Folding and Assembly Studies on the Components of Mammalian PDC and OGDC

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

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Declaration

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

R. Graham McCartney

May 1998

Dedication

As the wind collects my thoughts at twilight, father

I can feel the sun

Fill my body with cold nectar

Turn your head grey and reach, in a wordless way,

Inside of me.

With no need to speak of Yeats

And Joyce, just a girl that you once used to know.

Our bound remains life that, in moments,

We treat so full of grace and contempt

I can sit, still, in India and my smile would be for you.

Distance carries us close, these days.

O! that you could have stayed

For these days.

For Joe McCartney and Kitty Graham

Acknowledgements

Where to begin?
Its going to be hard to prevent this from beginning to sound like a lengthy Oscar acceptance speech, but, in truth, there have been a multitude of guides and spirits along the way who have made a difference:

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Abstract

Component enzyme purification and reconstitution studies have been conducted on the dihydrolipoamide acetyltransferase-protein X core subcomplex (E2/X) of bovine heart pyruvate dehydrogenase complex (PDC). Gel permeation chromatography, in the presence of 1M NaCl, produced an E2/X core and E1/E3 fraction. Subsequent, anion exchange chromatography successfully separated the E1 and E3 components into homogeneous fractions. Electrospray mass spectrometry (ES/MS) determined the M_r of bovine E2 and protein X.

GdnHCl induced dissociation of the E2/X core disclosed that it was an ordered co-operative event involving formation of specific lower M_r intermediates corresponding to dihydrolipoamide acetyltransferase trimers and monomers. Modulation of refolding conditions allowed for the reassembly of native E2 cores devoid or partially depleted of the protein X component, as determined by immunological analysis.

Reconstitution studies, with stoichiometric levels of the E1/E3 components, were unable to sustain overall complex activity with the E2 core devoid of protein X. In contrast 30-35% recovery of PDC activity could be obtained, under the same conditions, with the E2 core depleted of protein X, as compared to the native E2/X core assembly. Further reconstitution studies with excess (up to 100 fold) E3 were able, in both instances, to promote significant additional stimulation of PDC activity (25-30%). This effect was dependent on the source of E3 used and was optimal with parent bovine E3. These studies, using the refolded E2 cores, demonstrated the low affinity, but specific, binding of the dihydrolipoamide dehydrogenase (E3) component to sites on E2. This provided *in vitro* evidence to support the low levels of PDC activity observed in cell lines derived from patients who do not express protein X.

A protocol has been devised which facilitated the dissociation of the 2-oxoglutarate decarboxylase (E1) and E3 components from the 24meric dihydrolipoamide succinyltransferase (E2) core of the mammalian 2-oxoglutarate dehydrogenase complex (OGDC). By mixing the components in stoichiometric quantities, it was possible to reconstitute significant levels of OGDC activity. Gel permeation analysis of the E1/E3 fraction, performed under associative conditions, indicated the ability of the two homodimeric components to interact and form a stable subcomplex, comprising single copies of the two components.

N-terminal sequence analysis identified SP-22, a thiol specific antioxidant protein, which appears to associate with the E3 component of mammalian PDC. Using specific primers, the polymerase chain reaction (PCR) was employed to obtain double stranded SP-22 DNA from bovine brain cDNA. Following purification, the DNA was cloned, in a blunt ended fashion, into the pCR-script vector. Both single and double restriction enzyme analyses were performed to screen the clones obtained for the presence of the appropriate inset. Subsequent *in vitro* transcription /translation analysis of the clones succeeded in identifying one capable of synthesising a protein product of the predicted M_r.

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2.2 Protein Biochemistry Methods

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Abbreviations:

amp	Ampicillin
approx.	Approximately
BCOADC	Branched chain 2-oxo acid dehydrogenase complex
bp	Base pair
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulphonate
Da	Daltons
DNA	Deoxyribonucleic acid
DCPIP	Dichlorophenol-indophenol
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
E1	2-oxo acid decarboxylase
E2	Dihydrolipoamide acyltransferase
E3	Dihydrolipoamide dehydrogenase
EDTA	Ethylenediaminetetra-acetic acid
ES/MS	Electrospray mass spectrometry
Fig	Figure
GdnHCl	Guanidine hydrochloride
IPTG	Isopropyl-thio-galactopyranoside
kan	Kanamycin
kb	Kilobase
MOPS	3-(N-Morpholino)propanesulphonic acid

M_r	Relative molecular mass
MSUD	Maple syrup urine disease
OGDC	2-oxoglutarate dehydrogenase complex
PAGE	Polyacrylamide gel electrophoresis
PBC	Primary biliary cirrhosis
PCR	Polymerase chain reaction
PDC	Pyruvate dehydrogenase complex
PEG	Poly ethylene glycol
PMSF	Phenylmethylsulphonylfluoride
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethyeneldiamine
ThDP	Thiamine diphosphate
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
TX-100	Triton X-100
vol	Volume(s)
v/v	Volume to volume
w/v	Weight to volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1

Introduction

The 2-Oxoacid Dehydrogenase Multienzyme Complexes

1 Introduction

1.1 The 2-oxoacid dehydrogenase family of multienzyme complexes

The family of 2-oxoacid dehydrogenase multienzyme complexes, found both in prokaryotic and eukaryotic organisms, consists of three members: the pyruvate dehydrogenase complex (PDC); the 2-oxoglutarate dehydrogenase complex (OGDC) and the branched chain 2-oxoacid dehydrogenase complex (BCOADC). These three multienzyme complexes occupy important roles in the intermediary metabolism of carbohydrates and branched chain amino acids (see Patel & Roche, 1990; Perham, 1991; Mattevi *et al.*, 1992b; Behal *et al.*, 1993; Patel & Harris 1995, Berg & De Kok, 1997 for reviews).

Organisms wishing to obtain multiple catalytic functions in a single enzyme complex are faced with a choice. One solution is to incorporate the required activities in a single polypeptide chain. An example of this is the mammalian enzyme fatty acid synthase. The alternative is to aggregate the catalytic activities required into a multienzyme complex. Multienzyme complexes may be defined as an association of individual enzyme components, which typically catalyse successive steps in a metabolic pathway. These components are complexed in a non-covalent fashion and are usually present in multiple copies. The presence of multiple copies of the components in these complexes generates very large complexes (M_r of the 2-oxoacid dehydrogenase complexes 5-10x10^6). In multienzyme complexes, the close proximity of the enzymatic components increases the efficiency of the pathway: off pathway reactions are minimised and the overall rate of catalysis increased. Additionally, the multienzyme complexes may permit unusual active site chemistries and allow the channelling of

substrates from one active site to the next (Perham, 1975; Reed & Hackert, 1990; Perham, 1991; Wallis *et al.*, 1996).

1.2 Metabolic roles of the 2-oxoacid dehydrogenase complexes

The positions of the three 2-oxoacid dehydrogenase complexes in carbon and nitrogen metabolism are illustrated in Figure 1.1. All three complexes catalyse the irreversible oxidative decarboxylation of their respective substrates. From their positions in metabolism (Figure 1.1) and the irreversible nature of the reactions catalysed, it is evident that these complexes represent key control points in regulating metabolic flux within cells (for reviews of nutritional and /or hormonal regulation see Behal *et al.*, 1993; Sugden *et al.*, 1995; Harris *et al.*, 1995). Indeed, their multi-enzymatic nature permits more sensitive and efficient regulation of activity.

Positioned at the end of the glycolytic pathway, the pyruvate dehydrogenase complex (PDC) holds a pivotal position in carbon metabolism. The conversion of its three carbon substrate, pyruvate, to acetyl-CoA provides a source material for energy production, via further oxidation in the citric acid cycle, or the biosynthesis of other products. Acetyl-CoA serves as a substrate for the biosynthesis of important molecules notably fatty acids, sterols and, in neuronal tissue, the neurotransmitter acetylcholine (Behal *et al.*, 1993).

BCOADC has a important role to play in nitrogen homeostasis. The disposal of excess branched chain amino acids, which cannot be stored and are potentially pathogenic (section 1.6), is a vital process. BCOADC catalyses a key step in the catabolism of branched chain amino acids. However, these amino

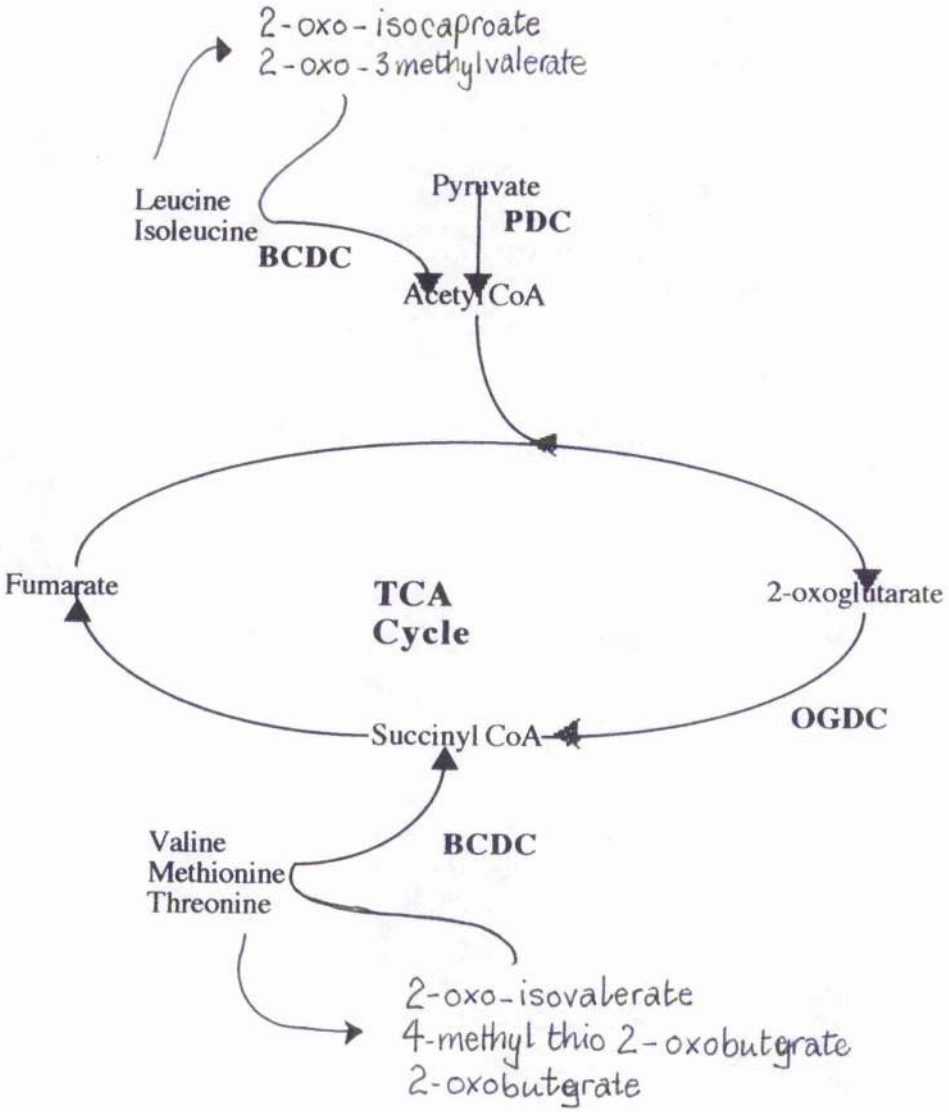


Figure 1.1 The role of the 2-oxoacid dehydrogenase multienzyme complexes in metabolism

acids are also essential for protein synthesis. Thus, tight regulation of their breakdown is required to ensure sufficient availability of these amino acids for biosynthesis of proteins (Harris *et al.*, 1986). The reaction catalysed by OGDC is central to oxidative metabolism and energy production in mitochondria. It represents the rate-limiting step in the flux of intermediates through the later stages of the citric acid cycle.

1.3 The reaction catalysed by the 2-oxoacid dehydrogenase complexes

These multienzyme complexes are responsible for the oxidative decarboxylation of their respective 2-oxoacid substrates as detailed in the general reaction scheme shown below:

2-oxoacid+ CoA + NAD^+ --> Acyl CoA + CO_2 + NADH + H^+

The reaction is catalysed by the sequential reaction of the three catalytic components: a 2-oxoacid decarboxylase (E1); a dihydrolipoamide acyltransferase (E2) and a dihydrolipoamide dehydrogenase (E3). The E1 and E2 components are substrate specific (with E1p, E1o, E1b and E2p, E2o and E2b referring to the components which belong to PDC, OGDC and BCOADC respectively). In contrast, the E3 component, which catalyses the same reaction in each multienzyme complex, is common to all three complexes. In most of the systems studied to date, E3 would appear to be a product of a single gene, maintaining the principle of genetic parsimony usually observed when assembling biological structures. However, exceptions have been discovered. There are three E3 genes in *Pseudomonas putida* (Palmer *et al.*, 1991) and

distinct isoforms of the E3 component have also been observed in mitochondria and chloroplasts of *Pisum sativum* (Conner *et al.*, 1996).

