipoamide dehydrogenase) transferring the reducing equivalents onto NAD^+ via its FAD cofactor.

1.3 CONSTITUENT ENZYMES OF THE 2-OXOACID DEHYDROGENASE COMPLEXES

1.3.1 2-OXOACID DEHYDROGENASE (E1)

The 2-oxoacid dehydrogenase (E1) component catalyses the only irreversible reaction in the multistep activity of the 2-oxoacid dehydrogenases, the initial decarboxylation of the 2-oxoacid. This is the rate limiting step of the overall reaction (Walsh *et al.*, 1976). This reaction is thought to proceed via a covalent adduct of TPP with the formation of 2-(1-hydroxyethylidene)-TPP and 1 molecule of CO_2 (Reed, 1974). Sequences available for E1 of OGDC and PDC show little homology even when extracted from the same source (Darlison *et al.*, 1984). However, a common motif indicative of a putative TPP binding site has been located on all known E1 sequences as well as other TPP-dependent proteins (Hawkins *et al.*, 1989).

In addition to catalysing decarboxylation of the 2-oxoacid substrate, E1 also catalyses the reductive acylation of the lipoyl acid moiety of E2. This results in the opening of the dithiolane ring generating a free thiol. Acylation occurs initially at the S^8 position of lipoic acid with the formation of an S^8-acetyldihydrolipoamide intermediate. The interchange of the acyl group between the two sulphurs of lipoamide is known to occur in *E. coli* (Yang & Frey, 1986). The isomerisation constant is 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.

The E1 components of bacterial and mammalian OGDC and Gram negative bacterial PDC are homodimers with M_r of approx. 100,000 (Koike & Koike, 1976). In contrast, the E1 components of eukaryotic and Gram positive bacterial PDC and BCDC are composed of two non identical subunits termed α and β which aggregate to form $\alpha_2\beta_2$ tetramers (Reed *et al.*, 1985). PDC E1α and E1β subunit M_r values are approx. 41,000 and 35,000 respectively (Matuda *et al.*, 1983) while the equivalent values for BCDC are 46,000 and 38,000 respectively (Pettit *et al.*, 1978). The location of the postulated TPP-binding site motif on the α-subunit of E1 suggests that E1α catalyses the decarboxylation reaction and E1β the reductive acylation of the lipoic acid moiety of E2 (Roche & Reed, 1972; Perham, 1991).

1.3.2 DIHYDROLIPOAMIDE ACETYLTRANSFERASE (E2)

The dihydrolipoamide acetyltransferase (E2) component of the 2-oxoacid dehyrogenase complexes not only forms a structural core to which E1 and E3 bind, coupling their activities by providing a flexible lipoyl group and domain which can visit the various active sites, but is also responsible for catalysing the acyltransferase reaction. All E2 enzymes are highly segmented structures consisting of several functional domains (Bleile *et al.*, 1979; Reed & Hackert, 1990; Perham 1991) (Fig. 1.4). The N-terminal region of the E2 polypeptide contains 1-3 lipoyl domains which carry the lipoyl groups, with each domain composed of approx. 80 amino acids (Dardel

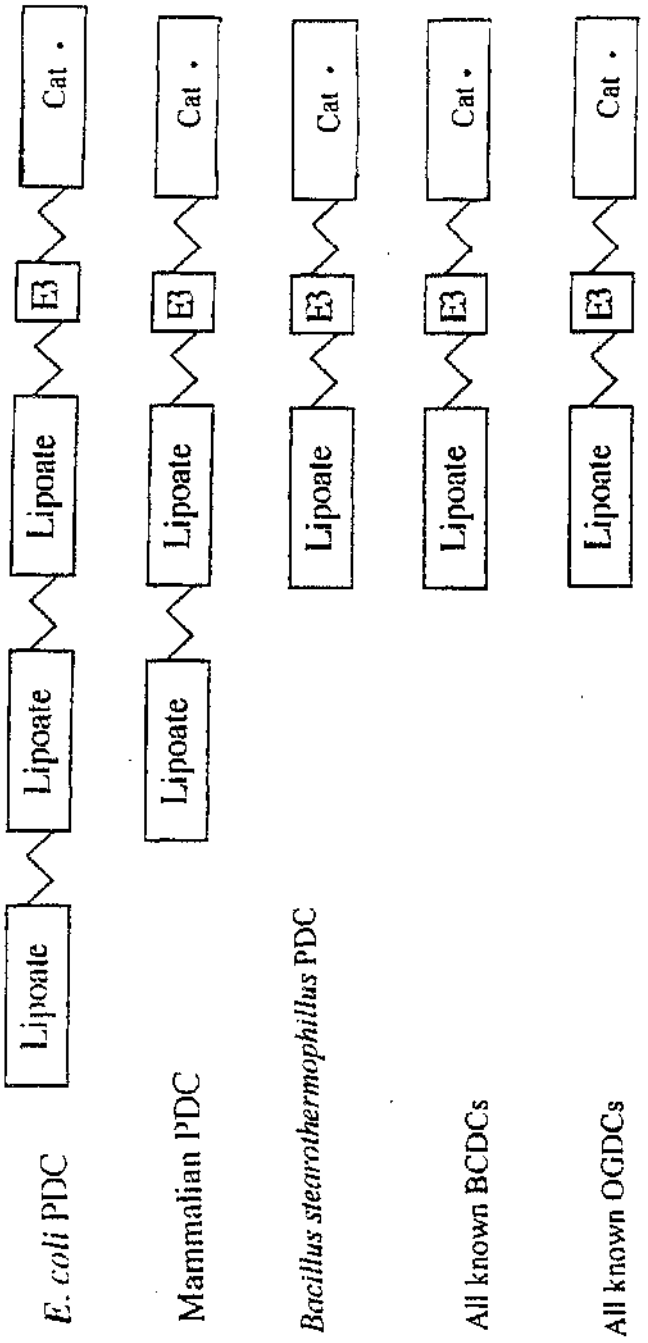


Figure 1.4: Schematic representation of the E2 component of *Escherichia coli* mammalian and *Bacillus stearothermophillus* PDC and all known OGDCs and BCDCs. The individual domains; inner catalytic domain (Cat), the E3 binding domain (E3) and the lipoic acid containing domain (Lipoate) are connected via linker regions (∿). The putative active site is denoted with a (⋆)

et al., 1991). This extended outer domain contains the lysine groups to which the lipoyl acid cofactors are covalently attached via an amide linkage. The number of lipoyl domains present are dependent on the source of the E2 enzyme. However, there seems to be no simple correlation between the number of lipoyl domains per E2 polypeptide, the symmetry of the E2 core and the source of the E2 core (Perham & Packman, 1989; Allen & Perham, 1991). These domains are linked together by conformationally flexible linker regions which facilitate active site coupling and are comprised of short stretches of 25-30 amino acids which are rich in alanine, proline and acidic residues. Adjacent to the lipoyl domain(s), there is a distinct region of polypeptide consisting of approx. 50 amino acids which is responsible for the binding of E3 and/or E1 to the core. This is linked in turn to the C-terminus of the E2 polypeptide which possesses a compact inner domain containing the E2 binding sites allowing the E2 polypeptides to interact with each other, thus forming the core of the complex. In addition, the C-terminus of the E2 polypeptide contains the catalytic site of the acetyl/acyl transferase (Packman *et al.*, 1988).

The lipoyl domains present at the N-terminus of the E2 polypeptide represent the site of acylation within the complex. As indicated previously, the number of lipoyl domains present on a particular E2 polypeptide can vary between the different multienzyme complexes of the family of 2-oxoacid dehydrogenases. Crosslinking experiments were initially used to determine the number of lipoyl domains on an E2 polypeptide. This involved the use of phenyl-o-bismalemide in the presence of the 2-oxoacid substrate resulting in the crosslinking of the E2 subunits via the single substrate-generated thiols of the covalently attached lipoic acid groups. The E2 component from bovine heart OGDC and BCDC produce a single cross-linked dimer indicating the presence of only one lipoyl domain. In contrast, the E2 subunit of mammalian PDC produced a variety of cross-linked products, dimers and trimers with higher M_r aggregates indicating that E2 of PDC is comprised of at least 2 lipoyl domains (Hodgson *et al.*, 1988).

Subsequent cloning and sequencing of the genes encoding the E2 components

from various sources has since allowed for the easy identification of the sequences associated with the presence of lipoyl domains (Russell & Guest, 1991). The question is raised as to why some of the E2 polypeptides have three lipoyl domains (PDC of *E. coli* & *A. vinelandii*), others two (mammalian PDC) and some like that of BCDC, OGDC and yeast PDC have only one lipoyl domain (for review see Perham, 1991). It appears that only one lipoyl domain is sufficient for complex activity since deletion of two of the three domains of *E. coli* E2 by site-directed mutagenesis does not affect its assembly or catalytic activity (Guest *et al.*, 1985). The reasoning behind this apparent surplus of lipoyl domains remains somewhat obscure. It is thought possible that these extra lipoyl domains come into play when substrate or cofactor concentrations are limiting. Mutants of *E. coli* have been produced containing 0 to 9 lipoyl domains per E2 chain of PDC. The complexes containing 1 to 3 lipoyl domains exhibited optimal PDC activity and linker mobility which decreased in the presence of more than three lipoyl domains (Machado *et al.*, 1992, 1993).

The crystallisation of the intact E2 subunit has been hindered owing to its highly segmented structure with its mobile lipoyl domains which prevents the growth of well-ordered crystals (De Rosier *et al.*, 1971; Fuller *et al.*, 1979). However, the structures of the individual domains and of the linkers that join them have now been determined. This is essential for a full understanding of the complex and its mechanism of active-site coupling. Large amounts of the individual domains were produced by means of genetic engineering or direct chemical synthesis and their structures solved by NMR techniques (Dardel *et al.*, 1991; Robien *et al.*, 1992; Schulze *et al.*, 1991). Additionally, the structure of the catalytic domain of E2 from *Azotobacter vinelandii* PDC has been determined by X-ray crystallography and has shown a similarity to chloramphenicol acetyltransferase (CAT) (Mattevi *et al.*, 1992). 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

resemblence between the mechanisms of E2 and CAT (Guest, 1987).

The role of the N-terminal lipoyl domains is that of "transporting" the cofactor lipoamide through the various active centres. In addition the lipoyl group must be attached to a specific lipoyl domain to promote reductive acylation of the dithiolane ring by the cognate E1 component, indicating that the decarboxylase subunit binds and recognises the domain itself and not only the lipoyl group (Graham *et al.*, 1988). The E2 domains of the 2-oxoacid dehydrogenase complexes form a flexible 14 A 'swinging arm' which interacts with the three active sites (Koike *et al.*, 1963). This flexibility is promoted by the presence of the linker regions which act to connect the lipoyl domains to each other and to the subunit-binding and catalytic domains. It is this extreme flexibility of the E2 enzyme of the 2-oxoacid dehydrogenases which aids the mechanism of active site coupling. This catalytic mechanism involves the rapid intramolecular transfer of acyl groups between lipoic acid moieties on different E2 components within an E2 core with more than one lipoyl domain able to service any given E1 active site (Hackert *et al.*, 1983). The resulting enhancement of the catalytic activity that accompanies such a mechanism is a feature of the multienzyme complexes where sequential reactions are coupled, thus minimising the occurrence of side reactions.

1.3.3 DIHYDROLIPOAMIDE DEHYDROGENASE (E3)

As discussed briefly in section 1.1, dihydrolipoamide dehydrogenase (E3) is a member of the group of flavoprotein disulphide oxidoreductases (Carothers *et al.*, 1989). It is responsible for the NAD^+ dependent re-oxidation of the dihydrolipoamide groups which are bound to lysine residues present on the acyltransferase component (E2) of the family of 2-oxoacid dehydrogenase complexes, or the hydrogen carrier protein (H protein) of the glycine decarboxylase complex (GDC), thus allowing the acyltransferase component to re-enter the catalytic cycle (Kikuchi & Hiraga, 1982; Carothers *et al.*, 1989; Yeaman, 1989). All E3 enzymes to date are reported to exist as

homodimers with a M_r of approx. 2 x 50,000 with each subunit containing one molecule of non-covalently bound FAD. The structure of E3 comprises four distinct domains; a FAD binding domain, a NAD binding domain, a central domain and an interface domain with the FAD and NAD binding domains having similar topology which suggests that they may have evolved from the duplication of a common ancestor. During the reaction catalysed by E3, electrons are transferred from lipoamide first via the disulphide/dithiol and then via its cofactor FAD to NAD^+ as the final electron acceptor (Ghisla & Massey, 1989).

E3 has been isolated from a wide variety of sources and is generally believed to be the identical gene product in all members of the 2-oxoacid dehydrogenase complexes as well as the glycine decarboxylase complex (for review see Carothers *et al.*, 1989). However, there is now increasing evidence for the existence of isoforms of E3. Two immunologically distinct E3s have been located in rat liver mitochondria where it is suggested that E3 associated with GDC is different from that associated with the 2-oxoacid dehydrogenase complexes (Carothers *et al.*, 1987). Two species of *Pseudomonas* (*P. putida* and *P. aeruginosa*) are known to express two genetically distinct forms of E3, one specific for the branched chain 2-oxoacid dehydrogenase complex (BCDC) and the other specific for both 2-oxoglutarate dehydrogenase complex (OGDC) as well as GDC and possibly PDC. A possible third E3 gene has also been identified, the function of which is still to be determined (Sokatch *et al.*, 1981, 1983; Sokatch & Burns, 1984; Burns *et al.*, 1989; Palmer *et al.*, 1991). *E. coli* is also reported to have two different genes encoding E3, one associated with the 2-oxoacid dehydrogenase complexes and the other with GDC (Richarme, 1989). The presence of isoforms of E3 is discussed in greater detail in section 4.1.

As mentioned earlier E3 is also present as the L-protein of the glycine decarboxylase complex which catalyses the reversible oxidation of glycine in plants, animal and bacteria (Klein & Sagers, 1966; Motokawa & Kikuchi, 1974; Walker & Oliver, 1986). E3 has also been found to exist as an independent enzyme in its own right, and not associated with the 2-oxoacid dehydrogenase complexes or GDC in the

bloodstream form of the parasitic protozoan *Trypanosoma brucei* (Richarme & Heine, 1986). It is thought that this E3 may be involved in the transport of sugars across the plasma membrane (see section 3.1). The halophilic archaebacteria which also lack the 2-oxoacid dehydrogenase complexes also contain an E3 (Danson *et al.*, 1984). E3 has been purified from *H. halobium* and was observed to be a homodimer with a subunit M_r of 58,000 (Danson *et al.*, 1986). The essential catalytic disulphide bond was identified by chemical modification of the E3 enzyme with the trivalent arsenical, p-aminophenyldichlorarsine. However, no identity was observed with either the *E. coli* or porcine heart E3 sequences despite the similarities in catalytic function (Danson *et al.*, 1986). This may be as a result of the organism's adaptation to high salt conditions.

The cloning and sequencing of the E3 enzymes from a wide variety of sources has provided much information which allows us to make comparisons between them (for review, Carothers *et al.*, 1989). Furthermore, the three dimensional structures of E3 have been determined from several sources including *P. fluorescens* (Mattevi *et al.*, 1992b), *P. putida* (Mattevi *et al.*, 1992a) and yeast (Takanaka *et al.*, 1988). It has been shown that E3 is a highly conserved enzyme across species, for example, the amino acid sequence of human liver E3 exhibits 96% identity with the amino acid sequence of E3 from porcine heart (Otulakowski & Robinson, 1987) and 44% identity with *E. coli* E3 (Pons *et al.*, 1988). E3 also exhibited both structural and functional similarities with several members of the pyridine nucleotide disulphide oxidoreductases, in particular with glutathione reductase (Takanaka *et al.*, 1988). This latter enzyme is responsible for catalysing the NADPH dependent reduction of glutathione disulphide. Therefore E3 and glutathione reductase act in opposite directions by passing reducing equivalents to NAD^+ or from NADPH respectively. Both enzymes contain a redox-active disulphide bridge, which show considerable sequence similarity, that undergoes oxidation-reduction during catalysis causing them to cycle between oxidised and 2 electron reduced forms (Williams *et al.*, 1989). The reaction catalysed by E3 proceeds through a ping-pong mechanism as described in section 5.1.

1.4 REGULATION OF THE 2-OXOACID DEHYDROGENASE COMPLEXES

The 2-oxoacid dehydrogenase complexes occupy key positions in intermediary metabolism and therefore it is extremely important that their activities be precisely regulated. All members of this family from bacteria and mammalian sources are subject to product inhibition. 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 & Koike, 1976; Yeaman, 1986).

A second control mechanism also exists for both mammalian PDC and BCDC which involves reversible phosphorylation of the E1α subunit (Yeaman, 1986, 1989) (Fig. 1.5). The effect of phosphorylation is to cause inactivation of the complex by a dramatic reduction of V_{max}. OGDC is not susceptible to covalent modification and instead its activity is demonstrated to be regulated by the intramitochondrial concentrations of free Ca^{2+} which decreases the K_m for 2-oxoglutarate (McCormack & Denton, 1979; Denton & McCormack, 1985). Moreover, ADP will also activate the complex by altering the affinity of the E1 enzyme for 2-oxoglutarate such that the K_m value is decreased. This effect is antagonised by ATP which will increase the K_m for 2-oxoglutarate (Lawlis & Roche, 1981; Hunter & Lindsay, 1986).

Regulation of mammalian PDC and BCDC by reversible phosphorylation is mediated by specific kinases and phosphatases (Yeaman, 1986, 1989). Inactivation follows the primary phosphorylation of a serine residue on the E1α subunit. There are three sites on the E1α subunit of PDC which can undergo phosphorylation and two sites on the BCDC E1α component (Cook *et al.*, 1983, 1984; Yeaman *et al.*, 1978). However phosphorylation of only one primary site is responsible for inactivation and it is suggested that the phosphorylation of the additional sites is involved in modulating the reactivation of inactive complex (Sugden *et al.*, 1978). The kinases and phosphat-

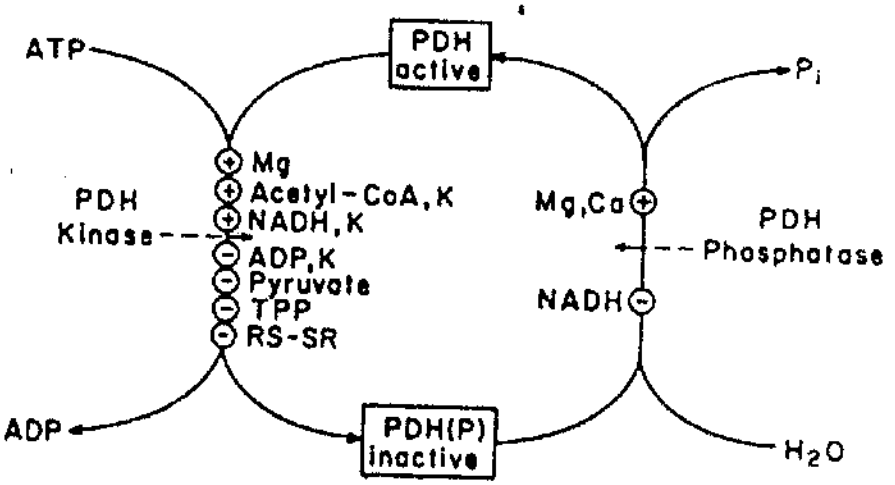


Figure 1.5: Schematic representation of PDC regulation via covalent modification and the control elicited by effector molecules.

ases acting on PDC and BCDC are specific for each complex.

The kinases are tightly bound to and copurify with their respective complexes, whereas the phosphatases are more loosely associated and are purified as distinct soluble enzymes. The kinase is tightly bound to the E2 component in PDC and consists of two non identical subunits termed Ka and Kb with M_r values 48,000 and 45,000 respectively with the former representing the catalytic subunit (Stepp *et al.*, 1983). The genes representing the two kinases have since been cloned, with both clones observed to be very closely related to each other (Popov *et al.*, 1993, 1994). Both subunits exhibit independent kinase activity, although Kb had a lower specific activity than that of Ka. PDC kinase is stimulated by the products of the complex, acetyl CoA and NADH, whereas the substrates CoA and NAD^+ are inhibitory (Pettit *et al.*, 1975). Its activity is also decreased by pyruvate and thiamine pyrophosphate (TPP) which presumably binds directly to the active site of the E1 substrate (Pratt & Roche, 1979). The phosphatase responsible for de-phosphorylation and subsequent activation of the complex is much more loosely associated with PDC. It has a M_r value of approx. 150,000 and consists of a catalytic subunit with a M_r value of 50,000 and a larger subunit of 97,000 which contains a molecule of FAD, the function of which is 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 & Yeaman, 1987). Acute hormonal Ca^{2+}-mediated control has been demonstrated for PDC from heart and liver tissue with adrenaline. However, the activation of PDC is via stimulation of phosphatase not via direct allosteric activation (Denton & McCormack, 1985). It would also seem that Ca^{2+} ions have no affect on the activity of the phosphatase at saturating concentrations of Mg^{2+} ions and that the major effect of Ca^{2+} ions is to lower the K_m for Mg^{2+} ions (Midgley *et al.*, 1987).

In BCDC, phosphorylation of the E1α subunit of the complex by its specific kinase causes inactivation of the complex, BCDC kinase has been purified from rat heart and liver and is a monomeric enzyme with an M_r value of 44,000 (Shimomuru *et*

al., 1990). BCDC kinase, unlike its PDC counterpart, is unaffected by Ca^{2+}, NAD^+/NADH or CoA/acetyl CoA levels (for review, Yeaman, 1989). The major regulatory mechanism influencing activity is inhibition by the 2-oxoacid substrates (Paxton & Harris, 1984; Jones & Yeaman, 1986). The BCDC phosphatase has also been purified with the native enzyme having a M_r value approx. 460,000 (Damuni *et al.*, 1984). The subunit composition has not been fully determined; however a catalytic subunit with a M_r value of 33,000 has been purified to homogeneity (Damuni & Reed, 1987).

1.5 2-OXOACID DEHYDROGENASE COMPLEXES IN PLANTS

Most research to date concerning the 2-oxoacid dehydrogenase complexes has focused on the mammalian and bacterial enzyme complexes 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 plant material (Randall *et al.*, 1990). Intact mitochondrial PDC has been purified to homogeneity only once from a plant source where it took a massive 500kg (half a tonne) of broccoli (*Brassica oleracea*) florets to produce 1mg of pure mitochondrial PDC (Rubin & Randall, 1977). However no SDS-PAGE analysis or definitive subunit composition was provided in this publication. Antisera were subsequently raised to broccoli mitochondrial PDC and used to probe other plant extracts.

The majority of the research has so far focused on PDC of the plant 2-oxoacid dehydrogenase complexes. While there are considerable similarities between the plant and mammalian/bacterial 2-oxoacid dehydrogenase complexes, they also differ significantly in terms of their structural, catalytic and regulatory characteristics. For example, plants are unique in that they possess two distinct, spatially separated types of PDC, one located in the mitochondrial matrix and the other in the plastid stroma. These two forms of PDC exhibit different structural, catalytic and regulatory properties (Miernyk *et al.*, 1985; Randall *et al.*, 1989). The plant mitochondrial PDC, like

mammalian PDC, serves as a primary entry point of carbon into the tricarboxylic acid cycle (TCA). The plastid PDC, however, provides the acetyl CoA and NADH required for fatty acid and isoprenoid biosynthesis (Randall *et al.*, 1989).

1.5.1 PLANT MITOCHONDRIAL PDC

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 & Randall, 1977), castor seed (Rapp *et al.*, 1987), spinach (Rao & Randall, 1980) and potato (Crompton & Laties, 1971). Based upon sedimentation analysis, plant mitochondrial PDCs like their mammalian counterparts are quite large with M_r values of several millions. Most of the plant PDCs have optimal activity at pH 7-8, for example, the *in vitro* activity of the pea leaf mitochondrial complex is optimal at pH 7.6. The susbstrate and cofactor requirements of plant mitochondrial PDCs 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+}. The only substrate, other than pyruvate, utilised by plant mitochondrial PDCs is 2-oxobutyrate, giving 10-20% of the rate with pyruvate (Rubin & Randall, 1977; Miernyk & Randall, 1987). Like mammalian complexes, plant PDCs also display a multisite ping-pong kinetic mechanism as described in section 5.1 (Rubin *et al.*, 1978).

Analysis of pea mitochondrial PDC by SDS-PAGE and Western Blotting was the first attempt to determine the subunit composition of plant mitochondrial PDCs (Camp & Randall, 1985). This was achieved using antibodies raised against mitochondrial PDC purified from broccoli florets (Rubin & Randall, 1977). This revealed subunits of M_r 97,000, 64,400, 58,100, 43,300 and 37,000. Since the 58,100 Da subunit also cross reacted with anti-porcine E3, it was assumed that it represented the E3 component and that the 43,000 and 37, 000 Da species corresponded to the E1α and β subunits respectively. The 64,400 polypeptide exhibits a similar M_r value to the mammalian E2 subunit (Miernyk *et al.*, 1985; Leuthy *et al.*, 1994, 1995). It is possible that the 97,000

Da band may be the E1 subunit of OGDC which is often a contaminant of mammalian PDC preparations.

Further immunological analyses of pea mitochondrial PDC using antibodies to specific components of bovine heart PDC has identified several prominent cross-reacting proteins (Taylor *et al.*, 1992). The pea mitochondrial extract did not show any appreciable cross-reactivity with antiserum to mammalian E1; however, it did cross react strongly with antiserum to yeast E1 highlighting a single band (M_r 41,000) which is thought to be E1α. This also correlates with a 43,000 Da pea mitochondrial protein identified as E1α by Miernyk *et al.*, (1985). Anti-bovine E2 from PDC cross-reacted with a 50,000 Da polypeptide which is lower than that of mammalian E2 (M_r 70,000) and is consistent with the presence of only a single lipoyl domain (Taylor *et al.*, 1992). The pea mitochondrial E3 enzyme was found to have an M_r value of 67,000 which is greater than the analogous mammalian enzyme, M_r value 55,000. In addition component X, not previously detected in plant PDC, was also found to be present with a subunit M_r value of 67,000. It is possible that both the E3 and X component that share the same M_r value of 67,000 could either represent distinct polypeptides, or it may be that component X sequences have become integrated into the E3 gene. A similar situation exists for mammalian OGDC where a component X-like domain is located at the N-terminal region of the E1 subunit (Rice *et al.*, 1992). However, recent immunological and protein chemical studies now indicate that the true M_r value for pea mitochondrial E3 is approx. 56,000-58,000, comparable to the M_r of 58,100 for pea mitochondrial E3 determined by Camp and Randall, (1985) (M. Conner, Glasgow University, personnal communication). It will also be necessary to test for the possible presence of protein X in pea mitochondrial PDC.

The E2 component has recently been cloned and sequenced from PDC of *Arabidopsis thaliana* exhibiting extensive homology with PDC-E2 from human, yeast and *E. coli* and was observed to contain two lipoyl domains like its human counterpart (Guan *et al.*, 1995). However, whether this clone encodes a mitochondrial or chloroplast enzyme was not proved definitively, although the presence of a putative

amino-terminal leader sequence was revealed which possessed the characteristics of a typical mitochondrial matrix targeting presequence.

Recent advances in the study of plant mitochondrial PDC have involved the development of a monoclonal antibody against the E1α subunit of the plant PDC (Leuthy *et al.*, 1995a). The antibody was raised in mice immunised against maize matrix proteins and will recognise the mitochondrial E1α subunit from a variety of plant sources but does not recognise plastid PDC. Subsequent cloning and sequencing of a cDNA encoding the E1α subunit of mitochondrial PDC from *Arabidopsis thaliana* was performed (Leuthy *et al.*, 1995b). This clone was observed to display 47-51% identity and 63-69% sequence similarity with other eukaryotic E1α sequences. A cDNA encoding the mitochondrial E1β subunit was also identified from the *A. thaliana* EST database (Leuthy *et al.*, 1994). The deduced amino acid sequence showed high homology with the *Saccharomyces cerevisiae* E1β subunit (65% sequence identity).

1.5.2 REGULATION OF PLANT MITOCHONDRIAL PYRUVATE DEHYROGENASE COMPLEX

Plant cells are typified by a greater complexity of subcellular compartmentalisation than mammalian or bacterial cells, thus it is not surprising that plants contain two distinct, spatially-separate types of PDCs located in the mitochondrial matrix and the plastid stroma. Like their mammalian/bacterial counterparts, fine control of plant mitochondrial PDC is achieved by end product inhibition where NADH and acetyl CoA inhibit competitively with respect to NAD^+ and CoA (Miernyk *et al.*, 1985). The plant mitochondrial PDC was observed to be more sensitive to the NADH/NAD^+ ratio than to acetyl CoA/CoA ratio demonstrated by the fact that the K_i value for NADH is 5-10 fold lower than the K_m for NAD^+, whereas the K_i for acetyl CoA is twice the K_m for CoA (Rubin *et al.*, 1978; Randall *et al.*, 1977).

Plant mitochondrial PDC is also subject to control by covalent modification via reversible phosphorylation (Randall *et al.*, 1981, 1989; Rubin & Randall, 1977; Rao &

Randall, 1980) (Fig. 1.6). This method of regulation occurs across a broad range of plant species including pea, spinach, broccoli and cauliflower. The existence of such a mechanism was first verified by demonstrating a Mg-ATP dependent inactivation of PDC (Rubin & Randall, 1977). When incubating with Mg-[γ-^{32}P]-ATP loss of activity is coupled with the incorporation of the label into a 43,300 Da subunit. This subunit cross reacts with anti-broccoli PDC highlighting a polypeptide that corresponds to the phosphorylated E1α subunit of mammalian PDC which has a slightly lower M_r value of 41,000. As with the mammalian complex, it is a serine residue that is phosphorylated during inactivation of the PDC (Miernyk *et al.*, 1985). Although evidence is available that indicates the presence of additional serine phosphorylations on the 43,300 Da subunit, these are not related to its activation state which is analogous to the situation in mammalian complexes (Randall *et al.*, 1989). Antibodies were prepared to a synthetic peptide corresponding to a 14 amino acid tryptic fragment containing phosphorylation sites 1 and 2 of bovine PDC (Miernyk & Randall, 1989). No cross reaction was observed with pea mitochondrial and plastid PDC in contrast to porcine, bovine and yeast PDC. This synthetic peptide also acts as a substrate for mammalian PDC kinase but not for the plant phosphorylation system, suggesting that the phosphorylation sites on mammalian and plant mitochondrial PDC are significantly different.

Mammalian PDC-kinase exists as an $\alpha\beta$ heterodimer where it is proposed that the α subunit (M_r 48,000) is directly involved in catalysis and the β subunit (M_r 45,000) regulates activity (Stepp *et al.*., 1983). The low abundance of the plant mitochondrial PDC kinase has prevented its isolation and purification. In addition the kinase is lost from the complex or is inactivated during purification; thus all studies to date have used partially purified PDC that retains kinase activity or mitochondrial extracts. The catalytic subunit of pea mitochondrial PDC kinase was estimated to have an approx. M_r value of 53,000 by photoaffinity labelling with 8-azido [^{32}P-α] ATP (Miernyk & Randall, 1987). Activity of plant PDC kinases is optimal at pH 7.5, with Mg-ATP as the preferred phosphoryl donor but with quite a broad nucleotide specificity.

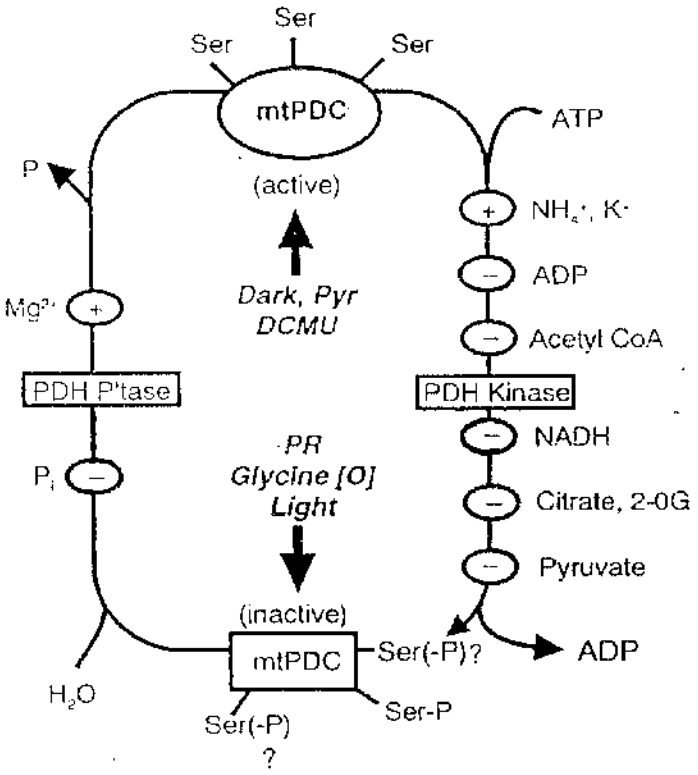


Figure 1.6: Schematic representation of pea leaf mitochondrial PDC regulation via covalent modification and the control elicited by effector molecules.

The kinase for plant mitochondrial PDC exhibits a K_m for Mg-ATP of 2.5μM, supporting the conclusion that if the PDC kinase is not regulated, the PDC would always be phosphorylated and therefore inactivated (Randall *et al.*, 1989; Miernyk & Randall, 1987).

In vitro effectors of the plant PDC kinase activity include acetyl CoA, NADH, citrate, Na^+ and pyruvate (Miernyk & Randall, 1987; Schuller & Randall, 1989). Inhibition by ADP is competitive with ATP as it is for mammalian PDC kinase. In contrast to their stimulatory effect on the mammalian kinase, acetyl CoA and NADH inhibit the plant enzyme. It is yet to be established why this is the case in plants. Inhibition by acetyl CoA is competitive with respect to Mg-ATP.

As with the mammalian PDC kinase, pyruvate has been identified as an inhibitor of the plant mitochondrial PDC kinase and is competitive with respect to ATP with a K_i of approx. 60μM. Inhibition by pyruvate is also enhanced by the presence of TPP (Budde & Randall, 1988; Schuller & Randall, 1989). Kinase activity is also stimulated by K^+ and NH_4^+, for example, *in vitro* pea leaf PDC kinase is stimulated by 10-80μM NH_4^+ and 10-100μM K^+ (Schuller & Randall, 1989). The NH_4^+ stimulation of the PDC kinase has significant regulatory potential as NH_4^+ is a product of photorespiratory carbon metabolism that occurs in mitochondria of illuminated leaves (Lorimer & Andrews, 1981).

The reactivation of phosphorylated plant mitochondrial PDC is catalysed by PDC phosphatase. This enzyme has an absolute requirement for divalent cations, with activation by $Mg^{2+}>Mn^{2+}>Co^{2+}$ and K_m values of 3.8, 1.7 and 1.4mM respectively (Miernyk & Randall, 1987b). Micromolar concentrations of Ca^{2+}, in contrast to the mammalian phosphatase, inhibit the plant phosphatase (Randall *et al.*, 1990). The only metabolite that has any effect on this phosphatase is orthophosphate which inhibits slightly.

A light dependent inactivation via phosphorylation of plant mitochondrial PDC has also been reported. Part of the photorespiration pathway involves the oxidation of glycine to serine in plant mitochondria with the concomitant production of NH_4^+ ions

(Lorimer & Andrews, 1981). It was noted that upon illumination of pea leaves , there was a decrease in the steady-state mitochondrial PDC activity to 10-20% of the levels exhibited by the dark adapted leaves. Mitochondria from illuminated tissues exhibit increased levels of ATP, glycine oxidation and NH_4^+ ions. It is these conditions that stimulate kinase activity and inactivate the complex. In studies where photosynthesis was chemically inhibited, the light-dependent inactivation of PDC was also prevented. In addition, when photorespiration was artificially inhibited, there was also reduction in the extent of light-dependent inactivation. This provides a mechanism for conserving carbohydrate when the cell is photosynthetically active by minimising the flux of carbon around the TCA cycle (Budde & Randall, 1990; Gemmel & Randall, 1992). The fact that both photosynthesis and photorespiration are required for the inactivation of PDC *in planta*, reflects the fact that mitochondrial glycine oxidation will only be significant when there is a functional photosynthetic apparatus to produce the precursor of glycine (Tolbiert, 1983). This inactivation is further enhanced by the stimulation of PDC kinase by NH_4^+ which is also produced by glycine oxidation during photorespiration.