The reaction scheme for PDC is detailed in Figure 1.2. The four steps in the overall reaction are catalysed by the three catalytic components E1, E2 and E3. The E1 (pyruvate decarboxylase) is responsible for the first two steps: the decarboxylation of pyruvate and the subsequent reductive acetylation of the lipoamide prosthetic group on the E2 component (Frey *et al.*, 1989). The cofactor thiamine pyrophosphate (ThDP) is required for this catalysis and its 2-hydroxyethylidine-ThDP form is the substrate for the subsequent reductive acetylation of the lipoamide moieties on E2. Lipoamide groups are covalently attached via the ε amino group of specific lysine residues on the E2 component. The attached lipoamide group forms an extended (14 Å) flexible arm which conveys the substrate between active sites. The E2 (dihydrolipoamide acetyltransferase) enzyme transfers the acetyl group from its lipoamide groups to coenzyme A. Finally, the E3 enzyme (dihydrolipoamide dehydrogenase) is responsible for regenerating the oxidised form of lipoamide on E2 and the transfer of reducing equivalents, via its FAD cofactor, to NAD^+. It is possible to differentiate between the catalytic cofactors, ThDP, lipoamide and FAD, which remain unaltered from the net reaction, and the stoichiometric cofactors CoA and NAD^+ (Reed, 1974).

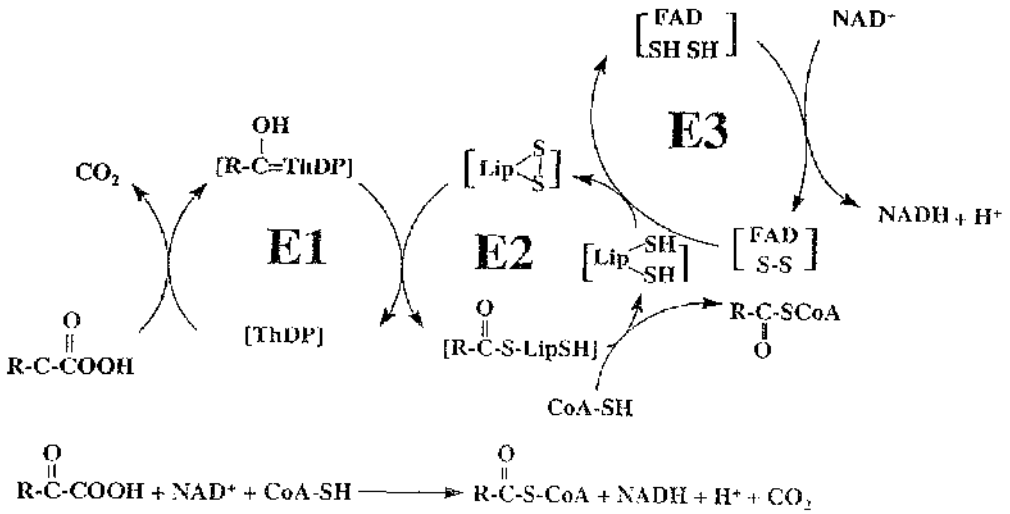


Figure 1.2 Reaction scheme for the oxidative decarboxylation of 2-oxoacids by the 2-oxoacid dehydrogenase complexes (adapted from Berg & de Kok, 1997)

R=CH_3 for PDC; CH_2CH_2COOH for OGDC and R=$CH(CH_3)_2$,

$CH_2CH(CH_3)_2$ or $CH(C_2H_5)CH_3$ FOR BCOADC

THDP, thiamindiphosphate

Lip, lipoic acid

1.4 Structure of 2-oxoacid dehydrogenase complexes

1.4.1 The dihydrolipoamide acyltransferase (E2) component

The E2 component performs a vital structural as well as catalytic role within the 2-oxoacid dehydrogenase complexes. This E2 component self assembles into a structural lattice to which the other enzymes are noncovalently attached. Two forms of the E2 core have been observed (i) a 24mer exhibiting octahedral symmetry and (ii) a 60mer which forms a pentagonal dodecahedron (see section 1.4.2 for details). The characteristic segmented structure of E2 polypeptide chains is central to their multi-functional nature. Attempts to crystallise the entire E2 component have not been successful, probably as a result of the inherent flexibility displayed by N terminal regions of these proteins. Instead, the structures of the individual domains have been determined separately. The segmented structure displayed by the E2 component of mammalian PDC is illustrated in Figure 1.3. Each of the domains has been shown to be an independent folding entity. E2 displays three types of globular domain separated by alanine/proline rich sequences termed linker regions. The domain structure shown is common to all E2 components studied to date (Perham, 1991; Mande *et al.,* 1996; Berg & De Kok, 1997), with the notable exception of mammalian OGDC (Rice *et al.,* 1992).

The lipoyl domains on mammalian E2 each contain a specific lysine residue to which a lipoic acid moiety is covalently attached (Fujiwara *et al.,* 1996). The role of this prosthetic group in overall complex catalysis has been discussed previously (section 1.3). The number of lipoyl domains on E2 varies between one and three, independent of complex type or tissue/species source. Interestingly, one lipoyl domain has been shown to be sufficient for catalysis,

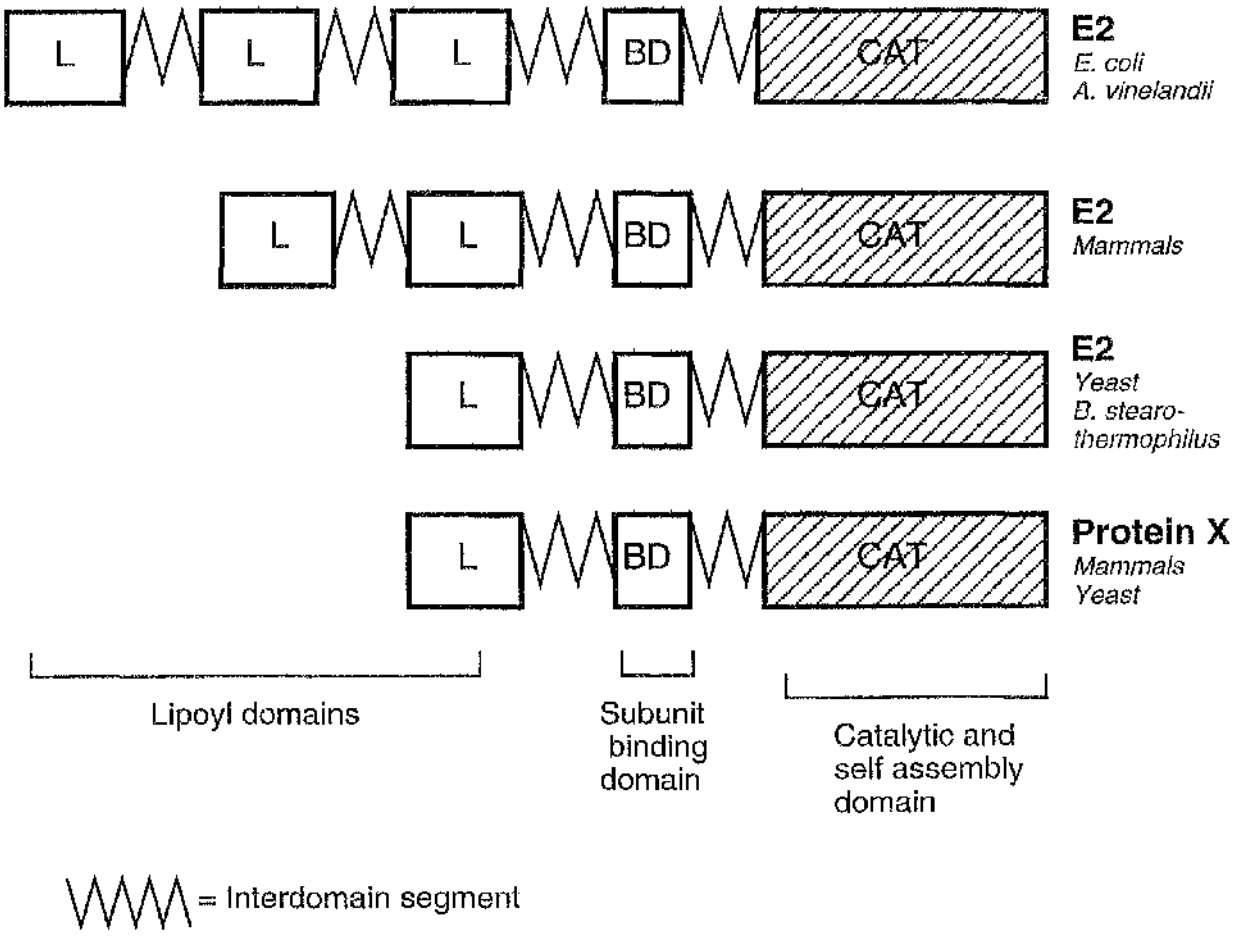


Figure 1.3 Domain layout of the E2 and protein X components of pyruvate dehydrogenase complexes (adapted from Perham, 1991).

(Allen *et al.*, 1989). A study in *E. coli* of PDC E2, which has three lipoyl domains, also showed that one lipoyl group was sufficient for full E2 catalytic activity *in vitro*. However in genetic studies, three lipoyl domains, with the outer being lipoylated and the two inner domains not requiring cofactor attachment, were considered optimal for complex activity. The authors conclude that the function of the two inner domains is to extend the reach of the lipoyl cofactor on the outer domain, generating the so called "swinging arm" of E2 (Guest *et al.*, 1997).

Solution structures, employing nuclear magnetic resonance (NMR), have been obtained for lipoyl domains from *Bacillus stearothermophilus* PDC (Dardel *et al.*, 1993) and OGDC (Ricaud *et al.*, 1996); *E. coli* PDC (Green *et al.*, 1995); *Azotobacter vinelandii* PDC (Berg *et al.*, 1997). Nuclear magnetic resonance (NMR) spectroscopy and site directed mutagenesis have identified a surface loop of the lipoyl domain which makes contact with the E1 component of *B. stearothermophilus* PDC, in addition to contacts at the back of the domain. It is believed that this interaction forms the molecular basis for substrate channelling (Wallis *et al.*, 1996). However, the basis of the specificity of such interactions (i.e. the fact that E1p will only react with E2p and not E2o/E2b) is not yet understood. Evidence also exists that, as an additional role, the lipoyl domain is involved in binding complex specific kinases and phosphatases in mammalian PDC and BCOADC (Liu *et al.*, 1995; Popov *et al.*, 1994). Activation/inactivation of these multienzyme complexes is thought to involve a mechanism which permits the transferral of the kinase/phosphatase enzymes between the lipoyl domains of the E2 subunits. This allows the relatively few copies of these regulatory enzymes present rapid access to their target substrate, the E1 component.

The binding domain on the E2 component (E2BD) consists of 30-50 amino acids. This size represents the smallest functional globular domain observed to date. It is then somewhat surprising that, in prokaryotic icosahedral E2 cores (see section 1.4.2), it is responsible for binding both the E1 and E3 components to the multienzyme complex. NMR structures have been obtained for E2BD from *B. stearothermophilus* PDC (Kalia *et al.*, 1993) and *E. coli* OGDC (Robien *et al.*, 1992). Elegant studies (Lessard & Perham 1995; Lessard *et al.*, 1996) employing surface plasmon resonance (SPR) measurements and native PAGE have probed the interaction of E2BD with both the E1 and E3 components. These investigations led to the conclusion that an individual E2BD of *B. stearothermophilus* PDC was unable to simultaneously bind E1 and E3 components. While the association constant for both enzymes with the E2BD was found to be identical, the two components exhibited different rate constants for their dissociation. Both E1 and E3 components were able to displace each other from complexes formed with the E2BD and the capacity for E1 to bind was shown to rest with the E1β component.

The catalytic (inner) domain of E2 represents, at typically 250 amino acids in length, the largest part of the protein. It is responsible for catalysing the acyltransferase reaction and contains sequences which mediate the self-assembly of the E2 lattice. In addition, the catalytic domain is involved in binding the E1 component to some complexes displaying octahedral E2 core symmetry. The domain has been crystallised and the catalytic mechanism investigated (Mattevi *et al.*, 1992a;1993b; Hendle *et al.*, 1995).

The flexible linker regions which segregate the domains in E2 are also critical to component function. Typically, linker regions are rich in alanine and proline residues. There are also a number of charged residues. These linkers provide the flexibility which allows the lipoyl moiety access to all three active

sites within the multienzyme complex (Radford *et al.,* 1989; Green *et al.,* 1992).

1.4.2 E2 core formation

2-oxoacid dehydrogenase complexes are built around an E2 oligomeric core to which multiple copies of the E1 and E3 components are noncovalently attached (see Mande *et al.,* 1996 for review). Electron microscopy (Oliver & Reed, 1972; Wagenknecht *et al.,* 1990, 1991; Stoops *et al.,* 1992, 1997) and X-ray crystallography (DeRosier *et al.,* 1971; Fuller *et al.,* 1979; Mattevi *et al.,* 1992a) have identified two forms of E2 core morphology. (1) Cubic structures, consisting of 24 E2 monomers exhibiting octahedral (432) symmetry, have been characterised in all OGDC, all known BCOADC, *E. coli* and *A. vinelandii* PDC, OGDC. (2) Pentagonal dodecahedron structures, consisting of 60 E2 monomers exhibiting icosahedral (532) symmetry, have been observed in PDC from mammalian, avian, fungal and Gram-positive bacterial (*B. stearothermophilus*) sources (Figure 1.4). In all cases, the basic building block of the core structures appears to be a trimeric E2 unit. In addition, the structural and functional similarity of E2 to chloramphenicol acetyltransferase, CAT (Guest, 1987), active as a trimer, has led to the suggestion that the 60-meric mammalian E2 core represents an association of twenty trimeric units, an idea which has received experimental support from recent publications (Behal *et al.,* 1994; De Marcucci *et al.,* 1995). These trimers interconnect to form the cage like inner core. Refolding and assembly studies on the E2 core of mammalian PDC (Behal *et al.,* 1994; McCartney *et al.,* 1997) have also indicated that this trimeric E2 unit serves as an assembly intermediate in mature E2 core formation.

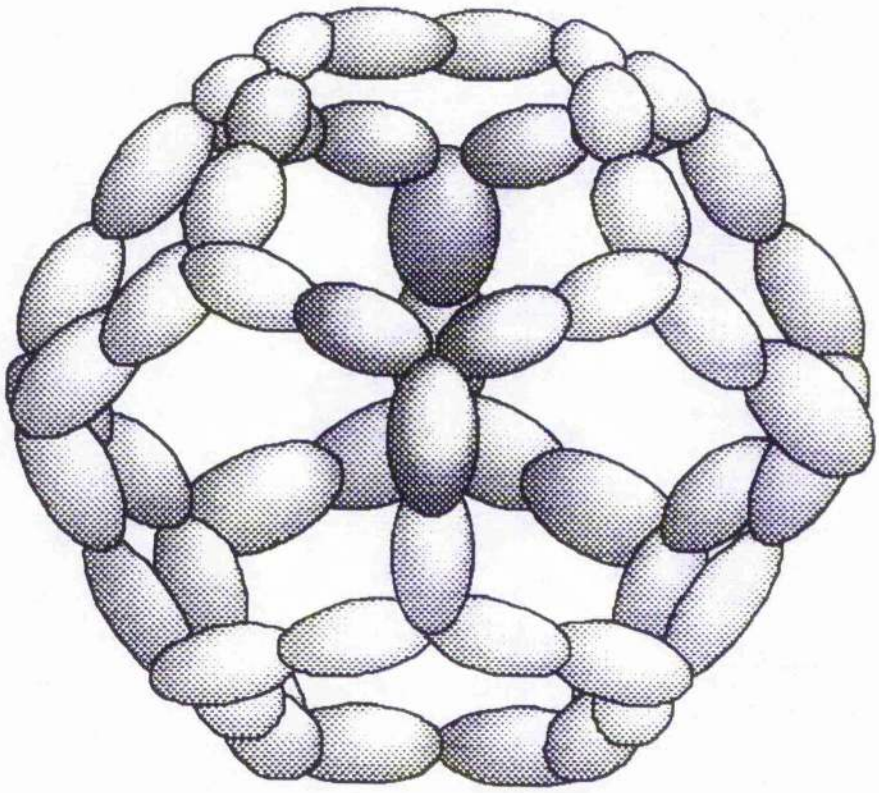


Figure 1.4 Schematic representation of the E2 core assembly of mammalian PDC

1.4.3 2-oxoacid decarboxylase (E1) component

Two forms of E1 have been observed: (1) an α_2 homodimer (subunit M_r = 100, 000) which is found with octahedral PDC and OGDC E2 cores; (2) an $\alpha_2\beta_2$ heterotetramer (subunit M_r values 40-46, 000 and 35-38, 000 respectively) associated with icosahedral PDC E2 cores and all BCOADC E2 cores characterised to date. In mammalian PDC, 20-30 heterotetramers of E1 are associated per complex. In complexes exhibiting octahedral symmetry, the number is around 6 E1 components per complex. There appears to be little correlation between the E2 core structure and the subunit structure of the associated E1 enzyme (Perham, 1991).

In contrast to the other catalytic 2-oxoacid dehydrogenase components, no crystal structure for any E1 is to be found in the literature. The three dimensional structure of another ThDP dependent enzyme, transketolase, has provided insights into the residues required in the binding of the cofactor to such enzymes (Lindquist *et al.,* 1992). The active site chemistry of ThDP requiring enzymes is well understood and recent work has highlighted the biphasic (first ThDP fast, second ThDP slow) nature of the binding of the two ThDP molecules to E1 (Yi *et al.,* 1996). In eukaryotes, E1 components associated with icosahedral PDC complexes and all BCOADC complexes play an additional role in the regulation of complex activity by a phosphorylation-dephosphorylation mechanism (Yeaman, 1989) (see sections 1.4.6 and 1.5 for details).

1.4.4 Dihydrolipoamide dehydrogenase (E3) component

E3 exists as an FAD containing homodimer with a subunit M_r = 55 000. It is part of a large family of pyridine disulphide oxidoreductases (Williams, 1992; Perham *et al.,* 1995). Evidence to date, indicates that the E3 component, present in all three mammalian multienzyme complexes, is the product of a single gene. In mammalian PDC, 6-12 copies of this homodimer are present in each mammalian PDC complex. The role of E3 is to reoxidise the reduced lipoamide prosthetic group and thus prepare it for another round of catalysis (Figure 1.2). Three dimensional structures, from X-ray crystallography studies, exist for E3 derived from *A. vinelandii* and *P. putida* sources (Mattevi *et al.,* 1991, 1992b;1993) and, more recently, yeast (Toyoda *et al.,* 1998). The structure of the protein has been well conserved throughout evolution. Despite this, in reconstitution studies with bovine PDC, pig and yeast E3 produced poor levels of PDC activity in comparison with levels obtained with native E3. This points to subtle differences in the subunit interactions between the heterologous E3s and the core preparations investigated (Sanderson *et al.,* 1996). Functional analysis of the human E3 has recently been performed (Lanterman *et al.,* 1996). In this study the human enzyme was able to restore function to a yeast strain deficient in this component.

1.4.5 Protein X

PDCs from mammalian and yeast sources, in addition to the E1, E2 and E3 catalytic components, have been shown to contain another polypeptide, termed protein X, (see Figure 1.3 for outline of domain structure). This subunit was first identified as an immunologically distinct polypeptide within mammalian PDC (De Marcucci & Lindsay, 1985). Subsequent studies have been successful in characterising the key role of protein X in the specific binding of E3 to the PDC complex in higher eukaryotes (Jilka *et al.*, 1986; Powers-Greenwood *et al.*, 1989; Neagle & Lindsay, 1991; Lawson *et al.*, 1991a,b; Maeng *et al.*, 1994). This has led to the protein also being termed the E3 binding protein (E3BP). Typically, binding of the E1 and E3 components is mediated by the E2 component in 2-oxoacid dehydrogenase multienzyme complexes from prokaryotic sources (section 1.4.1). The presence and function of protein X in mammalian PDC would appear to indicate a higher level of complexity. Another exception, appears to be in mammalian OGDC. The E2 component lacks any apparent E3 binding domain (Nakano *et al.*, 1991) and the E1 component is thought to be responsible for E3 binding (Rice *et al.*, 1992).

The protein X gene has been cloned from *Saccharomyces cerevisiae* (Behal *et al.*, 1989) and 12 copies of the protein have been shown to be associated per PDC complex (Maeng *et al.*, 1994; Sanderson *et al.*, 1996a). Protein X is tightly bound to the E2 core. In contrast to the other subunits of PDC, which dissociate readily from the E2 core under conditions of high ionic strength, protein X requires the use of denaturing concentrations of chaotropic reagents for its removal. While each E2 molecule has the ability to bind 1 protein X, a recent study points to steric hindrance by protein X itself as limiting the

numbers of this component associated with the E2 core to 12 (Maeng *et al.*, 1996). In support of this, evidence presented in a recent elegant study of mammalian PDC (Stoops *et al.*, 1997), employing cryo-electron microscopy, shows the presence of 12 large openings in the E2 core assembly which permit the molecules of protein X to bind to the E2 core.

Recently, the protein X gene from human PDC has been cloned and characterised (Harris *et al.*, 1997). The authors employed a search of the expressed sequence tag database at NCBI (National Centre Biological Information), based on reported protein X amino acid sequences (Rahmatullah *et al.*, 1989; Neagle *et al.*, 1989; Sanderson *et al.*, 1996b), to identify four possible clones. Two of these were sequenced and mature human protein X was characterised as a 448 amino acid protein with a calculated M_r of 48, 040. This M_r value agrees with that obtained by ES/MS for protein X from a bovine source 47, 982 (see section 3.2.3). The sequence data also confirmed the presence of only one lipoyl domain on protein X as indicated by a previous study (Neagle *et al.*, 1989).

In terms of its segmented structure, the protein is very similar to the E2 enzyme and displays high levels of sequence similarity throughout its length to this component. This is in contrast to yeast protein X where similarity to the E2 component is confined to the N terminal region only. The C- terminal regions of yeast and human protein X exhibit no sequence similarity. The sequence data also appeared to exclude the role of protein X as an acyltransferase. The protein was shown to lack a histidine residue in its C-terminal which is common to all other 2-oxoacid dihydrolipoamide acyltransferases and is thought to be involved in catalysis. Instead, the authors suggest an additional role for protein X in the regulation of the isoforms of pyruvate dehydrogenase kinase which have been

discovered (Popov *et al.,* 1994; Gudi *et al.,* 1995) and are known to regulate PDC activity (see section 1.4.6).

1.4.6 PDC kinase and phosphatase

The PDC and BCOADC multienzyme complexes are also subject to regulation via a phosphorylation (inactive) /dephosphorylation (active) mechanism. This involves specific, complex-associated kinase and phosphatase activities (see Behal *et al.,* 1993 for a review). Short and longer term regulation of their activities is discussed in section 1.6. Recent work has been successful in cloning four isoforms (PDK1, PDK, 2, PDK 3, PDK 4) of human PDC kinase and Northern blot analysis has provided evidence for their tissue specific expression (Gudi *et al.,* 1995; Harris *et al.,* 1995b; Rowles *et al.,* 1996). While PDK1 expression is confined in the main to the heart, PDK 2 appears to be the present in all human tissues tested. PDK 3 is expressed in the testis and PDK 4 mRNA is concentrated in skeletal muscle and heart tissue. The family of protein kinases exhibit no sequence similarity to other eukaryotic protein kinase groups but appear related to histidine protein kinases expressed in prokaryotes (Harris *et al.,* 1995b).

Phosphorylation of PDC by PDC kinase leads to inactivation of the complex activity, often in response to the prevailing metabolic conditions (see section 1.6). The target for the kinase and phosphatase enzymes is the E1α subunit, specifically 3 serine residues on E1p (E1 PDC) and 2 serine residues on E1b (E1 BCOADC). PDC kinase activity is tightly associated with the E2 core. In contrast, the association of the phosphatase component appears to be considerably weaker. Recent evidence suggests that the catalytic unit of PDC phosphatase does not behave as a soluble mitochondrial matrix protein. Instead,

it is strongly associated with the mitochondrial inner membrane (Simonot *et al.*, 1997). The number of kinase and phosphatase molecules associated with the multienzyme complexes is small, as few as 1 kinase per complex (Liu *et al.*, 1995).

1.5 Regulation of the 2-oxoacid dehydrogenase complexes

The position of all three multienzyme complexes (as detailed in section 1.2 and Figure 1.1) in relation to carbohydrate, protein and lipid metabolism underlies the importance of their regulation. Modulation of the levels of activity of the complexes may result from a change in nutritional circumstance (starvation/diabetes) and/or hormonal stimulation (Yeaman, 1989; Behal *et al.*, 1993; Sugden & Holness, 1994; Denton *et al.*, 1996). Levels of individual 2-oxoacid dehydrogenase complex activity may vary in a tissue specific fashion. For example, if gluconeogenic conditions prevail in the liver, the levels of PDC activity must be suppressed to prevent the irreversible decarboxylation of the gluconeogenic substrate pyruvate. Conversely, tissues carrying out lipogenisis would require high levels of PDC activity, to supply acetyl CoA for fatty acid and sterol synthesis.

All three multienzyme complexes respond to end product inhibition of their activities. In addition, PDC and BCOADC from higher eukaryotes contain complex specific kinases and phosphatases (section 1.4.6) which modulate the level of overall complex activity by a phosphorylation (inactivation)/dephosphorylation (activation) mechanism. The short-term regulation of the PDC specific kinase and phosphatase is summarised in Figure

1.5. The details of the regulation of BCOADC have been the subject of a recent review (Harris *et al.,* 1995) and displays parallels to that of PDC.

PDC kinase activity is stimulated by an increase in the ratios of [ATP:ADP], [acetyl CoA:CoA], [NADH:NAD^+]. The substrate for PDC, pyruvate, and the E1 cofactor, ThDP, both serve to inhibit the activity of this cAMP independent kinase (Sugden & Holness, 1994). The redox and acetylation status of the L2 (inner) lipoyl domain of mammalian PDC also modulates the activity of PDC kinase, with reduced and acetylated states stimulating the activity of the kinase (Ravindran *et al.,* 1996). The long term regulation of PDC kinase has also been studied. In response to prolonged starvation, elevated levels of PDC kinase activity have been observed (Randle *et al.,* 1988). In muscle it has been possible to attribute this as a response to elevated intracellular cAMP and the high levels of fatty acids in circulation (Ofali *et al.,* 1993).

PDC phosphatase activity is a member of the protein phosphatase 2C family. Its activity is stimulated by Ca^{2+} ions and, in adipocytes, the hormone insulin (Yeaman, 1989). PDC phosphatase interchange among mobile L2 (inner lipoyl) domains has been shown to be a Ca^{2+} mediated process. This increased mobility leads to enhanced dephosphorylation of E1α PDC and concomitant increase in complex activity (Liu *et al.,* 1995). Insulin activation of PDC phosphatase in adipocytes is believed to result from its effect on the regulatory subunit of the heterodimer (Yang *et al.,* 1996). Studies have also begun to investigate the longer-term regulation of the PDC complex, in response to prolonged hormonal stimulation and/or nutritional conditions (Hu *et al.,* 1983; Da Silva *et al.,* 1993). The evidence to date suggests that long-term regulation of mammalian PDC involves co-ordinate regulation of the components at transcriptional/ post-transcriptional levels.