1.5.3 PLASTID PYRUVATE DEHYDROGENASE COMPLEX

The fatty acid biosynthetic pathway is found in every cell of the plant and is essential to growth. All the carbon atoms found in fatty acid are derived from the pool of acetyl-CoA present in the plastid and as a result there must be a rapid system which produces acetyl-CoA in the plastid. How this pool of acetyl-CoA is generated is a point of contention. One of the most obvious pathways would be through the action of a plastidial PDC acting on pyruvate derived from the glycolytic pathway or produced as a side reaction of ribulose bisphosphate carboxylase activity (Andrews & Kane, 1991). However, PDC activity in isolated chloroplasts of some species is too low to account for rates of fatty acid synthesis (Lernmark & Gardestrom, 1994). The second proposed pathway converts acetate to acetyl-CoA through the action of acetyl-CoA synthetase (Zeiher & Randall, 1991). It was proposed that acetate produced from

acetyl-CoA which in turn was produced by a mitochondrial PDC is transported to the plastid (Zeiher & Randall, 1990). In addition, it was observed that plastids isolated from the developing embryos of oilseed rape were capable of synthesizing fatty acids from cytosolic malate, glucose-6-phosphate, pyruvate and acetate, with pyruvate being the most effective substrate (Kang & Rawsthorne, 1994). It is noted however that malate conversion to fatty acids follows a pathway that includes pyruvate and PDC. Acetate has been shown to be superior to pyruvate and other substrates as a precursor in fatty acid synthesis in other cases (Roughan & Slack, 1982; Springer & Heise, 1989; Masterson *et al.*, 1990). It has also been reported that the incorporation of label from malate into fatty acids occurred at a higher rate than either pyruvate or acetate (Smith *et al.*, 1992). It would appear that the rates of substrate incorporation into fatty acids is highly dependent upon species and tissue type investigated. It is likely that more than one pathway may contribute to maintaining the acetyl-CoA pool and that the pathway used may vary with tissue, developmental stage, light/dark conditions and species. All research to date has been performed on *in vitro* studies, therefore *in vivo* studies are required. Thus the existence of a plastid PDC and its possible functional significance has also been a matter of contention for many years.

A 75-fold purification of pea chloroplast PDC was achieved by Camp & Randall (1985). They unequivocally established that the observed oxidative decarboxylation of pyruvate was a function of PDC activity by showing an absolute requirement for NAD^+ and CoA. In addition, the pea plastid PDC was only partially inactivated by antibodies to broccoli mitochondrial PDC indicating that the observed plastid PDC did not arise from mitochondrial contamination. The possibility of a BCDC-mediated reaction utilising pyruvate as a substrate was eliminated also since the partially purified multienzyme complex was unable to interact with 2-oxoisovalerate or 2-oxoisocaproate. $NADP^+$ was also examined as a substrate because of the large quantity of it present in chloroplasts; however, only a minor $NADP^+$-dependent activity was observed. The products of the chloroplast complex were assayed and a 1:1:1 stoichiometry of CO_2: acetyl CoA: NADH was observed as was predicted for a PDC reaction mechanism.

The plastid PDC catalyses the same overall reaction and has the same kinetic mechanism as the mitochondrial PDC, but differing in their pH optima, Mg^{2+} requirements and structure. Subunit analyses of plastid and mitochondrial PDCs by SDS-PAGE plus immunoblotting showed significant differences (Camp & Randall, 1985). The only subunit of the plastid PDCs to be positively identified is the E3 component from pea, with an M_r value of 57,000-58,000 (Camp & Randall, 1985). Taylor and colleagues (1992) have since carried out extensive immunological analyses of pea chloroplast PDC using antibodies to specific components of bovine heart and yeast PDC. Chloroplast protein exhibited no cross reaction with antisera to mammalian or yeast E1. In contrast the the pea mitochondrial PDC protein exhibited a band with M_r value of 41,000 when exposed to antiserum to the E1 subunit of yeast PDC. Taking into consideration the different methods of regulation between the mitochondrial and plastid forms of PDC, it is perhaps not surprising that the respective E1 components from these organelles are immunologically distinct polypeptides in their own right. The E2 component from pea chloroplast was found to cross react with anti-E2 from bovine heart PDC to produce a band with a M_r value of 50,000, substantially smaller than its analogous mammalian enzyme (67,000) but similar in size to yeast E2. The M_r value of plastid PDC E2, like plant mitochondrial PDC E2, is consistent with the presence of only one lipoyl domain (Taylor *et al.*, 1992). A pea chloroplast protein of M_r 52,000 cross reacted with mammalian anti E3, substantially smaller than the pea mitochondrial PDC E3 M_r 67,000. This observation suggests the possibility of organelle specific E3 isoforms (Taylor *et al.*, 1992).

1.5.4 REGULATION OF PLASTID PYRUVATE DEHYDROGENASE COMPLEX

a) PRODUCT INHIBITION

As is the case with all other PDCs, the plastid complex is sensitive to product inhibition by NADH and acetyl CoA. Both products are linear competitive inhibitors with respect to CoA and NAD^+ with K_i values of 20μM (Camp *et al.*, 1988). The

plastid complex, like the mitochondrial complex, was much more sensitive *in vitro* to the ratio of NADH to NAD^+ than to that of acetyl CoA/CoA.

b) METABOLITE REGULATION

There is no evidence available to suggest that plastid PDC is prone to metabolite influence unlike plant mitochondrial PDC. The *in vitro* activity of pea chloroplast PDC is insensitive to intermediates of glycolysis, the reductive pentose-phosphate pathway and amino acid metabolism. Exceptions to the rule are inorganic phosphate and oleic acid which inhibit PDC activity and palmitic acid which stimulates chloroplast PDC (Camp *et al.*, 1988).

c) PROTEIN TURNOVER

Plastid PDC activity showed a discrete developmental programme during *Ricinus* endosperm and green tissue maturation. Plastid PDC activity was highest in the developing seeds during the period of maximum storage lipid accumulation, similarily in leaves, chloroplast PDC activity is maximal during leaf expansion during a period of rapid lipid synthesis. It was also noted 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).

c) LIGHT/DARK TRANSITIONS

The proposed role of plastid PDC is to supply acetyl CoA for fatty acid biosynthesis. All *de novo* fatty acid synthesis in higher plant cells from green tissues occurs within the plastid stromal phase and, in the case of chloroplast fatty acid synthesis, is light driven (Ohlrogge *et al.*, 1979; Roughan *et al.*, 1980; Treede & Heise, 1986). In the dark, the chloroplast stromal has a pH in the range 7.3-7.5, contains a low concentration of divalent cations and is deficient in "reductant charge". On illumination when the chloroplast is photosynthetically active, there is an alkalinisation of the stromal phase pH to about 8.0. In addition to this pH shift there is also an

increase in free Mg^{2+} and Ca^{2+} levels and production of reducing equivalents. Plastid PDC has been shown to have an alkaline pH optimum and a higher divalent cation requirement than mitochondrial PDC (Camp & Randall, 1985; Williams & Randall, 1979; Randall *et al.*, 1989). The K_m for pyruvate of the pea chloroplast PDC was lowest at pH 8.0, the optimum pH of the complex, and much higher at acidic or more alkaline pH values while the V_{max} was largely unaffected. Therefore, plastid PDCs differ from mitochondrial PDCs in that they require higher divalent cation concentrations for maximum *in vitro* activity. This regulatory regime can be observed even in plastid PDC from non-green tissue and is a unique regulatory mechanism amongst all PDCs studied to date (Camp & Randall, 1985; Treise & Heise, 1986; Miernyk *et al.*, 1985).

Plastid PDCs, like the bacterial PDC complexes are not regulated by reversible phosphorylation which is a property of mitochondrial PDCs (Randall *et al.*, 1989; Miernyk *et al.*, 1985). As described earlier, in green plant tissues, fatty acid synthesis is light driven and there is therefore a demand for acetyl CoA and NADH in the plastid stroma. At the same time there are increased levels of ATP produced by photophosphorylation. If the plastid PDC was subject to covalent modification , like the mitochondrial PDC, then an ATP dependent inactivation of PDC would be likely to occur during periods of illumination which would be counterproductive.

1.5.5 PLANT 2-OXOGLUTARATE DEHYDROGENASE COMPLEX (OGDC)

Very little research to date has been carried out on the plant 2-oxoglutarate dehydrogenase complex (OGDC) or branched-chain 2-oxoglutarate dehydrogenase complex (BCDC) in comparison to plant PDC. Therefore subsequent characterisation of these enzymes has been very limited. The presence of high levels of NADH oxidase activity in plant cells has made the detection of OGDC-catalysed reactions difficult owing to the masking effect of OGDC activity by NADH oxidation (Poulson & 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) concentrated on purifying a 'subcomplex' of cauliflower mitochondrial OGDC comprising the E1/E2 core. Consequently, intact OGDC activity could only be achieved by the addition of exogenous E3 purified from porcine heart. This 'subcomplex' of plant OGDC displayed very similar properties to the mammalian and bacterial counterparts as described earlier. It was found to have an absolute requirement for 2-oxoglutarate, CoA, TPP, 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 is suggested that this results in the tighter binding of Mg^{2+}-TPP to the E1 component (Wedding & Black, 1971a, 1971b; Craig & Wedding, 1980). Plant OGDC isolated from cauliflower also has an absolute divalent cation requirement in common with all other 2-oxoacid dehydrogenase complexes. Cauliflower 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 & Black, 1971b; Craig & Wedding, 1980). Both PDC and OGDC utilise CoA resulting in competition for available substrate between the two complexes. Pyruvate is reported to exert 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 CoA pool size in the mitochondrial matrix (Dry & Wiskich, 1985, 1987).

To date OGDC has only been reported to have a mitochondrial location in plant cells, since the only other organelle to be screened for OGDC activity is the chloroplast. To date enzymatic detection of OGDC in chloroplasts has never been reported. However, the lack of research in this area means that the presence of OGDC in other plant organelles cannot be excluded completely.

A study of the intracellular distribution of plant OGDC was carried out, however, by A. Carmichael (Ph.D. thesis, Glasgow University, 1994). A range of organelles,

isolated from pea, were screened for the presence of the 2-oxoacid dehydrogenase complexes by enzymatic and immunological techniques. This investigation was the first extensive screening of organelles for the presence of OGDC and activity was not detected in the peroxisomal or plastid compartments.

1.5.6 PLANT BRANCHED-CHAIN 2-OXOACID DEHYDROGENASE COMPLEX

BCDC catalyses the degradation of leucine, isoleucine and valine. It oxidatively decarboxylates the 2-oxoacids produced by the transamination of these compounds and is also thought to be involved in the catabolism of methionine and threonine. Therefore, BCDC plays an important role in protein turnover. Plant BCDC has never been thoroughly investigated with very little information available with regard to its intracellular location, structure, subunit composition and regulation.

BCDC activity has been detected only in the peroxisomes of mung bean hypocotyls (Gerbling & Gerhardt, 1988, 1989). It was reported to be a CoA and NAD^+ dependent reaction with the concomitant formation of NADH and 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 acyl CoAs. Their results suggest 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 appropiate acyl CoA from branched-chain 2-oxoacids, intermediates are further metabolised by β-oxidation to acetyl CoA.

Gerbling and Gerhardt (1988) reported the absence of BCDC activity in mitochondria and plastids of mung bean hypocotyls and suggested that plant BCDC is located exclusively in peroxisomes. However, a possible mitochondrial location for BCDC in pea has been reported by A. Carmichael (Ph.D. Thesis, Glasgow University, 1994). BCDC activity was recorded in solubilised pea mitochondrial fractions

concentrated by high speed centrifugation. This was also supported by the detection of a BCDC-catalysed reaction in potato mitochondria. Since BCDC activity produces CoA derivatives which could enter the TCA cycle then it would be advantageous to have a mitochondrial BCDC. It is possible that the reason for not detecting a mitochondrial BCDC in the past is related to the enzyme being predominantly inactivated by a phosphorylation mechanism. Certainly, it is known that in certain mammalian tissue, BCDC is in the inactive phosphorylated state. It may also be possible that BCDC activity or synthesis is governed by a developmental programme and therefore it may have been developmentally suppressed in the tissue screened. Surprisingly, no BCDC activity was observed in peroxisomes (A. Carmichael, Ph.D. Thesis, Glasgow University, 1994).

1.6 GLYCINE DECARBOXYLASE COMPLEX

Glycine decarboxylase (GDC) or the glycine cleavage system (EC 2.1.2.10) is a distant relation to the family of 2-oxoacid dehydrogenase complexes. This multienzyme system has been purified from a variety of sources including mammals, bacteria as well as plants (Klien & Sagers; 1966; Kikuchi & Hiraga, 1982; Walker & Oliver, 1986). It is located in the mitochondrial compartment and exists as a labile multicomponent complex maintaining a loose association with the inner membrane. GDC consists of four enzymes, the P, H, T and L proteins (termed P_1, P_2, P_3 and P_4 respectively in bacterial systems). GDC catalyses the reversible oxidation of glycine in a multistep process to produce N^5, N^{10}-methylene-H_4 folate, CO_2,and NH_4^+:

$$\text{Glycine} + H_4\text{folate} + NAD^+ \text{<----> Methylene-}H_4\text{folate} + NH_4^+ CO_2 + NADH + H^+$$

GDC is extremely important with respect to plant cells because of its involvement in photorespiration. Glycolate formed in chloroplasts is transported to peroxisomes where it is oxidised to glyoxylate which is then transaminated to glycine. Glycine, in

turn, migrates to leaf mitochondria where it is subsequently decarboxylated by GDC. It is reported that GDC may represent 30-50% of the total mitochondrial matrix protein. In contrast, GDC constitutes only a small fraction of the total mitochondrial matrix from mammalian sources (Kikuchi, 1973; Oliver *et al.*, 1990). Thus photorespiration involves the co-operation of three separate plant organelles (Fig. 1.7). Photorespiration results in the net consumption of energy where GDC is responsible for salvaging reducing power which is fed into the mitochondrial electron transport chain (Douce *et al.*, 1994).

The P Protein (P_1) is a pyridoxal phosphate-dependent enzyme which binds to and catalyses the decarboxylation of glycine, but only in concert with the low M_r value, acidic and heat stable H protein (P_2) (Fig. 1.8). The resulting aminomethyl moiety is attached to the lipoic cofactor of the H protein. The T protein (P_3) is then responsible for catalysing the release of ammonia from the methylamine intermediate that 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}-methylene-H_4folate. The lipoic acid of the H-carrier protein (H protein) is left in a reduced state and its subsequent reoxidation with formation of NADH is catalysed by the L protein, a flavin-requiring dihydrolipoamide dehydrogenase. The lipoic acid is attached via an amide linkage to the ε-amide group of a lysine residue on the H protein, 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. The N^5,N^{10}-methylene-H_4folate produced reacts with a second mole of glycine to form serine in a reaction catalysed by serine hydroxymethyltransferase (SHTM; EC 2.1.2.1) (Klein & Sagers, 1966; Kawasaki *et al.*, 1966; Fujiwara *et al.*, 1984; Walker & Oliver, 1986). Very low levels of glycine metabolism are observed in green leaf tissue in the dark. On illumination, however, there is a rapid synthesis of GDC, until it represents the major metabolic reaction in mitochondria in mature leaves (Oliver *et al.*, 1990).

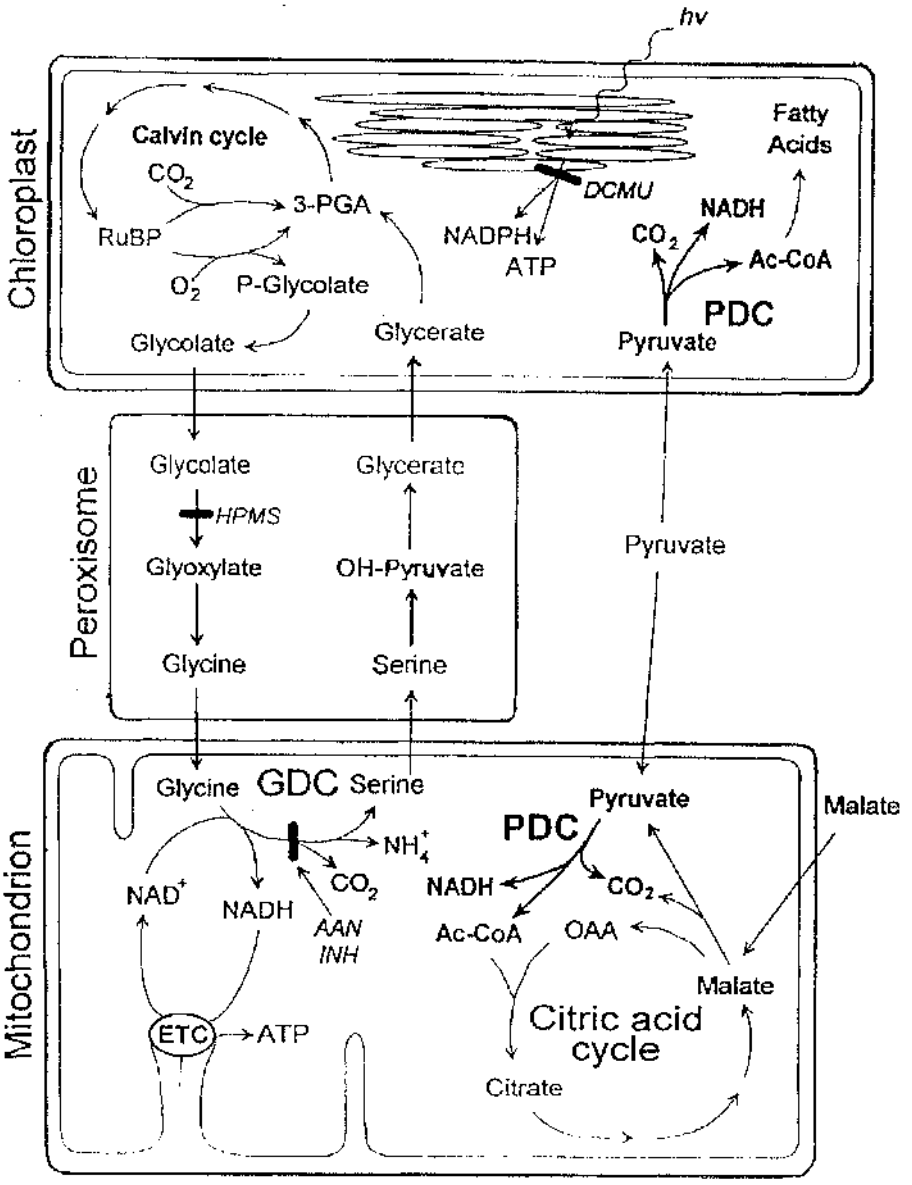


Figure 1.7: Interactions of the photosynthetic, photorespiratory, and the citric acid cycle (tricarboxylic acid cycle). Schematic diagram of the photorespiratory cycle, including portions of the Calvin and citric acid cycles. The site of inhibitor action (DCMU, HPMS, INH and AAN) are indicated. Not all of the intermediates are shown and stoichiometry has not been maintained. Figure is reproduced from Leuthy *et al*, (1995b).

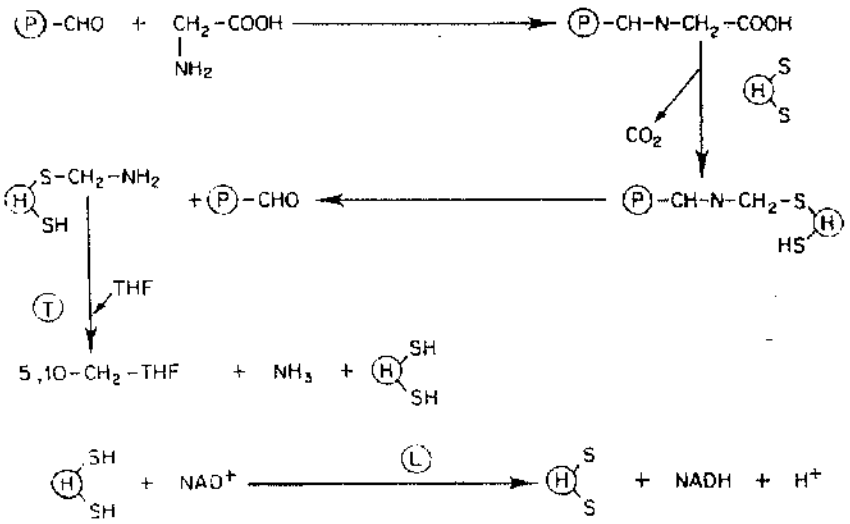


Figure 1.8: Reaction mechanism of glycine decarboxylase complex. P, H, T and L in the circles represnt the component enzymes involved in the catalysis of glycine. THF = tetrahydrofolate.

Characterisation of GDC has been limited to studies with either whole mitochondria or a crude protein extract since attempts to isolate the GDC activities intact, resulted in low yields owing to the instability of the complex (Sarojini & Oliver, 1983). However, cDNA clones encoding all four proteins of GDC (P, H, T and L) of pea mitochondria have now been isolated and characterised (Kim & Oliver, 1990; Macherel *et al.*, 1990; Turner *et al.*, 1992; Bourguinon *et al.*, 1992, 1993). All four proteins were shown to be encoded by unique nuclear genes which are translated on cytosolic ribosomes and imported into the mitochondrial matrix. The expression of the genes encoding the proteins of GDC, with the exception of the L protein, occurs specifically in mature leaf tissue. In addition, upon illumination the mRNA levels of the P, H and T 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 is related to the fact that dihydrolipoamide dehydrogenase can be found associated with two other mitochondrial enzymes namely, pyruvate dehydrogenase complex (PDC) and 2-oxoglutarate dehydrogenase complex (OGDC) as well as the glycine decarboxylase (GDC). This indicates that it is the same dihydrolipoamide dehydrogenase associated with each of these three complexes present in the mitochondria.

As mentioned above, both GDC and the 2-oxoacid dehydrogenase complexes utilise the same dihydrolipoamide dehydrogenase, designated the L-protein and E3 respectively. The L-protein of GDC has been successfully purified, cloned and sequenced from pea leaf (Turner *et al.*, 1992; Bourguignon *et al.*, 1992). This protein had a M_r value of approx. 60,000 when analysed by SDS-PAGE, similar to M_r values (57,000-58,000) obtained for pea mitochondrial and chloroplast E3 as described in sections 1.5.1 and1.5.3. Positive cDNA clones were isolated from pea leaf and embryo λgt11 expression libraries using an antibody raised against the purified dihydrolipoamide dehydrogenase. A difference in their open reading frames of 529 and 501 amino acids respectively was observed. It has since been confirmed that the correct sequence is that of Bourguignon and co-workers (S. Rawthorne, personnal

communication). The deduced amino acid sequence exhibited clear homology to the sequences of dihydrolipoamide dehydrogenase from *E. coli*, yeast and humans. Both groups concluded that from sequence comparison and the organisation and expression of the corresponding gene indicates that it is the same dihydrolipoamide dehydrogenase that is utilised by GDC, PDC and OGDC.

The primary aim of this thesis was to purify and characterise plant dihydrolipoamide dehydrogenases from pea and potato to determine if a single mitochondrial isoform was present which served as a common constituent of the multienzyme complexes present in plants (PDC, OGDC and GDC). Immunological evidence indicated that in plant tissue there was a possibility of organelle specific isoforms of dihydrolipoamide dehydrogenase in pea (Taylor *et al.*, 1992). Thus, this line of research was also subsequently investigated.

CHAPTER 2

MATERIALS AND METHODS

2.1 **MATERIALS**

2.1.1 PLANTS

Thirium treated pea seeds (*Pisum sativum* L., cv. Little Marvel) were purchased from Sharpes International Seeds Ltd. (Sleaford, Linconshire) and potato tubers (*Solanum tuberosum*, cv. Maris Piper) from Glasgow Fruit Market.

2.1.2 CHEMICALS

The following reagents were obtained from Sigma Chemical Co., Poole, Dorset, UK: substrates and coenzymes for enzymatic assays, phenylmethylsulphonyl fluoride (PMSF), benzamidine-HCl, Coomassie Brilliant Blue type R250, Tween-20, antifoam A concentrate, 3-(N-Morpholino) propane-sulphonic acid (MOPS), polyvinyl-pyrrolidone (PVP), N-tris[hydroxymethyl]-methyl-2-aminoethane-sulfonic acid (TES), Percoll, thioctic acid amide and L-ascorbic acid. Polyethylene glycol 6000 grade and Triton X-100 were obtained from Fisons, Loughborough, England. Pyronin Y dye was obtained from George T. Gurr Ltd., London. Folin and Ciocalteu phenol reagent was from FSA Laboratory supplies, Loughborough, England. Ultra pure acrylamide used for the protein sequencing was from Fluka, Derbyshire, England. 2-Mercaptoethanol was purchased from Prolabo, Paris, France. Leupeptin was from the Marketing Association, Herts., England. DTT was obtained from Boehringer Mannheim GmbH, Germany. All other chemicals including reagents for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were of Analar grade from BDH Chemicals Ltd., Poole, U.K., or were of the highest grade commercially available.

2.1.3 ENZYMES AND PROTEINS

Bovine serum albumin (BSA), protein A (*Staphylococcus aureus*, Cowan 1 strain), dihydrolipoamide dehydrogenase from porcine heart, bovine mucosa and yeast and molecular weight markers for gel filtration chromatography were obtained from Sigma Chemical Co., Poole, Dorset. Low molecular weight marker proteins for M_r determinations by SDS-PAGE were purchased from Pharmacia Ltd., Milton Keynes.

2.1.4 ANIMALS

Bovine hearts were obtained from Paisley Abbatoir, Sandyford Road, Paisley. New Zealand White Rabbits for antisera production were bought at 3 months old from MRC accredited resources.

2.1.5 PHOTOGRAPHIC MATERIALS

X-Omat S film, Kodak FX-40 liquid fixer and Kodak LX-24 X-ray developer were obtained from Kodak Ltd., Dallimore Road, Manchester, England.

2.1.6 MISCELLANEOUS

FPLC columns, gel chromatography materials and CNBr-activated Sepharose 4B were purchased from Pharmacia Ltd., Milton Keynes. Nitrocellulose filters (0.22μM) were from Millipore S.A., Molsheim, France. Rat serum, Freund's complete and incomplete adjuvant were obtained from Sigma Chemical Co., Poole, Dorset, U.K.. Hybond-C extra nitrocellulose was bought from Amersham International, Bucks., U.K. Immobilon PVDF membrane for protein sequencing was purchased from BioRad, Richmond, CA, U.S.A. Non-immune donkey serum was supplied by the Scottish Antibody Production Unit (SAPU), Lanarkshire, Scotland. Plast-X

autoradiography cassettes were from Anthony Monk (England) Ltd., Sutton-in-Ashfield, U.K. Centricon-10, 30 and centriprep-10, 30 concentrators were bought from Amicon Ltd., Stonehouse, Gloucestershire, England.

2.2 **METHODS**

2.2.1 GROWTH OF PLANTS

Pea seeds (*Pisum sativum* L., cv. Little Marvel) were grown in moist vermiculite at 200mEm^{-2}s^{-1} in a growth chamber with a 12h photoperiod (22°C light / 18°C dark). In order to avoid the development of an extensive lateral root system the pea roots were harvested after 4 days. Leaf tissue was collected 11 days after planting when the primary leaves were fully expanded.

2.2.2 ORGANELLE ISOLATION

(A) MITOCHONDRIAL ISOLATION

Mitochondria from both potato tuber and pea root/leaf tissue were isolated by a process described by Day *et al* . (1988) with all procedures carried out at 4°C. Pea root and leaf tissue were ground in a polytron homogeniser (System Tecknik, Ruschlikon, Switzerland) for four short bursts at setting five to limit organelle damage. Potato tuber tissue was ground using a Waring Commercial Blender at high speed for five second bursts approx. five times. In each case 3-4 volumes of grinding buffer were used containing 0.3M sucrose, 10mM KH_2PO_4 2mM EDTA, 2mM $MgCl_2$, 1% (w/v) polyvinylpyrrolidine 40 (PVP-40), 1% (w/v) bovine serum albumin (BSA) and 25mM sodium phosphate buffer. Iso-ascorbic acid (30mM) was added fresh on day of preparation and the pH adjusted to 7.6.

The homogenate was filtered through four layers of pre-wetted muslin into cold centrifuge buckets containing protease inhibitors (1mM phenylmethylsulphonyl fluoride

(PMSF), 1mM benzamidine-HCl and 10μM leupeptin) and centrifuged at 1000g for 5 min. The supernatant fluid was retained and centrifuged at 12000g for 20 min. The resulting crude mitochondrial pellet was resuspended using a hand homogeniser in 30ml of wash buffer containing 0.3M sucrose, 0.1% (w/v) BSA, 1mM glycine and 10mM (N-tris[hydroxymethyl]-methyl-2-aminoethane-sulphonic acid (TES), pH 7.2. Both spins were repeated and the resultant mitochondrial pellet resuspended in approx. 2ml of wash buffer. The mitochondrial extract was then layered onto a self generating gradient comprising 10mM potassium phosphate pH 7.2, 30% (v/v) Percoll, 1mM EDTA, 0.1% (w/v) BSA and centrifuged at 40,000g for 35 min. The mitochondria formed a distinct broad band near the bottom of the gradient. This could be removed using a Pasteur pipette carefully so as not to disturb the upper regions of the gradient which contain the membrane fractions.

The Percoll was then removed from the mitochondral fraction by diluting it in 5 volumes of wash buffer minus glycine prior to centrifugation at 12,000g for 15 min. The upper section of the supernatant fraction was removed and the pellet further diluted with wash buffer minus glycine and BSA and again centrifuged at 12,000g for 15 min. This last step was repeated until a firm mitochondrial pellet was formed which was then resuspended in a minimal volume of wash buffer minus glycine and BSA.

(B) CHLOROPLAST ISOLATION

Chloroplasts were harvested from pea leaf tissue according to a slightly modified method described originally by Murphy & Leech (1977, 1978). All procedures were carried out at 4°C. Approximately 100g of leaf tissue, in 3-4 volumes of grinding buffer (described in 2.2.2), was disrupted in 3-4 four second bursts at speed 3 on a polytron homogeniser. The homogenate was filtered through six layers of pre-wetted muslin with protease inhibitors added (1mM PMSF, 1mM benzamidine-HCl and 10μM leupeptin), then the samples were centrifuged at 200g for 3 min. The supernatant fluid was subsequently centrifuged at 3020g for 3 min. Mitochondria were located in the supernatant fraction while intact chloroplasts fraction were pelleted under these

conditions.

The crude chloroplast pellets were gently resuspended with a Pasteur pipette in approximately 5ml of buffer A containing 0.3M sorbitol, 4.4mM sodium pyrophosphate, 1mM EDTA, 3.5mM $MgCl_2$ and 50mM N-[2-hydroxy-1, 1-bis(hydroxymethyl)ethyl]-glycine (Tricine) pH 8.0; 2-mercaptoethanol (13.5mM) was introduced immediately prior to use. Chloroplast pellets were washed twice by resuspending in Buffer A and repelleting. The final pellet was resuspended in 10ml of Buffer A and layered onto a 10ml volume of Buffer B (as Buffer A, except 0.6M sorbitol) and centrifuged at 420g for 5 min. The resulting pellet was resuspended in a minimal volume of Buffer A with the addition of 1 mg/ml dithiothreitol (DTT).

2.2.3 PREPARATION OF CRUDE TISSUE EXTRACT

In an attempt to maximise yields of dihydrolipoamide dehydrogenase (E3) from potato tubers, a modification of the method described in 2.2.2(A) was employed. As before the potato homogenate was prepared and centrifuged at 1000g for 5 min and the supernatant then spun at 12,000g for 20 min. After resuspending each pellet in approx. 30ml of wash buffer minus BSA and glycine, the sample was centrifuged at 1000g for 5 min, the supernatant fraction retained and respun at 12,000g for 20 min. The pellets were resuspended in wash buffer minus BSA and glycine as before but with the addition of 0.1% (v/v) Triton X-100 and protease inhibitors (1mM PMSF, 1mM benzamidine-HCl and 10μM leupeptin). The resuspended material was left on ice for approx. 30 min to rupture any intact mitochondria present and therefore allow the release of dihydrolipoamide dehydrogenase. The sample was centrifuged finally at 12,000g for 20 min and the supernatant containing the crude extract of potato E3 retained.

2.2.4 PURIFICATION OF PYRUVATE DEHYDROGENASE COMPLEX (PDC) AND 2-OXOGLUTARATE DEHYDROGENASE COMPLEX (OGDC) FROM BOVINE HEART.

PDC and OGDC were purified according to the method of Stanley and Perham (1980) with the following modifications with all procedures performed at or near 4°C. Heart tisssue (600g) was homogenised for 5 min in approx. 1l of buffer containing 2.7mM EDTA, 0.1mM DTT, 3% (v/v) Triton X-100, 1mM PMSF, 1mM benzamidine-HCl, silicone antifoam (0.5 ml/l) and 50mM MOPS buffer, pH 7.0. The homogenate was then diluted with an equal volume of the same buffer and centrifuged at 10,000g for 20 min. The pellets were discarded and the pH of the supernatant adjusted to 6.45 using 10% (v/v) acetic acid, after which 0.15 vol of 35% (w/v) polyethylene glycol was added and the solution left stirring on ice for 30 min, followed by centrifugation at 18,000g for 15 min. The resulting pellets were resuspended by homogenisation with a loose fitting-Teflon glass homogeniser in 400 ml of 2.7mM EDTA, 0.1mM DTT, 1% (v/v) Triton X-100, 0.15μM leupeptin, 1mM PMSF, 1mM benzamidine-HCl and 50mM MOPS buffer, pH 6.8. The homogenised material was filtered through muslin to remove fat particles with 1M $MgCl_2$ and 1M sodium phosphate buffer pH 6.3 added to give final concentrations of 13mM and 50mM respectively. During this procedure the pH of the solution was not allowed to fall below 6.8. This was achieved by adding drops of 0.6M NaOH to keep the pH in check. The pH was then lowered to 6.45 using 10% (v/v) acetic acid, 0.12 vol. of 35% (w/v) PEG 6000 was added and the solution left to stir on ice for 30 min. This solution was centrifuged at 25,000g for 10 min, the pellets resuspended in 160ml of 2.7mM EDTA, 0.1mM DTT, 1% (v/v) Triton X-100 and stored overnight in the presence of 1mM PMSF,1mM benzamidine-HCl and 0.5% (v/v) rat serum. Rat serum was added to the buffer as a source of protease inhibitors to prevent losses of up to 50% OGDC activity from occurring overnight (Wieland, 1975).

The following day, the suspension of partially purified enzyme was homogenised

and clarified by spinning it at 35,000g for 1h. PDC and OGDC were separated from each other by selective precipitation which involved the addition of 0.06 vol of 35% (w/v) PEG 6000. OGDC was pelleted by spinning the sample at 25,000g for 10 min. PDC could be found in the supernatant and was pelleted by centrifuging at 100,000g for 150 min. Both OGDC and PDC pellets were resuspended in a minimal volume of 1% (v/v) Triton X-100 buffer and stored at 4°C.

2.2.5 BOVINE HEART MITOCHONDRIAL PREPARATION

To prepare bovine heart mitochondria, 600g of fresh lean heart muscle was required. This material should contain as little stringy, fatty tissue as possible. Heart muscle was minced in a blender at high speed for about 5 min and then added to 800ml of ice cold TS buffer containing 250mM sucrose and 10mM Tris-HCl, pH 7.8. The pH of this mixture was re-adjusted to approx. 7.8 using 2M Tris base. Excess fluid was drained off using muslin cloth and the minced material added to 800ml of TES buffer containing 0.2mM EDTA, 1mM succinate, 250mM sucrose and 10mM Tris-HCl, pH 7.8.

Half this material was added to a Waring commercial blender and blended for 20 sec at full speed. The pH of this mixture was adjusted to 7.8 with 2M Tris base as before and re-blended for a further minute. This procedure was repeated with the second half of the heart material and the two portions pooled and diluted with TES buffer to a volume of not greater than 2.4l. To remove nuclei and any unbroken cells, the homogenate was centrifuged for 20 min at 2000g. After centrifuging the supernatant fraction at 20,000g for 20 min to pellet the mitochondria, the pellets were resuspended in TES buffer to a total volume of 300ml and centrifuged at 17,000g for 20 min. Tubes were finally sealed with cling film prior to storage of the purified mitochondrial pellets at -20°C.

2.6.6 CONCENTRATION OF PROTEIN SAMPLES

Large volumes of protein samples were concentrated using Amicon Centriprep disposable ultrafiltration devices with a molecular weight cut-off of 10,000 Da. For smaller volumes Amicon Centricon-10 and 30 were used. These concentrators were also used to desalt column eluates and gradient fractions.

Samples for electrophoresis were concentrated by trichloroacetic acid (TCA) precipitation. TCA 10% (w/v) was added to the sample from a 100% (w/v) stock solution of TCA and kept at 4°C for 1h. The samples were then centrifuged at 11,600g for 2 min and the pellets washed with ether to remove any remaining traces of TCA. After the pellets were allowed to dry at room temperature, they were resuspended in Laemmli sample buffer as described in section 2.2.10(A). Samples could also be alternatively concentrated by the addition of 4 vol of acetone prior to storage at -20°C for 1h. As before the samples were centrifuged at 11,600g for 2 min, the acetone removed and the pellet resuspended in Laemmli sample buffer.

2.2.7 ENZYME ASSAYS

(A) 2-OXOACID DEHYDROGENASE COMPLEXES

The overall activities of the 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase complexes were assayed according to the method of Brown and Perham (1976). The reaction mixture comprised 50mM potassium phosphate buffer (pH 7.6) containing 3mM NAD^+, 1mM $MgCl_2$, 0.2mM TPP, 2.6mM cysteine-HCl, 0.13mM CoA, 2mM 2-oxoglutarate (or 2mM pyruvate when assaying for PDC activity). All assays were performed at 30°C in a final volume of 0.7ml. The assay was initiated by addition of enzyme and the reaction followed by monitoring the formation of NADH spectrophotometrically at 340nm (Danson *et al.*, 1978). The extinction coefficient for NADH is $6.22mM^{-1}cm^{-1}$.

(B) DIHYDROLIPOAMIDE DEHYDROGENASE (E3)

Assays were performed according to the method of Jackman *et al.* (1990) at 30°C in a final volume of 0.7ml. The assay mixture contained 50mM potassium phospate buffer, pH 7.6, containing 3mM NAD^+, 1mM $MgCl_2$, 0.2mM TPP and 20μg of dihydrolipoamide (for preparation see 2.2.8). The reaction was initiated by addition of enzyme and activity followed by monitoring NADH formation at 340nm. All enzymatic data presented in subsequent chapters of this thesis represent an average of duplicate determinations differing by less than 5%.

2.2.8 PREPARATION OF DIHYDROLIPOAMIDE

Dihydrolipoamide was prepared according to the method described by Reed *et al.* (1958). 400mg of lipoamide (thio-octic acid amide) was dissolved in 80% (v/v) methanol at 4°C. 400mg of sodium borohydride was dissolved in 2ml of distilled water, also at 4°C, and then added to the lipoamide/methanol. The solution was stirred at room temperature until it became clear (approx. 45 min). The pH of the solution was then lowered to 2.0 with 0.25M HCl and extracted with 40ml of chloroform. The lower layer containing the dihydrolipoamide was removed and dried down under N_2. This material was redissolved by stirring on a hot plate in 40-60ml of toluene/heptane (2.5:1.0). After drying down once more under N_2 until a minimal volume of liquid remained, the dihydrolipoamide was allowed to crystallise naturally and stored at -20°C. For use approx. 20.7mg of dihydrolipoamide was dissolved in 1ml of ethanol.

2.2.9 MEASUREMENT OF PROTEIN CONCENTRATION

Protein concentration was determined by the method of Lowry *et al.* (1951) as modified according to Markwell *et al.* (1976).

The following stock solutions were prepared:

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

SOLUTION B containing 4% (w/v) copper sulphate.

SOLUTION C containing 1ml of solution B added to solution A to a final volume of 100ml.

SOLUTION D containing Folin and Ciocalteu phenol reagent diluted 1:1 with distilled water.

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

2.2.10 SODIUM DODECYL SULPHATE 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 10 or 15% (w/v), polyacrylamide gel slabs (pH 8.8, 16 x 19.5 x 0.15cm), with a 5% (w/v) stacking gel (pH 6.8).

The following stock solutions used in the making of the polyacrylamide gels were prepared:

ACRYLAMIDE STOCK containing 29.2% (w/v) acrylamide and 0.8% (w/v) N,N'-methylenebisacrylamide.

RESOLVING GEL BUFFER containing 0.75M Tris-HCl (pH 8.8) and 0.2% (w/v) SDS.

STACKING GEL BUFFER containing 0.17M Tris-HCl (pH 6.8) and 0.14% (w/v) SDS.

10% (w/v) AMMONIUM PERSULPHATE.

The resolving gel was prepared according to the percentage of acrylamide required as shown in the following table.

	% (w/v) Acrylamide	
	10% Gel (ml)	15% Gel (ml)
Acrylamide Stock	24	35
Resolving Gel Buffer	36	36
Ammonium persulphate	0.72	0.72
Distilled H_20	11.28	0.28
TEMED	0.072	0.072

All solutions were mixed and degassed before the addition of NNN'N'-tetramethylethylenediamine (TEMED), which initiated polymerisation, and then poured into the casting apparatus. A layer of isopropan-2-ol was poured on top of the resolving gel to ensure an even surface and removed once the gel had set. Before pouring the stacking gel, any residual isopropan-2-ol was removed by washing with distilled water.

The 5% stacking gel was prepared as follows:

	Volume (ml)
Acrylamide Stock	3.6
Stacking Gel Buffer	16.4
Ammonium Persulphate	0.24
Distilled H_2O	3.76
TEMED	0.024

Stacking gel solutions were mixed, degassed, TEMED added and then poured into the casting apparatus on top of the resolving gel and allowed to set. Gel plates were wrapped in damp tissue and cling film and stored at 4°C (for no more than 2 weeks) until use.

Gels were subjected to electrophoresis at a constant current of 60mA (8mA if running overnight) in buffer containing 1mM glycine, 0.1% (w/v) SDS, 25mM Tris base, pH 8.3 in vertical gel electrophoresis apparatus purchased from Bethesda Research Laboratories. Electrophoresis was continued until the Pyronin Y tracker dye reached the end of the gel (approx. 2.5h/60mA, 16h/8mA).

(A) PREPARATION OF PROTEIN SAMPLES FOR SDS/POLYACRYLAMIDE GEL ELECTROPHORESIS

Protein solutions were mixed with an equal volume of Laemmli sample buffer which contained 2% (w/v) SDS, 10% (w/v) sucrose, 0.001% (w/v) Pyronin Y and 62.5mM Tris-HCl, pH 6.8. DTT (10mM) was added to the samples which were boiled for 3 min prior to loading onto the gels.

(B) STAINING OF GELS WITH COOMASSIE BLUE

Gels were stained after electrophoresis in 0.04% (w/v) Coomassie Brilliant Blue R250, 10% (v/v) acetic acid, and 25% (v/v) methanol for 1h at room temperature with gentle agitation.

(C) DESTAINING OF GELS

Unbound stain was removed by washing the gel in 10% (v/v) acetic acid and 20% (v/v) methanol. The destain solution was changed at regular intervals until all excess stain was removed (1-2h).

(D) SILVER STAINING

Silver staining of SDS-PAGE gels was used as an alternative to Coomassie blue

staining when very small amounts of protein (0.5-2ng/band) were being analysed by gel electrophoresis. The procedure was carried out following a method described by Wray *et al.*, (1981). Protein was fixed by immersing the gel for 8h in 50% (v/v) methanol at room temperature. The gel was then placed for 20 min in staining solution consisting of 0.8% (w/v) silver nitrate, 0.21M NH_4OH and 0.08% (w/v) NaOH. The gel was washed with several changes of distilled water over the course of 1h and developer, prepared no more than 5 min previously, was added consisting of 0.24mM citric acid and 0.02% (v/v) formaldehyde. Once all protein bands could be seen, the gel was again treated with repeated washings with distilled water to remove all traces of developer.

(E) DETERMINATION OF M_r VALUES BY SDS-PAGE

The M_r values of protein species was determined by comparing their mobility to those of a set of molecular weight standards which included phosphorylase b (M_r 94,000), albumin (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 31,000), soyabean 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 yields a calibration curve from which the subunit molecular weight of the unknown species can be determined.

2.2.11 WESTERN BLOTTING (IMMUNOLOGICAL DETECTION OF SPECIFIC PROTEINS)

After proteins were separated by gel electrophoresis, they were electrophoretically transferred onto Hybond-C nitrocellulose paper in the presence of buffer containing 192mM glycine, 0.02% (w/v) SDS, 20% (v/v) methanol and 25mM Tris pH 8.3 as described by Towbin *et al.* (1979) and Batteiger *et al.* (1982). This procedure was carried out in a BioRad Trans-Blot cell either at 40mA overnight or at 400mA for 2.5h.

To check that the transfer procedure was successful, the nitrocellulose membrane was briefly stained with Ponceau S solution which allowed the visualisation of transferred proteins. The membrane was washed free of this stain with distilled water and excess binding sites were blocked by washing the membrane for 1h in wash buffer containing 0.15M NaCl, 0.5% (v/v) Tween-20 and 20mM Tris-HCl, pH 7.2. This membrane was then incubated for 90 min with the desired antibody diluted 1:100 with fresh wash buffer in the presence of 5% (v/v) heat inactivated donkey serum. After the incubation the membrane was washed with at least 4 changes of wash buffer over a period of 1h to remove any unbound antibody.

^{125}I-labelled protein A ($3x10^6$ c.p.m.) was added to the nitrocellulose membrane with 50ml of wash buffer for 1h, at the end of which the membrane was washed with at least 4 changes of wash buffer as before. This nitrocellulose immunoblot was allowed to dry in air, mounted in cassettes in close contact with X-Omat S film and exposed at -80°C for 1-7 days. After the cassette was removed from the freezer and allowed to reach room temperature, the film was removed and developed.

2.2.12 N-TERMINAL PROTEIN SEQUENCING

A conventional SDS/polyacrylamide gel of 15% (w/v) ultrapure acrylamide was prepared as described in section 2.2.10, with the exception that both the stacking and resolving gels were prepared using the resolving gel buffer and not the stacking gel buffer. The bottom reservoir was filled with normal electrophoresis buffer while the upper reservoir was filled with a 1 in 4 dilution of stacking gel buffer with 0.03mM glutathione added to remove free radicals. The gel was run at 15mA until a sample of dye reached the stacking/resolving gel interface. The reservoir buffers were then replaced by normal electrophoresis buffer containing 0.1mM sodium thioglycolate and this gel after loading of samples, was run as normal at 65mA.

Immobilon PVDF membrane was soaked in 50% (v/v) methanol for 30 min and then the resolved proteins were electrophoretically transferred as described in section

2.2.11 onto this membrane. After transfer the membrane was washed in distilled water for about 10min and then stained for 30min in special buffer 0.1% (w/v) Amido black in 50% (v/v) methanol. Using 50% (v/v) methanol, the membrane was destained and allowed to dry at room temperature and the protein band of interest then cut from the membrane for sequencing at the BBSRC sequencing facility at the University of Aberdeen.

2.2.13 PREPARATION OF ANTISERUM

Antisera to the native 2-oxoacid dehydrogenase complexes isolated from bovine heart were prepared as in the method reported by De Marcucci *et al.*, (1985). Subunit specific antisera were prepared as follows. 0.25-1.0mg of desired antigen dissolved in 0.75ml of 0.9% (w/v) NaCl mixed with 0.75ml of complete Freund's adjuvant was injected subcutaneously into rabbits at various sites in the neck, back and thighs. A booster dose was then administered four weeks later consisting of 0.1-0.5mg antigen in 0.75ml of 0.9% (w/v) NaCl and 0.75ml of Freund's incomplete adjuvant. 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 was removed using a Pasteur pipette. Antiserum was centrifuged at 700g for 5 min to remove any remaining red blood cells and stored in 1ml aliquots at -80°C. Further collections of antisera were made at monthly intervals, two weeks after additional booster injections, using the regime described above.

2.2.14 PREPARATION OF CNBr-ACTIVATED SEPHAROSE 4B AFFINITY COLUMN

CNBr-activated Sepharose 4B is a preactivated gel for immobilisation of proteins, peptides and nucleic acids. Here purified E2/X core from mammalian PDC was coupled to this preactivated gel matrix and employed as an affinity column in the

purification of E3 from plant sources.

The required amount of CNBr-activated Sepharose 4B was weighed out (with 1g of the freeze-dried powder giving about 3.5ml final vol of gel) and suspended in 1mM HCl. The gel swelled immediately and was washed with at least 10 column volumes of 1mM HCl on a sintered glass filter. E2/X (1-2mg protein/ml of gel) was then dissolved in coupling buffer containing 0.5M NaCl, 0.1M $NaHCO_3$, pH 7.5 and mixed with the gel in a stoppered vessel. This mixture was rotated end over end for 2h at room temperature. Uncoupled protein was removed by washing the gel with at least 5 column volumes of coupling buffer. To block any remaining active groups the gel was washed with 5 column volumes of 25mM Tris-HCl buffer, pH 8.0, and left at 4°C overnight. The next day the gel was poured into a column and washed with sample buffer containing 1mM EDTA, 20mM NaCl, 50mM potassium phosphate buffer, pH 7.6. This column used for the purification of E3 was stored at 4°C in sample buffer with the addition of 0.1mM sodium azide.

CHAPTER 3

PURIFICATION OF DIHYDROLIPOAMIDE DEHYDROGENASE FROM POTATO AND PEA

3.1 INTRODUCTION

Dihydrolipoamide dehydrogenase (E3) is a member of the group of flavin-containing pyridine nucleotide-disulphide oxidoreductases (Carothers *et al.*, 1989). This group also includes: thioredoxin reductase, bis-γ-glutamyl cystine reductase, glutathione reductase, trypanothione reductase, pantethine 4'4"-diphosphate reductase and mercuric reductase (Pigiet & Conley, 1977; Sundquist & Fahey, 1988; Shames *et al*, 1986; Swerdlow & Setlow, 1983; Fox & Walsh, 1983). All these enzymes are homodimers containing 1 flavin adenine dinucleotide (FAD) per subunit and a redox-active disulphide. They are resistant to heat and proteolysis and are sensitive to some divalent cations (e.g., Hg^{2+}, Cu^{2+}) and some arsenical derivatives (Holmgren, 1980; Knowles, 1985; Williams, 1976). In eukaryotes and eubacteria, dihydrolipoamide dehydrogenase is an integral component of the 2-oxoacid dehydrogenase complexes and the glycine decarboxylase complex. It catalyses the NAD^+ dependent reoxidation of dihydrolipoamide bound to the acyltransferase (E2) component of the 2-oxoacid dehydrogenase complexes (Yeaman, 1989) or the hydrogen-carrier protein (H protein) of the glycine decarboxylase complex (Kikuchi & Hiraga, 1982; Douce *et al.*, 1994).

The refined crystal structures of dihydrolipoamide dehydrogenase of *Azotobacter vinelandii* (Mattevi *et al.*, 1991) and *Pseudomonas putida* (Mattevi *et al.*, 1992) have been obtained and provided much information on E3. Each monomer of the E3 dimer comprises four domains with the catalytic centre located at the interface between the two subunits. The flavin ring which separates the NAD^+ and the dihydrolipoamide binding sites, has an adjacent disulphide bridge generating the redox centre involved in the electron transfer reaction between the substrates, dihydrolipoamide and NAD^+.

E3 has been isolated and characterised from a wide variety of prokaryotic and eukaryotic species, with *E. coli* and pig heart E3s being the most extensively studied of them all. The cloning and sequencing of these enzymes as well as other E3s has provided much information which allows us to make comparisons between them (Stephens *et al.*, 1983; Otulakowski & Robinson, 1987). The amino acid sequences

of E3 from *E. coli*, yeast, pig and human have shown considerable conservation of amino acid sequence, in particular in the specific catalytic and structural domains (Carothers *et al.*, 1989). Until recently, it was believed that dihydrolipoamide dehydrogenase was the identical gene product in all members of the 2-oxoacid dehydrogenase family and possibly also the glycine decarboxylase complex from mammalian and bacterial sources. For example, a single gene, the *lpd* gene in *E. coli*, encodes for E3 of both pyruvate dehydrogenase complex (PDC) and 2-oxoglutarate dehydrogenase complex (OGDC)). However, there is now increasing evidence for the existence of isoenzymes of E3 in some species (see section 4.1).

In *Trypanosoma brucei*, E3 has been found in the mammalian bloodstream form of this parasitic protozoan, despite the absence of functional mitochondria and also, therefore, the multienzyme complexes with which E3 is normally associated. This E3 enzyme is also found in an unusual location, being loosely associated with the inner surface of the plasma membrane and it is thought that it may be involved in the shuttling of sugars across the membrane (Richarme & Heine, 1986). The exact physiological function of this enzyme is still unknown, although kinetic analysis and chemical modification studies indicate it to be a true dihydrolipoamide dehydrogenase. In the insect procyclic form of *T. brucei*, E3 can be found in the mitochondrion and is probably associated with pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes present there also (Danson *et al.*, 1987; Jackman *et al.*, 1990; Cook *et al.*, 1990). Therefore it appears that this species of E3 can exist both independently and associated with the multienzyme complexes. Interestingly, it appears that both forms are encoded by the same gene and that there is little if any difference between the two enzymes (Else *et al.*, 1994).

As is evident from the previous discussion, dihydrolipoamide dehydrogenase is a very thoroughly investigated protein, however, very little information is available on this enzyme from plant sources. In comparison to their mammalian and bacterial counterparts, there is greater difficulty in purifying these multienzyme complexes and their associated E3 components from plants. This is owing to the fact that there is a

low abundance of mitochondria per unit of fresh weight plant material (Randall *et al.*, 1990). Mitochondrial PDC has only ever been purified to homogeneity on the one occasion, where 1mg of pure mitochondrial PDC was purified from 500kg of broccoli (*Brassica oleracea*) florets (Rubin & Randall, 1977). Interestingly, no SDS-PAGE analysis or definitive subunit composition was provided in this particular publication. In addition to the difficulty in purifying plant mitochondria, large amounts of phenols and endogenous proteases are present in plant tissue which cause major difficulties in the purification of intact multienzyme complexes and other plant proteins in general.

PDC is the most extensively characterised of the plant 2-oxoacid dehydrogenase complexes. Plant PDC is unusual in that there are two distinct spatially separated types, one located in the mitochondrial matrix and the other in the plastid stroma. Plant mitochondrial PDC, like mammalian PDC, acts as the primary entry point of carbon into the tricarboxylic acid cycle. The subunit and cofactor requirements of plant mitochondrial PDC are typical of the complex from non plant sources. However plastid PDC has different structural, catalytic and regulatory characteristics and is thought to provide acetyl CoA and NADH for fatty acid and isoprenoid biosynthesis, see section 1.5.3 (Camp & Randall, 1985 & Randall *et al.*, 1989). Plant OGDC, a component enzyme of the TCA cycle itself, has only been identified in mitochondria where it displays approx. 20% the activity of the mitochondrial PDC (Cho *et al.*, 1988).

It has been reported that BCDC is absent from plant mitochondria and plastids and instead located in peroxisomes (Gerbling & Gerhardt 1988). More recent research, however, indicates the possibility that BCDC (branched chain 2-oxoacid dehydrogenase complex) 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). GDC, like PDC, 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 & Oliver, 1986; Bourguignon *et al.*, 1988 & Oliver *et al.*,

1990). GDC plays an important role in photorespiration in plants where it catalyses the decarboxylation of glycine.

Two cDNA clones have been isolated and characterised from pea leaf/embryo λgt11 expression libraries and said to represent dihydrolipoamide dehydrogenase (L protein) of glycine decarboxylase complex (Bourguignon *et al.*, 1992; Turner *et al.*, 1992) In pea mitochondria, it appears that pyruvate dehydrogenase complex, 2-oxoglutarate dehydrogenase complex and glycine decarboxylase complex all use the same dihydrolipoamide dehyrdogenase component. This chapter deals with the purification of E3 from potato and pea leaf/root mitochondria and chloroplasts, except that total E3 present in these organelles was purified rather than E3 from individual multienzyme complexes (PDC, OGDC and GDC).

3.2 RESULTS AND DISCUSSION

3.2.1 ISOLATION OF ORGANELLES

Mitochondria and chloroplasts from potato (*Solanum tuberosum*, cv. Maris Piper) and pea (*Pisum sativum* L., cv. Little Marvel) were isolated as described in Materials and Methods (section 2.2.2). These procedures were based on differential centrifugation, with mitochondria being further purified on self generating Percoll gradients. These methods of organelle isolation have been tested for purity of sample (A. Carmichael, Ph.D. thesis, Glasgow University, 1994). Each preparation was assessed for cross-contamination using marker enzymes for mitochondria (succinate dehydrogenase), peroxisomes (hydroxypyruvate reductase) and chloroplasts (ADP-glucose pyrophosphorylase). In addition to this, the purity of the isolates was assessed by electron microscopy. It has been shown that the individual organelle preparations were relatively free from contaminating species and a high level of organelle intactness was maintained. However, there was slight contamination from broken membranes which may have originated from ruptured tonoplast, plasmalemma or other ruptured organelles.

3.2.2 RELEASE OF THE MULTIENZYME COMPLEXES FROM PLANT MITOCHONDRIA AND CHLOROPLASTS

To release the multienzyme complexes, and therefore the E3 associated with them, the plant organelles have to be ruptured. Samples were frozen by placing in dry ice and methanol for approx. 2 min and allowed to thaw at room temperature. This procedure was repeated 4 times and then the membrane fractions removed by centrifugation at 12,000g for 10 min. Alternatively the organelles could be ruptured by solubilising in 0.1% (v/v) Triton X-100. After the addition of the detergent Triton X-100, the samples were left on ice for 30min and then were centrifuged as before at 12,000g for 10 min to remove the membrane fractions. The use of Triton X-100 to rupture the organelles was found to be more effective in the release of E3, yielding approx. twice the amount of enzyme as compared to the cyclical freeze-thaw treatment. This reflects a similar situation in mammalian mitochondria where E3 is associated with the multienzyme complexes located here (PDC and OGDC), which are in turn tightly bound to the mitochondrial membrane. As can be seen from Materials and Methods (section 2.2.4), Triton X-100 is also employed in the efficient release of these multienzyme complexes from mammalian mitochondria.

3.2.3 PURIFICATION OF DIHYDROLIPOAMIDE DEHYDROGENASE

(A) HEAT TREATMENT OF E3

As mentioned in the introduction to this chapter, E3 from a variety of sources is resistant to heat. It was therefore an obvious first stage in the purification of E3 to expose the plant enzyme to high temperatures, thus allowing its release from the plant complexes, assuming a similar heat stability in the plant E3 enzyme. Another advantage to this method was that by exposing the organellar extract to high temperatures, it would also inactivate proteases present in the sample. This was performed by placing the samples in a water bath at 65°C for 10 min. Since many

proteins present in the crude extract of E3 are not resistant to heat, they are precipitated and can be removed by centrifuging the samples at 12,000g for 10 min. This step in the purification of E3 did not result in any significant loss of E3 activity but a 20-50% loss in protein (Table. 3.1). In fact the plant enzyme is so heat resistant, that E3 from pea leaf mitochondria and chloroplasts can be incubated at 65°C for 1h to remove the majority of contaminating proteins and chlorophyll with no loss in E3 activity (personal communication, M. Conner, Glasgow University).

(B) E2/X AFFINITY CHROMATOGRAPHY

It is desirable the purification of the E3 enzyme involves as few steps as possible since there is an inevitable loss of E3 at every stage. This is especially important when purifying plant enzymes, as there are extremely limited amounts of the enzyme of interest available at the start of the purification procedure compared to enzymes from mammalian sources. A novel affinity column was devised in order to purify E3 from potato and pea in a one-step process. The structure of mammalian PDC is such that 60 E2 polypeptides form a pentagonal dodecahedral core to which six E3 homodimers and thirty E1 tetramers bind non covalently. Under conditions of high salt, the E1 and E3 components of bovine heart PDC are dissociated from the E2/X core assembly, and this immobilised core assembly can be employed as an affinity column for the purification of E3. As E3 is such a highly conserved enzyme, it is feasible that E3 from plant sources can still recognise and bind with sufficient affinity to the E2/X core assembly of mammalian PDC to permit its one step purification from a relatively crude (heat treated) mitochondrial extract. Intact plant 2-oxoacid dehydrogenase and glycine decarboxylase complexes would not be present in the crude E3 extract, since they would have been dissociated by the heat treatment prior to application to the affinity column.

	Volume (ml)	mg/ml	Total mg	Enzyme units/ml	Total units	% recovery	Specific activity	Purification factor
Triton X-100 extract	7.90	1.83	14.50	5.20	41.10	100.00	2.80	1.00
Triton X-100 extract + heat	7.50	1.20	9.00	5.10	38.3	93.00	4.30	1.50
Affinity column pool	2.20	0.10	0.22	9.80	21.60	53.00	98.20	35.00

Table 3.1: Purification table of potato mitochondrial E3

E3 assays and determination of protein concentrations were carried out as described in Materials and Methods (sections 2.2.7(B) and 2.2.9).

Approximately 42% of mammalian PDC is composed of E2/X and 7% is E3 by weight. From these values the theoretical binding capacity of E2/X for E3 was calculated, with 1mg of E2/X having the potential for binding a maximum of 0.17mg of E3. However this value does not take into consideration the residual contamination of the E2/X core by E1. In addition a proportion of the binding sites on the immobilised E2/X core may also be inaccessible to E3 and the coupling procedure itself may actually inactivate some of the E2/X, rendering it incapable of interacting with E3. Therefore, the true binding capacity of E2/X for E3 will be somewhat lower than its theoretical value.

Bovine heart PDC was prepared as described in Material and Methods (section 2.2.4.) An equal volume of running buffer containing 4M NaCl, 1mM DTT, 0.01% (v/v) Triton X-100, 50mM Tris-HCl, pH 9.0 was added to a 1ml sample of PDC (approx. 30mg/ml) so that the final concentration of NaCl was 2M. This sample was then incubated at 4°C for 1h prior to centrifugation at 6250g for 5min to remove any non-soluble protein that may cause any blockage during subsequent FPLC analyses. A Superose 6 FPLC column (100ml) was equilibrated with running buffer plus 1M NaCl, then pre-injected with 4ml of the running buffer containing 4M NaCl prior to the loading of the PDC sample. The column was run in running buffer containing 1M NaCl with the high M_r E2/X oligomeric core found to elute at the void volume (approx. 32ml) and the E1/E3 components eluting at approx. 62ml (Fig. 3.1). This E2/X core assembly was then dialysed into a buffer suitable for coupling it to CNBr-Sepharose 4B as described in Materials and Methods (section 2.2.14), and this was then used as the affinity column.

(C) ANALYSIS OF BOVINE HEART MITOCHONDRIAL E3 BINDING TO THE E2/X AFFINITY COLUMN

Bovine heart mitochondrial E3 was used to test the suitability of the affinity column for the purification of the E3 component. It was much easier to prepare large amounts of crude E3 from mammalian sources than plant sources. Bovine heart mito-

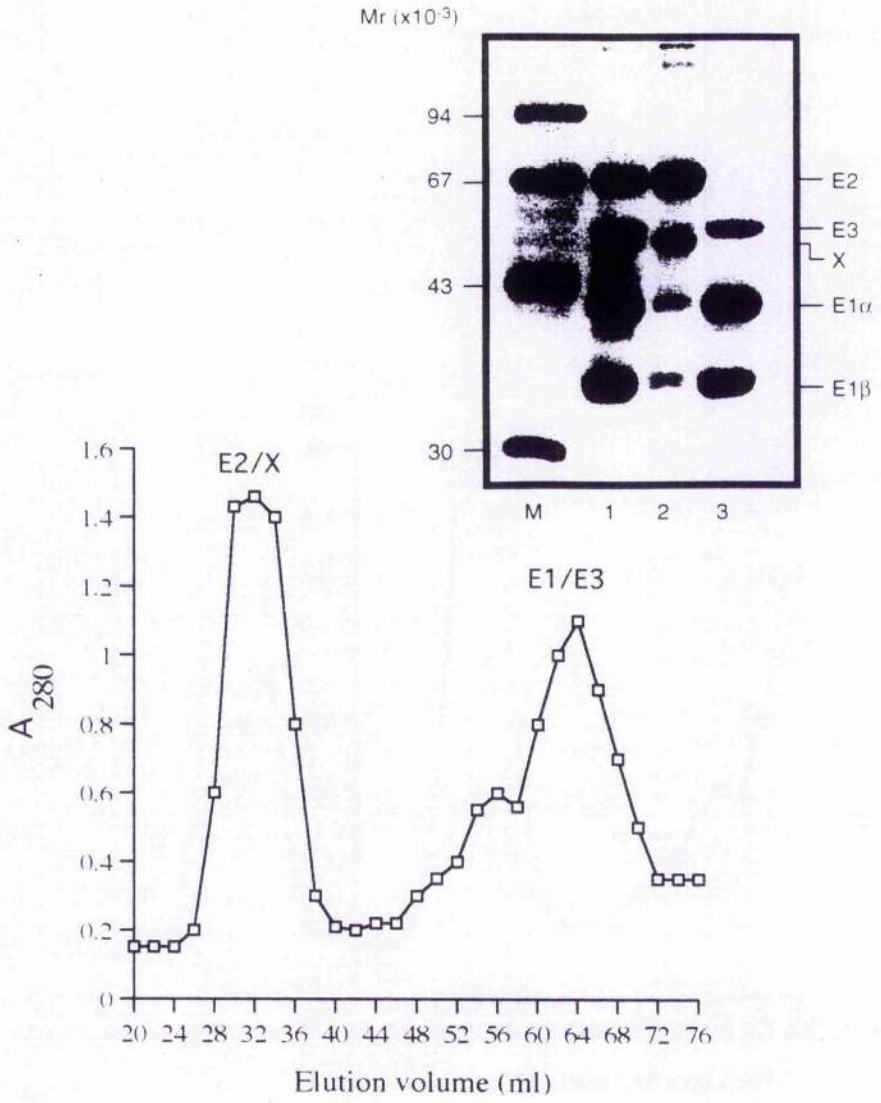


Figure 3.1: Dissociation of bovine heart PDC.

PDC (30mg) was treated with 2M NaCl prior to loading onto a 100ml Superose 6 FPLC column. Running buffer (1mM DTT, 1M NaCl, 0.01% (v/v) Triton X-100 and 50mM Tris-HCl, pH 9.0) was pumped through the column at a flow rate of 1ml/min. Fractions (2ml) were collected and their absorbance measured at 280nm.

Inset: SDS-PAGE analysis of dissociated PDC (Lane M, M_r marker proteins; Lane 1, bovine heart PDC; Lane 2, isolated E2/X core; Lane 3, dissociated E1/E3)

chondria were prepared as described in Materials and Methods (section 2.2.5). E3 was released from the mitochondria by the addition of Triton X-100 and heat treated (65°C/10 min) as described in sections 3.3 and 3.4.1. This crude extract of E3 was dialysed into sample buffer containing 1mM EDTA, 20mM NaCl, 50mM KPi buffer pH 7.6 and then applied to an affinity column (10ml, 1cm x 12.7cm), with approx. 9mg of E2/X coupled to it, which had been equilibrated with at least 5 column vol of sample buffer prior to loading. Optimal binding was observed when the E3 sample was retained on the column for 5-10 min, after allowing the sample to run into the gel matrix, permitting time for interaction with subunit binding domains on the immobilised E2/X core assembly. The column was then washed with 5 column vol of sample buffer to remove any unbound proteins. Bound proteins were then eluted with high ionic strength eluting buffer containing 1mM EDTA, 1M NaCl, 50mM KPi pH 7.6. Fractions (1ml) were collected and assayed for E3 activity as described in Materials and Methods (section 2.2.7 (B)).

The heat treated bovine heart mitochondrial extract, approx. 20 units of E3 enzyme activity (1 unit = 1μmol NADH/min), was loaded onto the column in a total vol of 0.5ml. A small amount of E3 (15%) did not bind to the E2/X affinity column; however the vast majority of the E3 activity (84.1%) eluted as a single peak in the presence of 1M NaCl (Fig. 3.2). For further analysis, samples were then TCA precipitated, resuspended in Laemmli sample buffer and separated by SDS-PAGE (Fig. 3.3). This procedure was carried out as described in Materials and Methods (sections 2.2.6 and 2.2.10). It is evident from inspection of the SDS polyacrylamide gel that the E3 component represents one of several prominent species in the heat treated mitochondrial extracts (lanes 4 & 5), but is exclusively retained on the E2/X affinity matrix (lanes 6 & 7) and is subsequently eluted with buffer containing 1M NaCl (lanes 8 & 9). In the E3 bound fraction, only one major band (M_r 55,000) could be visualised. This corresponds with the subunit M_r value for porcine heart E3 present as a marker (lanes 1 & 10). Indeed, when commercially prepared porcine heart E3 was applied to the column it also bound and could be eluted under the same conditions as

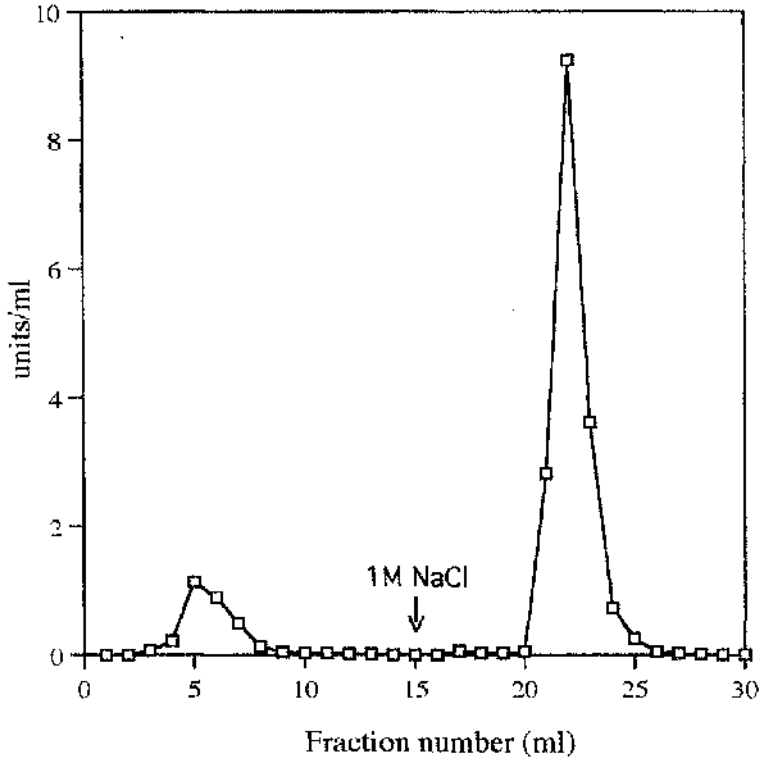


Figure 3.2: Isolation of bovine heart mitochondrial E3 on E2/X affinity column.

Approx. 1.85mg of heat treated (65°C/10min) crude extract of bovine heart E3 was loaded onto an affinity column (10ml, 1cm x 12.7cm). The column was washed with sample buffer (20mM NaCl, 1mM EDTA, 50mM KPi pH 7.6). Fractions (1ml) were collected and assayed for E3 activity and expressed as units of enzyme activity/ml.(1unit=1μmol NADH/min) Bound protein was eluted with sample buffer containing 1M NaCl and each fraction assayed for E3 activity.