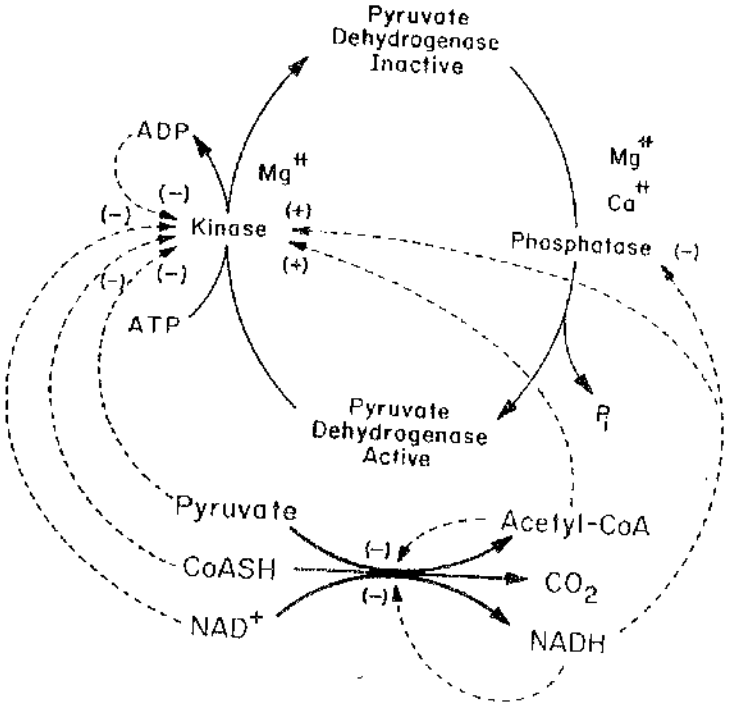


Figure 1.5 Metabolic regulation of mammalian pyruvate dehydrogenase complex (Adapted from Behal *et al.*, 1993)

1.6 Gene structure and disease states associated with 2-oxoacid dehydrogenase complexes

For the majority of the component enzymes of the 2-oxoacid dehydrogenase complexes cDNA, or genes coding for the protein product, from human or other eukaryotic sources, have been cloned (see Patel & Harris 1995 for details). Studies of gene structure and regulation for PDC (Johanning *et al.*, 1992; Chang *et al.*, 1993), BCOADC (Lau *et al.*, 1992; Chuang *et al.*, 1993) and OGDC (Koike, 1995) have been carried out. All of the genes studied to date have the appearance of housekeeping genes. The exception appears to be the PDC E1α gene located on the X chromosome (there are known to be two E1α genes, one on the X chromosome and the other on chromosome 4). In this case, the presence of TATA and CAAT boxes upstream are suggestive that expression has the potential to be regulated in a tissue specific or temporal fashion. It has also been observed that the human E3 promoter contains putative hormone responsive elements.

PDC deficiency is a common, but by no means exclusive, cause for the metabolic disorder congenital lactic acidosis. The disease is associated with effects on the central nervous system and clinical presentation can range from mild ataxia to severe neuroanatomical lesions (Stacpoole, 1997; Patel & Harris, 1995; Kerr *et al.*, 1996). Defects in all the individual components of human PDC, with the exception of E1β, have been documented, but the majority of cases to date (>90%) have involved lesions in the E1α component. The nature of the mutations in this gene studied have indicated that missense mutations represent over half of those detailed. Limited genetic analysis of familial

histories has been unable to define patterns of inheritance and it appears that the majority of cases represent a new mutational event.

PDC is also implicated in the auto-immune disease primary biliary cirrhosis (PBC). Final stage PBC is the commonest indication for liver transplantation in the UK. In its early stages the disease is characterised by immune-mediated destruction of the bile ducts (O' Donohue & Williams, 1996; Yeaman & Diamond, 1996). Protein X and E2 components of PDC have been identified as the major auto-antigens (Fussey *et al.*, 1988), although E2 of all multienzyme complexes and E1α of PDC also elicites a significant immunological response in a significant proportion of patents. The disease is thought to arise from environmental factors acting upon a person who is genetically sensitive to them. Bacterial infection has been suggested as a possible trigger for the disease. The hypothesis relies on the cross-reactivity of human antibodies, raised against the PDC E2 component of rough forms of *E. coli* or strains of mycobacteria, with the major autoantigens. This has been termed the molecular mimicry hypothesis (Burroughs *et al.*, 1992). However, the autoantibodies produced appear to only cross-react strongly with the mammalian complexes. Thus, the cause of the disease, or indeed the significance of the auto-antigen response in its pathology, remains unclear.

Genetic defects in the subunits of BCOADC result in maple syrup urine disease (MSUD). This results in the inability of the body to catabolise excess branched chain amino acids and their 2-oxoacids, leading to their accumulation in blood, tissue and urine of patients (Patel & Harris, 1995; Indo & Matsuda 1996). If untreated, MSUD can lead to mental retardation, coma or death. A wide variety of insertion deletions, substitutions and missense mutations have been detected in the components of BCOADC (Chuang *et al.*, 1991; Zhang *et al.*, 1991). Treatment has traditionally relied on dietary control. However, the

correction of the disorder by retroviral gene transfer of normal E1α precursor into cultured lymphoblasts from an MSUD patient has been reported (Chuang *et al.*, 1995).

1.7 Protein folding

The translation of the linear, two dimensional genetic code, from its mRNA form, into an active, three dimensional protein structure remains one of the most intensely studied areas of biochemistry. The body of work, accumulated from the early experiments of Anfinsen and colleagues, to present day investigations into the role of molecular chaperones, is certainly impressive. However, despite these considerable advances, attempts to produce a definitive "protein folding code" have not been successful, although the field of structure prediction continues to improve (for recent reviews see Fenton & Horwich, 1997; Lithgow *et al.*, 1997; Visick & Clarke, 1995; Jaenicke, 1996 and references therein)

To be of any use to an organism, the proteins that it synthesises must fold to their native form within a biofeasible time frame. This is usually taken to mean a time frame much shorter than the lifetime of the organism. Based on this premise, an entirely random search of protein conformational space can be excluded and one is forced to consider kinetic pathways of protein folding. The central dogma of protein studies comes from Anfinsen's work on ribonuclease. It states that the active three dimensional protein structure is an inherent quality of the primary amino acid sequence (Anfinsen, 1973).

Typically, native proteins exhibit a very low free energy of stabilisation (ΔG_{stab} of around 50kJ/mol), corresponding to a few weak interactions in a protein molecule (Jaenicke, 1995). This apparent low level of stability is

important for protein function. Higher stability would lead to a decrease in flexibility, a feature essential to protein function in terms of catalysis, regulation and turnover.

In vitro studies of protein folding have traditionally relied upon either heat, acid or chemical denaturants as a means of perturbing protein structure. Despite a long history of use, details of the molecular mechanism by which they exert their effects remains limited. In the case of chemical denaturants, such as urea, guanidinium hydrochloride (GdnHCl), and potassium thiocyanate (KSCN), two effects need to be considered: (i) the effect of denaturant on solvent behaviour (Breslow & Guo, 1990); (ii) the direct interaction of denaturant with the protein itself (Makhatadze & Privalov, 1992). Recent crystallographic studies of dihydrofolate reductase with urea and ribonuclease A with GdnHCl (Dunbar *et al.,* 1997) point to a decrease in protein flexibility as a result of binding denaturant molecules.

A general mechanism for the folding and assembly of proteins is detailed in Figure 1.6 (adapted from Jaenicke, 1987). In this scheme *M*, M', M represent unfolded, intermediate and structured monomer, N is a native dimer and k_1 and k_2 represent first and second order rate constants respectively.

The mechanism corresponds to:

1) Formation of elements of super-secondary structure

2) Hydrophobic collapse to a domain/subdomain, leading to the formation of a structured monomer

3) The assembly of the structured monomer into the dimer

4) Molecular rearrangement of the near-native oligomer to maximise packing density and minimise hydrophobic surface area.

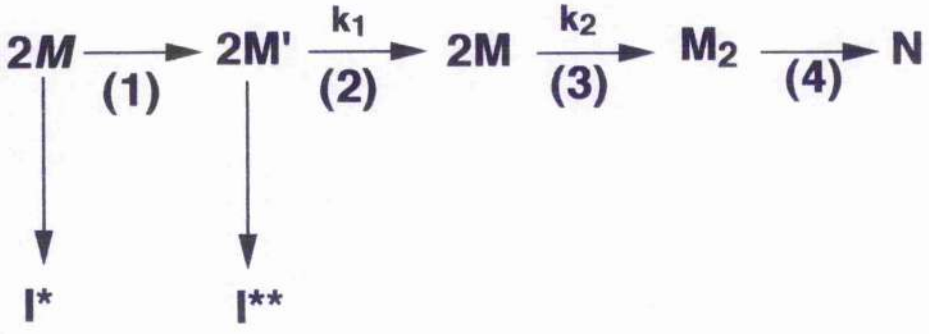


Figure 1.6 Proposed kinetic pathway for the folding and association of a dimeric protein
(Adapted from Jaenicke, 1995)
M, **M'**, and **M** represent unfolded, intermediate and structured monomeric states
N is the native dimeric protein
I* and **I**** are kinetically trapped intermediates, prone to aggregation
k_1 and k_2 are first and second order rate constants respectively

The rate limiting step in the kinetic pathway proposed above is believed to occur during its later stages. Possible rate limiting steps include disulphide bond formation, proline isomerisation and subunit association. Steps 1-3 focus on intramolecular events and, as the localised concentration of the reactive elements is relatively high, these tend to exhibit first order kinetics. They also represent the steps in the pathway during which the nascent polypeptide is vulnerable to incorrect intra or inter molecular associations which result in aggregation. However subunit association, as it is governed by collision theory, is a concentration dependent process and typically displays second order kinetics. The oligomeric assembly process requires correct intermolecular recognition to occur to be successful. This requires that monomers are in a native/near native state. As a consequence of this, one would expect incorrect aggregation is minimised during this step (Goldberg *et al.,* 1991; Jaenicke, 1995).

The mechanism for protein folding proposed above can be extended to larger multidomain proteins, such as the component enzymes of the 2-oxoacid dehydrogenase complexes, by virtue of evidence to date that proteins fold in parts. Studies of proteins both *in vitro* and in the cellular context (Jaenicke 1993a) reveal that proteins can be considered to fold and unfold on a domain by domain basis; each domain appears to act as an independent folding entity.

1.8 Role of molecular chaperones

The first successful attempt to address the protein folding question was made by Anfinsen nearly thirty years ago (reviewed in Anfinsen 1973; Jaenicke, 1987). He and his co-workers were able to show that despite destruction, *in vitro*, of the 3D structure of ribonuclease by denaturing agents, it was still possible to refold the protein to its active and therefore native state. No additional protein factors, or energy input were required to promote reconstitution of this enzyme. Thus, it appeared, all the information required for protein assembly/folding was contained in its primary amino acid sequence, specified originally by the genetic code.

However, work over the past decade, beginning with studies on ribulose 1,5 bisphosphate carboxylase (Barraclough & Ellis, 1980), has shown clearly that *in vivo* protein folding is not so straightforward and is neither spontaneous nor energy independent. In fact, a very complex cellular mechanism involving sequential action by a series of molecular chaperones has been shown to be essential for effective cellular protein folding in both prokaryotic and eukaryotic systems (for reviews see Ellis, 1987; Landry & Gierasch, 1994; Martin & Hartl 1994; Hartl, 1996). The novel observation that protein assembly *in vivo* was protein assisted led to a proliferation of biochemical studies which demonstrated that molecular chaperones were involved in: the association with nascent/unfolded protein chains (Bole *et al.,* 1986), enhancement of *in vitro* folding and assembly reactions (Zheng *et al.,* 1993), protection against aggregation/heat denaturation (Lindquist, 1992) and improved yields of heterologous proteins co-expressed with the molecular chaperones of *E. coli* (Wynn *et al.,* 1992). Meanwhile, genetic approaches (Zeilstrya-Ryalls *et al.,*

1991) yielded data which illustrated that strains with mutant chaperone phenotypes often failed to fold and assemble proteins. It was also possible to demonstrate using a combined genetic and biochemical approach that chaperones were essential for the translocation of proteins across biomembranes (Stuart *et al.,* 1994; Martinus et al., 1995).

Protein studies carried out *in vitro* often seek to minimise the problem of protein aggregation by ensuring that the concentration of protein is controlled, typically to levels lower than 1mg/ml. However, this does not reflect conditions within the cell. In this environment the concentration of macromolecules, including protein and RNA, has been estimated as being in the 200-400mg/ml range (Zimmerman & Minton 1993). This leads to what has been termed macromolecular crowding. This phenomenon is predicted to increase the effective protein concentration, at a ribosome, active in protein synthesis, to levels estimated at 100-1000mg/ml (Ellis, 1997).