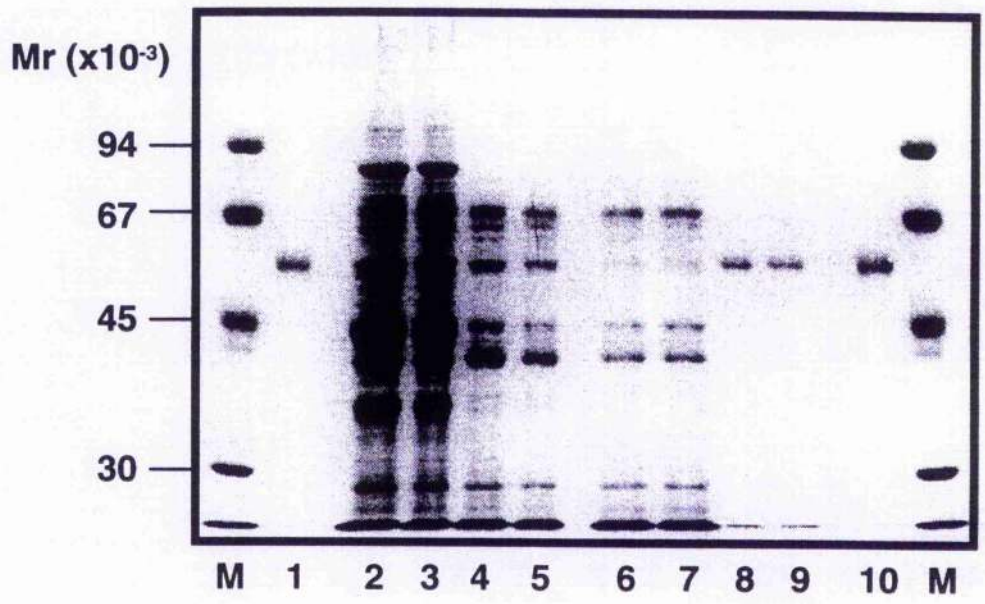


Figure 3.3: Analysis of purification of bovine heart mitochondrial E3 by SDS-PAGE.

Protein from various steps in E3 purification on the E2/X affinity column from bovine heart mitochondria was electrophoresed in 10% (w/v) SDS polyacrylamide slab gels and stained with Coomassie blue. Lane M, Mr marker proteins; lanes 1 and 10, purified pig heart E3; lanes 2 and 3, crude mitochondrial extract; lanes 4 and 5, heat treated (65°C/10min) mitochondrial extract; lanes 6 and 7, unbound protein; lanes 8 and 9, peak E3 activity fractions from eluant (see Figure 3.2).

the bovine heart E3. These results indicate that the E2/X affinity column provides a convenient approach in the purification of heterologous E3s, at least from mammalian sources. The column could also be used repeatedly remaining stable for approx. six months, although the initial capacity of the column which was determined to be approx. 1mg of bovine E3, was found to decline gradually over several months. It was treated with eluting buffer containing 2M NaCl between runs so as to ensure that no protein remained bound to the column that could contaminate the next sample. The next step was then to attempt the purification of E3 from plant sources, namely potato and pea.

(D) PURIFICATION OF POTATO TUBER MITOCHONDRIAL E3 BY E2/X AFFINITY CHROMATOGRAPHY

E3 was extracted from mitochondria with 0.1% (v/v) Triton X-100 and heat treated at 65°C/10min as before. This resulted in a 1.5-fold purification of E3, even before the use of the affinity column (Table 3.1). It has since been discovered that this purification factor can be improved further by increasing the length of time the sample is exposed to 65°C as described in section 3.4.1. After dialysis into sample buffer containing 1mM EDTA, 20mM NaCl and 50mM KPi, pH 7.6, the E3 sample was loaded onto the pre-equilibrated affinity column. Unbound protein was then washed through the column with 5 vol of sample buffer and bound proteins were released from the column matrix in eluting buffer containing 1mM EDTA, 1M NaCl and KPi buffer, pH 7.6.

As with the bovine and pig heart E3s, the majority of the potato mitochondrial E3 activity (86.7%) loaded onto the affinity column bound to it in low ionic strength buffer but was released subsequently in the presence of 1M NaCl (Fig. 3.4). Peak E3 fractions were pooled and concentrated by the use of Centricon-10 tubes as described in Materials and Methods (section 2.2.6). A small portion of these samples were subjected to TCA precipitation and analysed by SDS-PAGE (Fig. 3.5). Lane 4 represents a sample from the pooled fractions 4 to 8 (inclusive) and lane 5 represents a sample taken from the pooled fractions of 24 to 28 (inclusive). As can be seen from

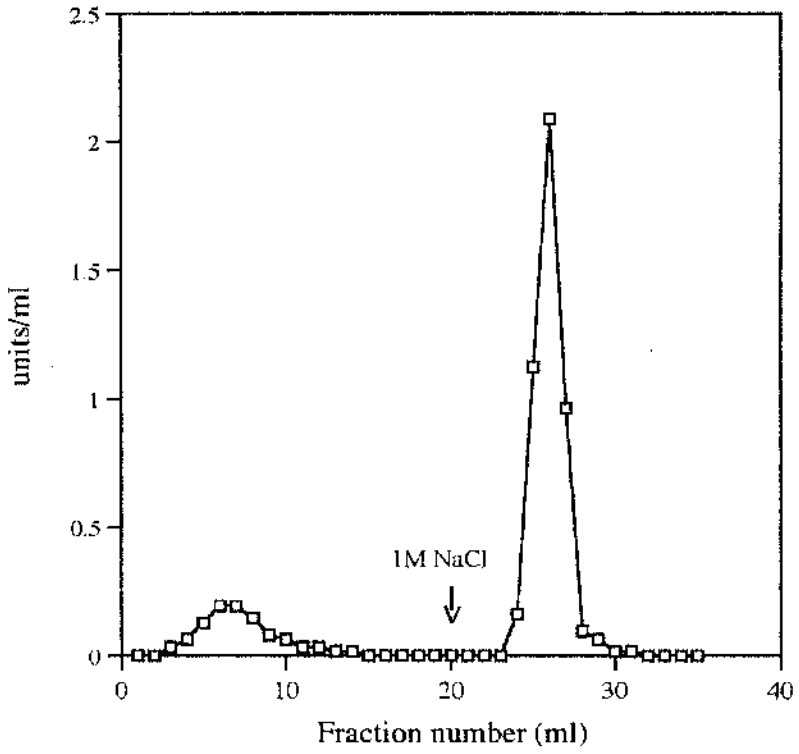


Figure 3.4: Purification of potato E3 on E2/X affinity column.

Approx. 15.7mg of heat treated (65°C/10 min) crude extract of potato tuber mitochondrial E3 was loaded onto an affinity column (10ml, 1cm x12.7cm). The column was washed with sample buffer (20mM NaCl, 1mM EDTA, 50mM KPi pH 7.6). Fractions (1ml) were collected and assayed for E3 activity and expressed as units of enzyme activity/ml (1unit=1μmol NADH/min). Bound protein was eluted with sample buffer containing 1M NaCl and each fraction assayed for E3 activity.

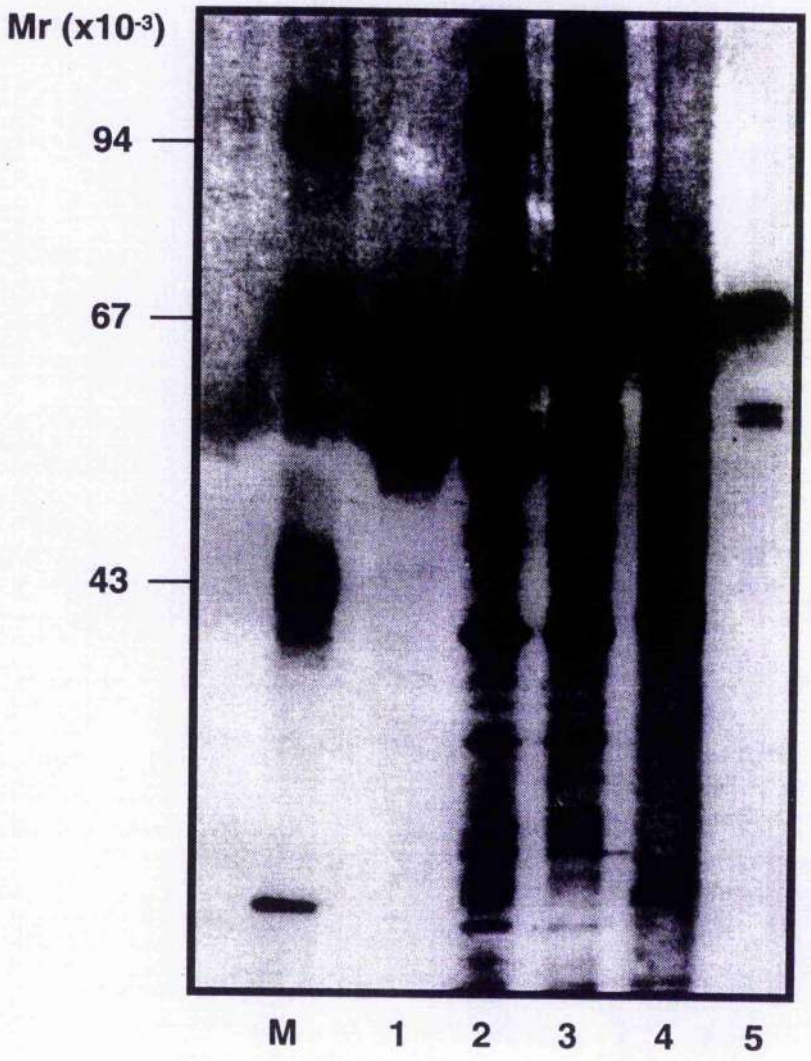


Figure 3.5: Analysis of purification of potato tuber mitochondrial E3 by SDS-PAGE.

Protein from various steps in E3 purification on the E2/X affinity column from potato tuber mitochondria was electrophoresed on a 10% (w/v) SDS polyacrylamide slab gel and silver stained. Lane M, molecular weight marker proteins; lane 1, purified pig heart E3; lane 2, crude potato mitochondrial extract; lane 3, heat treated mitochondrial extract (65°C/10min); lane 4, unbound protein; lane 5, peak E3 activity fractions from eluant (see Figure 3.4).

this gel, the bound E3 fraction in lane 5 has 2 distinct protein bands with M_r of 58,000 and 56,000. Either both bands represent different forms of E3 or another protein, present in the crude mitochondrial E3 sample, was able to recognise and bind to the E2/X core assembly. A band with a subunit M_r value of about 67,000 can also be observed, not only in every lane, but between them as well. This band is generally believed to be an artefact since it is very commonly observed by silver staining plant extracts after resolution by SDS-PAGE. It has been suggested that this artefact is caused by keratin contamination of the gel buffers, although in our experiments it is most prominent in tracks containing plant mitochondrial extracts as compared to chloroplast samples. When purified potato E3 was exposed to anti-E3 IgG raised to the pig heart enzyme in Western blot studies, it cross reacted strongly displaying a band with a subunit M_r of approx. 56,000 (see chapter 4, Fig 4.4). It is difficult to detect if both bands or only a single species has cross reacted with the E3 antiserum owing to the lack of resolution of the bands following detection of immune complexes with ^{125}I-labelled protein A in normal exposure of the immunoblot. However, with short exposure times (not shown) it was clear that both these subunits cross-reacted strongly and with similar intensity with anti-E3 serum.

From the SDS-polyacrylamide gel analysis (Fig. 3.5), it can be seen that the affinity column has succeeded in purifying the E3 component successfully. Track 4 showing the column wash fractions, i.e. the unbound proteins, has many distinct proteins present, whereas the E3 bound fraction has only the 2 major bands with Mr values of approx. 58,000 and 56,000. From the purification table (Table 3.1), it can be seen that the E2/X affinity column has succeeded in purifying the potato mitochondrial E3 to at or near homogeneity in a single step (35-fold purification). The percentage recovery of E3 was 53%; this can be partly explained by the fact that approx. 19% of the E3 loaded onto the column failed to bind (Fig. 3.4). In addition, there is some loss in E3 activity during the concentration procedure, with some protein absorbing to the sides of the centricon tube. This loss is correspondingly greater during concentration of small amounts of protein as is usually the case when dealing with plant extracts.

However, this E2/X affinity chromatography has proved to be a very successful one step procedure for the purification of E3. As mentioned earlier it is especially important when dealing with plant enzymes to have as few steps involved in their purification as possible to prevent inevitable loss of enzyme at each stage.

(E) ATTEMPTED PURIFICATION OF PEA ROOT MITOCHONDRIAL E3 BY E2/X AFFINITY CHROMATOGRAPHY

Mitochondria were isolated from pea roots following the method descibed in Materials and Methods (section 2.2.2 (A)) and E3 extracted by the use of 0.1% (v/v) Triton X-100. As with the bovine and potato mitochondrial E3, this crude extract was heat treated at 65°C for 10 min and denatured protein removed by centrifugation. The pea mitochondrial E3 sample was dialysed into buffer containing 1mM EDTA, 20mM NaCl and 50mM KPi buffer, pH 7.6 and 0.4ml of sample containing 2.9 units of enzyme activity was loaded onto the pre-equilibrated affinity column. The column was washed with sample buffer to remove any unbound proteins and then the eluting buffer containing 1mM EDTA, 1MNaCl and 50mM KPi pH 7.6 was applied to remove proteins which specifically bound at low ionic strength.

When the fractions (1ml) were assayed for E3 activity, it was found that the E3 did not bind stably to the E2/X affinity column (Fig. 3.6). However, there was a high degree of interaction between pea mitochondrial E3 and the column matrix. E3 activity was significantly retarded, gradually eluting with wash buffer over a wide range of fractions with the mean peak of E3 activity eluting between 14 and 16 ml which is significantly greater than the total column volume. A small amount was finally released on treatment with high ionic strength buffer. If the E3 enzyme had failed to interact with the column, then it would be expected to peak at its characteristic elution volume (Ve) at approx. 7-8ml since the Sepharose 4B matrix also exhibits the properties of a gel filtration matrix under these conditions. The conditions used here were identical to that of section 3.4.5 where potato mitochondrial E3 was successfully purified on the affinity column, yet it failed to bind and therefore purify the pea mitochondrial E3.

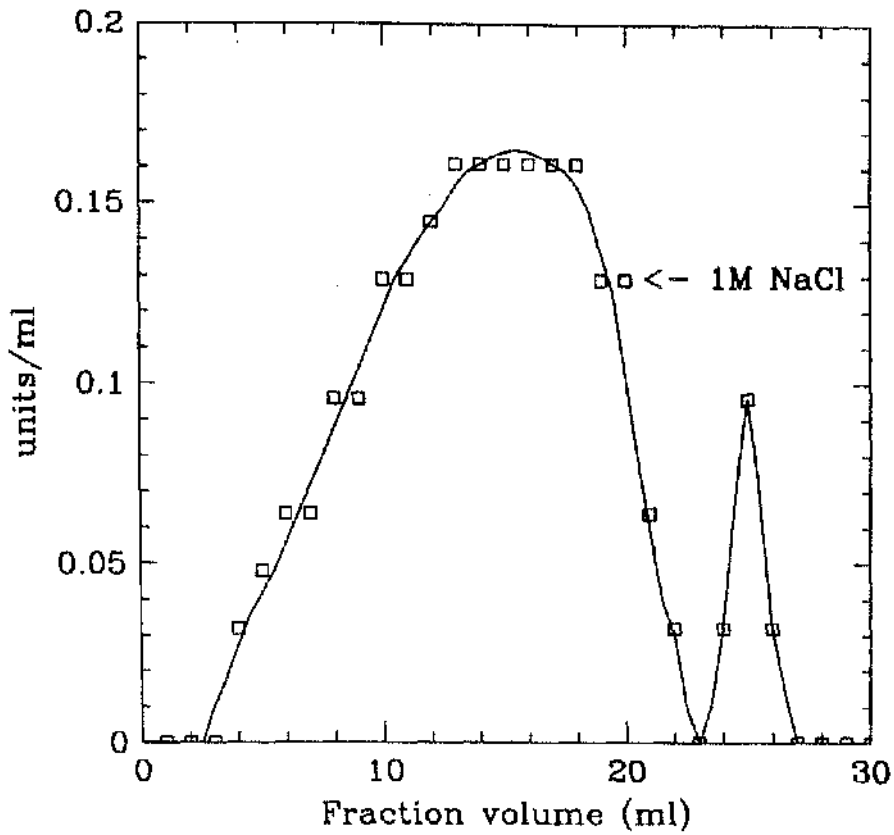


Figure 3.6: Elution profile of pea root mitochondrial E3 on E2/X affinity column.

Approx. 5mg of heat treated (65°C/10 min) crude extract of pea mitochondrial E3 was loaded onto an affinity column (10ml, 1cm x 12.7cm). The column was washed with sample buffer (20mM NaCl, 1mM EDTA, 50mM KPi pH 7.6). Fractions (1ml) were collected and assayed for E3 activity and expressed as units of enzyme activity/ml (1unit=μmol NADH/min) Bound protein was eluted with sample buffer containing 1M NaCl and each fraction assayed for E3 activity.

This would suggest that mitochondrial E3 differs sufficiently between different plant species so as to affect its recognition and binding to the mammalian PDC E2/X core assembly.

(F) ELUTION PROFILE OF PEA LEAF CHLOROPLAST ON E2/X AFFINITY CHROMATOGRAPHY

Chloroplasts were isolated from pea leaf as described in Materials and Methods (section 2.2.2 (B)). E3 was extracted with 0.1% (v/v) Triton X-100 and heat treated at 65°C for 10 min as described before. The chloroplast E3 sample was then dialysed into sample buffer containing 1mM EDTA, 20mM NaCl and KPi pH 7.6 and 0.8ml of this sample containing 1.48 units (μmol NADH/min/ml) of activity was loaded onto the affinity matrix. This time, however, E3 eluted in a single peak with maximal elution occurring at 7-8ml, the expected elution volume for E3 exhibiting little or no interaction with the column matrix (Fig. 3.7). Some retardation of E3 was evident from the trailing shoulder following elution of the main E3 peak, possibly owing to mitochondrial contamination.

These results indicate that only the mammalian and potato mitochondrial E3 will successfully bind to the affinity column and that the pea mitochondrial and chloroplast E3 appear to differ significantly in their abilities to interact with immobilised E3. This provides some indication that dihydrolipoamide dehydrogenase in plants from different species differ significantly in their biochemical properties. It would also appear that E3 in plants may also be organelle specific since there seems to be a greater retardation of pea root mitochondrial E3 on the affinity column than pea leaf chloroplast E3, providing preliminary evidence that these two activities have distinctive properties. Both were applied to the same E2/X affinity column under identical conditions, however, whereas the peak of elution of pea mitochondrial E3 was markedly retarded by the immobilised E2/X core indicating a significant degree of interaction, the majority of the E3 activity in pea chloroplast extracts was eluted immediately with a Ve characteristic of 'free' E3.

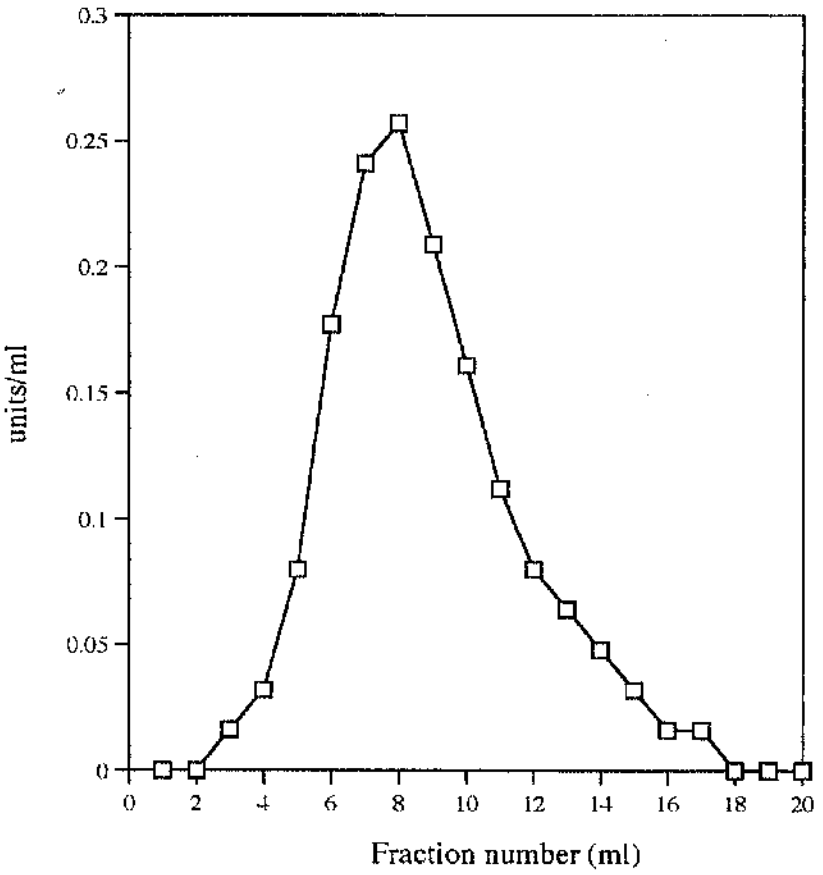


Figure 3.7: Elution profile of pea leaf chloroplast E3 on E2/X affinity column.

Approx. 2mg of heat treated (65°C/10 min) crude extract of pea chloroplast E3 was loaded onto an affinity column (10ml, 1cm x 12.7cm). The column was washed with sample buffer (20mM NaCl, 1mM EDTA, 50mM KPi pH 7.6). Fractions (1ml) were collected and assayed for E3 activity and expressed as units of enzyme activity/ml (1unit=1μmol NADH/min).

There is additional evidence available to confirm that the pea mitochondrial and chloroplast E3 are indeed different enzymes. In Western blot studies the pea mitochondrial E3 was shown to be a distinct band of M_r 58,000 and pea chloroplast E3 to have an M_r of 52,000 (Taylor *et al.*, 1992; A. Carmichael. Ph.D. Thesis, Glasgow University. 1994).

3.2.4 THE EFFECT OF SALT (NaCl) CONCENTRATION ON E3 ACTIVITY

To determine whether the biochemical properties of the various E3s differed significantly, the effect of salt concentration on E3 activity in bovine heart, potato and pea mitocondria and pea chloroplasts was investigated. Heat-treated E3 extracts from these sources were exposed to increasing concentrations of NaCl in their assay buffer and the effect on activity measured and expressed as a percentage of E3 activity in absence of salt. (Fig. 3.8). Bovine heart mitochondrial E3 was found to be the least sensitive to salt, with inhibition starting at 0.5M NaCl and about 50% of its activity remaining even at concentrations of 3.5M NaCl. At low NaCl concentrations (0-0.25M), pea root, potato tuber mitochondrial E3 and pea leaf chloroplast E3 display similar susceptibilities to NaCl treatment. Above 0.25M NaCl, E3 from pea and potato mitochondria were more severely inhibited than that from pea chloroplasts. At 3.5M NaCl only about 10% of the plant mitochondrial E3 activity remained, whereas 24% of the pea chloroplast E3 activity remained. These results lend weight to the conclusion that plant E3s are both organelle and species specific as discussed in section 3.4.7.

3.2.5 MOLECULAR WEIGHT DETERMINATION OF POTATO MITOCHONDRIAL E3 BY GEL FILTRATION

The M_r value of potato tuber mitochondrial E3 was determined by gel filtration techniques using a Pharmacia FPLC Superose 6 column. The standard molecular weight markers were; Blue Dextran (M_r 2,000,000); β amylase (M_r 200,000); alcohol

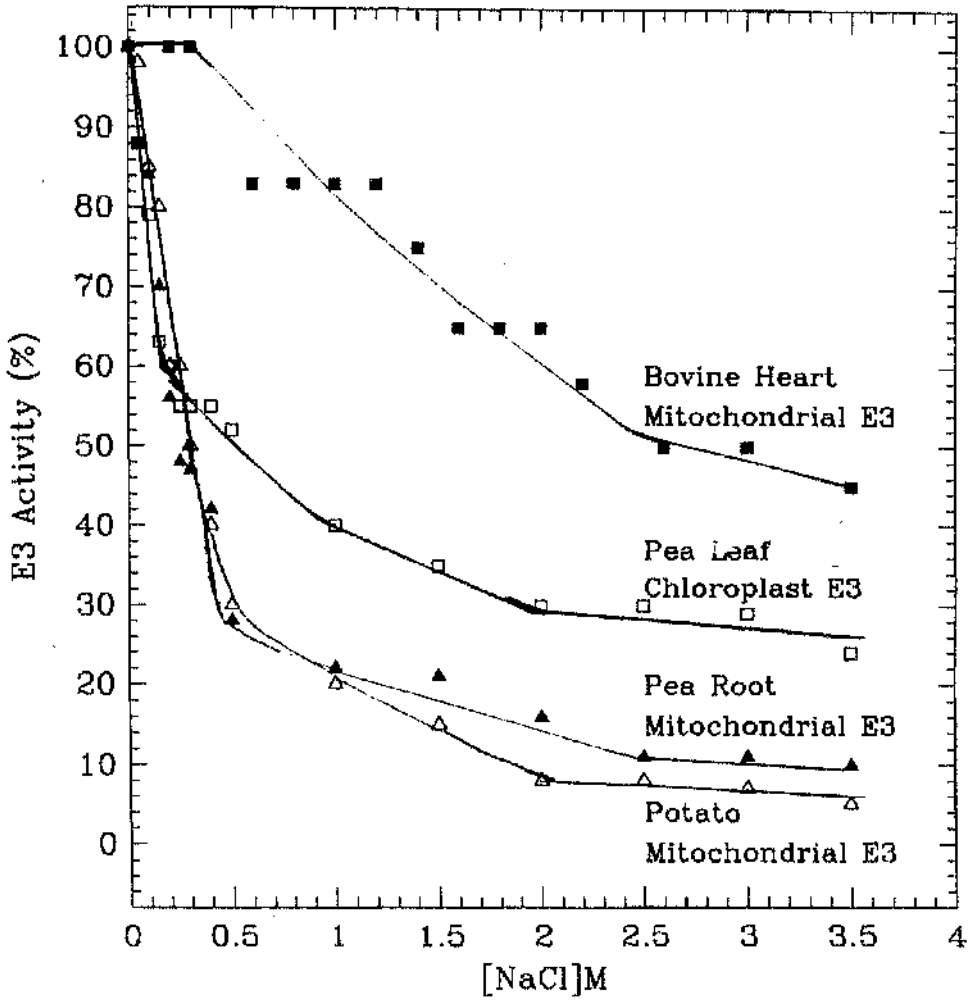


Figure 3.8: The effect of NaCl on E3 activity.

Samples of E3 from bovine heart, potato tuber and pea root mitochondria and pea chloroplasts were assayed as described in section 2.2.7(B) after a 5min preincubation at 31°C in the presence of increasing concentrations of NaCl as denoted in the graph. The reaction was initiated by addition of 20µg of dihydrolipoamide.

dehydrogenase (M_r 150,000); albumin, bovine serum (M_r 66,000); carbonic anydrase (M_r 29,000) and cytochrome c (M_r 12,400). Blue Dextran (2mg) was loaded onto the column and eluted at 1ml/min in running buffer containing 25mM NaPi and 0.1M NaCl pH 7.5 and its elution volume determined by measuring the A_{280} for each of the 1ml fractions collected. This volume represented the void volume of the column. The elution volume was then determined in the same way for the other molecular weight markers. The ratio of elution volume to the void volume (Ve/Vo) was then plotted against the log of their M_r values and a standard curve drawn (Fig. 3.9).

A heat-treated extract of potato mitochondrial E3 (approx. 5 units of E3 enzyme) was then loaded under the same conditions as before, and its elution volume determined by assaying each 1ml fraction for E3 activity. From the standard curve the approx. M_r of the native form of potato E3 was calculated to be in the range 100-110000. This value is consistent with its organisation as a dimer as is reported for all other E3 enzymes analysed to date.

The results of this chapter suggest that dihydrolipoamide dehydrogenase in plants is a dimer as are all other E3s from mammalian and bacterial sources described in the literature. It may also be concluded tentatively that chloroplast E3 is distinct from the mitochondrial enzyme, as the results of the salt sensitivity experiments, the use of the E2/X affinity column where they bind with a range of affinities and Western blot studies suggests that organelle-specific forms of E3 are expressed. The existence of the 58,000M_r and 56,000M_r polypeptides in affinity purified preparations of potato tuber mitochondrial E3 is of particular interest since E3 has been reported to be present as a homodimeric species in all other organisms studied to date. The determination of the M_r value (100,000-110,000) for native potato mitochondrial E3 is consistent with its organisation as a dimer. However, it may also be possible that one of the bands is another non-related protein that copurifies with E3, or that there is more than one E3 (i.e. isoforms) present in potato mitochondria. These two E3 polypeptides may differ only slightly from each other, explaining why they have been overlooked previously.

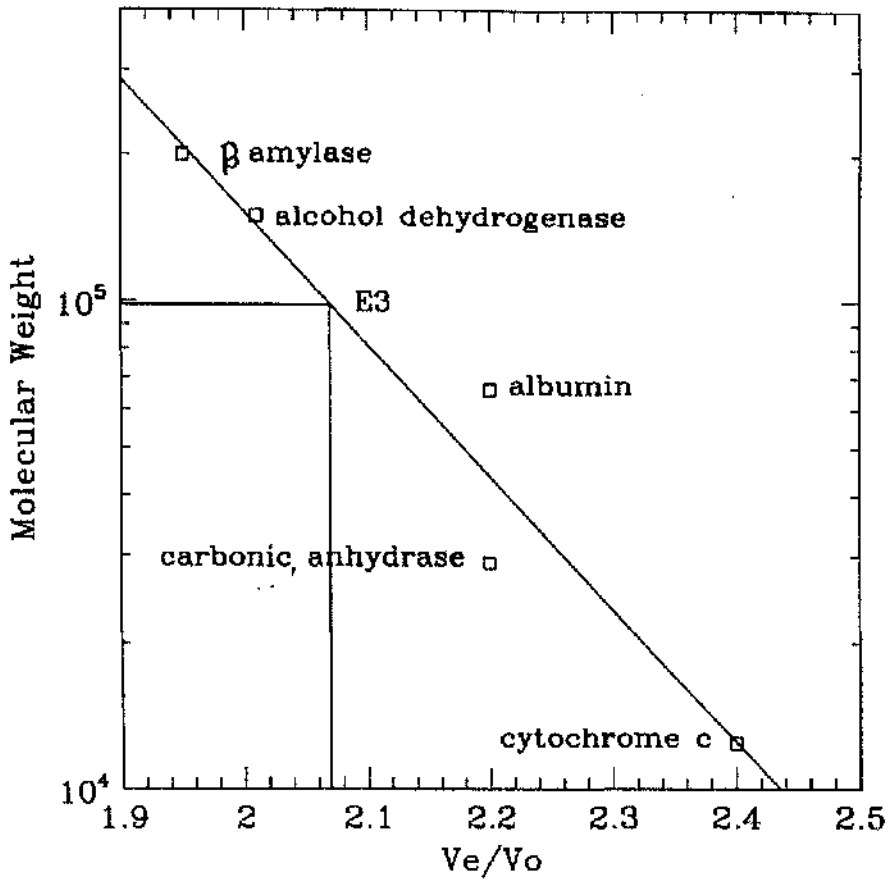


Figure 3.9: Calibration curve for the molecular weight determination of potato tuber mitochondrial E3 by gel filtration.

The void vol of the FPLC superose 6 column was determined by calculating the elution volume of 2mg of Blue Dextran applied to the column. The elution vols of each of the molecular weight markers were divided by the void vol and plotted against the log of their molecular weight. The Ve/Vo was calculated for the potato mitochondrial E3 and its molecular weight determined from the standard curve.

The next stage in this research project was to investigate further the unusual presence of the polypeptide chains in highly purified potato mitochondrial E3 preparations and to screen for the possible presence of isoforms of plant E3 in potato mitochondria and to characterise them if possible.

CHAPTER 4

CHARACTERISATION OF DIHYDROLIPOAMIDE DEHYDROGENASES FROM POTATO TUBER MITOCHONDRIA

4.1 INTRODUCTION

Dihydrolipoamide dehyrogenase (E3) is generally believed to be the identical gene product in all members of the 2-oxoacid dehydrogenase family of multienzyme complexes from mammalian sources. There is much evidence in the literature to support this theory based on reconstitution experiments, immunological reactions, end-group amino acid analyses and human mutations of E3 affecting all three complexes (Sakurai *et al.*, 1970; Matuda & Saheki, 1985; Stansbie *et al.*, 1986; Carothers *et al.*, 1987). It has been assumed that this E3 is also shared with the glycine decarboxylase complex; however, there is also evidence in the literature which contradicts this idea. Certainly, the results of chapter 3 suggest that E3 in plants may exist as organelle-specific forms. Pea mitochondria and pea chloroplast E3 were observed to have different susceptibilities to NaCl, the pea mitochondrial E3 interacted to a greater extent with the affinity column and most convincingly, when studied by Western Blotting the pea mitochondrial E3 was shown to be a distinct band of M_r 58,000 and pea chloroplast E3 to have an M_r of 52,000 (A. Carmichael, Ph.D. Thesis, Glasgow University, 1994).

Two immunologically distinct E3s have been observed in rat liver mitochondria (Carothers *et al.*, 1987). Antibodies were raised against E3 isolated from purified PDC and also from a commercial preparation of porcine heart which did not first involve purification of the 2-oxoacid dehydrogenase complex(es). The two sera varied in their reaction with rat liver mitochondrial extracts in that only the pig heart E3 antiserum inhibited GDC activity. The PDC E3 antiserum also failed to inactivate E3 completely from pig heart. It would appear, therefore, that E3 associated with GDC may be different from that associated with the 2-oxoacid dehydrogenase complexes.

Two species of *Pseudomonas* (*P. putida* and *P. aeruginosa*) are known to express two genetically distinct forms of dihydrolipoamide dehydrogenase known as LPD-val and LPD-glc. LPD-val was found to be the specific E3 subunit for the branched-chain 2-oxoacid dehydrogenase complex (BCDC), and LPD-glc the E3

subunit for 2-oxoglutarate dehydrogenase complex (OGDC) as well as the L component of glycine decarboylase complex (GDC). Both forms of E3 are specific for their functions and are not interchangeable (Sokatch *et al.*, 1981,1983; Sokatch & Burns, 1984; McCully *et al.*, 1986). So far it is not clear whether LPD-glc is also the E3 subunit of pyruvate dehydrogenase complex, as a mutant was isolated which lacked LPD-glc but had almost normal levels of PDC(Sykes *et al.*, 1985). A third E3 gene has been identified which differs in its M_r value, amino acid composition and N-terminal sequence from LPD-val and LPD-glc. The exact function of this third gene is still unknown although it has been demonstrated that it will restore PDC and OGDC activities in mutants defective in LPD-glc (Burns *et al.*, 1989).

Two different genes encoding E3 have also been identified in *E. coli*, one of which codes for E3 associated with the 2-oxoacid dehydrogenases and the other produces an E3 which has been implicated in the transport of certain sugars (galactose and maltose) and may be plasma-membrane associated (Richarme, 1989). This second E3 has a very low abundance which may explain why it has been overlooked in previous studies. Similarities can be drawn between this new E3 with that of LPD-val, the second dihydrolipoamide dehydrogenase, of *P. putida* described earlier. Both enzymes have similar subunit M_r values (47,000) and are induced by the presence of isoleucine and valine. In addition, antibodies raised against this postulated new form of E3 gave a faint reaction in immunoblots with crude extracts of *P. putida* producing LPD-val. This suggests that it may be possible for E3 to have an additional role other than that associated with the multienzyme complexes.

The existence of possible E3 isoenzymes is supported by evidence obtained from human genetic disorders of 2-oxoacid and glycine metabolism. In patients who have an E3 deficiency, if the same E3 were common to PDC, OGDC, BCDC and GDC, it would be expected that there would be elevated levels of lactate, 2-oxoglutarate, branched-chain amino acids and glycine. However, elevated glycine levels have not been reported in patients who have an E3 deficiency (Yoshino *et al.*, 1986).

Two groups have isolated and characterised cDNA clones encoding the E3

component from pea leaf/embryo λgt11 expression libraries and said to represent the dihydrolipoamide dehydrogenase component (L protein) of GDC (Bourguignon *et al.*, 1992; Turner *et al.*, 1992). Their Northern blot analysis and immunological studies also suggest that a single E3 gene might code for a common E3 component of all the multienzyme complexes (the 2-oxoacids and GDC). However, the experimental techniques used would not necessarily have detected the presence of closely related polypeptides of similar sequence arising from separate genes or via differential splicing of a single E3 gene. Turner and co-workers provided evidence indicating the possible presence of two closely related E3 genes from copy number analysis of pea DNA probed with a 1.3Kb fragment corresponding to 60% of the open reading frame of their cDNA clone, which also contains a typical mitochondrial targetting sequence. This suggests that it is possible that 2 genes may be being expressed in peas but one at very low levels, or that the two genes are so very closely related that it is difficult to distinguish between the two.