The term molecular chaperone was first used to describe nucleoplasmin, a histone binding protein involved in nucleosome assembly (Laskey *et al.,* 1978). These proteins function to prevent aggregation of partially folded protein intermediates. The chaperone systems studied to date have clearly indicated that several chaperones act in concert during protein folding; with individual chaperones having evolved to specifically deal with discrete steps along the pathway. It is also important that they co-operate with one another to deliver the folding protein to the next step in the process. It would appear the molecular chaperones do not act to violate the protein self-assembly principle, rather they function to prevent incorrect protein-protein interactions from occurring, which would result in incorrectly folded/aggregated forms of the protein.

Two key types of molecular chaperones have been identified during the course of work in the field, which has accelerated in the last decade. These have been termed the small and large molecular chaperones (Ellis, 1997). Each has, within the cell, a distinct function; but they do interact with each other and constitute the molecular chaperone pathway (Hartl, 1996). Small chaperones of the DNA J and DNA K (Hsp 70) families are stress proteins which function to prevent premature folding of the nascent polypeptide as it emerges from the ribosome. They also serve to prevent hydrophobic aggregation of the polypeptide with other proteins (see table 1.1 for details).

A second type of molecular chaperone, often termed chaperonins, (Ellis, 1994) include the (GroEL/ES) family (Fenton & Horwich, 1997). Genetic studies (Horwich *et al.,* 1993) into the role of the GroEL/ES chaperonin family in *E. coli* have demonstrated that they serve to prevent protein aggregation. A possible mechanism for this action has been elucidated *in vitro* (Jackson *et al., 1993* ; Todd *et al.,* 1997). It is proposed that aggregation prevention permits a partial unfolding of kinetically trapped protein intermediates, and this in turn is sufficient to promote their return to productive folding pathways. The model allows for successive iterations of binding and release of the polypeptide to occur before the native state is reached (see table 1.1 for details).

In addition to preventing aggregation, this chaperonin family directly facilitates productive protein folding. The central cavity of the GroEL oligomer, with its GroES cap bound, provides an isolated space in which the aggregation sensitive protein can fold. Binding of the chaperonin GroES to one end of the GroEL oligomer promotes release of the bound, folding protein from its contact sites on GroES. Protein residence time within this Anfinsen cage (Ellis, 1994), is determined by the ATPase activity of the GroEL molecules and subsequent GroES release (see Figure 1.7 for proposed mechanism). The two central

Components of the hsp70 and chaperonin system.

Bacteria	Eukaryotes	Compartment (in eukaryotes)	Properties
hsp70 system			
DnaK	hsp70, BiP	Cytosol, mitochondria, endoplasmic reticulum lumen	70 kDa protein with ATPase activity, binds extended peptides, interacts with hsp40 or its homologues and GrpE, required for post-translational protein import
DnaJ	hsp40	Cytosol, mitochondria, endoplasmic reticulum lumen	40 kDa protein, binds unfolded proteins, interacts with hsp70 or its homologues, stimulates their ATPase activity
GrpE	GrpE	Mitochondria	20 kDa protein, nucleotide exchange factor for hsp70 or its homologues
Chaperonin system			
Group I			
GroEL–GroES	hsp60–hsp10	Mitochondria	Homo-oligomer, two rings of 760 kDa subunits, ATPase activity, binds folding intermediates, mediates protein folding together with co-chaperonin GroES (hsp10)
	RBP	Chloroplasts	GroEL homologue with two homologous subunit species, α and β, ATPase activity, interacts with cpn20 (the cofactor of cpn60) in folding of Rubisco subunits
Group II			
TF55			Hetero-oligomer, two rings of 9 × 55 kDa subunits, two subunit species with homology to CCT, ATPase activity, binds folding intermediates, promotes folding in Archaea
Thermosome			Hetero-oligomer, two rings of 8 × 55 kDa subunits, two subunit species homologous to TF55, ATPase activity
	CCT	Cytosol, nucleus	Hetero-oligomer, two rings of 8 or 9 × 55 kDa subunits, 7–9 different subunits, ATPase activity, binds folding intermediates, promotes folding and possibly assembly of cytosolic proteins

Table 1.1 Components of the hsp 70 and chaperonin system (taken from Braig, 1998)

cavities within the GroEL molecule are thought to exhibit positive intra domain co-operativity, but negative interdomain co-operativity. Thus, as one half of the oligomer binds to ATP the other side will favor ATP hydrolysis and ADP formation. ATP binding has been shown to increase cavity size. This enlargement is aided by GroES binding and results in a cavity up to 65Å high and 80Å wide. This facilitates protein entry into the central cavity where the ADP-bound state of the oligomer has an enhanced affinity for the polypeptide. The folding polypptide is thought to undergo a number of iterations before it gains biological activity and is released from the chaperonin machinery (Todd *et al.*, 1996; Martin & Hartl, 1997a; Fenton & Horwich, 1997).

Recent work has begun to investigate the effects of molecular crowding, conditions expected within the cell, on chaperonin function (Martin & Hartl, 1997b). In this study the molecular crowding agents, such as ficol, dextran 70 and cytoplasmic xenopus egg extracts, were used to mimic cytoplasm conditions. In such a system release of the partially folded protein from the uncapped GroEL cavity was minimised. This greatly enhances the possibility of the partially folded protein rebinding the same GroEL oligomer. This limited diffusion and rapid recruitment of the partially folded protein intermediate serves to minimise the danger of aggregation.

Thus, molecular chaperones can be defined as functional classes of unrelated families of protein that mediate the correct non-covalent assembly of other polypeptide-containing structures. However, they do not serve as components of these assembled structures when the latter are carrying out their normal biological functions. All of the molecular chaperones studied to date act not by providing steric information essential for assembly, but by preventing incorrect interactions which would produce non-functional structures and in some cases large insoluble aggregates.

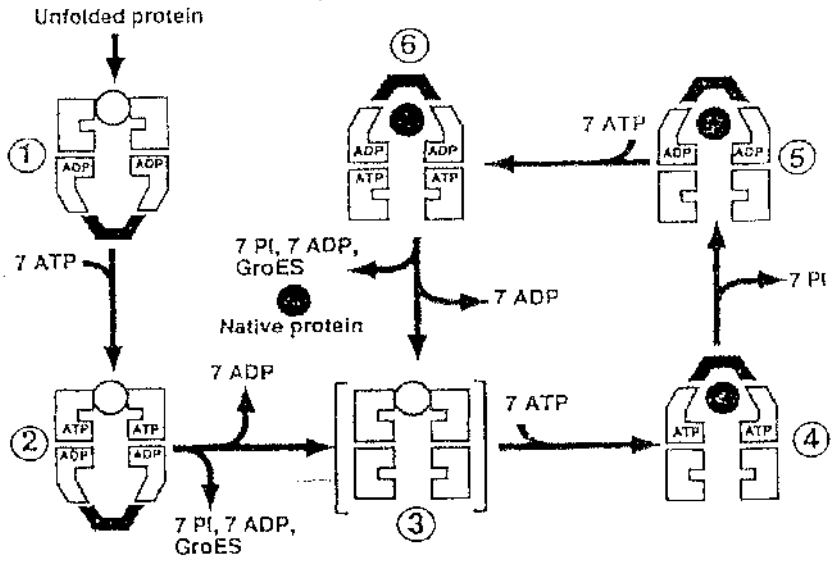


Figure 1.7 Proposed model for a GroEL-GroES mediated folding reaction (adapted from Hartl, 1994)

The production of native protein may require that the unfolded polypeptide undergoes successive iterations (cycles of unbinding and rebinding), involving steps 3-6 outlined above, before this can be achieved.

1.9 Folding and assembly of 2-oxoacid dehydrogenase complexes

In eukaryotes, the 2-oxoacid dehydrogenase complexes are located in the mitochondrial matrix. However, they are not coded for by the mitochondrial genome, which, in mammals, only codes for 13 polypeptides involved in the electron transport chain and ATP synthesis. Instead, all components of the 2-oxoacid dehydrogenase complexes are the products of nuclear encoded genes. This has important implications for their synthesis and delivery to the correct cellular compartment.

The delivery of proteins to mitochondria and their subsequent import and functional maturation has been an area of intense study (see Glover & Lindsay 1992; Martinus *et al.*, 1995; Lithgow *et al.*, 1997 for reviews). In particular, the modulation of the above by a molecular chaperone mediated pathway, exhibiting a division of labour and functional complementation, has been demonstrated (see Figure 1.8). Thus, proteins destined for mitochondria, undergo cytosolic synthesis and, decorated in cytosolic hsp70 molecules to prevent premature folding, are transferred to receptor sites situated on the mitochondrial outer membrane. Import into the mitochondria is driven by energy (ATP), membrane potential (ψ) and the mitochondrial hsp70 molecules, which appear to "pull" the precursor protein into the matrix (Kang *et al.*, 1990). Maturation of the precursor protein involves the removal of the mitochondrial targeting sequence and the folding and assembly of the protein into its active form. In many instances, the folding and assembly process is thought to require the assistance of the mitochondrial hsp60/cpn10 machinery. These proteins, homologues of the well characterised bacterial GroEL/ES system (see Hartl, 1996; Fenton &

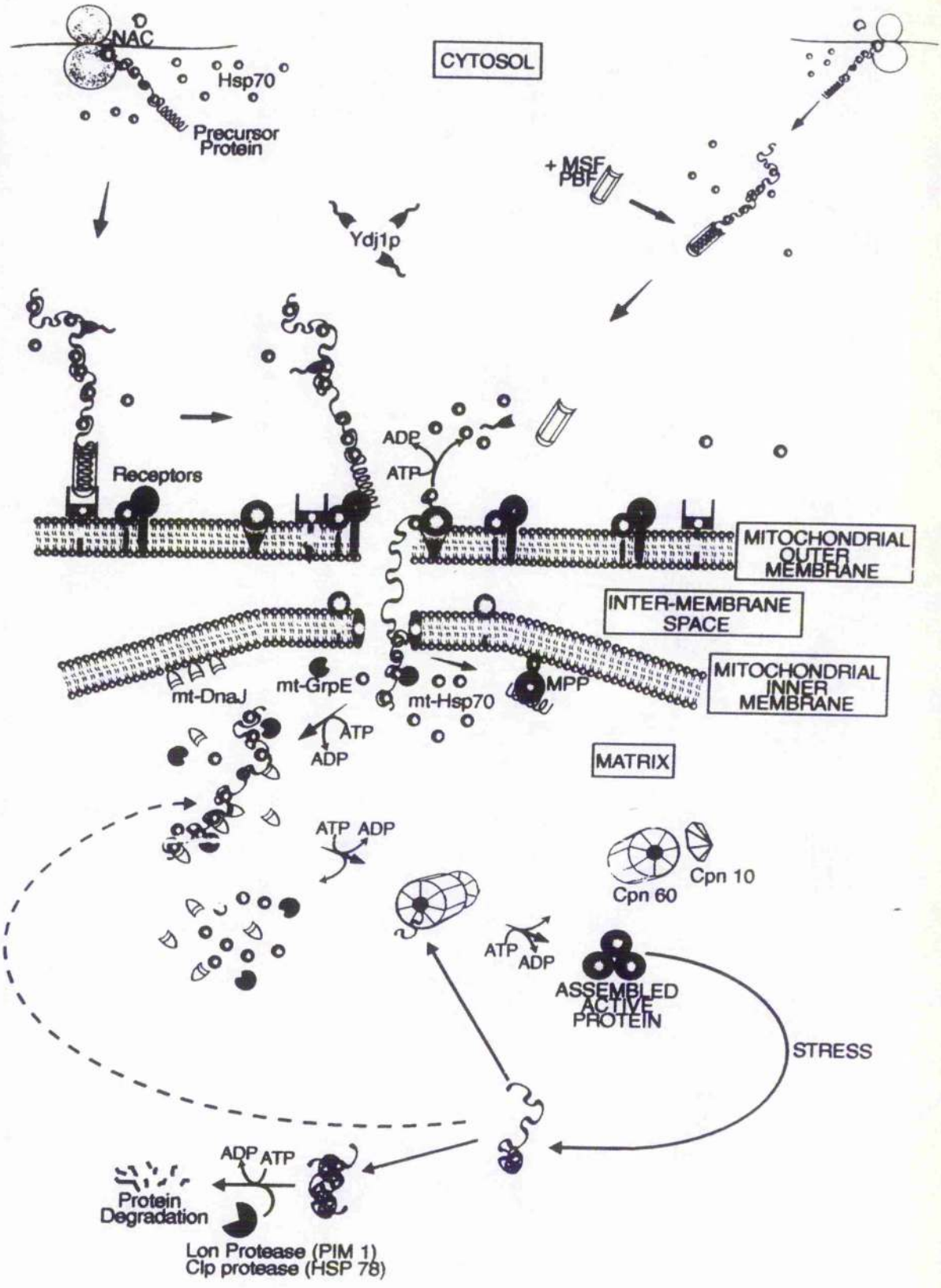


Figure 1.8 Role of molecular chaperones in the biogenesis of mitochondrial proteins (adapted from Martinus *et al.*, 1995).

Horwich, 1997 for recent reviews) are thought to promote the correct folding of the polypeptide via repeated ATP driven cycles of binding and release.

It is probable that the pathway used to produce functional 2-oxoacid dehydrogenase multienzyme complexes in the mitochondrial matrix bears a good deal of similarity to the one outlined above. However, the regulatory mechanisms required to ensure co-ordinated expression of all the individual polypeptides necessary for complex formation is not as yet understood. *In vitro* refolding studies of some of the individual components of the multienzyme complexes employing (a) a truncated E2 of BCOADC (Wynn *et al.*, 1994) and (b) E1 of BCOADC (Wynn et al., 1992) have used the GroEL/ES chaperonin machinery to successfully refold chemically denatured/unfolded forms.

1.10 Oxidative stress

As a consequence of the aerobic environment in which most organisms live, oxidative damage to biological macromolecules is a potential hazard. Protein oxidation, lipid peroxidation, and DNA damage, incorporating both base modifications and strand breaks, represent the major modifications observed when cellular components are exposed to reactive oxygen species (for a recent review see Stacpoole, 1997). Accumulation of these damaged macromolecules contributes to, or has the potential to accelerate, any senescence or disease process. In addition to its role in the normal ageing process (Harman, 1981), oxidative stress has been implicated in a number of age related diseases, including Parkinson's disease and Alzheimer's disease.

To combat this destructive process, organisms have evolved mechanisms to sense and prevent oxidative stress situations, as well as repair any damage

which may arise. A complex system of defence involving chemical agents and enzyme families, which appear designed specifically for the task, is required to achieve this (Stacpoole, 1997; Fridovich, 1997; Berlett & Stadtman 1997).

Hydrogen peroxide, hydroxide radicals and reactive thiol species all pose a threat to biological macromolecules. However the commonest cause of component damage is the superoxide anion radical ($O_2^{\cdot-}$) and its reactive progeny. This radical can be generated either in a spontaneous fashion (i.e. light, radiation), or by enzymatic reactions, such as those catalysed by xanthine oxidase (Fridovich, 1997). In the cellular environment, mitochondria, where 90% of the oxygen utilised is reduced to water (Stacpool, 1997), serve as a major source for this and other reactive oxygen species (Nohl & Hegner, 1978; Farmer & Sohal, 1989). Thus, mitochondrial proteins, many of which rely on their redox state for their cellular function and mitochondrial DNA which is not afforded the protection of histone proteins, are particularly vulnerable to damage (Wallace 1992; Shigenaga *et al.*, 1994). Indeed, recent work has identified the mitochondrial enzyme aconitase as a target for oxidative damage during ageing (Yan *et al.*, 1997).

Chemical agents, such as ascorbate, glutathione and lipoic acid, along with the catalayse, peroxidase and superoxide dismutase families of enzymes, play important roles in the defence against oxidative damage. In eukaryotes, the superoxide dismutase (SOD) family of enzymes constitutes the main line of defence (Fridovich, 1997). In addition to the cytosolic Cu-Zn form of the enzyme, two other forms, an extracellular Cu-Zn form and a mitochondrial Mn form, have been characterised. Extracellular SOD (EC-SOD) is found principally in the extracellular spaces and the function of this glycosylated heterotetramer appears to be to inactivate superoxide anion radicals generated by phagocytic leukocytes (Sandstrom *et al.*, 1992). Work on the Cu-Zn form of the

enzymes implicates mutations in the gene(s) as being a causative agent for the disease amyotrophic lateral sclerosis (ALS), which involves motor neurone degeneration, skeletal muscle atrophy, paralysis and death (Gurney, 1997; Shaw *et al.,* 1998; Kong & Xu, 1998). Mitochondrial Mn-SOD serves as a primary defence mechanism against the high levels of reactive oxygen generated by the organelle. The importance of mitochondrial SOD to organism viability has been highlighted by gene knockout studies performed in mice (Li *et al.,* 1995; Lebovitz *et al.,* 1996). Mice lacking this enzyme displayed a dramatic shortening of life expectancy to 1-2 weeks and faulty mitochondrial activity was detected in tissues investigated, especially the heart.

Studies in *E. coli* of the SoxRS regulon, representing a group of genes expressed in response to oxidative stress, has begun to provide information on the molecular mechanism underlying the sensing of such events and mobilisation of a concerted defence (Ding *et al.,* 1996).The protein SoxR acts as a redox sensor. Specifically, oxidation of the protein's [2Fe-2S] cluster causes it to act as a transcriptional activator of the SoxS gene; which is responsible for switching on the entire regulon. Another regulon, directed against peroxide stress has also been identified in *E. coli* (Storz *et al.,* 1990). It is possible to speculate that similar control systems may be implicated in the concerted response of eukaryotes to such stress conditions.

Organisms require systems to sense and then prevent, or limit, damage caused to cellular components by reactive oxygen species. Systems which rely on both enzymatic and non enzymatic antioxidants have been described. The list includes glutathione and thioredoxin systems; the enzymes superoxide-dismutase, catalase and glutathione peroxidase. These and others, depending on their specific function and localisation, have important roles in protection, prevention, interception and repair of oxidative damage

1.11 Aims of this thesis

The principal area of investigation contained in this thesis relates to the subunit interactions between the component enzymes of the 2-oxoacid dehydrogenase multienzyme complexes. To carry out the studies outlined below, it was first necessary to develop purification protocols which facilitated the production of the individual components of the 2-oxoacid dehydrogenase complexes in their active forms, and in sufficient quantities (Chapter 3)

Studies on the E2 component of mammalian PDC concern the folding and assembly of the 60meric core structure. The ability of refolded E2 cores to reconstitute PDC activity, upon the addition of the E1 and E3 components, is examined and the presence of specific, low affinity binding sites on the E2 component for E3 is demonstrated.

Previous work in the laboratory had implicated a possible role for the E1 component of mammalian OGDC in binding the E3 component to this multienzyme complex (Rice *et al.*, 1992). Further studies, which include component purification, whole complex reconstitution and gel permeation analysis, confirm that the E1 and E3 components of mammalian OGDC do interact directly with each other (Chapter 5).

During the course of purification development work, a protein was discovered which appeared to associate with mammalian PDC. N-terminal amino acid analysis identified it as SP-22, a thiol antioxidant protein. To allow

future study of the role of this protein, and its proposed relationship with mammalian PDC, a clone was obtained , from bovine brain cDNA (chapter 6).

CHAPTER 2: MATERIALS AND METHODS

Section A: Protein Biochemistry

2.1 Protein Biochemistry Materials

2.1.1 Chemical reagents

The majority of reagent grade chemicals were obtained from either Fisher Scientific, Loughborough, Leicestershire, U.K. or BDH laboratory supplies, Poole, Dorset, U.K. These included:
glycine, sodium dodecyl sulphate (SDS), sodium hydroxide, magnesium chloride, polyethylene glycol 6000, acrylamide, N,N-methylenebisacrylamide, ammonium persulphate, citric acid, trichloroacetic acid (TCA).

The following reagent grade chemicals were purchased from the Sigma Chemical Co, Poole, Dorset, U.K.
Acetyl phosphate (lithium potassium salt), glutaraldehyde (grade 1), 3-[N-morpholino]-propane-sulphonic acid (MOPS), imidazole base, triethanolamine, Coomassie Brilliant Blue R250, Pyronin Y, antifoam A (concentrate), L-cysteine-HCl (anhydrous), β-nicotinamide adenine dinucleotide (oxidised form, β-NAD^+), thiamine diphosphate (ThDP), DL-6,8-thioctic acid amide (DL-lipoamide, oxidised form), benzamidine-HCl, phenylmethylsulphonyl fluoride (PMSF), coenzyme A (lithium salt), acetyl coenzyme A (C2:0) lithium salt, pyruvic acid (sodium salt) and 2-oxoglutaric acid (disodium salt), 2,6- dichlorophenolindophenol (DCPIP)

Ultrapure guanidine hydrochloride (GdnHCl) and dithiothreitol (DTT) were supplied by Gibco BRL, Paisley, Scotland. Additional chemicals were of the highest grade commercially available.

2.1.2 Proteins and enzymes

The majority of proteins and enzymes were supplied by Sigma Chemical Co., Poole, Dorset, U.K.

Lipoamide dehydrogenase, type VI, E.C. 1.8.1.4, isolated from bovine intestinal mucosa (170 U/mg protein); lipoamide dehydrogenase, E.C. 1.8.1.4, from *Candida utilis* (40-100U/mg protein); phosphotransacetylase, E.C. 2.3.1.8 isolated from *Bacillus stearothermophillus* (4,000-10,000 U/mg protein); bovine serum albumin, (fraction V); leupeptin protease inhibitor.

Low molecular mass electrophoresis marker proteins were purchased from PHARMACIA LKB Biotechnology, Milton Keynes, U.K.

Bovine gamma globulin was purchased from BIO-RAD Laboratories Ltd., Herts., U.K.

2.1.3 Biological materials

Bovine hearts were obtained from Paisley Abbatoir, Sandyford Rd., Paisley, U.K. shortly after slaughter, transported on ice to the laboratory and diced before storage at -80°C. New Zealand rabbits, used in the production of antibody, were purchased from Medical Research Council accredited sources. Freund's adjuvant (incomplete & complete) was purchased from Sigma Chemical Company Ltd., Dorset, U.K.; and heat inactivated donkey serum supplied by the Scottish Antibody Production Unit (SAPU), Glasgow & West of Scotland Blood Transfusion Service, Law Hospital, Carluke, U.K.

2.1.4 Chromatographic materials

Mono Q strong anion exchange resin, Superose 6 (prep grade) and Superose 12 gel matrices were purchased from PHARMACIA LKB Biotechnology, Milton Keynes, U.K.

2.1.5 Photographic materials

High performance Hyperfilm-ECL was purchased from Amersham International plc., Buckinghamshire, U.K. LX-24 X-ray developer, FX-40 X-ray liquid fixer and X-omat S film were purchased from IBI Ltd. (Kodak), Cambridge, U.K. Autoradiography cassettes and high speed- intensifying screens were supplied by Genetic Research Institute Ltd., Essex, U.K.

2.1.6 Miscellaneous items

Slide-a-lyser dialysis cassettes and buoyancy aids were obtained from Pierce Chemical Co., Chester, U.K.

PVDF protein sequencing membrane was purchased from BIO-RAD Laboratories Ltd., Hertfordshire, U.K.

HYBOND-C extra supported nitrocellulose (0.45μm pore size) was purchased from AMERSHAM International plc., Buckinghamshire, U.K.;

Centricon microconcentrator tubes were purchased from AMICON, Danvers, Ma., U.S.A.

2.2 Protein Biochemistry Methods

2.2.1 Determination of protein concentration

Protein concentration was determined using the method of Bradford (1976). In this instance, bovine gamma globulin was found to be a better relative standard than bovine serum albumin. For each set of samples a standard curve was constructed and absorbances read at 595nm.

2.2.2 Dialysis of protein samples

Visking tubing was boiled in 10mM sodium bicarbonate pH 8.0, 1mM EDTA for 30 min to remove chemical contaminants. It was then rinsed and boiled in distilled water for a further 10min, before storage in 100%(v/v) ethanol. Dialysis was carried out at 4°C at a minimum ratio of 1:1000 (sample: dialysis buffer) and usually employed multiple changes of dialysis buffer.

2.2.3 Concentration of protein samples

For sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of dilute protein samples, or those which contained interfering material (i.e., guanidine hydrochloride), trichloroacetic acid (TCA) precipitation was carried out (Hames, 1981). Typically, following the addition of 10%(w/v) TCA and incubation of the sample for 15min at 4°C, precipitated protein was pelleted by microcentrifugation at 10,000g in a bench top centrifuge for 15min. Subsequently,

the pellets were washed with ice cold acetone before resuspension in Laemmli sample buffer and the pH adjusted to 7.0 using 2M Tris base.

Pooled fractions from the preparative dissociation of PDC (see section 2.2.9) into E2/X and E1/E3 were concentrated, after extensive dialysis to remove salt, by a second dialysis step directly into storage buffer which contained 50%(v/v) glycerol. Occasionally the desalted pools were concentrated by submerging the dialysis tubing in fresh, dry poly(ethylene glycol) (PEG 6000) flakes at 4°C until the desired decrease in volume was achieved.

Ultrafiltration/concentration of protein samples was carried out using the Amicon Centricon system when very small sample volumes were required. This involved, multiple 20-30min spins at 5000g.

2.2.4 Protein analysis by gel electrophoresis under denaturing conditions

Proteins were compared and characterised using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Resolution was achieved using the discontinuous Tris/glycine system of Laemmli (1970).

Protein sample preparation

After the addition of an equal volume of Laemmli sample buffer (62.5mM Tris/HCl pH6.8, 2%(w/v) SDS, 10%(w/v) sucrose, 0.2%(w/v) Pyronin Y) and DTT to a final concentration of 100mM, samples were boiled for 5min. Prior to

loading, samples were spun briefly in a benchtop microcentrifuge to remove any particulate material.

Analytical SDS-PAGE gel preparation

Gels with dimensions 170mm x 145mm x 1.5mm were prepared from the stock solutions below:

a) Acrylamide stock solution (22.2% (w/v) acrylamide, 0.6%(w/v) bis acrylamide).

b) 2x Resolving gel buffer (0.75M Tris/HCl pH 8.8, 0.2%(w/v) SDS).

c) 1.4x Stacking gel buffer (0.17M Tris/HCl pH 6.8, 0.14%(w/v) SDS).

d) Ammonium persulphate (0.1mg/ml)

Stock solutions were filtered and degassed prior to use. Polymerisation of the acrylamide was initiated by the addition of TEMED yielding resolving gels of 7-15% (w/v) acrylamide (0.375M Tris/HCl pH 8.8, 0.1%(w/v) SDS, 0.1%(w/v) ammonium persulphate, 0.08%(v/v) NNN'N'-tetramethylethylenediamine (TEMED); and stacking gels of 4.4% (w/v) acrylamide (0.12M Tris/HCl, pH 6.8, 0.1%(w/v) SDS , 0.1%(w/v) ammonium persulphate, 0.08% (v/v) TEMED).