The results of chapter 3 of this thesis provide tentative evidence that in plants E3 may be organelle specific. In addition, affinity purified potato tuber mitochondrial E3 was observed to be composed of two protein bands with M_r values of 56,000 and 58,000. Thus the aim of this chapter was to investigate this further so as to determine whether one of the bands is a non-related protein that copurifies with the E3 enzyme, if E3 exists as a heterodimer or whether both bands represent separate isoforms of E3.

4.2 RESULTS AND DISCUSSION

4.2.1 FURTHER ANALYSIS OF POTATO TUBER MITOCHONDRIAL DIHYDROLIPOAMIDE DEHYDROGENASE (E3)

Heat treated potato tuber mitochondrial E3 was first purified using the E2/X affinity column (as described in chapter 3 section 3.4.4) and then dialysed into 1mM DTT, 50mM imidazole buffer, pH 6.8. This sample of E3 was then applied to a Mono

Q HR5/5 column equilibrated with 5 column vol of buffer A containing 1mM EGTA, 1mM β-mercaptoethanol, 10mM KPi buffer, pH 6.8, as described by Turner *et al.*, (1992). Proteins were eluted at room temperature with a linear gradient (30ml) of increasing potassium phosphate (10-400mM in buffer A) at a flow rate of 0.5ml min^{-1}. Fractions (1ml) were collected and assayed for E3 activity as described in Materials and Methods (section 2.2.7 (B)). The E3 activity eluted as a single peak at a phosphate concentration of 220mM (Fig. 4.1) similar to that of Turner and co-workers.

Extending the salt gradient when resolving proteins of similar charge on a Mono Q HR5/5 FPLC column is a method commonly used to improve the separation of polypeptides which tend to elute coincidentally. Thus the previous experiment was repeated under identical conditions except for the extension of the gradient to an overall vol. of 60ml. Potato tuber E3 eluted this time in three distinct peaks of activity, one small peak followed by two larger ones (at approx. 150mM, 180mM and 210mM KPi respectively), rather than as a single peak as observed previously (Fig. 4.2). Samples from the three individual peaks were concentrated using Centricon-10, TCA precipitated, resuspended in Laemmli sample buffer and separated by SDS-PAGE for further analysis (Fig. 4.3). This procedure was carried out as described in Materials and Methods (sections 2.2.6 and 2.2.10). Tracks 4,5 and 6 of Fig. 4.3 represent peaks 1,2 and 3 that have eluted from the Mono Q column respectively. Peak 1 was found to be represented by a single band with a M_r of 58,000, peak 3 by a band with a M_r of 56,000 and peak 2 by a combination of these two bands. A band with a M_r of about 67,000 can also be seen in every track as well as between them. This is generally believed to be an artefact as discussed in chapter 3 (section 3.2.3 (D)). When samples of the three peaks were exposed to anti-E3 serum in Western blot studies, they all cross-reacted strongly with the antiserum that was raised against porcine heart E3 (Fig. 4.4). Although it was difficult to resolve the two bands of peak 2 after detection of immune complex formation with ^{125}I-labelled protein A, both 58,000 and 56,000 M_r species (peaks 1 and 3 respectively) could be seen to cross react with equal intensities when the autoradiograph was under exposed (not shown).

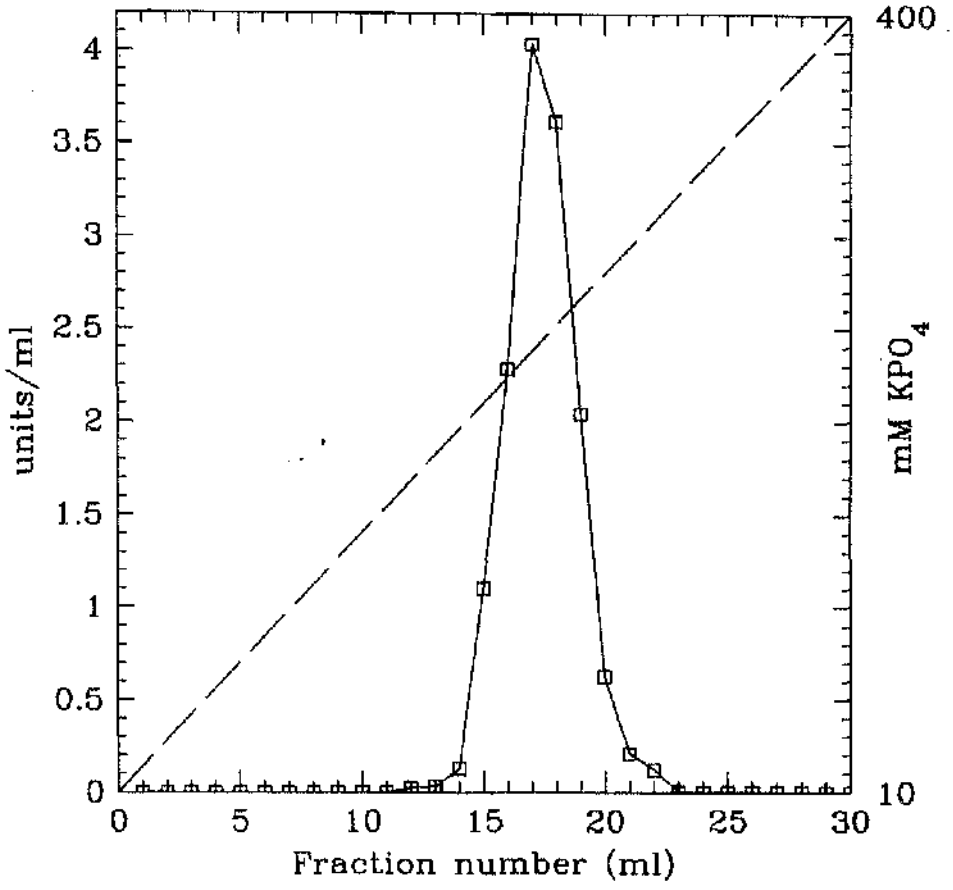


Figure 4.1: Application of potato tuber mitochondrial E3 to a Mono Q column and elution with a 30ml KPi gradient

A E2/X purified (see section 3.4.4) sample of potato tuber mitochondrial E3 was loaded onto a FPLC Mono Q HR5/5 ion exchange column. The sample was eluted from the column in a 30ml gradient with buffer (1mM mercaptoethanol, 1mM EGTA, 10-400mM KPi pH 6.8) at a flow rate of 0.5ml/min. Fractions (1ml) were collected and assayed for E3 activity (–□–) and expressed as units of enzyme activity/ml (see Materials and Methods section 2.2.7 (B)).

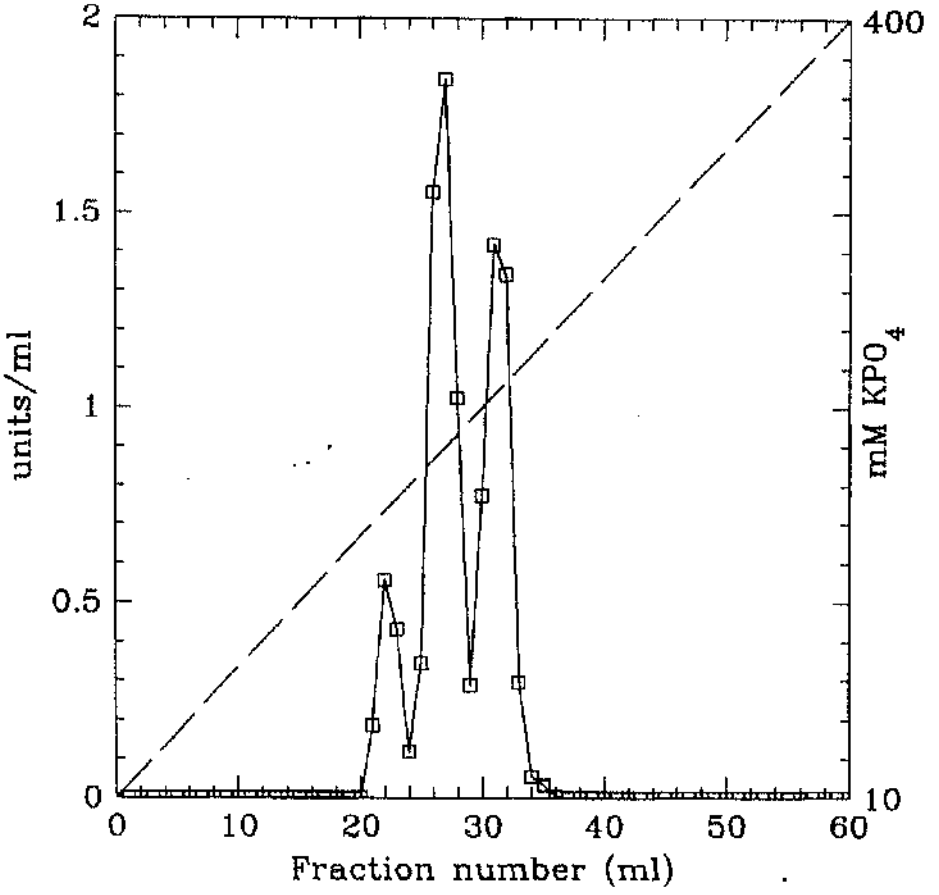


Figure 4.2: Application of potato tuber mitochondrial E3 to a Mono Q column and elution with a 60ml KPi gradient

A E2/X purified (see section 3.4.4) sample of potato tuber mitochondrial E3 was loaded onto a FPLC Mono Q HR5/5 ion exchange column. The sample was eluted from the column in a 60ml gradient with buffer (1mM mercaptoethanol, 1mM EGTA, 10-400mM KPi pH 6.8) at a flow rate of 0.5ml/min. Fractions (1ml) were collected and assayed for E3 activity (–□–) and expressed as units of enzyme activity/ml (see Materials and Methods section 2.2.7 (B)).

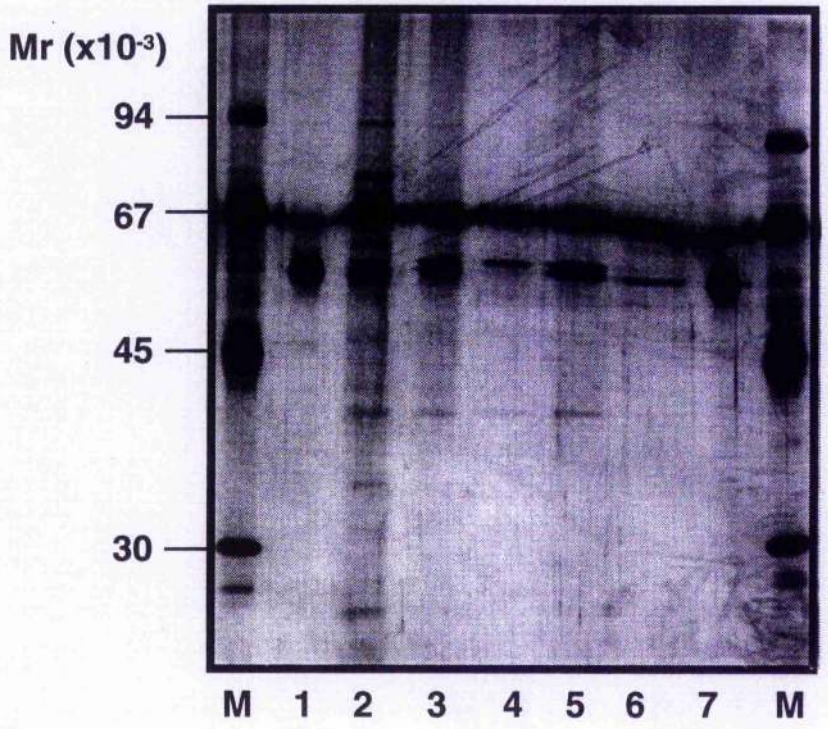


Figure 4.3: Analysis of the three peaks of potato E3 activity by SDS-PAGE

The three peaks of E3 activity eluted from the Mono Q column (see Figure 4.2) were analysed by SDS-PAGE and detected by silver staining as described in Materials and Methods (section 2.2.10 (D)). Lanes M, M_r markers; lanes 1 & 7, pig heart E3 controls; lane 2, heat-treated potato mitochondrial extract; lane 3, affinity-purified E3; lanes 4, 5 & 6, peaks 1, 2 & 3, respectively of affinity-purified E3 activity eluting from the FPLC-Mono Q column.

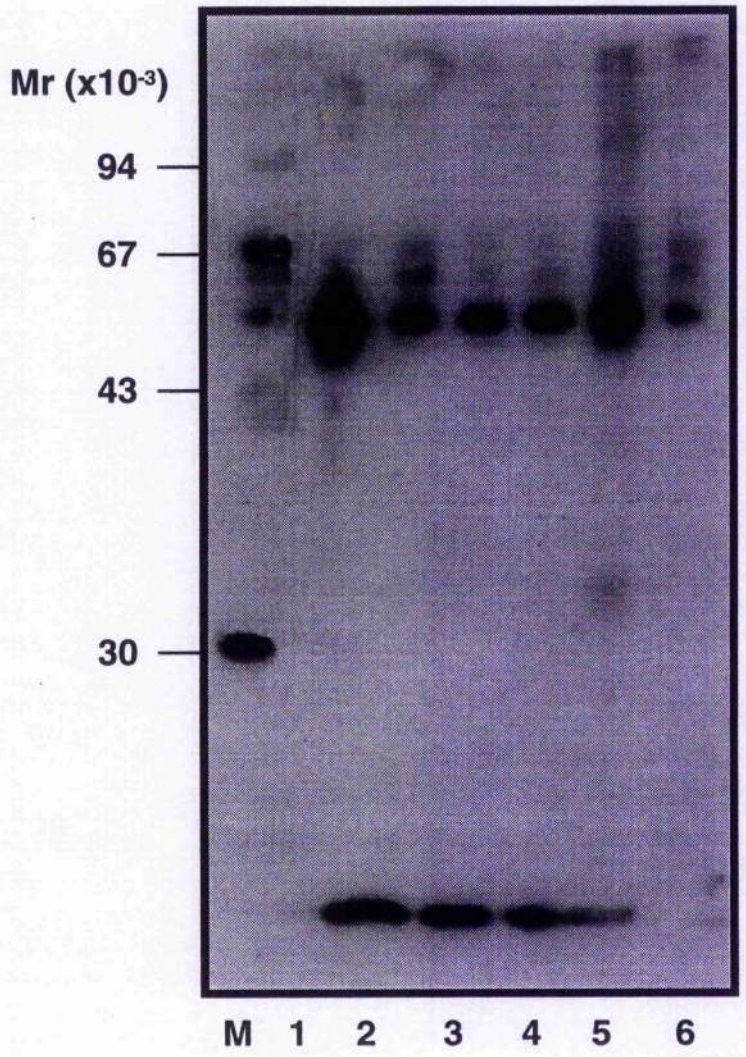


Figure 4.4: Analysis of the three peaks of E3 activity by Western Blot studies

The three peaks of E3 activity from the Mono Q column (see Figure 4.2) were analysed by Western Blotting as described in Materials and Methods (see section 2.2.11). Samples were blotted against antiserum raised against pig heart pig heart E3. Lane M, M_r markers; lane 1, porcine heart control; lane 2, heat-treated potato mitochondrial E3 extract; lane 3, E2/X affinity-purified potato E3; lanes 4,5 & 6, peaks 1, 2 & 3, respectively of affinity-purified E3 activity eluted from the FPLC Mono Q column.

4.2.2 ANALYSIS OF THE THREE MONO Q ELUTION PEAKS

To test whether the three peaks of potato tuber mitochondrial E3 activity eluted from the Mono Q column may represent aggregates of a single form of E3, the three peaks were reloaded onto the Mono Q column individually under the same conditions as described in section 4.2. If the three peaks did represent aggregates of a single form of E3, then it might be expected that the individual peaks would be interconvertible and elute once more as three peaks when reapplied to the ion exchange column. However, the peaks eluted as single peaks at the same stage in the gradient as they did previously, indicating that they did not simply represent aggregates of a single form of E3 (Figs. 4.5, 4.6 & 4.7).

The Mono Q profile was found to differ significantly when comparing potato tuber E3 extracts that had been released from mitochondria by freeze/thaw action, to that of E3 released by the addition of 0.1% (v/v) Triton X-100 to the crude mitochondrial extract. A preparation of potato tuber mitochondria was divided into two with one half subjected to freeze thaw treatment and the other with 0.1% (v/v) Triton X-100 to release E3 from the mitochondria as described in section 3.2.2. Both samples were applied to a Mono Q HR5/5 ion exchange column and eluted with a 60ml 10-400mM KPi gradient as described in section 4.2.1 of this chapter. When comparing the two elution profiles one can see that both methods of extraction of E3 from mitochondria result in the production of three peaks of E3 activity; however the first peak was observed to elute in a greater yield when the potato mitochondrial sample has been treated with the Triton X-100 (Fig. 4.8). In addition, there was slight enhancement of the release of peaks two and three.

It was also necessary to check that the three peaks of E3 activity did not arise as a result of proteolysis of the potato E3 enzyme. Conditions were altered so as to promote the degradation of the plant tuber mitochondrial E3 to see if this would alter the profile of this enzyme eluting from the Mono Q column. The mitochondrial extracts were incubated at 31°C for 3h with no protease inhibitors present prior to loading on to a

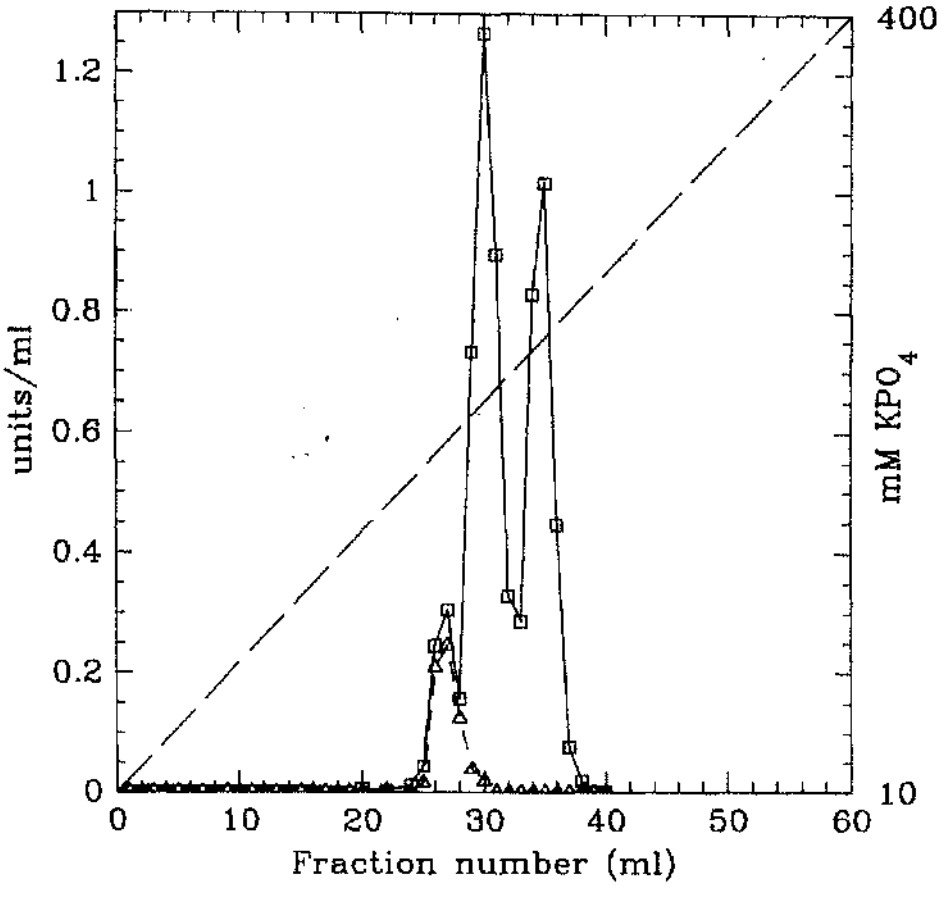


Figure 4.5: Peak 1 re-chromatographed on Mono Q column

Potato tuber E3 was separated into three peaks of E3 activity (–□–)(see Fig. 4.2) and the individual peaks then diaylsed into 1mM DTT, 50mM imidazole buffer pH 6.8. Peak 1 was reapplied to the Mono Q column under the same conditions as previously (--Δ--). Fractions (1ml) were collected and assayed for E3 activity and expressed as units of enzyme activity/ml (see Materials and Methods section 2.2.7 (B)).

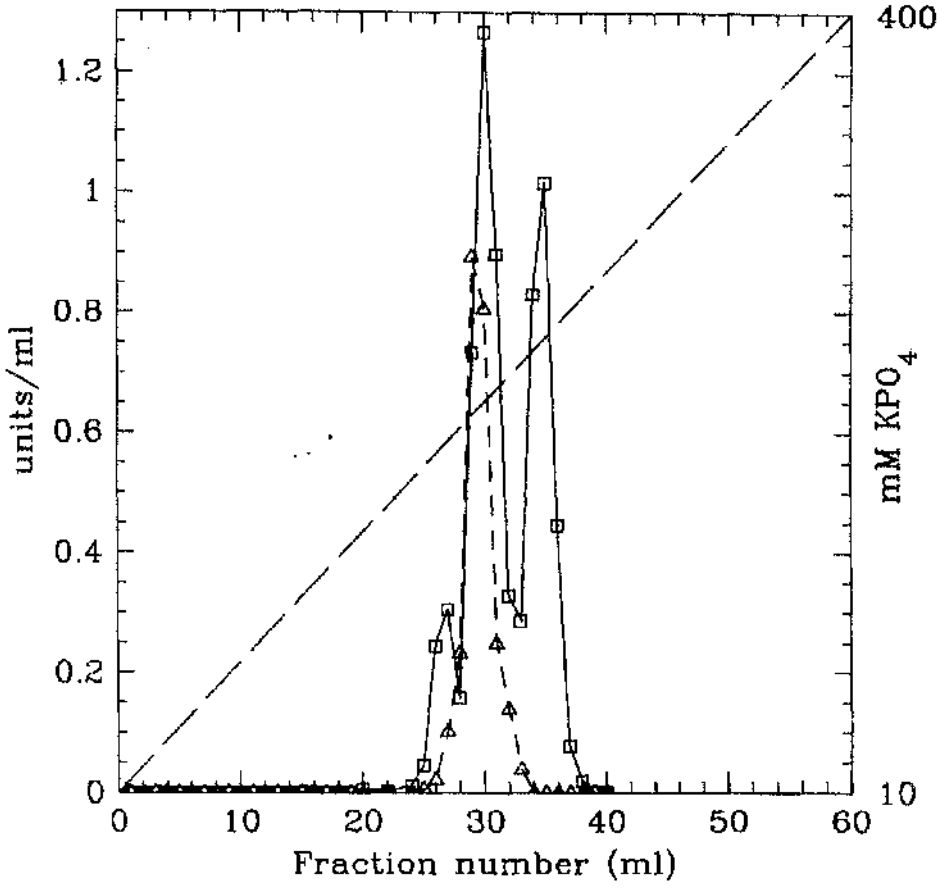


Figure 4.6: Peak 2 re-chromatographed on Mono Q column

Potato tuber E3 was separated into three peaks of E3 activity (–□–)(see Fig. 4.2) and the individual peaks then diaylsed into 1mM DTT, 50mM imidazole buffer pH 6.8. Peak 2 was reapplied to the Mono Q column under the same conditions as previously (--△--). Fractions (1ml) were collected and assayed for E3 activity and expressed as units of enzyme activity/ml (see Materials and Methods section 2.2.7 (B)).

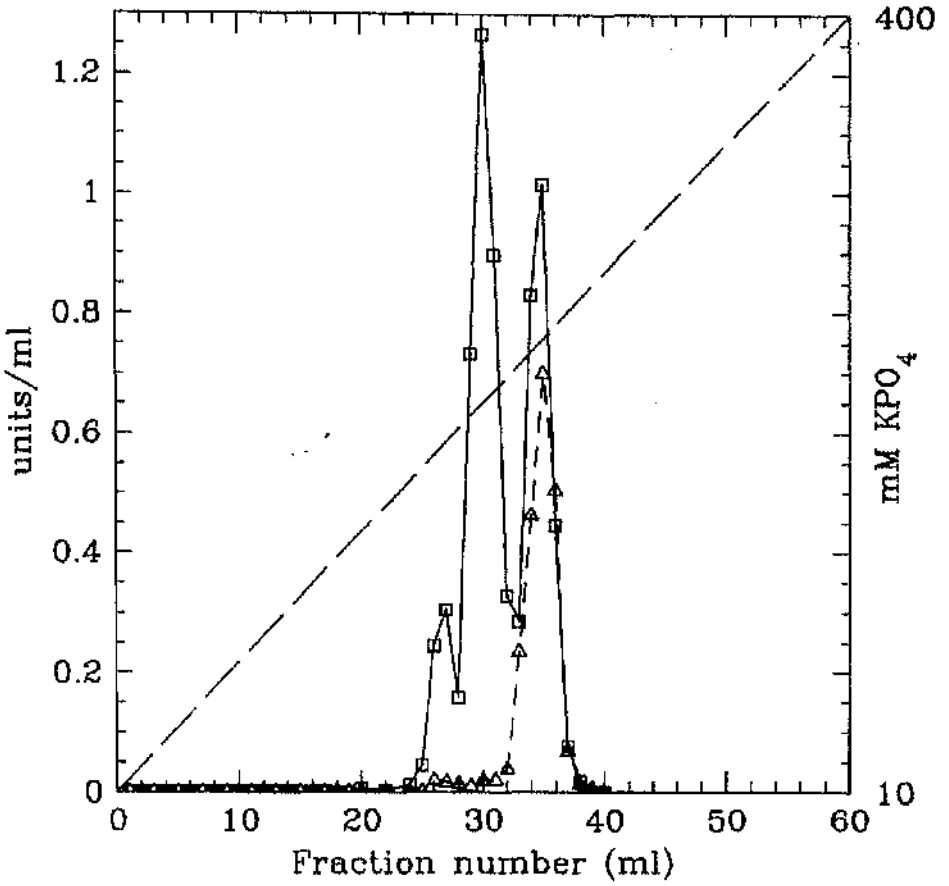


Figure 4.7: Peak 3 re-chromatographed on Mono Q column

Potato tuber E3 was separated into three peaks of E3 activity (–□–)(see Fig. 4.2) and the individual peaks the diaylsed into 1mM DTT, 50mM immidazole buffer pH 6.8. Peak 3 was reapplied to the Mono Q column under the same conditions as previously (--△--). Fractions (1ml) were collected and assayed for E3 activity and expressed as units of enzyme activity/ml (see Materials and Methods section 2.2.7(B)).

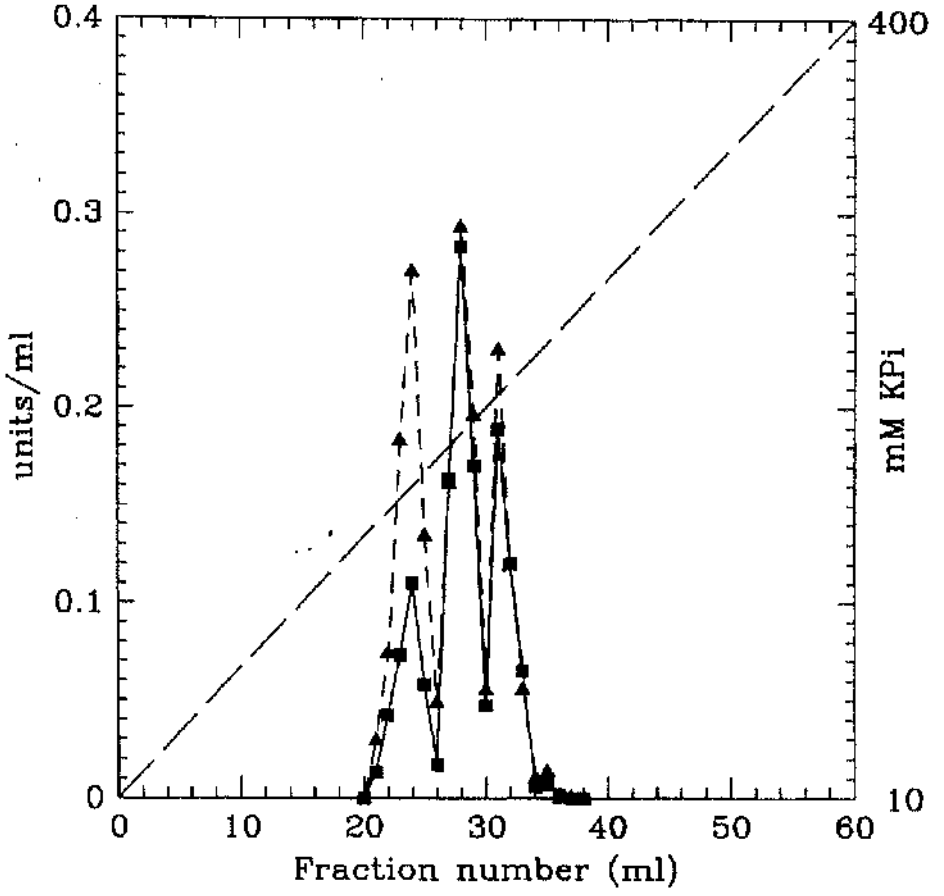


Figure 4.8: The effect of freeze/thaw treatment as compared to Triton X-100 extraction of E3 on the Mono Q elution profile

A preparation of potato tuber mitochondria was divided into two with one half being treated by freeze/thawing (-■--) and the other with 0.1% (v/v) Triton X-100 (--▲--) to extract the E3 enzyme. Individually these two samples were loaded onto a Mono Q column and eluted with a 60ml gradient with buffer (1mM mercaptoethanol, 1mM EGTA, 10-400mM KPi pH 6.8) at a flow rate of 0.5ml/min. Fractions (1ml) were collected and assayed for E3 activity and expressed as units of enzyme activity/ml (see Materials and Methods section 2.2.7 (B)).

Mono Q column with all other conditons being identical to that described in section 4.2. The resulting profile was found to be unaltered by this treatment when compared to the previous runs where Triton X-100 extracted potato mitochondrial E3 extracts where applied to the ion exchange column with three peaks of E3 activity being observed at the same stage in the potassium phosphate gradient (Fig. 4.9), indicating that the three activities are not readily interconvertible. It should be noted that E3 is an extremely heat stable enzyme known for its resistance to proteolytic digestion.

From the results of Chapter 2 preliminary evidence was obtained for organelle specific isoforms of E3 from pea with differing biochemical properties. The results of this chapter would suggest that E3 from potato tuber mitochondria may also exist as isoforms, possibly serving as complex-specific enzymes in the three related multienzyme complexes (PDC, OGDC and GDC) present in this organelle. The three isoforms are likely to arise from the various combinations of the 58,000 (α) and the 56,000 (β) M_r subunits. For example, peak 1 and 3 could represent an α_2 and β_2 homodimers and peak 2 a $\alpha\beta$ heterodimer as is evident from section 3.6, gel filtration studies indicate that the M_r value of the native potato E3 enzyme is in the range of 100-110,000 consistent with its organisation as a dimer. A crude extract of potato E3 was used in this M_r value determination and therefore all three isoenzymes were present. The observation that peak 1 is only partially released by the freeze/thaw treatment but is extracted in a higher yield with Triton X-100, suggests that the three isoforms bind with differing affinities to the inner mitochondria membrane, or that it is the multienzyme complexes with which they are associated that are extracted selectively.

4.2.3 N-TERMINAL SEQUENCING OF PEAK 1 AND 3 OF POTATO TUBER MITOCHONDRIAL E3

N-terminal sequencing was carried out for the two bands of potato mitochondrial E3 with M_r values of 56,000 and 58,000 to determine if they were indeed different proteins. The three peaks of potato E3 were loaded onto a 15% (w/v) SDS-polyacryl-

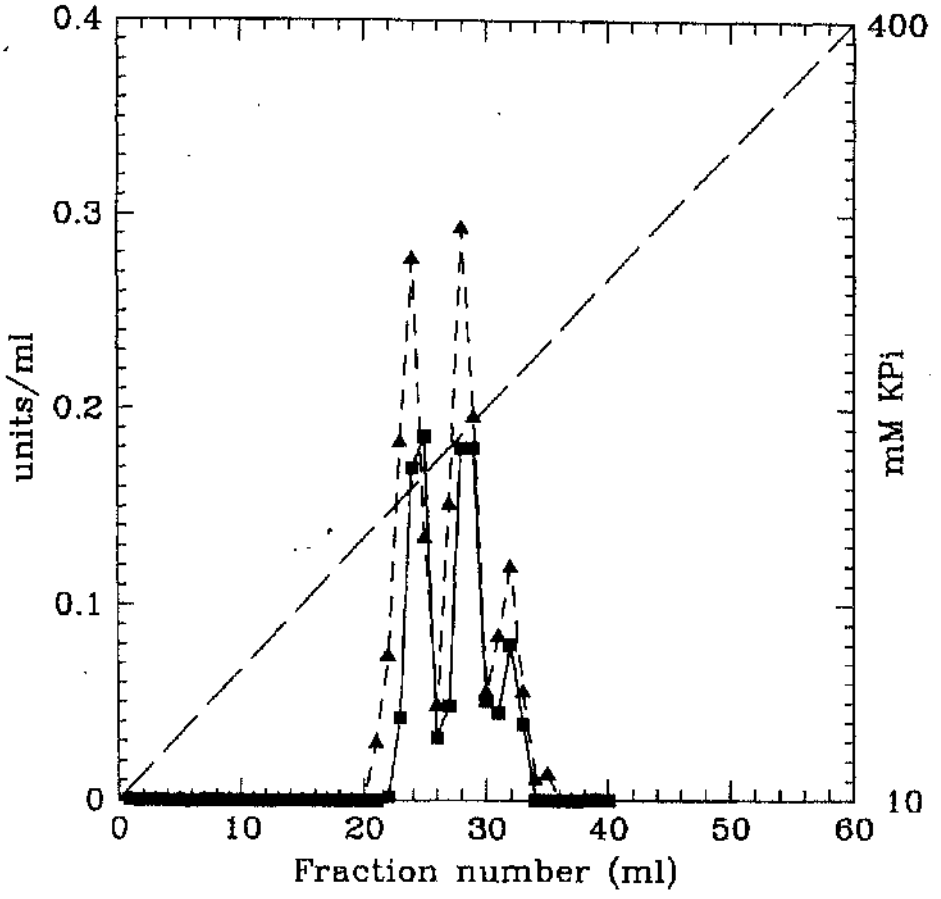


Figure 4.9: The effect of extended incubation at 31ºC on the elution profile of potato tuber mitochondrial E3 eluted from the Mono Q column

A sample of potato tuber mitochondrial E3 that had been treated with Triton X-100 was incubated at 31ºC for 3h (-□-). This sample was loaded onto the Mono Q column and eluted with a 60ml gradient with buffer (1mM mercaptoethanol, 1mM EGTA, 10-400mM KPi pH 6.8) at a flow rate of 0.5ml/min. This sample was compared to the previous Triton X-100 extract potato mitochondrial E3 sample run (--△--) (see Fig. 4.8). Fractions (1ml) were collected and assayed for E3 activity and expressed as units of enzyme activity/ml (see Materials and Methods section 2.2.7 (B)).

amide gel that was prepared using ultra pure acrylamide as described in Materials and Methods (section 2.2.10). SDS-PAGE was performed and the protein bands representing the three peaks of E3 activity were transferred onto PVDF membrane for sequencing as described in section 2.2.12 of Materials and Methods. The actual sequencing was carried out at the BBSRC funded Aberdeen Amino Acid Sequencing Facility, Aberdeen University. Only peaks 1 and 3 with M_r values 58,000 and 56,000 respectively were sequenced. The results of the sequencing showed that the N-termini of peaks 1 and 3 were identical to each other as well as to that of the pea leaf E3 sequence available (Bourguignon *et al.*, 1992; Turner *et al.*, 1992), with exception of one amino acid at residue 11 where a isoleucine was replaced by a valine (Fig. 4.10). From only 3 picomoles (approx.) of both peaks 1 and 3, 28 amino acid residues were identified which is a record for this small quantity of sample for the Aberdeen sequencing facility. It can be concluded therefore that both bands do represent the E3 enzyme, however, although the two sequences were identical to each other it still does not rule out the possibility that the two proteins differ as they could differ at their C-termini or internally.

4.2.4 ELUTION PROFILE OF VARIOUS PLANT E3s ON MONO Q ION EXCHANGE CHROMATOGRAPHY

Both pea mitochondria and chloroplast E3 when subjected to analysis by ion exchange chromatography on a 60ml gradient (10-400mM KPi) were found to elute as single homogeneous species (M. Conner, Glasgow University, Personnal communication). Experiments were performed to identify if any other plant species may elute as 3 peaks of activity. Turnip and horseradish and pea mitochondrial E3 were all found to elute as a single peak when applied to the Mono Q column with a 60ml gradient (10-400mM KPi buffer, pH6.8). Each sample when studied by SDS-PAGE was also found to contain only one protein band representing the E3 subunit (Fig. 4.11).

Potato mitochondrial E3	ASGSDENDVVVIGGGPGGYVAAIKAAQL
(peaks 1 & 3)	
Pea mitochondrial E3	ASGSDENDVVIIGGGPGGYVAAIKAAQL

Figure 4.10: N-terminal sequence of potato tuber mitochondrial E3 isoforms

Peak 1 and 3 of potato E3 eluted from Mono Q column (see Figure 4.2) were transferred onto PVDF membrane as described in Materials and Methods (section 2.2.12) and their N-terminal sequence determined at BBSRC-funded Protein Sequencing Unit at the University of Aberdeen.

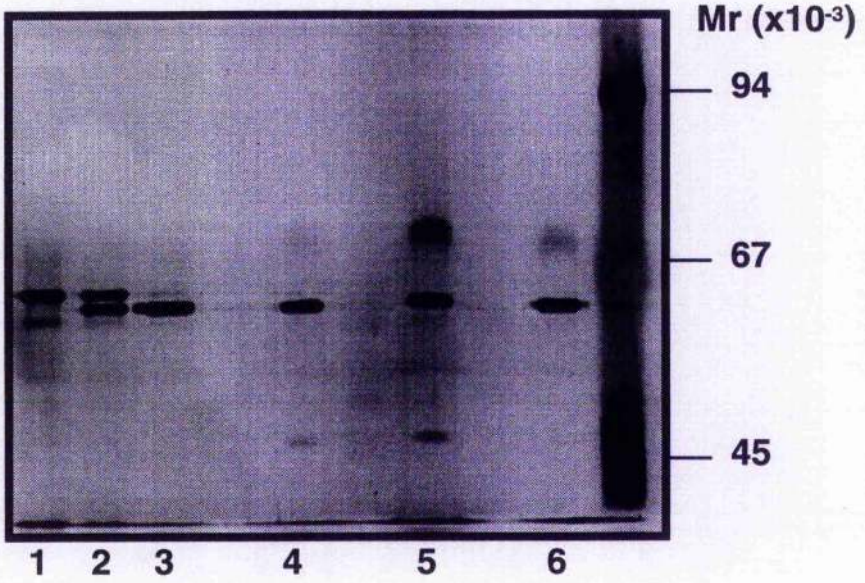


Figure 4.11: Analysis of various plant mitochondrial E3s by SDS-PAGE

Lanes 1, 2 & 3, potato tuber E3 peaks eluted from Mono Q ion exchange column (see Figure 4.2). Lane 4, 5 & 6, turnip, horse radish and pea mitochondrial E3 respectively that eluted from the Mono Q column.

Thus the presence of multiple isoforms, consisting of the three possible combinations of two closely-related polypeptide chains forming an α_2 and β_2 homodimers and an $\alpha\beta$ heterodimer has only been detected in potato tuber. It is possible that each of the three individual E3 isoforms may function preferentially or exclusively with one of the three mutienzyme complexes located in plant mitochondria (PDC, OGDC and BCDC). Differential extraction of the three peaks was also observed where peak 1 in particular was extracted in a higher yield with Triton X-100 compared to the less harsh freeze/thaw treatment. This suggests that the the three isoforms bind with differing affinities to the inner mitochondrial membrane, or perhaps it is the multienzyme complexes with which they are associated with that are extracted selectively. Although the N-terminal sequences of peak 1 and 3 are identical to each other, it is possible that the two E3 polypeptides differ at their C-termini or internally, and that any differences between the two may be only slight. However we can conclude that both protein bands (M_r 58,000 and 56,000) represent E3 and therefore eliminating the possibility that one may be a non-related protein that copurifies with E3. It may also be the case that these isoforms of E3 are restricted to tetraploid species, like potato, which are a result of cross-breeding. However, it is possible that the heterogeneity also occurs in other plant species but that the individual subunits are not readily resolved by SDS-PAGE. As mentioned in section 4.1, Turner and co-workers (1992), provided evidence indicating the possible presence of two closely related gene products in pea mitochondrial E3 which could not be resolved. It is also interesting that evidence has been obtained for the existence of an E3 heterodimer for the first time as all E3s to date are homodimers, even in bacteria (e.g. *P. putida and E. coli*) where there are two genetically distinct forms of E3 (as described in section 4.1).

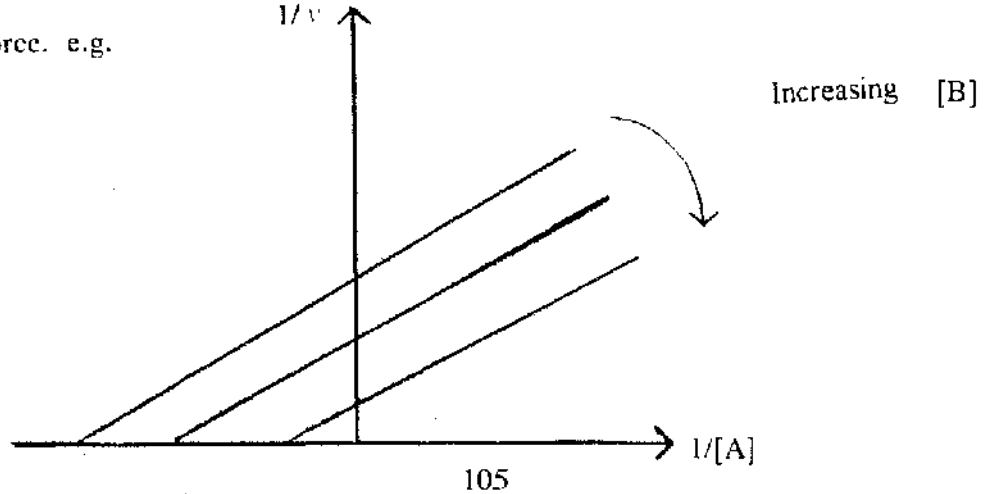
CHAPTER 5

KINETIC CHARACTERISATION OF ISOFORMS OF DIHYDROLIPOAMIDE DEHYDROGENASE FROM POTATO MITOCHONDRIA

5.1 INTRODUCTION

The family of 2-oxoacid dehydrogenase complexes consists of three multienzyme complexes, pyruvate dehydrogenase complex (PDC), 2-oxoglutarate dehydrogenase complex (OGDC) and the branched chain 2-oxoacid dehyrogenase complex (BCDC). In turn these multienzyme complexes are comprised of multiple copies of three separate enzyme components, a 2-oxoacid dehdrogenase (E1), a dihydrolipoamide acyltransferase (E2) and a dihydrolipoamide dehydrogenase (E3). The 2-oxoacid dehydrogenase complexes occupy key positions in intermediary metabolism and catalyse the irreversible oxidative decarboxylation of 2-oxoacids by the coordinated action of the individual enzymes (E1, E2 and E3).

Kinetic characterisation of the 2-oxoacid dehydrogenase complexes from mammalian sources (Tsai *et al.*, 1973; Hamada *et al.*, 1975; Boyer & Odessey, 1991), has shown that their reaction sequences proceed via a three site ping-pong mechanism first described by Cleland (1973). This mechanism can be described as one where the enzyme reacts with one substrate to give a covalently modified enzyme and releases one product, prior to reacting with the second substrate i.e., one or more products are released before all the substrates are bound. The reaction sequence of the 2-oxoacid dehydrogenase complexes involves a number of reactions catalysed at distinct active sites on each of their component enzymes (E1, E2 and E3). A ping-pong mechanism can be confirmed for an enzyme catalysed reaction by analysing 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 double reciprocal plots if a ping-pong mechanism is in force. e.g.



To date, kinetic characterisation of the plant 2-oxoacid dehydrogenase complexes has focused primarily on PDC which exemplifies a ping-pong reaction mechanism like its mammalian counterpart. Both mitochondrial and chloroplast PDC from a wide range of plant species including pea, broccoli and cauliflower exhibit a three site ping-pong reaction mechanism (Randall *et al.*, 1977; Thompson *et al.*, 1977a &1977b; Miernyk & Randall, 1987). Typical K_m and K_i values are presented in Table 5.1 which are taken from Randall & Miernyk (1990). Both plant mitochondrial and plastid PDCs are subject to inhibition by their reaction products as are their mammalian and bacterial counterparts. NADH and acetyl-CoA exhibit competitive inhibition with their respective substrates (NAD^+ and CoA respectively) as predicted by the multisite ping-pong mechanism (Cleland, 1973). From Table 5.1 it can be seen that the K_i(NADH) is lower than the K_m(NAD^+) demonstrating that the plant mitochondrial and plastid PDCs are very sensitive to the NAD^+:NADH ratio, in common with their mammalian and bacterial counterparts. It has been suggested that the primary effect of this ratio is on the dihydrolipoamide dehydrogenase (E3) itself (Wilkinson &Williams, 1981).

Kinetic characterisation of dihydrolipoamide dehydrogenase has concentrated on E3 from non plant sources such as pig heart and *E. coli*. E3 is a homodimer which contains 1 molecule of FAD per subunit, a redox-active disulphide, a histidine residue in each active site and catalyses the following reaction:

$$\text{DIHYDROLIPOAMIDE} + NAD^+ \rightleftharpoons \text{LIPOAMIDE} + \text{NADH} + H^+$$

The two substrate (NAD^+ and lipoamide) binding sites of E3 are situated on opposite faces of the flavin, with the disulphide bridge on the same side as the lipoamide binding pocket. A histidine side chain was reported to be located in front of the reactive disulphide bridge and is thought to be responsible for abstracting a proton from lipoamide (Matthews *et al.*, 1977). In the 2-oxoacid dehydrogenase complexes, E3 is responsible for the re-oxidation of the dihydrolipoamide groups which are bound to lysine residues present on the acyltransferase component (E2) and this in turn is coup-

Plant tissue	K_m(NAD) μM	K_i(NADH) μM
Pea leaf mPDC[a]	122	18
Broccoli floret mPDC[b]	110	13
Cauliflower floret mPDC[c]	125	34
Castor seed endosperm mPDC[d]	51	15
Etiolate maize shoot mPDC[e]	60	8
Pea leaf pPDC[f]	36	9
Castor seed endosperm pPDC[g]	130	27
Etiolated maize shoot pPDC[e]	16	12

[a]Miernyk and Randall, 1987
[b]Rubin and Randall, 1977
[c]Randall *et al* ., 1977
[d]Rapp *et al* ., 1987
[e]Cho *et al* ., 1988
[f]Camp *et al* ., 1988

Table 5.1: Michaelis constants for a range of mitochondrial and plastid PDCs from a variety of plant tissues

led to the reduction of NAD^+ to NADH, which can then be immediatcly utilised in the respiratory chain. This reaction proceeds via a ping-pong mechanism as described above for the 2-oxoacid dehydrogenase complexes, where electrons follow a complex pathway from lipoamide to the disulphide/dithiol complex and then via the flavin to finally reach NAD^+ with the production of NADH (Massey *et al.*, 1960; Reed, 1973; Williams, 1992; Ghisla & Massey, 1989).

Chapter 4 of this thesis is concerned with the purification of three isoforms of potato tuber mitochondrial E3. The application of E2/X affinity column purified potato tuber mitochondrial E3 to a FPLC Mono Q HR5/5 column resulting in the elution of three distinct E3 peaks of activity was described in section 4.2.1. These three peaks of potato E3 activity were then analysed by SDS-PAGE. Peaks 1 and 3 were found to be represented by distinct polypeptides with M_r values of 58,000 and 56,000 respectively and peak 2 by a combination of these two bands. It is suggested that these three peaks of potato mitochondrial E3 represent three isoforms which are likely to arise from the various combinations of the 58,000 (α) and the 56,000 (β) M_r subunits. Thus, peaks 1 and 3 appear to represent α_2 and β_2 homodimers and peak 2 a $\alpha\beta$ heterodimer. Since E3 has only previously been reported to exist as a homodimer, it is therefore highly unusual to detect a E3 heterodimer. This is also the first time that the possible existence of E3 isoforms has been reported from plant sources, thus the physiological significance of these isoforms requires further analysis. It is possible that these three isoforms of potato mitochondrial E3 may differ subtly in their biochemical properties and their ability to interact with the individual multienzyme complexes (PDC, OGDC and GDC) present in plants. In order to further characterise these isoforms of E3, studies were performed with the aim of determining whether the three isoforms differ in their basic kinetic parameters.

5.2 RESULTS AND DISCUSSION

5.2.1 KINETIC CONSTANTS FOR DIHYDROLIPOAMIDE DEHYDROGENASE-CATALYSED REACTIONS

Kinetic constants were estimated for the three isoforms of potato tuber mitochondrial E3, pig heart E3 , bovine mucosal E3 and yeast E3 and comparisons made between them. K_m values were calculated for each E3 by varying the concentrations of NAD^+ while maintaining the concentration of its other substrate dihydrolipoamide, at a fixed saturating level. The three potato E3 isoforms were prepared as described in Chapter 3, section 4.2.1. All E3 assays were performed in duplicate as described in Materials and Methods, section 2.2.7(B) at pH 7.6. The apparent Michaelis constants were derived from Lineweaver-Burke plots and are presented in Table 5.2 with the standard mean errors calculated from the least squares fit to the data points.

$K_m(NAD^+)$ values for the potato E3 isoforms were found to be significantly higher than those obtained for pig heart, bovine and yeast E3, with the bovine E3 having the lowest K_m value of 178μM ±9. The K_m value for peak 2 of potato E3 (547μM ±26) was found to be higher than that for both peak 1 (461μM ±28) and peak 2 (491μM ±46). However, the difference is greater between peak 1 and peak 2 than there is between peak 2 and 3 when the statistical errors are taken into consideration. These values are representative of a single preparation of potato tuber mitochondrial E3, however, kinetic constants were determined for a second preparation and were found not to differ by more than ±5%. Published $K_m(NAD^+)$ values for E3 vary dramatically according to the pH at which their experimental analysis was performed. For example, Sahlman &Williams (1989) studying *E. coli* E3 found that its $K_m(NAD^+)$ values varied from 170μM at pH 7.98 to 1010μM at pH 5.49. All kinetic constants calculated in this chapter represent experiments carried out at pH 7.6. At pH 7.51, Sahlman &Williams calculated a $K_m(NAD^+)$ value for *E.coli* of 230μM which is very close to our value of 227μM for pig heart E3 (Table 5.2).

	Michaelis Constant	
	$K_m(NAD^+)$ μM	$K_i(NADH)$ μM
Pig Heart E3	227 ±15	96.9 ±6.2
Bovine E3	178 ±9	67.8 ±3.3
Yeast E3	358 ±13	82.9 ±6.3
Potato tuber E3 Peak 1	461 ±28	104.5 ±6.3
Potato tuber E3 Peak 2	547 ±26	142.9 ±6.8
Potato tuber E3 Peak 3	491 ±46	128.0 ±4.6

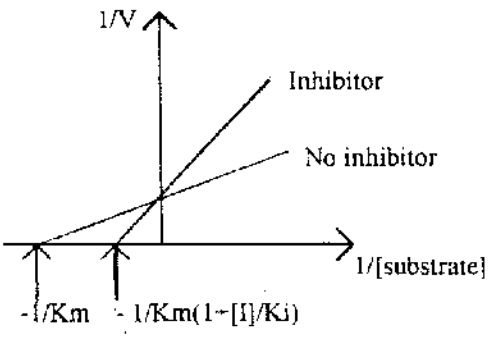
Table 5.2: Michaelis constants for dihydrolipoamide dehydrogenase catalysed reactions.

K_m and K_i values were calculated from Lineweaver-Burke plots for pig heart, bovine, yeast E3 and the potato tuber mitochondrial E3 isoforms as described in section 5.2.1 and 5.2.2.

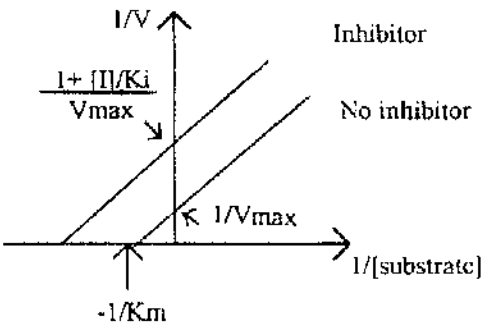
5.2.2 PRODUCT (NADH) INHIBITION OF DIHYDROLIPOAMIDE DEHYDROGENASE-CATALYSED REACTIONS

As with the 2-oxoacid dehydrogenase multienzyme complexes , E3 is inhibited by the products of the overall reaction. This reaction proceeds via a ping-pong mechanism as described in the introduction to this chapter (section 5.1). An experiment was designed to investigate inhibition of E3 from potato tuber mitochondrial isoforms, pig heart, bovine and yeast. The initial velocity of the reaction was measured varying the concentration of NAD^+ as described in section 5.2. The inhibitor NADH was introduced at two different concentrations (100 & 200μM) immediately prior to the initiation of the reaction.

The Michaelis-Menten model accounts for the kinetic properties of these E3 enzymes. K_i values can be calculated by estimating the K_m value in the presence of inhibitor for competitive and uncompetitive inhibition respectively:



COMPETITIVE INHIBITION



UN-COMPETITIVE INHIBITION

K_i(NADH) values for the potato mitochondrial E3 isoforms, porcine heart, bovine and yeast E3 are presented in Table 5.2. These values were calculated from Figures 5.1 to 5.6, where it can be seen that NADH competitively inhibits all the E3 species (pig heart, bovine heart, yeast and the three isoforms of potato).

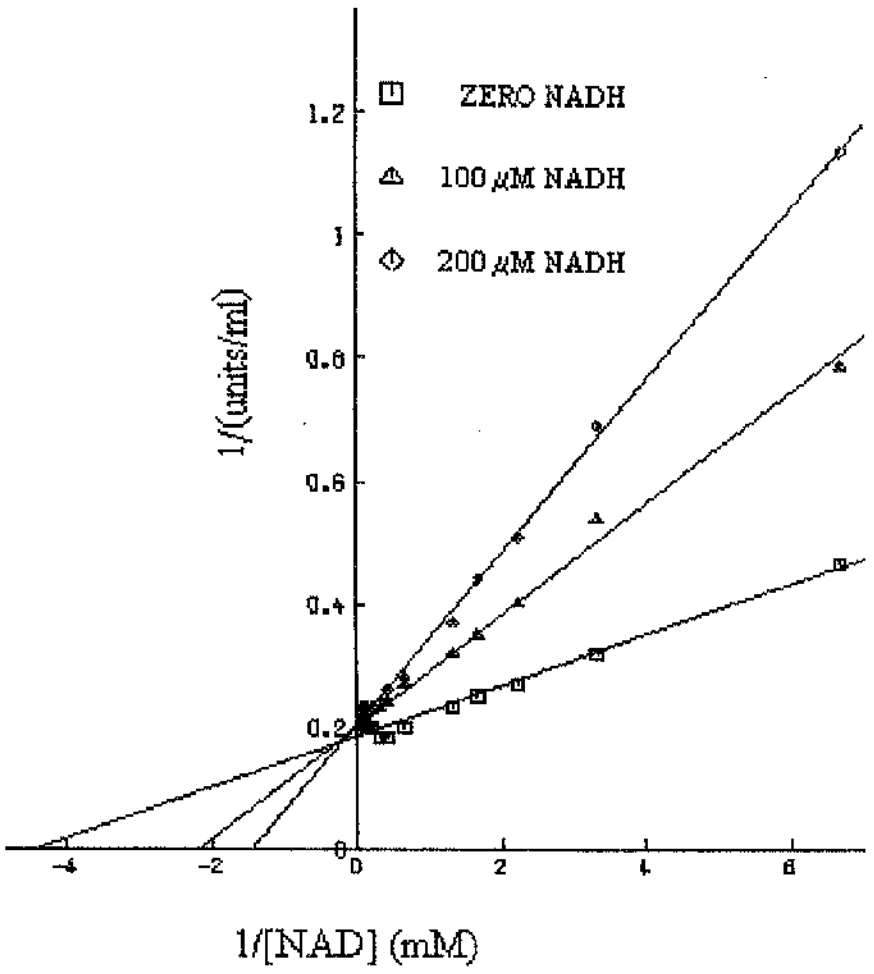


Figure 5.1: Double reciprocal plot of velocity of a porcine heart E3-catalysed reaction versus NAD^+ at zero, 100μM and 200μM NADH.

E3 activity expressed in μmol NADH/min/ml, was determined as described in Materials and Methods section 2.2.7(b) except with increasing concentration of NAD^+. Best fit lines were calculated by regression analyses using a least squares method (National Algorithms Group).

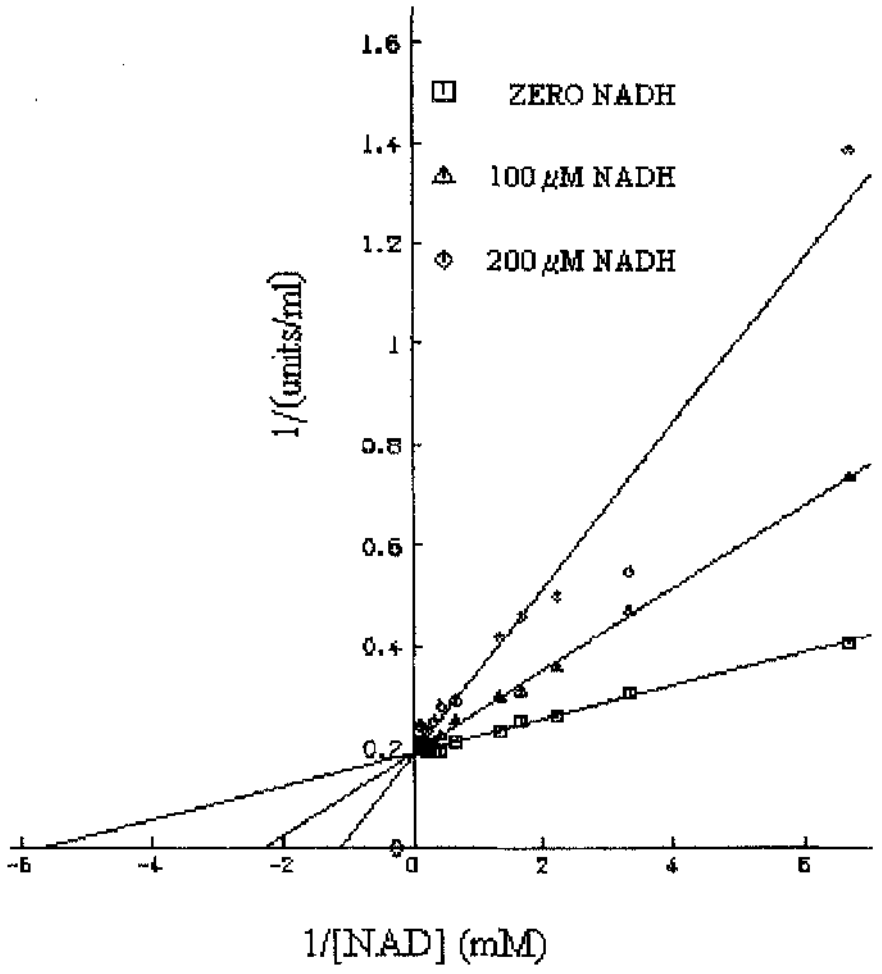


Figure 5.2: Double reciprocal plot of velocity of a bovine E3-catalysed reaction versus NAD^+ at zero, 100μM and 200μM NADH.

E3 activity expressed in μmol NADH/min/ml, was determined as described in Materials and Methods section 2.2.7(b) except with increasing concentration of NAD^+. Best fit lines were calculated by regression analyses using a least squares method (National Algorithms Group).

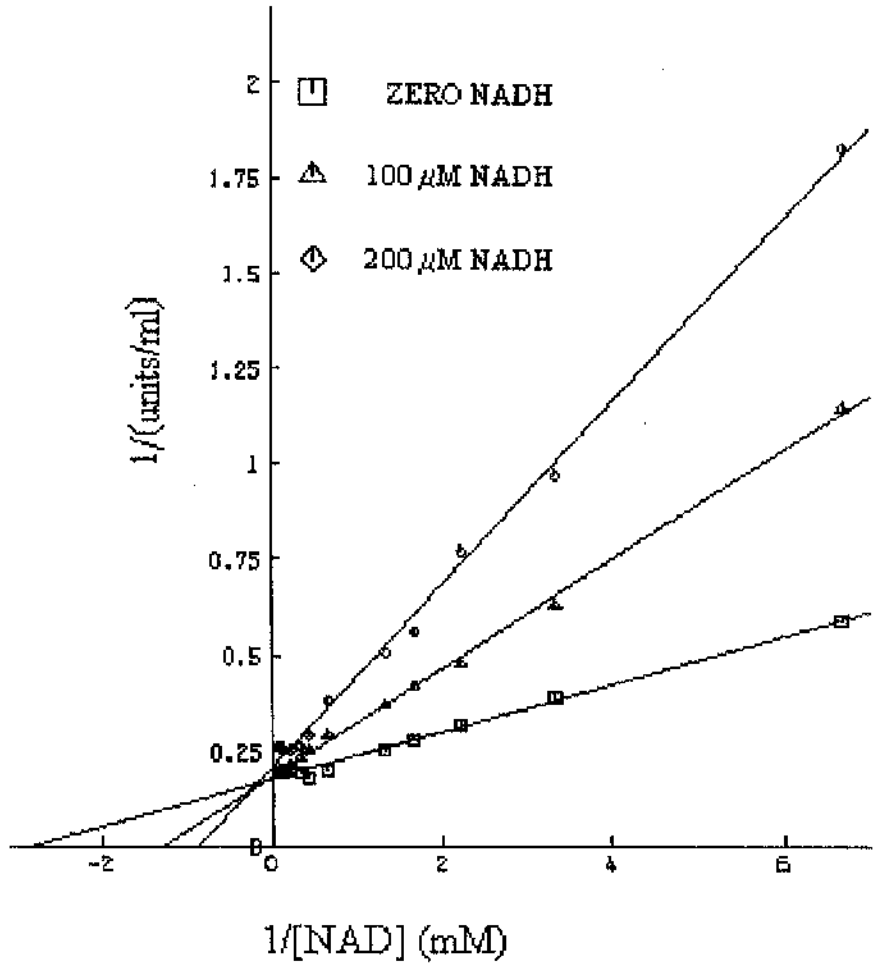


Figure 5.3: Double reciprocal plot of velocity of a yeast E3-catalysed reaction versus NAD^+ at zero, 100μM and 200μM NADH.

E3 activity expressed in μmol NADH/min/ml, was determined as described in Materials and Methods section 2.2.7(b) except with increasing concentration of NAD^+. Best fit lines were calculated by regression analyses using a least squares method (National Algorithms Group).

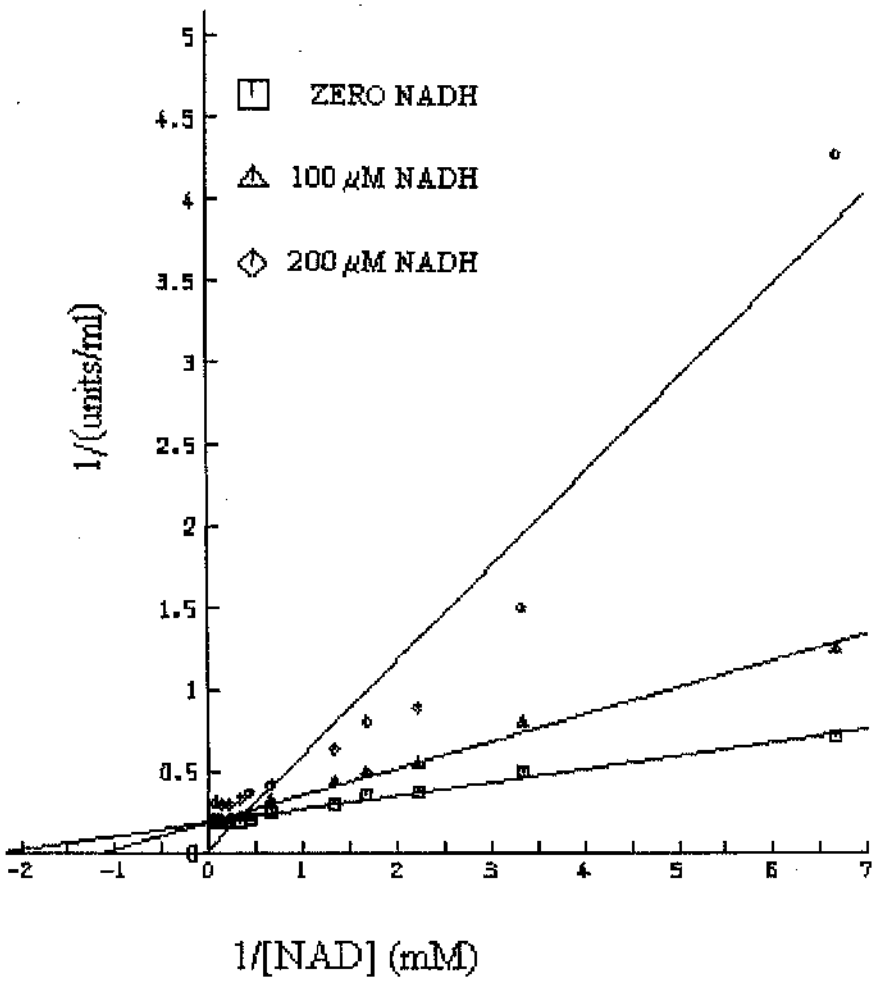


Figure 5.4: Double reciprocal plot of velocity of a potato tuber mitochondrial E3 (peak 1)-catalysed reaction versus NAD^+ at zero, 100μM and 200μM NADH.

E3 activity expressed in μmol NADH/min/ml, was determined as described in Materials and Methods section 2.2.7(b) except with increasing concentration of NAD^+. Best fit lines were calculated by regression analyses using a least squares method (National Algorithms Group).

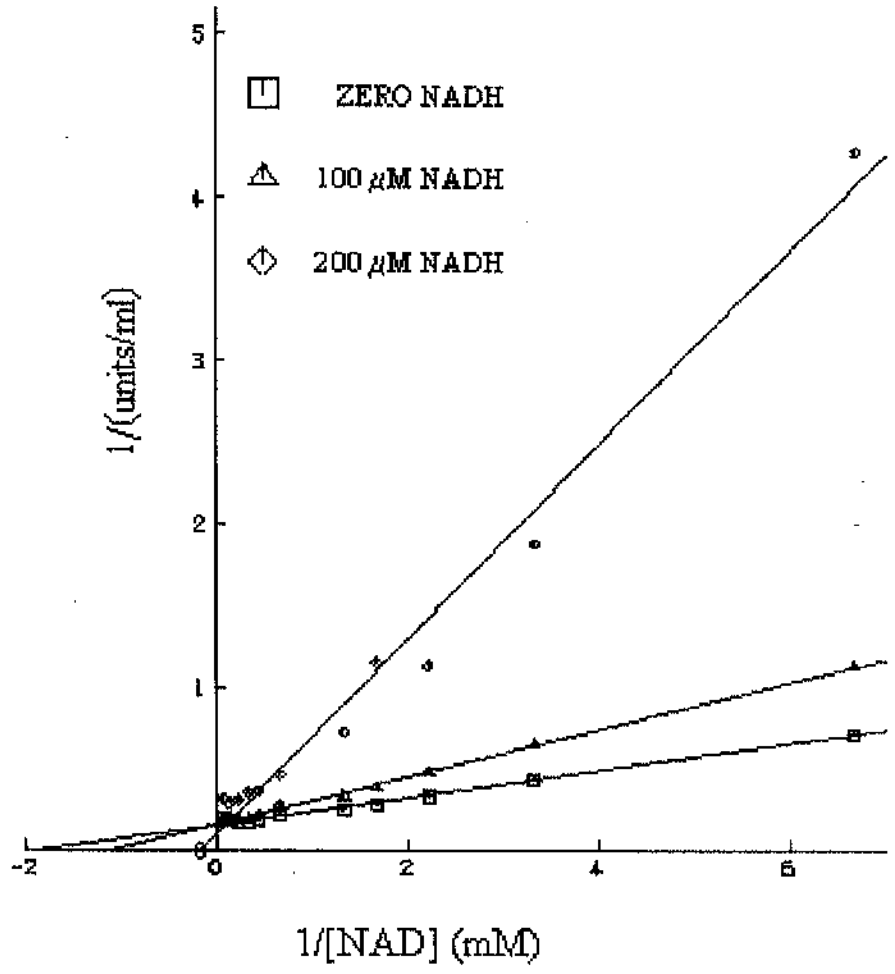


Figure 5.5: Double reciprocal plot of velocity of a potato tuber mitochondrial E3 (peak 2)-catalysed reaction versus NAD^+ at zero, 100μM and 200μM NADH.

E3 activity expressed in μmol NADH/min/ml, was determined as described in Materials and Methods section 2.2.7(b) except with increasing concentration of NAD^+. Best fit lines were calculated by regression analyses using a least squares method (National Algorithms Group).

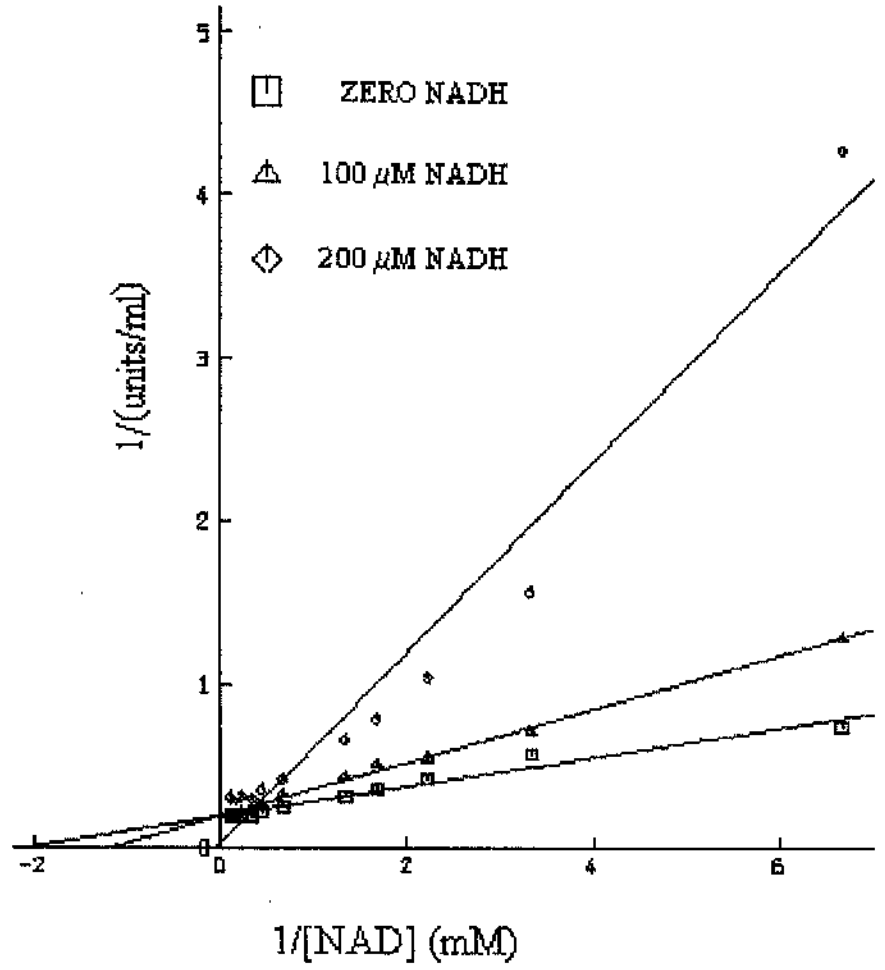


Figure 5.6: Double reciprocal plot of velocity of a potato tuber mitochondrial E3 (peak 3)-catalysed reaction versus NAD^+ at zero, 100μM and 200μM NADH.