Conditions for electrophoresis

Gels were run using vertical electrophoresis kits purchased from BRL employing a constant 40-70 mA current in running buffer (24mM Tris/192mM glycine pH 8.3, 0.1%(w/v) SDS) for 3-4 h. Gels were stained for protein bands (>0.1μg) overnight using 0.04% (w/v) Coomassie Brilliant Blue R, 10%(v/v) acetic acid and 25%(v/v) methanol, and destained employing multiple changes of 20%(v/v) methanol, 10%(v/v) acetic acid. Addition of 10% (v/v) glycerol to the destain allowed storage of the gel before photography.

Relative molecular mass estimation of SDS-PAGE resolved proteins

Since protein migration in SDS-PAGE is generally proportional to subunit M_r value it was possible to estimate the relative molecular mass by constructing a standard curve of the log of known protein molecular mass as a function of their R_f values (relative mobilities) where:

$$R_f = \frac{\text{distance of protein migration}}{\text{distance of tracking dye migration}}$$

Distances were measured from the beginning of the resolving gel to the leading edge of the protein band.

Two sets of standard proteins were employed: (note that the M_r values below refer to those quoted by the manufacterers).

1) Low molecular weight calibration kit (Pharmacia):

Protein	**Subunit M_r value**
Phosphorylase b	94, 000
Bovine serum albumin	67, 000
Ovalbumin	43, 000
Carbonic anhydrase	30, 000
Soya bean trypsin inhibitor	20, 000
α-lactalbumin	14, 000

2) Wide range protein standards (Novex)

Protein	**Subunit M_r value**
Myosin	200, 000
β–galactosidase	116, 000
Phosphorylase b	97, 000
Bovine serum albumin	67, 000
Lactate dehydrogenase	36, 500
Carbonic anhydrase	31, 000
Soya bean trypsin inhibitor	20, 000
Lysozyme	14, 000

2.2.5 Immunoblotting

Proteins, after resolution by SDS-PAGE, were transferred to and immobilised on a matrix for subsequent immunological detection (Towbin *et al.*, 1979; Gershoni and Palade, 1983)

The first step involved electrophoretic transfer of the proteins from the polyacrylamide gel to nitrocellulose using a wet tank blotting method. Pre-wetted Hybond-C immobilizing matrix was used to overlay the gel, sandwiched between wet filterpapers and placed in a transfer cassette. This cassette was then submerged in transfer buffer (25mM Tris, 0.19M glycine, pH 8.2-8.4, 0.02%(w/v) SDS, 20%(v/v) methanol) contained in a Bio-Rad Trans Blot apparatus and a current of 40mA applied overnight. The non-fixative stain, Ponceau S, was used to determine efficiency of transfer to the nitrocellulose.

Subsequently, the membrane was blocked overnight at 4°C, or all day at room temperature, with blocking buffer (20mM Tris-HCl, pH 7.2, 15mM NaCl, 5%(w/v) non fat milk, 5%(v/v) normal donkey serum, 0.2%(w/v) Tween 20). Incubation with the primary antibody (diluted between 1:10,000 and 1:50,000 in 20mM Tris-HCl, pH 7.2, 1%(w/v) non fat milk, 5%(v/v) normal donkey serum, 0.1%(w/v) Tween 20) took place overnight at 4°C. Following extensive, multiple washes with low salt buffer (20mM Tris, pH 7.2, 15mM NaCl, 1%(w/v) non fat milk, 1%(v/v) normal donkey serum) to remove unbound primary antibody, the secondary anti-rabbit horseradish peroxidase (HRP) conjugate antibody was applied (1:1000 dilution in 20mM Tris-HCl, pH 7.2, 150mM NaCl, 1%(w/v) non fat milk, 1%(v/v) normal donkey serum). After a 2h incubation at room temperature, blots were subjected to an extensive wash procedure employing multiple washes with the low

salt buffer followed by a single high salt wash (20mM Tris-HCl, pH 7.2, 150mM NaCl). Detection was carried out using enhanced chemiluminescence (ECL reagents Amersham) as per the manufacturer's instructions.

2.2.6 Resolution and preparation of proteins for N-terminal sequencing

Protein resolution was performed as described in section 2.2.4 using reagents of the highest purity available. Following SDS-PAGE protein bands were transferred to Hybond C as described in section 2.2.5. After transfer, the IMMOBILON was rinsed in distilled water, stained for 5min with 0.1%(w/v) Coomassie blue, 50%(v/v) methanol and destained for 10 min with 50%(v/v) methanol followed by thorough rinsing with distilled water before the membrane was allowed to dry. Protein bands were excised using a scalpel and sealed in plastic bags before delivery to the BBSRC Protein Sequencing Facility at Aberdeen University (Matsudaira, 1987). Sequencing performed by Brian Dunbar.

2.2.7 Negative ion electrospray mass spectrometry (ES/MS)

Protein samples were analysed in the negative ion mode at a concentration of 5-10pmol/µl. They were introduced into the electrospray source at 5µl/min. Typically, scanning was in the range mass:charge ratio (*m/z*) 900-1700 and data were summed over approx. 5 min to obtain final spectra. These data, on an *m/z* scale, were transformed onto a true molecular mass scale using maximum entropy (Max-Ent) software. Mass scale calibration employed the multiply charged negative ion series from separate introductions of horse heart myoglobin (Sigma) sequence molecular mass=16951.5 Da (see Nairn *et al.,* 1995 for details). Molecular masses are based

upon the following atomic weights of the elements: C=12.011, H=1.00794, N=14.00674, O=15.9994, P=30.97376, S=32.066 (IUPAC commission on atomic weights and isotopic abundances, 1993). ES/MS operated by Tino Krell.

2.2.8 Purification of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes from bovine heart

Isolation of bovine heart PDC and OGDC, performed, unless otherwise stated, at 4°C, was based upon the standard protocols (Stanley & Perham, 1980; De Marcucci *et al.*, 1985) incorporating the modifications as detailed below:

Fresh, diced ox heart, trimmed of fat deposits/connective tissue, was either used on the day, or stored frozen at -80°C and thawed overnight before use. To 600g of the prepared and washed ox heart, two volumes of ice cold extraction buffer (50mM MOPS, pH 7.0, 3%(v/v) Triton X-100, 3mM EDTA, 1mM DTT, 1mM PMSF, 1mM benzamidine, 0.1%(v/v) anti-foam A) was added. Following homogenisation for 5 min, a cell/nuclear free supernatant was prepared by centrifugation at 10,000g for 20 min. The pH of the supernatant was adjusted to 6.45 using 10%(v/v) acetic acid and the first PEG precipitation step was performed by the addition of 0.12 volumes of 35%(w/v) PEG. After stirring the solution on ice for 30 min to ensure complete precipitation, the pelleted material was recovered by centrifugation at 18,000g for 15 min.

Pellet resuspension was in 300-400ml of 1%(v/v) Triton buffer (50mM MOPS, pH 6.8, 1%(v/v) Triton X-100, 3 mM EDTA, 1mM DTT, 1mM PMSF, 1mM benzamidine, 1.5μM leupeptin) using a loose fitting glass Teflon homogeniser. Material which failed to resuspend was removed by the subsequent clarification spin at 25, 000g for 40 min. After filtration of the resulting supernatant through layers of

muslin to remove fat droplets, 0.05 volumes of 1M sodium phosphate buffer and 0.013 volumes of 1M $MgCl_2$ were added dropwise. Throughout this addition the pH was maintained at 6.8 by the addition of 0.5M NaOH. The pH was readjusted to 6.45 with 10%(v/v) acetic acid before a second PEG precipitation was performed. This involved the addition of 0.12 volume of 35%(w/v) PEG to the stirring supernatant on ice and, as previously, after 30 min, the material precipitated was pelleted by centrifugation at 25, 000g for 10 min. Before storage overnight at 4°C, the pellets were resuspended in 1%(v/v) Triton buffer (150-200ml) with an additional cocktail of protease inhibitors: 1mM PMSF, 1mM benzamidine, 1μM leupeptin, 0.5%(v/v) rat serum.

Prior to further purification the following day, it was necessary to re-homogenise the material and subject it to another clarification spin at 25,000g for 1h. The third and final PEG fractionation was then carried out. After adjusting the pH to 6.45, with 10%(v/v) acetic acid, 0.06 volumes of 35%(w/v) PEG was added and the solution left to stir on ice for 30 min. This differential cut enabled the separation of OGDC, insoluble at the prevailing PEG concentration, to be removed by centrifugation at 25,000g from the PDC which retains its solubility and was thus present in the supernatant. Ultracentrifugation of the PDC/PEG supernatant in a Ti 70 Rotor (Beckman) at 200,000g for 2.5 h resulted in the pelleting of the PDC and thus allowed for removal of the remaining PEG. Both PDC and OGDC were then resuspended in 1%(v/v) Triton buffer, containing no protease inhibitors, and stored, at 4°C with no significant loss of activity, for over 2 weeks for OGDC and 3-4 weeks in the case of PDC.

2.2.9 Preparative dissociation of PDC into enzymatically active E2/X and E1/E3 fractions

Preparative dissociation of PDC into active E2/X and E1/E3 fractions was performed on an FPLC system (Pharmacia) using a prep grade Superose 6 column (100ml) equilibrated with 50mM imidazole/HCl, pH 7.0, 1M NaCl, 0.01%(v/v) Triton X-100.

PDC (15.0mg/ml) in 100mM MOPS/NaOH, pH 6.8, 5mM EDTA, 1%(v/v) Triton X-100 was microfuged in a benchtop centrifuge at 10,000g for 15min, to remove any particulate material, before incubation at a 1:1(v/v) ratio with 50mM imidazole/HCl, pH 7.0, 4M NaCl on ice for 1h. A 2ml pre-injection of the 4M NaCl buffer was then performed prior to PDC sample loading (2ml) and subsequent elution at a flow rate of 1ml/min. Fractions were pooled on the basis of A_{280} and dialysed and concentrated, at 4°C, against multiple changes of buffer into 50mM imidazole/HCl, pH 7.4, 1mM EDTA, 50%(v/v) glycerol and stored at 4°C. Both pools were stable under these conditions for over one month (see stability data 3.2.2).

2.2.10 Purification of the E1 and E3 components of mammalian PDC by preparative anion exchange chromatography

Following the preparative dissociation of PDC as detailed in section 2.2.9, it was possible, using the E1, E3 fraction, to isolate the individual component enzymes. E1/E3, prepared as detailed in the previous section, was exchanged into 20mM triethanolamine/HCl buffer, pH 7.4, 10mM NaCl, 1mM CHAPS either by extensive dialysis or centricon ultrafiltration. A microfuge spin in a benchtop centifuge at 10,000g for 15min was used to remove any precipitated/particulate material before the sample was loaded (maximum load 4mg of protein) onto an 8ml Mono Q column (Pharmacia) pre-equilibrated with 20mM triethanolamine/HCl buffer, pH 7.4, 10mM NaCl, 1mM CHAPS (buffer A). The column was washed with 2 column volumes (8ml) of the buffer A to remove any unbound material before being developed with a linear gradient of 20mM triethanolamine/HCl buffer, pH 7.4, 500mM NaCl, 1mM CHAPS (buffer B) over 200ml.

The Mono Q column was then washed with 2 column volumes of buffer B to remove any strongly retained material. In addition to monitoring the eluent continuously at 280 nm, individual fractions were assayed for E1 or E3 activity. Pools of fractions which contained significant levels of non-contaminated activity were constructed and subsequently desalted using centricon-30 apparatus (Amicon) into storage buffer 50mM MOPS/KOH, pH 6.8, 10mM NaCl, 3mM EDTA before use.

2.2.11 Association state analysis of the E2/X subcomplex

Association state analysis of the E2/X subcomplex was performed using the FPLC system (Pharmacia). A Superose 6 column (24ml) was equilibrated, at 0.2ml/min, with increasing concentrations of GdnHCl in 50mM imidazole/HCl, pH 7.4, 200mM NaCl. E2/X subcomplex in column equilibration buffer, in the absence of GdnHCl, was centrifuged at 10,000g for 15min and pre-incubated at room temperature for 15 min in the appropriate concentration of GdnHCl before re-centrifugation and subsequent loading of 200µl onto the column at 0.05ml/min. Protein elution was detected at 280nm and the column calibrated using gel filtration molecular mass markers (Sigma).

Following the construction of a Ve/Vo versus log M_r plot, the relative molecular mass of the E2/X species observed was estimated by interpolation

Proteins used to construct Ve/Vo vs log molecular mass calibration curve:

Protein	**Protein M_r**
bovine serum albumin	66, 000
β-amylase	200, 000
Apoferritin	443, 000
Thyroglobulin	669, 000

2.2.12 Enzyme assays

All enzyme assays were perfomed on a Shimadzu UV-2101 PC uv-vis scanning spectrophotometer. Activities were expressed as U/ml unless otherwise stated (where one unit (U) of enzyme catalyses the conversion of 1μmol of substrate to product per minute under the specified conditions of pH, temperature, ionic strength and substrate concentration).