E3 activity expressed in μmol NADH/min/ml, was determined as described in Materials and Methods section 2.2.7(b) except with increasing concentration of NAD^+. Best fit lines were calculated by regression analyses using a least squares method (National Algorithms Group).

Literature available in this area also provides evidence for competitive inhibition for NADH (Reed, 1973). Competitive inhibition occurs where products bind at the same active site as the substrate molecule (e.g. NADH and NAD^+). It was observed, however, that at 200μM NADH the inhibition pattern differs from that of 100μM NADH. Normally for competitive inhibition only the K_m value is altered and the V_{max} value remains the same as is observed with 100μM NADH inhibition. At 200μM NADH, however, both the K_m value and the V_{max} value is altered producing mixed inhibition. It is known that an excess of NADH can overreduce the E3 enzyme to a catalytically inactive form of the enzyme (EH_4) and therefore V_{max} is not reached (Sahlman & Williams, 1989; Matthews *et al.*, 1976). All K_i values were therefore calculated from the results obtained for 100μM NADH inhibition.

As with the $K_m(NAD^+)$ values, the K_i constants for NADH are found to be higher for the potato isoforms than the pig, bovine or yeast E3 (Table 5.2). The K_i(NADH) for potato mitochondrial E3 peak 2 (142.9 ±6.8μM) is higher than those of peak 1 (104.5 ±6.3μM) and 3 (128.0 ±4.6μM), however the difference between peaks 1 and 2 is greater than that for peaks 2 and 3. These indicate that the three isoforms of potato tuber mitochondrial E3 show minor but significant variations in their kinetic constants, although no marked differences were noted as might be expected from such closely related isoenzymes.

5.2.3 EFFECT OF pH ON THE ACTIVITY OF DIHYDROLIPOAMIDE DEHYDROGENASE

The pH optima for pig heart, bovine, yeast E3 and the potato mitochondrial E3 isoforms were determined by assessing the effect on the E3 catalysed reaction of pHs ranging from 5.5 to11 (Figs. 5.7 & 5.8). The buffers used to maintain the assay mixture at the correct pH were 2-(N-morpholino)ethanesulfonic acid (MES), 3-(N-morpholino)propanesulfonic acid (MOPS), Tris-HCl, 2-(N-cyclohexylamino)-ethanesulfonic acid (CHES) and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS)

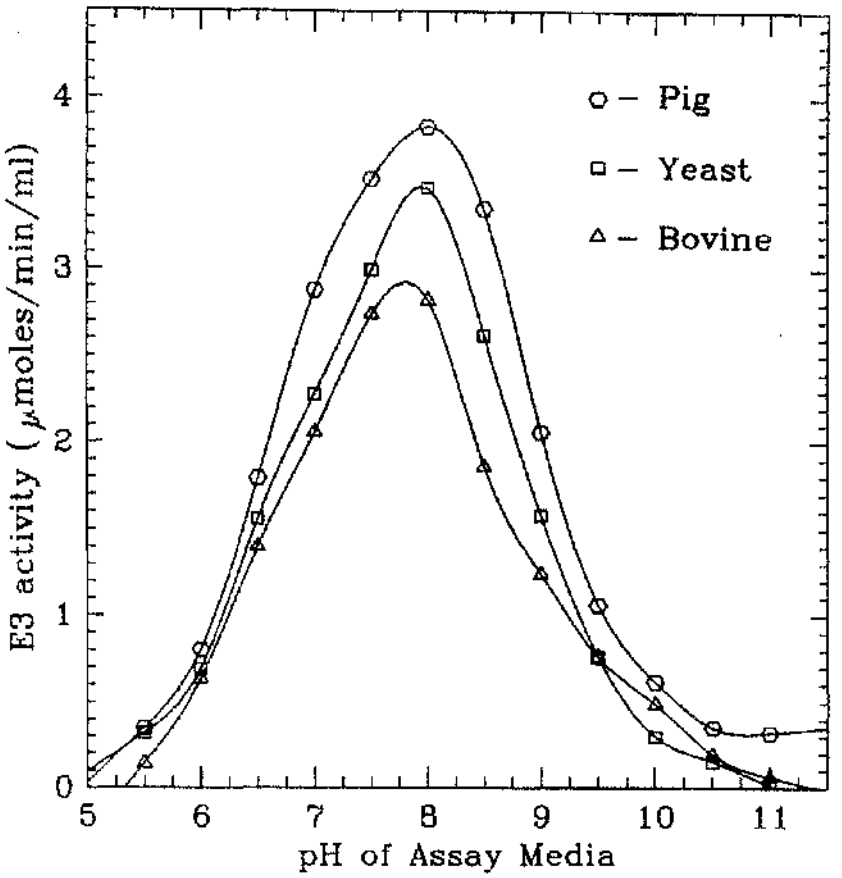


Figure 5.7: Effect of pH on pig heart, bovine and yeast E3 activity.

E3 activity was determined as described in Material and Methods section 2.2.7 (B) with the following buffers used to hold the assay medium at the appropiate pH: pH 5.5-6.5, MES, pH 6.5-7.5, MOPS, pH 7.5-8.5, Tris-HCl, pH 8.5-10.0, CHES, 8.5-10.0 and pH 10.0-11.0, CAPS.

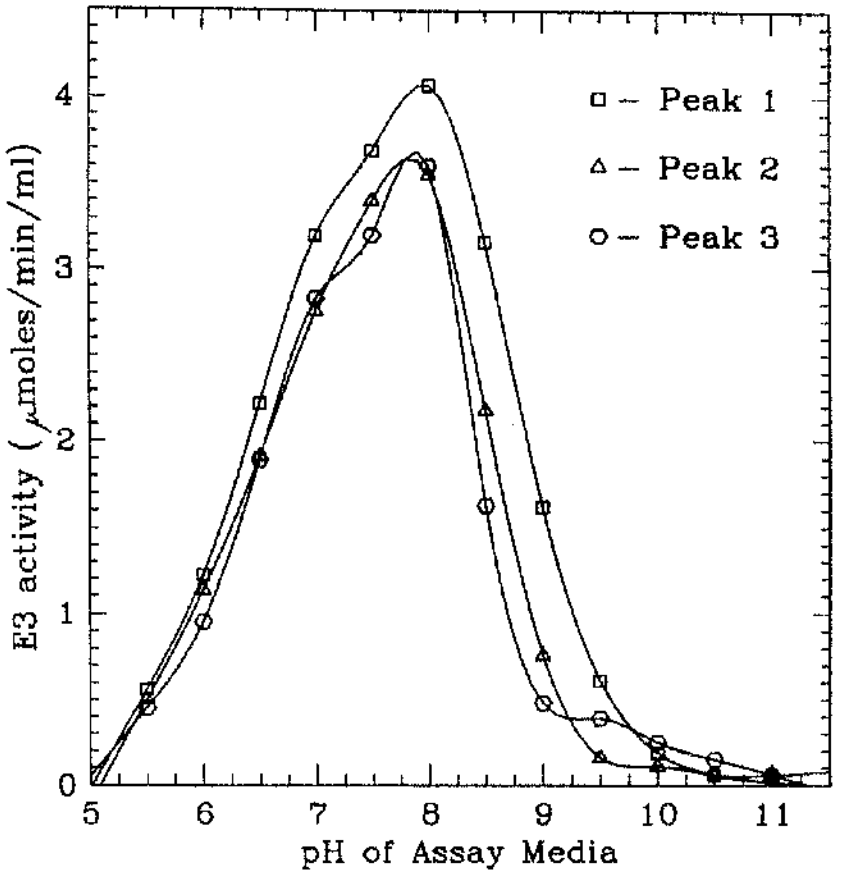


Figure 5.8: Effect of pH on the three potato tuber mitochondrial isoforms of E3 activity.

E3 activity was determined as described in Material and Methods section 2.2.7 (B) with the following buffers used to hold the assay medium at the appropiate pH: pH 5.5-6.5, MES, pH 6.5-7.5, MOPS, pH 7.5-8.5, Tris-HCl, pH 8.5-10.0, CHES, 8.5-10.0 and pH 10.0-11.0, CAPS.

adjusted to the correct pH with HCl or NaOH. E3 activity is measured at the same pH whenever there is a change in buffer to ensure that the E3 activity is only affected by the change in pH and not by the buffer itself. No major differences were observed between any of the E3s in their pH optima with each E3 showing peak activity at pH 8.0. The literature reports maximum velocity at approx. pH 8.0 for rat liver mitochondrial E3 (Reed, 1973) and human liver E3 (Ide *et al.*, 1967).

5.2.4 TREATMENT OF DIHYDROLIPOAMIDE DEHYDROGENASE WITH p-AMINOPHENYL DICHLOROARSINE (APA)

APA is an aromatic trivalent arsenical compound which specifically inhibits the redox active thiol disulphide group at the active site of E3. These compounds have an interesting chemical property in that they will not react well with single thiols, but will form stable complexes with pairs of appropriately spaced thiols in their reduced state. Thus only in the presence of its substrate, dihydrolipoamide or product, NADH, will APA inhibition of E3 be observed. This phenomenon, whereby substrate and product render active-site residues susceptible to chemical modification rather than protect them, is most unusual and characteristic of dihydrolipoamide dehydrogenases and related enzymes containing a redox-active disulphide pair at their respective active sites. Thus, inhibition by this reagent under appropriate conditions is diagnostic for E3 and related enzymes with similar catalytic mechanisms (Danson *et al.*, 1984; Loring *et al.*, 1992).

The three potato tuber mitochondrial E3 isoforms were prepared as described in section 4.2. These isoforms of E3 along with pig heart E3 were dialysed overnight into 50mM potassium phosphate buffer, pH 7.6. A stock solution of APA was prepared by dissolving it into 95% ethanol to a final concentration of 10mM. NADH supplied by Sigma Chemical Co. was dissolved in 50mM potassium phosphate buffer, pH 7.6 to a concentration of 1mM. Each of the E3s were assayed as described in Materials and Methods (section 2.2.7(B)) over a time course of 30min in the presence/absence of APA (0.5mM & 0.05mM) and NADH (25μM). Since E3 is itself inhibited by NADH

(as can be seen in section 5.3), these experiments were repeated until a suitable concentration of NADH was found where it did not inhibit E3 activity too severely but was present at the appropriate level to induce reduction of the putative cysteine pair at the catalytic site and thus permit APA induced inhibition of E3. Results are presented in Tables 5.3 to 5.6, where E3 assays are carried out at 0 min, 15 min and 30 min. E3 activity is expressed as a percentage of the control activity at zero time. The control represents the E3 enzyme with neither APA or NADH present.

From the results represented in Tables 5.3 to 5.6, it can be seen that all three isoforms of potato tuber mitochondrial E3 and the pig heart E3 are inhibited by APA in the presence of NADH. As expected APA does not significantly inhibit the E3 enzymes in the absence of NADH.

All the E3s (porcine, bovine, yeast and the three potato mitochondrial isoforms) were found to exhibit maximum velocity at pH 8.0. However differences between these enzymes were observed for their K_m and K_i values with the potato E3 isoforms exhibiting significantly higher values than the mammalian or the yeast E3 enzymes. Peak 1 (α_2 isoform) of potato mitochondrial E3 was observed to have lower K_m and K_i values than peak 2 ($\alpha\beta$ isoform) and peak 3 (β_2 isoform). These experiments would have to be repeated to confirm that these isoforms differ significantly in their kinetic constants. Recent research, however, indicates that there are marked differences between the three potato mitochondrial E3 isoforms in terms of their kinetic constants when measured under appropriated conditions (R. Cook, Glasgow University, personal communication). In this instance, K_m values were calculated for the three potato E3 isoforms as described in this chapter, with the exception that they were performed at 25°C and pH 7.5. The results of this chapter are representative of experiments performed at 31°C and pH 7.6. The K_m values of the latter experiments, although lower than those presented in Table 5.2 with K_m (NAD^+) values reported to be approx. 100μM. 200μM and 500μM for peaks 1, 2 and 3 respectively, exhibited significant differences between the three isoforms with peak 1 yet again having the

	Time (min)		
	0	15	30
Control	100%	101%	98%
NADH (25μM)	95%	90%	79%
APA (0.05mM)	102%	97%	94%
APA (0.5mM)	103%	97%	98%
NADH (25μM) + APA (0.05mM)	86%	14%	0%
NADH (25μM) + APA (0.5mM)	90%	12%	0%

Table 5.3: Treatment of potato tuber mitochondrial E3 (peak 1) with p-aminophenyl dichloroarsine (APA)

Peak 1 (isoform 1) of potato tuber mitochondrial E3 was treated with APA in the absence/presence of NADH. E3 activity was determined in duplicate as described in Materials and Methods section 2.2.7(B) and expressed as a percentage of E3 activity for the control.

	Time (min)		
	0	15	30
Control	100%	99%	98%
NADH (25μM)	99%	84%	66%
APA (0.05mM)	96%	96%	95%
APA (0.5mM)	97%	96%	96%
NADH (25μM) + APA (0.05mM)	51%	3%	0%
NADH (25μM) + APA (0.5mM)	56%	2%	0%

Table 5.4: Treatment of potato tuber mitochondrial E3 (peak 2) with p-aminophenyl dichloroarsine (APA)

Peak 2 (isoform 2) of potato tuber mitochondrial E3 was treated with APA in the absence/presence of NADH. E3 activity was determined in duplicate as described in Materials and Methods section 2.2.7(B) and expressed as a percentage of E3 activity for the control.

	Time (min)		
	0	15	30
Control	100%	97%	99%
NADH (25μM)	95%	75%	54%
APA (0.05mM)	98%	99%	98%
APA (0.5mM)	100%	97%	96%
NADH (25μM) + APA (0.05mM)	90%	0%	0%
NADH (25μM) + APA (0.5mM)	83%	0%	0%

Table 5.5: Treatment of potato tuber mitochondrial E3 (peak 3) with p-aminophenyl dichloroarsine (APA)

Peak 3 (isoform 3) of potato tuber mitochondrial E3 was treated with APA in the absence/presence of NADH. E3 activity was determined in duplicate as described in Materials and Methods section 2.2.7(B) and expressed as a percentage of E3 activity for the control.

	Time (min)		
	0	15	30
Control	100%	98%	101%
NADH (25μM)	86%	75%	71%
APA (0.05mM)	102%	98%	98%
APA (0.5mM)	99%	100%	98%
NADH (25μM) + APA (0.05mM)	70%	18%	0%
NADH (25μM) + APA (0.5mM)	58%	3%	0%

Table 5.6: Treatment of porcine heart E3 with p-aminophenyl dichloroarsine (APA)

Porcine heart E3 was treated with APA in the absence/presence of NADH. E3 activity was determined in duplicate as described in Materials and Methods section 2.2.7(B) and expressed as a percentage of E3 activity for the control.

lowest K_m value. It is not clear at present whether these striking variations in kinetic constants represented a marked temperature-dependence on these measurements, or may reflect ion strength or choice of buffers, since both sets of estimations were carried out at similar pH values. It is also known that K_m values are very dependent on pH (Sahlman & Williams, 1989); therefore it would be interesting to repeat these basic kinetic experiments at different pHs to determine if differences in the kinetic constants between the three potato E3 isoforms are also greater at a particular pH. These kinetic analyses tend to confirm that the three E3 isoforms have similar, but distinct enzymatic properties which may reflect the possibility that they play different physiological roles *in vivo*.

CHAPTER 6

RECONSTITUTION STUDIES ON PYRUVATE DEHYDROGENASE COMPLEX AND 2-OXOGLUTARATE DEHYDROGENASE COMPLEX

6.1 INTRODUCTION

The results described in chapters 3 to 5 of this thesis provide evidence for the existence of distinctive isoforms of dihydrolipoamide dehydrogenase (E3) in potato tuber mitochondria. Chapter 3 describes how the purification of E3 by a novel affinity procedure involving its specific interaction with the mammalian E2/X core resulted in the appearance of two bands when analysed by SDS-PAGE with M_r of 58,000 and 56,000 which were observed to cross react strongly with antiserum raised against mammalian E3. In chapter 4 the further purification of this E3 to resolve these two bands by Mono Q anion exchange chromatography resulted in the production of three peaks of E3 activity, which again cross-reacted strongly with anti-E3 serum. Chapter 5 provides evidence from preliminary kinetic characterisation experiments that the three forms of potato mitochondrial E3 may differ in their enzymatic properties. It is suggested therefore that these three peaks of E3 activity may represent complex-specific isoforms of E3 and that each species is selectively or exclusively employed by the various multienzyme complexes found in potato mitochondria (PDC, OGDC and GDC). This is the first time that evidence for the existence of multiple isoforms of E3 from a plant source has been presented, however E3 isoforms have been reported to exist in bacterial species such as *P. putida*, *P. aeruginosa* and *E. coli* and possibly also rat liver mitochondria as described in section 4.1.

Previous research, originally performed by Ailsa Carmichael (Ph.D. Thesis. Glasgow University, 1994) also provides support for the theory that three potato mitochondrial isoforms of E3 may function selectively with each interacting specifically with one of the individual multienzyme complexes as described above. When a crude extract of potato mitochondrial protein was separated by gel filtration, two separate peaks of E3 activity were found to elute. (Fig. 6.1). Some E3 (approx. 20%) activity co-eluted with PDC and OGDC in the void volume, with the second peak corresponding to dissociated, 'free' E3. It is known that unlike the 2-oxoacid dehydrogenases, associations between component enzymes of GDC are weak and they

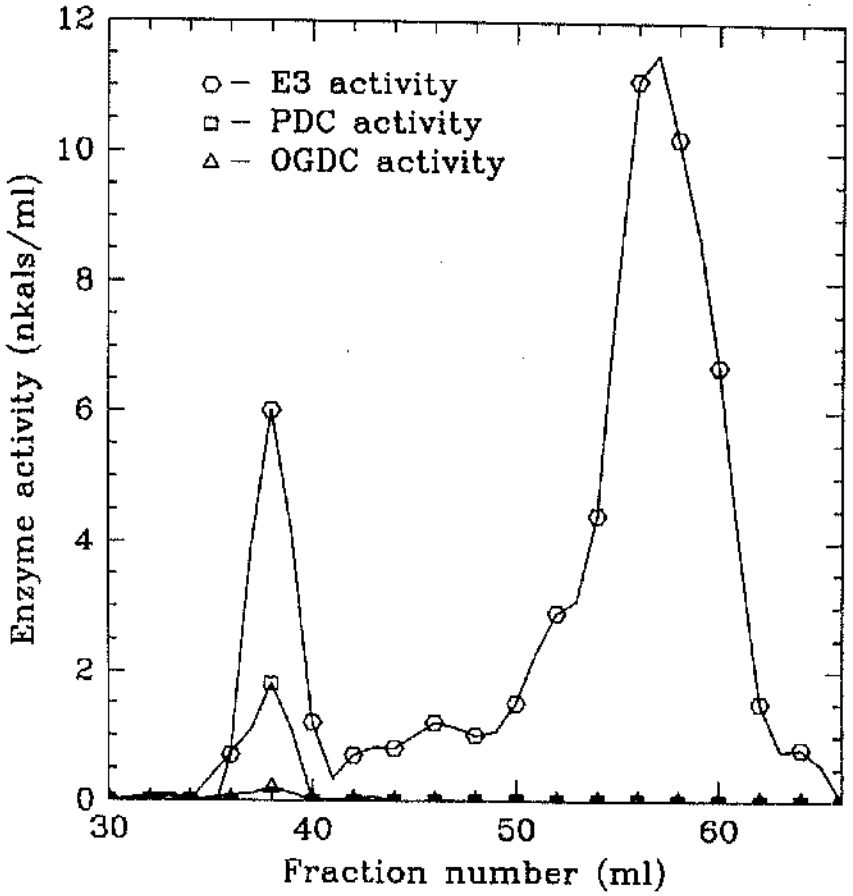


Figure 6.1: Separation of potato tuber mitochondrial E3 by gel filtration

Potato mitochondrial extracts were solubilised with 0.25% (w/v) Triton X-100 prior to loading onto a 100ml Superdex 200 gel filtration column. Protein was eluted with 100ml of elution buffer (1mM EGTA, 1mM DTT, 50mM imidazole, pH 6.8) with a flow rate of 1ml/min and E3 activity determined as described in Materials and Methods section 2.2.7(B).

will therefore elute separately in relation to their molecular mass on gel filtration (Bourguignon *et al.*, 1988). Consequently, it is thought likely that the void volume E3 is that which is associated with PDC and OGDC, and that E3 associated with GDC (L-protein) would be exclusively located in the 'free' E3 peak. It was also noted that although there was 100% recovery of E3 activity on gel filtration, only 60% and 45% of PDC and OGDC activity respectively was recovered. It was, therefore, assumed that a proportion of E3 dissociated from the PDC and OGDC complexes during preparation of the potato mitochondrial extract, and subsequently elute along with the E3 originating from GDC in the 'free' E3.

Both the void volume and the 'free' E3 fractions were heat treated and applied in turn to a Mono Q HR5/5 column and protein eluted with a 60ml 10-400mM linear gradient of potassium phosphate (pH 6.8). When the void volume fractions were separated by anion exchange chromatography, two peaks of E3 activity were resolved. In contrast three E3 activity peaks were resolved from the 'free' E3 fractions indicating that this third E3 activity peak may represent exclusively or selectively the GDC L-protein. Unfortunately attempts to confirm these results proved somewhat irreproducible, since on several occasions when crude extracts were separated by gel filtration little or no E3 activity coeluted at the void volume and as a consequence, analysis of this void volume E3 by anion exchange chromatography could not be performed. This may be a consequence of the variability of the biological material in that perhaps the age or length of storage of the potatos may affect the stability of these labile multienzyme complexes. In these circumstances it proved impossible to confirm these results unambiguously.

Comparison of the E3 profiles resulting from Mono Q resolution of mitochondrial proteins from photosynthetic (potato leaf) and non-photosynthetic tissue (potato tuber), performed under conditions as described above, was performed as an alternative method to gain information on the possible complex-specific nature of these E3s and their tissue-specific expression (Fig. 6.2). GDC is very abundant in mitochondria of leaves but very low in root tissues, thus a presence of a predominant form of E3 in

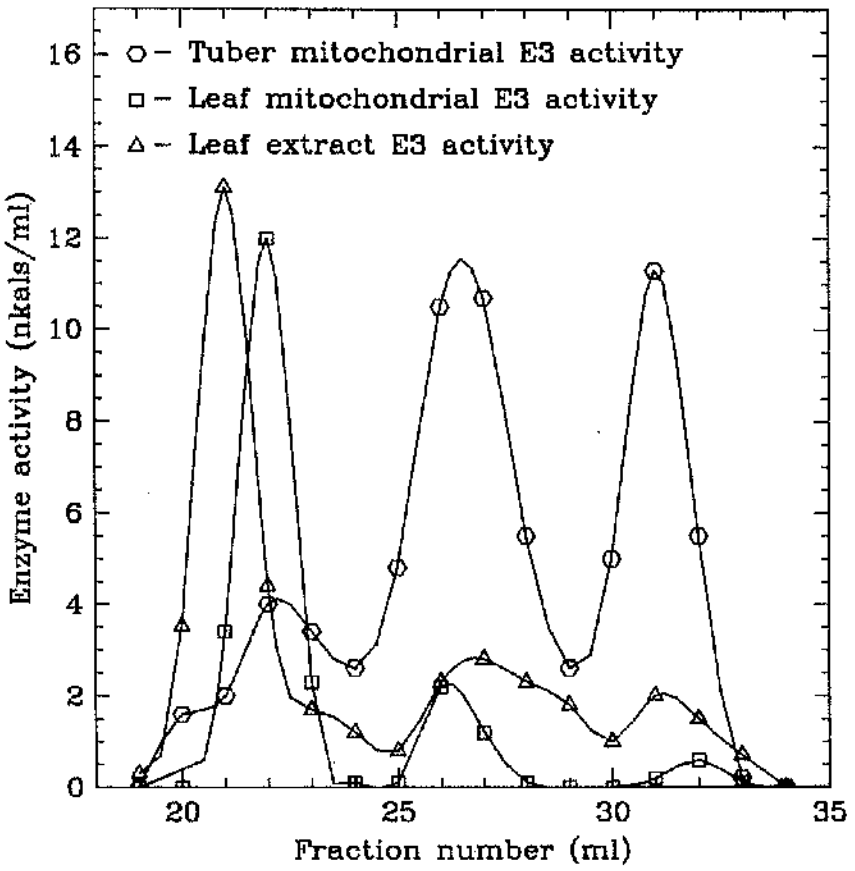


Figure 6.2: Resolution of potato tuber mitochondrial, leaf mitochondrial and total leaf E3 by anion exchange chromatography.

Extracts were solubilised with 0.25%(v/v) Triton X-100 and heat treated prior to loading onto a Mono Q HR5/5 column connected to a Pharmacia FPLC system. Protein was eluted with a 60ml phosphate gradient as described in Chapter 4. E3 activity was determined as described in Materials and Methods section 2.2.7(B).

leaves might indicate a specific function in a GDC-catalysed reaction. In both cases, three E3 activities were resolved with a substantial increase in the magnitude of peak one being observed for the potato leaf mitochondrial protein when compared to the potato tuber mitochondrial extract, with approx. 70% and 15% of the E3 activity loaded onto the column eluting as peak 1 respectively. It has been previously reported that GDC constitutes about 30% of leaf mitochondrial protein and approx. 2-5% of protein in leaves (Walker & Oliver, 1986). This preliminary experiment may indicate that the increased levels of peak one may be related to increased levels of GDC present in potato leaf mitochondria. However it was possible that chloroplast E3 associated with the plastid PDC may have contaminated the leaf mitochondrial extract. As a result E3 from total potato leaf extract was also resolved by anion exchange chromatography and found to display a profile similar to leaf mitochondria except peak two displays a shoulder which may reflect the presence of chloroplast E3 since the potato leaf extract will contain both chloroplast and mitochondrial protein (Fig. 6.2). It has been discovered subsequently that when pea leaf mitochondrial and chloroplast E3 are applied to a Mono Q ion exchange column under the same conditions as previously, two peaks of E3 activity elute, with the mitochondrial E3 eluting with a lower point in the KPi gradient than the chloroplast E3, the latter comprising 10-15% of total E3 in pea leaf extract (M. Conner, Glasgow University, personnal communication). Consequently, differences in potato tuber and leaf mitochondrial E3 profiles indicate there may be tissue-specific expression of E3 in that different relative amounts of each E3 isoform are expressed in tuber and photosynthetically active tissue, with peak one possibly representing E3 of GDC.

To determine any possible complex specificity of the three isoforms of potato mitochondrial E3, it is necessary to conduct studies where their ability to reconstitute the complex activity of the E1/E2 subcomplex of PDC and OGDC stripped of their native E3 subunits was compared. These reconstitution experiments require partial purification of the 2-oxoacid dehydrogenase complexes from potato and subsequent fractionation. However, in view of the low levels of organellar protein and the lability

of the complexes when the organelles are disrupted, this approach has proved technically difficult (Poulson & Wedding, 1970; Randall *et al.*, 1990). In addition, potato tubers have even lower levels of the complexes than actively growing plant tissue. This approach was abandoned after consultation with the group of Douce and coworkers, Grenoble, France who confirmed our initial findings that, although PDC and OGDC activity were detectable in crude potato extracts, on purification such activities are rapidly lost indicating that they exist as labile complexes. This is comparable to the related multienzyme complex glycine decarboxylase complex that exists as a labile multicomponent complex, although its high abundance has allowed partial purification in this case (Walker & Oliver, 1986). As a result little is known about the subunit composition of the plant 2-oxoacid dehyrogenase complexes. However, reconstitution studies have been carried out successfully for the 2-oxoacid dehydrogenase complexes from mammalian and bacterial sources in which purified components were mixed to form the intact catalytically active assembly (Sakurai *et al.*, 1970; Jaenicke & Perham, 1982).

It was observed from the results of Chapter 3, that the potato tuber mitochondrial E3 will recognise and bind to the immobilised E2/X core assembly of mammalian PDC on the affinity column. Since E3 is highly conserved across species, it was thought possible that the potato tuber mitochondrial E3 isoforms might reconstitute the activity of the mammalian complexes in a selective fashion. Any preferential stimulation by the individual potato E3s of the mammalian complexes will help to determine any potential complex specificity of the isoforms. A parallel study on OGDC reconstitution was performed by S. Khan, Glasgow University and results were included in this chapter as a useful comparison with the data on PDC reconstitution.

6.2 RESULTS AND DISCUSSION

6.2 1 DISSOCIATION OF PYRUVATE DEHYDROGENASE COMPLEX (PDC)

Selective depletion of mammalian PDC of its constituent E3 proved to be more difficult than first envisaged. As described in Chapter 3 (section 3.4.2), under conditions of high salt, the E1/E3 components of PDC can be dissociated from its E2/X core assembly. It must be noted also that a significant amount of E1 does remain bound to the E2/X subcomplex under these conditions. For the purpose of reconstitution studies, it was then necessary to separate E3 from E1 so as to re-introduce the E1 component to the E2/X core assembly, to enable testing of the ability of heterologous E3s to reconstitute overall PDC activity.

Initial attempts to separate the E1 component from E3 involved the use of the affinity column once more. Instead of immobilising the E2/X core assembly from PDC on Sepharose 4B, the E1/E2 subcomplex of OGDC was employed on this occasion. As mentioned earlier, E3 is a highly conserved enzyme and is reported to be the same gene product in both OGDC and PDC. It was thought that by applying the E1/E3 fraction from PDC to the E2/E1-OGDC affinity matrix, E3 would bind with the E1/E2 subcomplex of OGDC and that the E1 component would not, owing to its specificity for its parent mutienzyme complex (PDC). This, therefore, required the dissociation of OGDC to prepare E1/E2 subcomplex required for the affinity column. This was performed in the same manner as described in section 3.4.2 for the dissociation of PDC with the success of the dissociation of OGDC confirmed by SDS-PAGE (Fig. 6.3). The E1/E3 fraction of PDC to be separated into its individual E1 and E3 components, was dialysed into sample buffer containing 1mM EDTA, 20mM NaCl, 50mM KPi buffer, pH 7.6 and then applied to the column under the same conditions as described in Chapter 3 (section 3.4.3). Fractions (1ml) were collected and assayed for E3 activity as described in Materials and Methods (section 2.2.7(B)).

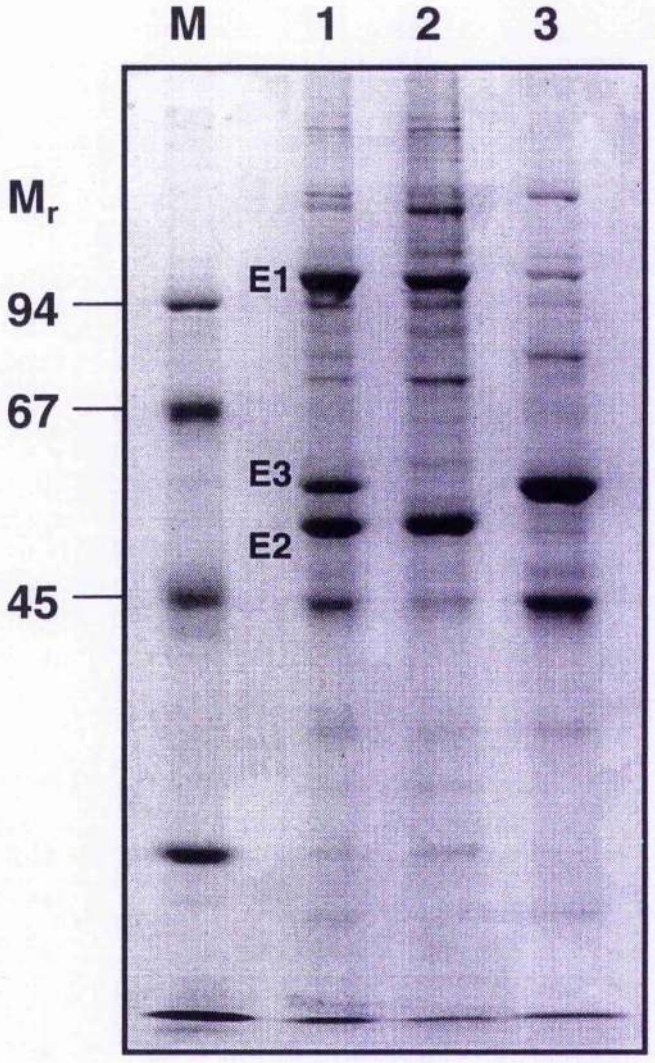


Figure 6.3: Dissociation of bovine heart OGDC analysed by SDS-PAGE.

The E1/E2 subcomplex of OGDC was separated from the E3 component as described in section 6.2.1. Lane M, M_r marker proteins; lane 1, bovine heart OGDC; lane 2, isolated E1/E2 subcomplex; lane 3, dissociated E3

All the E3 was observed to bind to this OGDC E2 core assembly immobilised on the affinity column (Fig. 6.4). For further analysis, samples from the column wash fraction and the eluant fractions were then TCA precipitated, resuspended in Laemmli sample buffer and separated by SDS-PAGE (Figs. 6.5 and 6.6). This procedure was performed as described in Materials and Methods (sections 2.2.6 and 2.2.10). Fig. 6.5 represents the column wash fractions displaying the proteins that failed to bind to the affinity matrix. It is apparent fractions 6-15 contain the E1α and E1β subunits of PDC with no detectable contamination by the E3 component. Fig. 6.6 represents the E3 bound fractions eluted from the column in 1M NaCl. However, it is observed that these fractions also contain E1α and E1β subunits that have eluted from the column along with the E3. This non specific interaction of E1 with the E1/E2 core of OGDC probably represents electrostatic binding owing to the low ionic strength of the wash buffers required when applying the E1/E3 fraction to the affinity column. Subsequent attempts to raise the salt concentration of the buffers led to a failure of E3 binding to the column, thus it was not possible to find an optimal salt concentration which prevented non-specific interactions of the E1 enzyme of PDC whilst also permitting quantitative retention of the E3 component.

These results, however, indicate that the separation of E1 from E3 of PDC has been partially successful, despite some loss of E1 which has bound to the column. By pooling and concentrating the E1 fractions that failed to bind to the column and adding them back to the E2/X core assembly of PDC, it should be possible to proceed with the reconstitution studies. Thus this appears to be a viable method for the separation of E3 from mammalian PDC; however, it was observed that yields of the E1 component of PDC were low, with a proportion sticking to the affinity column and further losses occurring during dialysis and concentration procedures. Moreover, the affinity column has a limited binding capacity and so only small amounts of purified E1 can be produced conveniently by this technique limiting the usefulness of this approach in reconstitution studies.

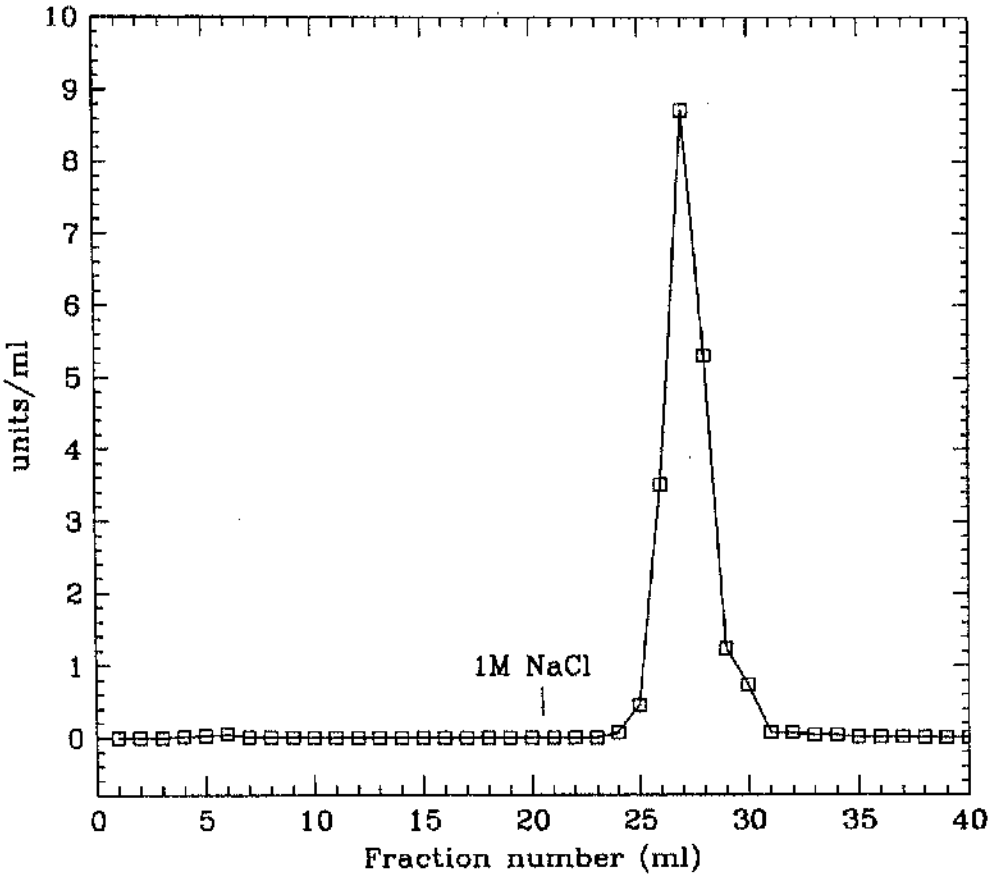


Figure 6.4: Separation of E3 from E1 of bovine heart PDC on OGDC-E2/E1 affinity column.

The E1/E3 fraction from bovine heart PDC containing approx. 19 units of E3 enzyme activity (1 unit = 1μmol NADH/min) was loaded onto an OGDC-E1/E2 affinity column (10ml, 1cm x 12.7cm). The column was washed with sample buffer (20mM NaCl, 1mM EDTA, 50mM KPi, pH 7.6). Fractions (1ml) were collected and assayed for E3 activity and expressed as units of enzyme activity/ml. Bound protein was eluted with sample buffer containing 1M NaCl and each fraction assayed for E3 activity.

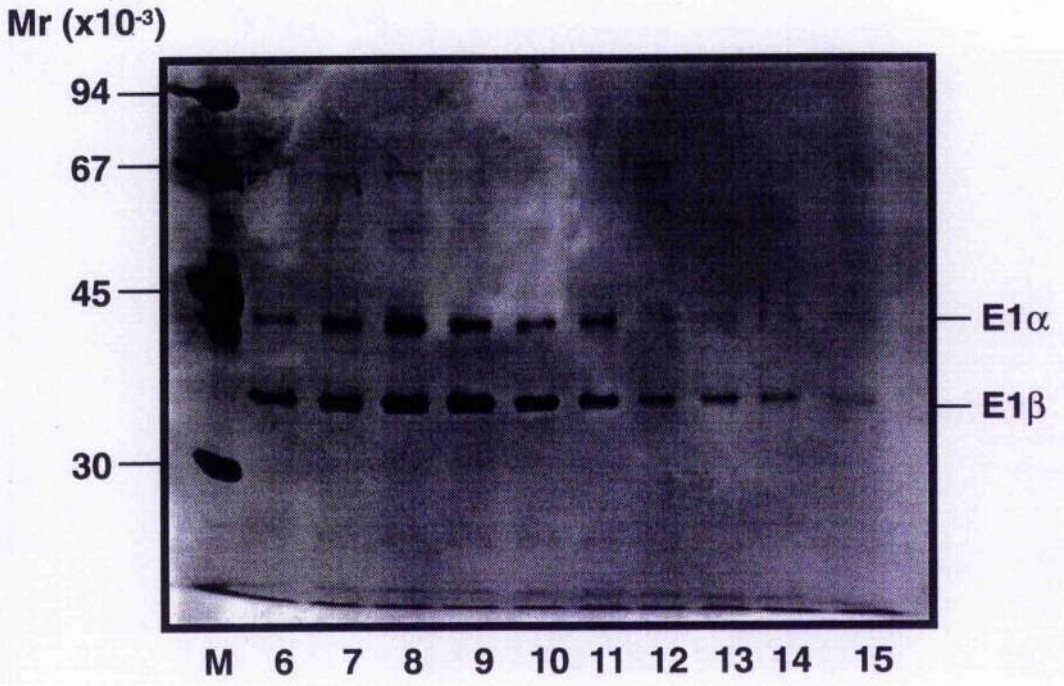


Figure 6.5: Analysis of wash fractions from the E1/E2-OGDC affinity column by SDS-PAGE.

The E1/E3 fraction of bovine heart PDC dissociated under conditions of high salt (as described in section 3.4.2) was loaded onto a E2 affinity column where the E2 component was purified from bovine heart OGDC (as described in section 6.2). Samples from the column wash fractions (6-15) representing unbound protein were separated by SDS-PAGE and silverstained. Lanes 6-15 representing column wash fractions 6-15 and lane M the M_r marker proteins.

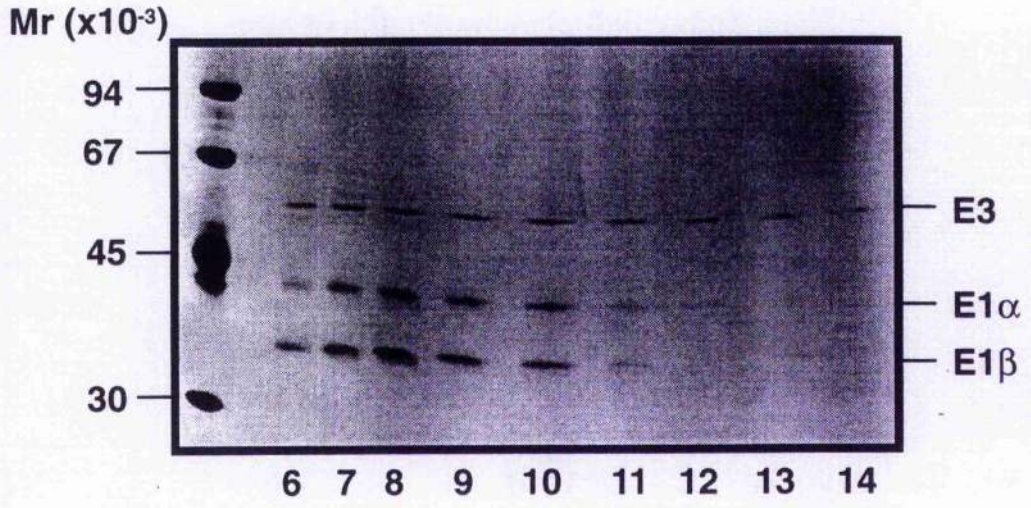


Figure 6.6. Analysis of the eluant fractions from the E1/E2-OGDC affinity column by SDS-PAGE

The E1/E3 fraction of bovine heart PDC dissociated under conditions of high salt (as described in section 3.4.2) was loaded onto a E2 affinity column where the E2 component was purified from bovine heart OGDC (as described in section 6.2). Samples from the eluant fractions (6-14) representing bound protein were separated by SDS-PAGE and silverstained. Lanes 6-14 representing column wash fractions 26-34 and lane M_r the marker proteins.

In view of inherent difficulties in obtaining high levels of pure E1 and E3 components in an active form, it was decided to devise an alternative strategy for the selective removal of E3 from PDC, whilst leaving the rest of this multienzyme complex intact. Thus, selective dissociation of PDC was attempted under conditions of high salt and subsequent separation of the E3 enzyme from the E2/X/E1 subcomplex by the Superose 6 FPLC gel permeation as described in Chapter 3 (section 3.4.2). By optimising the conditions under which PDC was dissociated, it proved possible to release the E3 component selectively from PDC leaving the rest of the (E2/X/E1) complex intact. This proved to be a more suitable method than that employing the E2/X affinity column as mentioned above, since it involved fewer steps and resulted in good yields of E3-deficient complex.

Bovine heart PDC was prepared as described in Material and Methods (section 2.2.4). An equal vol. of running buffer containing 2M NaCl, 1mM DTT, 1mM $MgCl_2$, 0.2mM TPP, 0.01% (v/v) Triton X-100, 50mM KPi, pH 7.0 was added to a 1ml sample of PDC (approx. 30mg/ml) so that the final concentration of NaCl was 1M. The presence of $MgCl_2$ and TPP in the buffer is to protect E1 activity and to stabilise the interaction of the E1α and β. E1 is also more stable and less readily released from the E2 core assembly at pH 7.0 rather than at pH 9.0 which is normally used in the dissociation of PDC. The sample was then incubated at 4°C for approx. 30 min prior to centrifugation at 6250g for 5 min to remove any non-soluble protein that may cause blockages during subsequent FPLC analyses. The Superose 6 FPLC column (100ml) was equlibrated with running buffer containing 0.6M NaCl, then pre-injected with 2M NaCl prior to the loading of the PDC sample. The column was run in buffer containing 0.6M NaCl with the high M_r E2/X/E1 core found to elute at the void vol. (approx. 32ml) and the E3 component eluting at approx. 62ml. For further analysis, samples from both the E2/X/E1 and E3 fractions were TCA precipitated, resuspended in Laemmli sample buffer and separated by SDS-PAGE (Fig. 6.7). Lane 3 contains the E2/X/E1 fraction and lane 4 the E3 fraction. It can be seen, however, that some E1 has dissociated from the core complex and eluted with the E3 component. Residual PDC

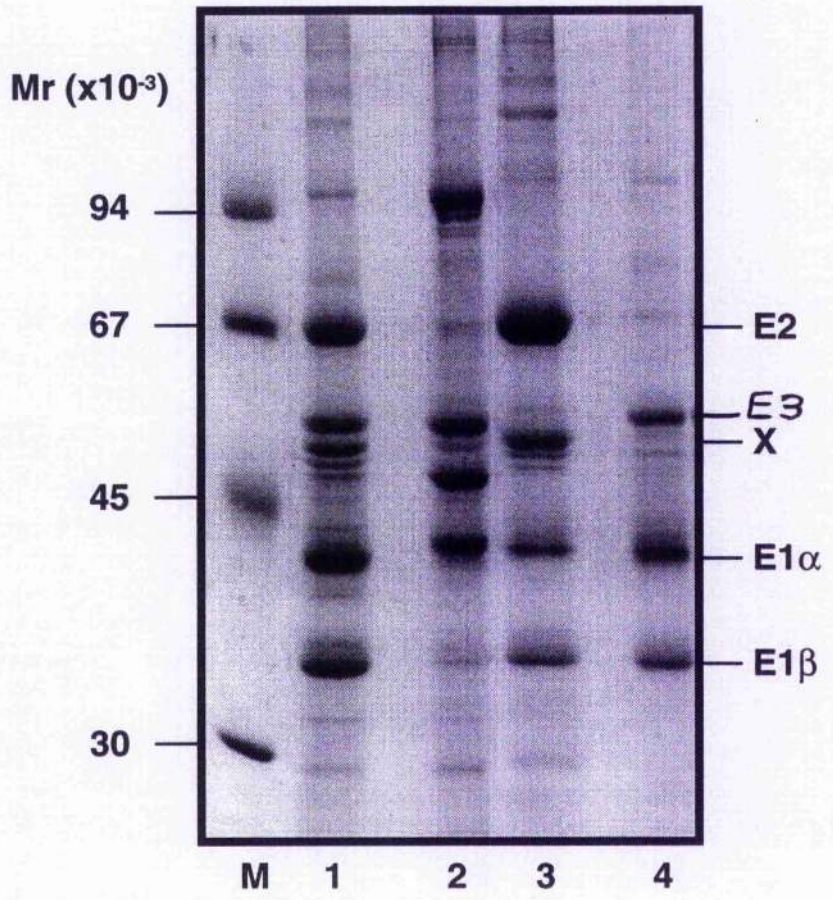


Figure 6.7: Dissociation of E3 from the E2/X/E1 components of bovine heart PDC analysed by SDS-PAGE

The dissociation of E3 from E2/X/E1 of PDC was carried out as described in section 6.2. Samples were analysed by SDS-PAGE and silverstained as described in Materials and Methods (sections 2.2.10 and 2.2.10 (D)). LaneM, Mr marker proteins; Lane 1, PDC; lane 2, OGDC; lane 3, E2/X/E1 and lane 4, E3/E1.

activity in the E2/X/E1 fraction was observed to be approx. 10% of the original PDC sample prior to dissociation.

6.2.2 PURIFICATION OF THE E3 ISOENZYMES

Potato E3 isoforms are generally purified from Percoll-purified potato tuber mitochondria. The E3 enzymes are extracted from mitochondria by treating with 0.1% (v/v) Triton X-100 and then heat treated at 65°C for 10 min as the first step in its the purification. The three peaks of E3 activity (the three isoforms) are resolved on Mono Q column as described in Chapter 3 (section 3.4.4). However, this method produces a very small amount of E3, so it was modified by extracting the enzyme with 0.1% (v/v) Triton X-100 from a crude organelle preparation and heat treated, producing an increase in yield as described in Materials and Methods (section 2.2.2(A)). The three isoforms were then resolved in sufficient yield on the Mono Q ion exchange chromatography to permit reconstitution studies.

6.2.3 PURIFICATION OF BOVINE HEART PDC AND OGDC E3

PDC and OGDC were prepared from bovine heart as described in Materials and Methods (section 2.2.4) and checked for purity by SDS-PAGE. These complexes were treated with 1M NaCl at 4°C for approx. 30min, heat treated at 65°C for 10min and centrifuged at 6250g for 5 min to remove denatured protein. Pure E3 was left in the supernatant fraction and checked for contamination by SDS-PAGE.

6.2.4 PDC RECONSTITUTION STUDIES

A time course was performed first to determine the optimal conditions required for maximal reconstitution of PDC activity after mixing the E2/X/E1 fraction and an excess of parent (homologous) E3. Re-assembly was found to occur after an

incubation period of between 5 and 10 min at 31°C. Routinely all reconstituted PDC samples were incubated for 15min in the presence of heterologous E3 before PDC assays were carried out as described in Materials and Methods section 2.2.7(A).

Reconstitution assays were performed by incubating increasing amounts of E3 samples from various sources (0-40μg) with a fixed amount (30μg) of E2/X/E1 subcomplex at 31°C for 15 min with 10μg removed and assayed for PDC activity. All E3s were dialysed into solution A minus NAD^+ (see Materials and Methods, section 2.2.7(B)) prior to use. The native PDC, OGDC, porcine and yeast E3, along with the three potato E3 isoforms were analysed for their ability to reconstitute PDC activity (Fig. 6.8). Each of the E3 enzymes were found to bind tightly to the E1/E2/X subcomplex, with the exception of the plant E3s which did not bind as tightly, with each varying in their ability to reconstitute PDC activity in terms of the actual level of reconstitution reached. When compared to intact PDC activity (the initial activity of PDC prior to dissociation), porcine heart E3 was found to reconstitute 50% of the original intact PDC activity, while, surprisingly its parent E3 only showed 35% reconstitution. This result is interesting as it was expected that optimal reconstitution would be obtained with parent E3 not the porcine heart E3 as a result of species specificity. It is possible that in the purification of E3 from bovine heart PDC, the enzyme was altered in such a way so as to affect its ability to reconstitute PDC activity. It may be that the bovine heart E3, although exhibiting a high affinity for the core subcomplex of PDC, is binding in an orientation which does not permit optimal access of lipoyl domains to its active site.

E3 purified from bovine heart OGDC showed a 22% reconstitution of PDC, however the yeast E3 showed no reconstitution at all. All three isoforms of potato mitochondrial E3 showed a degree of reconstitution of PDC (approx. 6%), although the low levels of activity make it difficult to assess the levels required for maximal reconstitution and thus to determine any differences between the three isoforms. In each case, only a slight excess of E3, compared to the stoichiometric amounts present in intact complexes, was required for maximal reconstitution. This demonstrates that

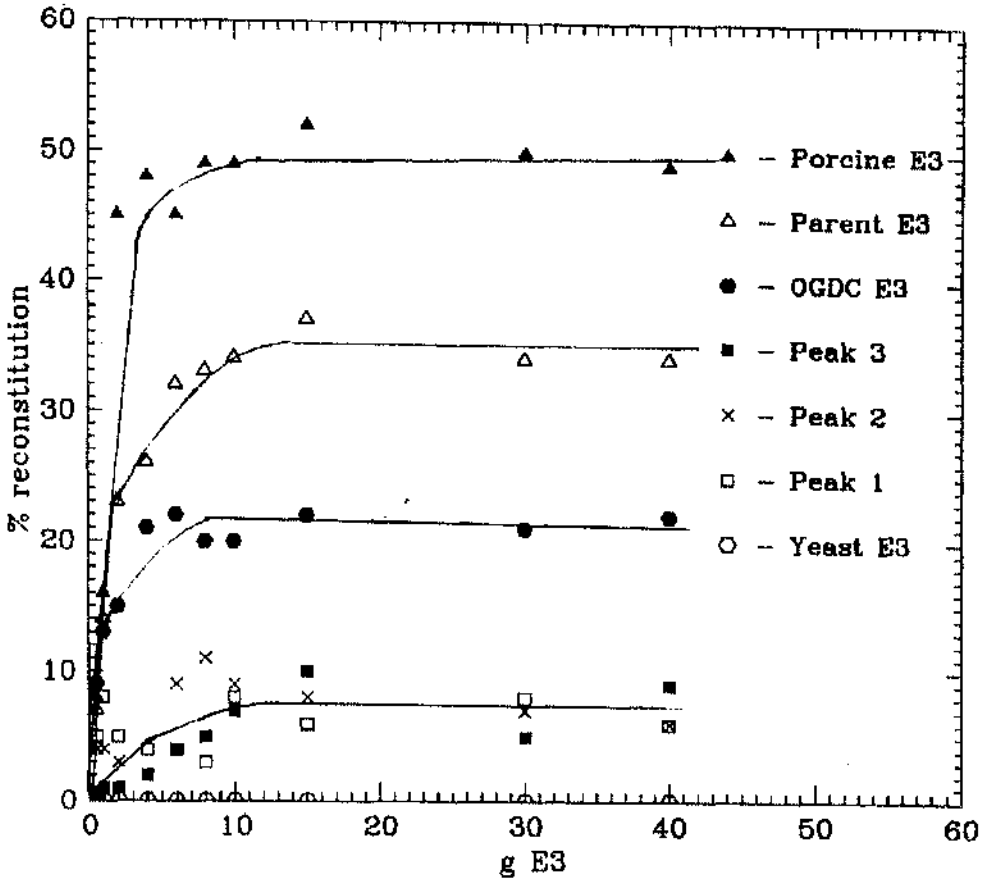


Figure 6.8: Profile of % reconstitution of PDC activity with different preparations of E3

Increasing amounts of E3 from the original bovine heart PDC sample, bovine heart OGDC, commercial preparations of porcine heart and yeast E3 and the three potato E3 isoforms were incubated for 15 min at 31°C with a fixed amount of bovine heart PDC E1/E2/X core (30µg). Intact PDC (10µg) activity was then assayed in duplicate (as described in Materials and Methods, section 2.2.7(A)) and reconstituted activity expressed as a % of original complex activity.

these complexes can assemble efficiently in high yield, even in dilute solution. For example, approx. 7-10μg of E3 was required for maximal reconstitution of 30μg of core enzyme and since E3 is approx. 7% of PDC by weight then saturation would have been expected at approx. 2.1μg of E3. Further experiments are necessary to ascertain why pig heart E3 is more successful in reconstituting PDC activity than parent E3 from bovine heart.

6.2.5 OGDC RECONSTITUTION STUDIES

The E3 subunit from bovine OGDC was dissociated from the E1/E2 core using 1M NaCl at pH 7.6 with a 20 min incubation on ice. E1/E2 and the dissociated E3 were then separated on a Superose 6 FPLC gel filtration column. A small proportion of E1, however, dissociated with the E3 fraction and was removed by heat treatment at 65°C for 10 min with no loss in E3 activity. The two peaks of protein were dialysed into 50mM KPi buffer pH 7.6 and concentrated in PEG. E3 contamination of the purified E1/E2 subcomplex was found to be minimal producing a very low background OGDC activity. Purification of the three potato isoforms of E3 and PDC and OGDC E3 were carried out as described in sections 6.2.2 and 6.2.3.

Reconstitution assays were performed by incubating increasing amounts of E3 samples from various organisms (0-50μg) with a fixed amount of E1/E2 core and assayed for OGDC activity. When compared to intact activity, native E3 from PDC and OGDC consistently gave 60-80% reconstitution (Fig. 6.9). Pig heart and bovine mucosal E3 were only able to sustain 20% and 15% activity of reconstitution respectively. In each case, reconstitution began to saturate at approx. 1.5μg of E3 with 5μg of the core component. This correlates well with the fact that E3 constitutes approx. 20% of intact OGDC by weight. No reconstitution was observed with yeast E3 or any of the three isoforms of potato mitochondrial E3, even at very high levels of E3. It was expected, however, that the potato E3 isoforms would bind as this was the method employed to purify it as described earlier (section 3.4.4). The effect of heat

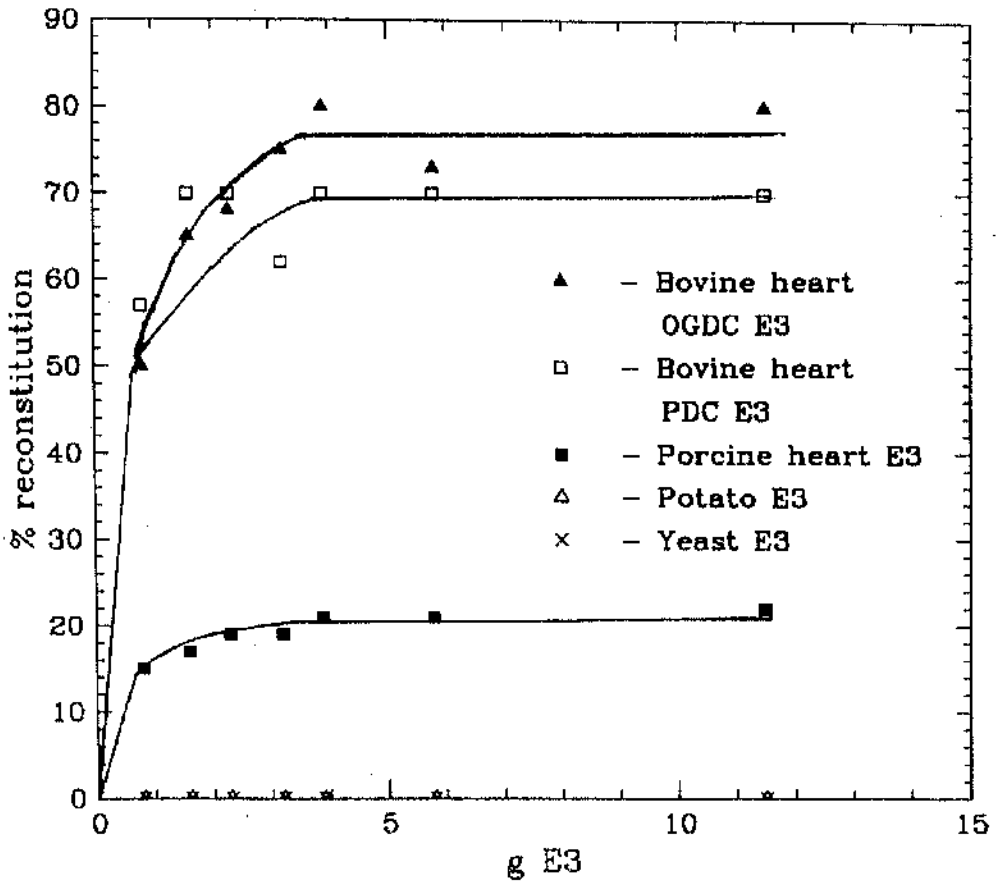


Figure 6.9: Profile of % reconstitution of OGDC activity with different preparations of E3

Increasing amounts of E3 from the original bovine heart OGDC sample, bovine heart PDC, commercial preparations of porcine heart and yeast E3 and the three potato E3 isoforms were incubated for 12 min at 31°C with a fixed amount of bovine heart PDC E1/E2/X core (14µg). Intact PDC (5µg) activity was then assayed in duplicate (as described in Materials and Methods, section 2.2.7(A)) and reconstituted activity expressed as a % of original complex activity.

treatment and freezing the E3 enzyme and the presence of Triton X-100 on reconstitution was also investigated and were found to have no adverse effects.

The ability of the components of the 2-oxoacid dehydrogenase complexes to self assemble spontaneously into a functioning complex is an inherent property of multienzyme complexes. This is demonstrated in the results of this chapter by the ability of both the PDC and OGDC components from mammalian sources to reassemble into catalytically active complexes. It is interesting to note that the pig heart E3 reconstitutes PDC activity to a greater extent than its native E3, yet native E3 reconstitutes OGDC activity more successfully than pig heart E3. In both cases the E3 enzymes were prepared identically. Further reconstitution experiments are required on the PDC complex to determine whether this difference is real or is a result of the method used for the purification of E3. The potato E3 isoforms were also found to reconstitute approx. 7% of PDC activity but failed to reconstitute OGDC activity. However no direct comparisons could be made between the three isoforms owing to the low levels of PDC reconstitution achieved with the plant enzymes. Therefore no definitive conclusions can be drawn concerning complex specificity of the three potato E3 isoforms.

It is unclear at present why the plant isoforms will reconstitute mammalian PDC activity, however slightly, but will not reconstitute the mammalian OGDC activity. In both cases, yeast E3 failed to reconstitute either PDC or OGDC activity, even at very high levels of E3. However, this result does not eliminate the possibility that the yeast E3 does recognise and bind to the mammalian OGDC/PDC core assembly but is incapable of reconstituting the catalytic activity of the complexes. This was confirmed by competition experiments where increasing amounts of competing yeast E3 led to a rapid decrease in the activity of OGDC reconstituted with stoichiometric amounts of its parent E3 (data not shown). Competition experiments were also performed with the plant E3 isoforms in which all three found to compete with the native E3 for binding to the OGDC core, although much less effective. It appears that although the yeast and

the potato E3 isoforms will recognise and bind to the core assembly, the flexible lipoyl domain of E2 is not able to optimally interdigitate with the active site of heterologous E3 components, therefore, restoration of complex activity is non-existent or markedly reduced. Time would not allow similar competition experiments to be carried out on PDC, as a result such experiments are essential for future work.

From the reconstitution and competition studies involving both PDC and OGDC, it may be concluded that all the E3s bind to the OGDC and PDC subcomplexes, with the plant E3s having a lower affinity than the mammalian and yeast E3s. Due to subtle stereospecific effects there may not be optimal orientation in the binding of the heterologous E3s to the subcomplexes of PDC and OGDC, therefore accounting for the different abilities of the E3s to reconstitute complex activity as exhibited by their different levels of reconstitution observed with several E3s which appear to bind with similar affinities (Figs. 6.8 and 6.9).

CHAPTER 7

DISCUSSION AND FUTURE RESEARCH

7.1 PURIFICATION OF DIHYDROLIPOAMIDE DEHYDROGENASE FROM POTATO AND PEA

The possible existence of organelle specific isoforms present in plant E3 was first reported by Taylor *et al.*, (1992). From Western blot studies the pea mitochondrial E3 was shown to be a distinct band of M_r 67,000 and pea chloroplast E3 to have an M_r of 52,000. Subsequent purification and characterisation of E3 from plant sources was therefore required for further analysis.

A new affinity column was developed specifically for the purification of the E3 from plant sources, exploiting the conserved nature of E3 and its ability to bind to the immobilised E2/X subcomplex of PDC or the E1/E2 subcomplex of OGDC. This procedure proved to be successful for the purification of potato mitochondrial E3, but neither the pea chloroplast or the pea mitochondrial E3 would bind effectively to the affinity matrix. Although this procedure does not represent a general approach for the purification of plant E3s, it is highly successful for the purification of E3 from potato mitochondria in a one step process, often essential because of low abundance of many enzymes in plant tissues. It is interesting to note that the potato E3 binds to both the core sub-complexes of PDC and OGDC, which is consistent with mammalian E3 bing the identical gene product in each of the members of the 2-oxoacid dehydrogenase complexes, as is generally believed to be the case (for review see Carothers *et al.*, 1989).

Application of this methodology has also provided supplementary evidence for the existence of distinct mitochondrial and chloroplastic forms of E3, as exhibited by their differing affinities for the immobilised E2/X core subcomplex of PDC. In agreement with this, the activities of the E3 enzymes vary in their susceptibility to salt and thus confirm previous immunological evidence for separate mitchondrial and chloroplastic forms of E3 (Taylor *et al.*, 1992).

Recent progress in this area has led to the identification and purification of a chloroplast specific E3 with a M_r value of 52,000 from pea, in agreement with Taylor *et al.*, (1992) (M. Conner, Glasgow University, personal communication). In

addition, the pea mitochondrial E3 was also identified and subsequently purified and observed to have an M_r value of approx. 56,000, significantly lower than M_r value of 67,000 reported previously for pea mitochondrial E3 (Taylor *et al.*, 1992). It is now believed that the 67,000 Da band is either an artefactual immune response or is the result of a miscalculation of its correct M_r value. The chloroplastic E3, which comprises approx. 10% of the total E3 in pea leaves, was again observed not to bind to the E2/X-PDC affinity matrix and to have a higher affinity for the Mono Q anion exchange column than its mitochondrial counterpart. Confirming the results reported in this thesis, the chloroplastic E3 was observed to be less sensitive to salt than the mitochondrial E3, and in addition to this, was found to be more heat stable than the mitochondrial E3. The K_m (NAD) value was also found to be six fold lower for the chloroplastic E3, lending weight to the conclusion that these two E3s are organelle specific with differing biochemical properties. Recent conclusive evidence for the existence of organelle-specific isoforms of E3 in pea is that the N-terminal sequence of the pea chloroplast E3 is distinct from its mitochondrial counterpart, and that antibodies to the mitochondrial enzyme show little or no cross-reaction with its chloroplast equivalent.

Analysis of the affinity purified potato tuber mitochondrial E3 by SDS-PAGE showed the presence of two bands with M_r 58,000 and 56,000 which cross reacted strongly with anti-E3 IgG raised to the porcine heart E3, although it was difficult to detect initially if both bands or only a single species were present on immunoblots owing to the lack of resolution of the bands. If both bands were shown to be constituent subunits of E3 of a heterodimeric form of E3, this represents a unique situation in that E3 has only ever been previously reported to exist as a homodimer (Carothers *et al.*, 1989). It was also possible however, that one of the bands was a non-related protein that copurified with E3, or alternatively, there was more than one E3 (i.e. isoforms) present in potato mitochondria. Further analysis was therefore required to establish the nature of these two bands and their physiological relevance in relation to E3 activities in potato mitochondria.

7.2 CHARACTERISATION OF DIHYDROLIPOAMIDE DEHYDROGENASE FROM POTATO

Affinity purified potato mitochondrial E3 eluted as three peaks of E3 activity when applied to Mono Q anion exchange column. When analysed by SDS-PAGE, peak 1 was observed to be represented by a single band of M_r 58,000, peak 3 by a band of M_r 56,000 and peak 2 by a combination of the two. All three peaks were observed to cross react strongly with E3 antiserum raised to porcine heart, indicating that they all are indeed E3 enzymes. Both proteolysis and aggregation of a single form of E3 were eliminated as possible explanations for the presence of these three peaks of E3. The N-terminal sequences of peaks 1 and 3 were also found to be identical to each other , and with the exception of one amino acid, identical to the N-terminal sequences reported for the pea mitochondrial E3 (Turner *et al.*, 1992). These two polypeptides may differ from each other however, at their C-termini or internally.

It is possible that the three peaks of E3 activity of potato mitochondrial E3 represent isoforms of the E3 enzyme, with the three isoforms likely to arise from the various combinations of the two-closely related polypeptide chains with M_r values of 58,000 (α) and 56,000 (β), with peak 1 and peak 3 representing an α_2 and a β_2 homodimer respectively and peak 2 a $\alpha\beta$ heterodimer. The presence of analogous complex-specific E3 isoforms has not been detected in any other plant species tested to date, however it is possible that the individual subunits are not readily resolved in other plant species. Interestingly, evidence for the presence of two closely related genes was provided from copy number analysis of pea DNA probed with a 1.3Kb fragment corresponding to 60% of the open reading frame of their cDNA clone (Turner *et al.*, 1992). It was suggested that one of the genes is either expressed at very low levels or not at all, thus explaining why it had escaped detection previously.

Further analysis was required to determine the exact interrelationship of the two polypeptides of potato mitochondrial E3 and to determine whether they had any physiological relevance. Certainly, the three peaks of E3 activity were found to differ

in their extractability from mitochondria, suggesting various modes of interaction with the inner mitochondrial membrane, or perhaps it is the multienzyme complexes with which they are associated with that are extracted selectively.

7.3 KINETIC CHARACTERISATION OF DIHYDROLIPOAMIDE DEHYDROGENASE FROM POTATO

Basic kinetic analyses was performed (at 31°C and pH 7.6) to determine whether the three isoforms of potato tuber mitochondrial E3 differed in their biochemical properties. All three isoforms were observed to vary in their K_m (NAD) and K_i (NADH) values, although further experimental readings were required to confirm that these preliminary results were statistically significant. However, recent progress has since confirmed these results (R. Cook, Glasgow University, personal communication). K_m (NAD) values were observed to vary between the three potato E3 isoforms although the values were lower than those reported in this thesis owing to the different experimental conditions employed (25°C and pH 7.5). It is known that K_m values are dependent on pH (Sahlman & Williams), therefore at a particular pH the difference in the three potato E3 isoforms kinetic constants may be more significant.

The three potato mitochondrial E3 isoforms were found to be inhibited by p-aminophenyldichlorarsine (APA) which specifically inhibits the redox active thiol disulphide group at the active site of E3 in the presence of its substrate dihydrolipoamide dehydrogenase or product, NADH. These results confirm that these three isoforms behave as conventional dihydrolipoamide dehydrogenases containing a redox-active disulphide as part of their overall catalytic mechanism.

7.4 RECONSTITUTION STUDIES ON PYRUVATE DEHYDROGENASE COMPLEX AND 2-OXOGLUTARATE DEHYDROGENASE COMPLEX

Tissue specific expression of the three potato E3 isoforms was first reported by A. Carmichael (Ph.D.Thesis, Glasgow University, 1994). The α_2 (peak1) isoform of potato mitochondria was observed to be predominant in potato leaves as is GDC the predominant multienzyme complex in leaves. This result suggests that the α_2 isoform may be specific for a GDC catalysed reaction. Reconstitution experiments were subsequently performed to identify further any complex-specificity of the three potato E3 isoforms.

The data presented in this thesis show that all three isoforms will bind to the E2/X-PDC or E1/E2-0GDC subcomplexes but were unable to promote reconstitution of OGDC activity and very limited reconstitution of PDC activity. Owing to the very low levels of PDC reconstitution by the three E3 isoforms of potato mitochondria no direct comparisons could be made and therefore there was no evidence for preferential usage of individual E3s.

Recent research, however, does help to identify complex-specificity of the three potato E3 isoforms (R. Cook, Glasgow University, personal communication). Crude potato mitochondrial extracts were prepared and E3 removed by salt treatment and gel filtration. The potato mitochondrial E3 isoforms were added back individually and the effect on PDC and 0GDC activity measured. Peak 1 and peak 2 were observed to inhibit both PDC and OGDC activity whereas peak 3 was observed to slightly stimulate both PDC and OGDC activity. This result indicates that peak 3 therefore may represent E3 associated with both OGDC and PDC activity. The possibility of a GDC-specific response when the three isoforms are added back to a crude extract of potato mitochondrial proteins is currently under investigation.

7.5 FUTURE RESEARCH

There is increasing evidence at present to indicate that these individual E3 isoforms in potato may interact selectively or exclusively with individual mitochondrial complexes. Functional analyses of their relative abilities to promote PDC, OGDC and GDC activities in *vitro* are still in progress.

The precise interrelationship between the two major polypeptides detected in these α_2, β_2 and $\alpha\beta$ dimeric forms of potato mitochondrial E3 has still to be established, although it is clear that the two subunits display complete sequence identity at their N-termini. Recent progress in our laboratory has resulted in the isolation of 2 (or possibly 3) full-length DNA clones corresponding to potato mitochondrial E3 polypeptides. Sequencing of these clones is currently in progress which should provide definitive information on the precise nature of the three potato E3 isoforms and exactly how they differ from each other.

Future research will also include the cloning and sequencing of the pea leaf chloroplastic E3 which has been successfully purified and characterised, and subsequently shown to be distinct from its mitochondrial counterpart. The cloning and sequencing of these plant genes is of particular importance for the production of transgenic plants in the future. For example, this would allow us, by selective inhibition of GDC or plastid PDC to assess their role in fatty acid metabolism using antisense technology.

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