Overall PDC/OGDC complex activity

PDC and OGDC activities were monitored spectrophotometrically at 340nm following the production of NADH (Brown & Perham, 1976). Typically 1-5μg of the complex was added to an assay cuvette pre-warmed to 30°C containing 670μl solution A (50mM potassium phosphate buffer, pH 7.6, 3mM NAD^+, 2mM $MgCl_2$, 0.2mM ThDP) and 14μl of solution B (0.13M cysteine-HCl, 0.13mM Li_2CoASH) and 14μl solution C (100mM pyruvic acid for PDC assays or 100mM 2-oxoglutarate for OGDC assays).

Pyruvate dehydrogenase (E1)

This was measured by following the reduction of 2,6-dichlorophenolindophenol (DCPIP) to its colourless form at 600nm (Khailova *et al.,* 1976). Varying amounts of either purified E1 or intact PDC complex were added to a cuvette containing 670μl solution A (50mM potassium phosphate buffer, pH 7.6, 3mM NAD^+, 2mM $MgCl_2$, 0.2mM ThDP) and 14μl of DCPIP (1mg/ml) pre-warmed to 30°C. The reduction of the dye was initiated upon the addition of 14μl solution C (100mM pyruvate).

Dihydrolipoamide acetyltransferase (E2)

The activity of the E2 component of PDC was assayed in the direction of formation of acetyldihydrolipoamide (Reed & Willms, 1966; Ono *et al.,* 1993; Yang *et al.,* 1996). The following assay conditions were used: 85mM Tris-HCl, pH 8.0, 10mM acetyl phosphate, 1.8mM dihydrolipoamide, 260μM Co A, phosphotransacetylase (4U/ml). After the absorbance became constant (10-15sec), due to the formation of acetyl CoA, the E2 source was added. Activity was measured directly following the increase in absorbance at 240nm, at 30°C and were recorded as change in absorbance/min, as the extinction of the immediate product, 8-acetyl-dihydrolipoamide, cannot be determined.

Dihydrolipoamide dehydrogenase (E3)

Dihydrolipoamide dehydrogenase activity, from a variety of sources, was determined spectrophotometrically following the increase in absorbance at 340nm, at 30°C, due to the formation of NADH from the oxidation of dihydrolipoamide (Jackman *et al.,* 1990). Routinely 1-2μg of either intact PDC or isolated E3 were added to a cuvette containing 670ul solution A (50mM potassium phosphate buffer, pH 7.6, 3mM NAD^+, 2mM $MgCl_2$, 0.2mM ThDP) and 14μl freshly dissolved dihydrolipoamide (2mM in 70%(v/v) ethanol) and activity determined from the increase in absorbance at 340nm.

2.2.13 Preparation of dihydrolipoamide

Dihydrolipoamide substrate was prepared in the laboratory from DL-lipoamide according to the method of Kochi and Kikuchi (1976). Typically, 60mg DL-lipoamide was dissolved in 1.2ml 1M potassium phosphate buffer, pH 8.0, 50%(v/v) ethanol. Reduction to the dihydrolipoamide form was achieved upon the addition of 2.4ml fresh 5%(w/v) sodium borohydride in 10mM NaOH. The reaction was terminated after 10 min by the addition of 1.2ml of 3M HCl to neutralise the reaction and destroy excess reducing agent. The product, dihydrolipoamide, could then be extracted into toluene (3x3ml) and, after solvent evaporation under nitrogen, stored at -20°C as a white crystalline solid.

2.2.14 Reconstitution of PDC activity from E2/X sub-complex and E1/E3 fractions

Following dissociation of PDC by gel permeation chromatography (see section 2.2.9), the E2/X and E1/E3 fractions were dialysed into 50mM imidazole/HCl, pH 7.4, 1mM EDTA, 50%(v/v) glycerol and stored at 4°C.

For reconstitution studies E2/X and E1/E3 fractions were activated by a 1:1 (v/v) dilution in 50mM potassium phosphate/KOH, pH 7.6, 3mM NAD^+, 2.7mM cysteine-HCl, 2mM $MgCl_2$, 0.2mM ThDP, 0.02mM CoASH (buffer AB) and incubation at 4°C for 15 min. At this stage, 2-10µg of E2/X was added to an assay cuvette containing 670µl 50mM potassium phosphate/KOH, pH 7.6, 3mM NAD^+, 2mM $MgCl_2$, 0.2mM ThDP (solution A); 14µl 130mM cysteine-HCl, 1.35mM CoASH (Solution B) and 2-30µg diluted E1/E3 pre-warmed to 30°C. The

production of NADH was initiated by the addition 14µl 100mM pyruvate (solution C) and monitored at 340nm. Activities were recorded as U/ml and expressed as a percentage recovery relative to undissociated PDC.

Reconstitution studies on refolded E2/X cores, employing an excess of E3, were performed as above, except that, following reconstitution with the correct stoichiometric quantity of E1/E3, an additional 5 min incubation with purified E3 (1-100µg) was incorporated, before the PDC activity was determined.

2.2.15 Unfolding and refolding studies on mammalian PDC and its component enzymes

GdnHCl denaturation studies

The determination of denaturation curves for PDC and its component enzymes was performed over a range of protein concentration and in a range of buffers using methods outlined in West & Price (1988) and Pace *et al.* (1990).

On the day of the experiment three fresh stock solutions were prepared:

A) a protein stock solution at the desired concentration

B) a buffer solution containing the desired additives

C) a denaturant stock solution prepared in the above buffer.

The molarity of the GdnHCl stock solution can be subsequently determined experimentally using recorded weights or refractive index measurements (Nozaki, 1972). Protein samples at the desired final concentration, in the molarity of denaturant required, were prepared by mixing solutions A, B and C in the appropriate ratios and allowing this unfolding solution to come to equilibrium at the

chosen temperature. After a sufficient time lapse to ensure the complete effect of the denaturant on the protein sample, typically 15min at 20°C, it was possible to measure the degree of unfolding using the parameter under investigation i.e. activity or fluorescence. In the case of activity measurements, it was necessary to remove an aliquot (10-100µl) from the unfolding solution and assay for the activity of interest, in the presence of an identical denaturant concentration present in that particular unfolding sample. After careful measurement of the experimental parameter, the result was plotted on a graph from which it was possible to determine if any additional points were required. Typically, at least three unfolding samples were prepared for each denaturant concentration and their individual activities determined. Details of specfic conditions, or deviations from the above, are presented in the relevant chapters.

Reactivation of PDC and its component enzymes after pre-conditioning with GdnHCl

Intact mammalian PDC, or the individual component enzyme under investigation, was incubated for a fixed time in the desired concentration of GdnHCl as described in the previous section. Reactivation was initiated by rapid dilution, (1:50 or 1:100), either, directly into a suitable assay buffer, or into a renaturation buffer which contained no denaturant. Alternatively, refolding and reactivation were investigated by dialysis of the denatured protein for periods ranging from 20 min to 24h. Enzyme assays were carried out immediately or after a suitable time-lapse. Care was taken to ensure that residual levels of chaotrope were lower than those which were determined to have an inhibitory effect on the enzyme (normally ≤ 30mM). In generating refolding curves, the measurement of the parameter of interest, at each GdnHCl concentration, was performed in triplicate. Activity was expressed as a percentage of the activity of an equal amount of undenatured enzyme. Details of

specfic conditions or deviations from the above are presented in the relevant chapters.

2.2.16 Preparative dissociation of mammalian OGDC

Following purification of OGDC from bovine heart as detailed previously (De Marcucci *et al.*, 1985), OGDC was dissociated into its E2 core and E1/E3 fractions by the following method: OGDC (30mg/ml) was incubated at a 1:1(v/v) ratio with dissociation buffer (50mM MOPS/KOH, 7.6, 2M $MgCl_2$, 20mM DTT, 0.2%(v/v) Triton X-100) for 1.5h on ice. The sample was spun at 10,000g in a bench top centrifuge for 15min to remove any particulate material. The E2 core of OGDC was separated from the E1/E3 fraction on an FPLC system (Pharmacia) using a 100ml bed volume prep grade Superose 6 column (1.6x50cm) equilibrated with 50mM MOPS/KOH, pH 7.6, 1M $MgCl_2$, 0.1%(v/v) Triton X-100. A pre-injection of the dissociation buffer (2ml) was performed prior to OGDC sample loading (2ml) and subsequent column elution at a flow rate of 1ml/min. The eluent was monitored on-line at 280nm. Pooled fractions were dialysed extensively, at 4°C, against multiple changes of buffer, into 20mM MOPS/KOH, pH 7.0, 10mM KCl, 0.1% (v/v) Triton X-100.

2.2.17 Reconstitution of OGDC activity from E2 core and E1/E3 fractions

The method used to reconstitute OGDC activity was derived from the one optimised previously for PDC (Sanderson *et al.*, 1996b). Reconstitution was started by adding quantities of E1/E3 (0-30µg) to cuvettes containing E2 (10µg) and the appropriate assay solutions. Following a 15 min incubation at 20°C, the production of NADH was initiated by the addition of 14µl 100mM 2-oxoglutarate. NADH production was monitored at 340nm and activities are expressed either as Units/ml or a percentage relative to the specific activity of undissociated OGDC (see chapter 5) .

2.2.18 Association state analysis of E1 and E3 OGDC

Gel permeation analysis of E1/E3 quaternary structure was performed on a 24ml bed volume Superose 12 column (1x30cm) attached to an FPLC system (Pharmacia). The method employed derived from one reported previously (McCartney *et al.*, 1997). The column was calibrated with gel filtration molecular mass markers (Sigma) for the construction of a calibration curve of V_e/V_o versus log M_r. The column was equilibrated, at 0.3ml/min, in 20mM MOPS/KOH, pH 7.0, 10mM KCl, 0.1%(v/v) Triton X-100. E1/E3 samples were clarified for 15min at 10,000g in a benchtop centrifuge prior to loading onto the column (500µl of sample). The elution profiles obtained were from protein detection at 280nm and from E3 activity assays on the eluted fractions.

Section B: Molecular Biology

2.3 Molecular biology materials

2.3.1 Chemicals

Agarose was purchased from Appligene Oncor (Birtley, UK). Bacterial growth media was obtained from Merck (Darmstadt, Germany). Bovine brain cDNA was obtained from Clonetech (Cambridge, UK). [^{35}S]-methionine (10mCi/ml) and Amplify fluorescent detection reagent were obtained from Amersham Life Sciences Ltd (Amersham UK). All other chemicals were of analytical grade or better and are detailed in section 2.1.1.

2.3.2 Bacterial strains and plasmid vectors

pCR-Script Amp SK(+) Cloning kit was obtained from Stratagene Ltd (Cambridge, UK). This contained Epicurian Coli XL1-Blue MRF' Kan supercompetent cells. The vector pCR-Script Amp SK(+) was used to clone the purified products amplified by PCR (polymerase chain reaction) using bovine brain cDNA as a template (see Figure 2.1 for vector map and polylinker sequence). pBluescript II KS(+/-) was also purchased from Stratagene.

2.3.3 Synthetic oligonucleotides

Oligonucleotides used as primers for PCR amplification of the SP-22 sequence were synthesised by Gibco BRL (Paisley, Scotland). M13 forward and reverse primers were purchased from Pharmacia Ltd (Milton Keynes, UK)

Vector Map and Polylinker Sequence of the pCR-Script Amp SK(+) Cloning Vector

The pCR-Script™ Amp SK(+) cloning vector is a 2961-bp plasmid designed by incorporating a *Srf* I site into the pBluescript® SK(+) phagemid. The SK designation indicates the polylinker is oriented such that *lacZ* transcription proceeds from *Sac* I to *Kpn* I.

f1 (+) origin (3–459 bp) f1 filamentous phage origin of replication allowing recovery of the sense strand of the *lacZ* gene when a host strain containing the plasmid is co-infected with helper phage.

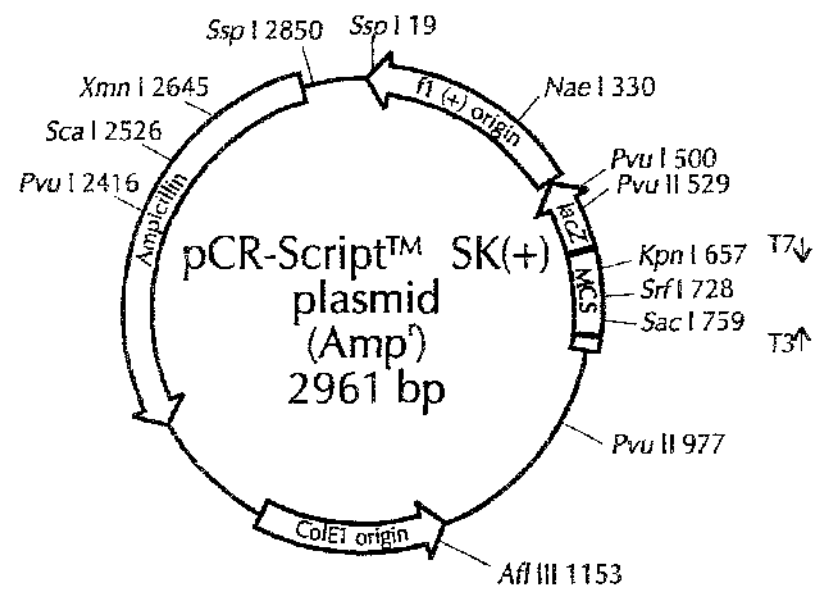
ColE1 origin (1032-1972 bp) Plasmid origin of replication used in the absence of helper phage.

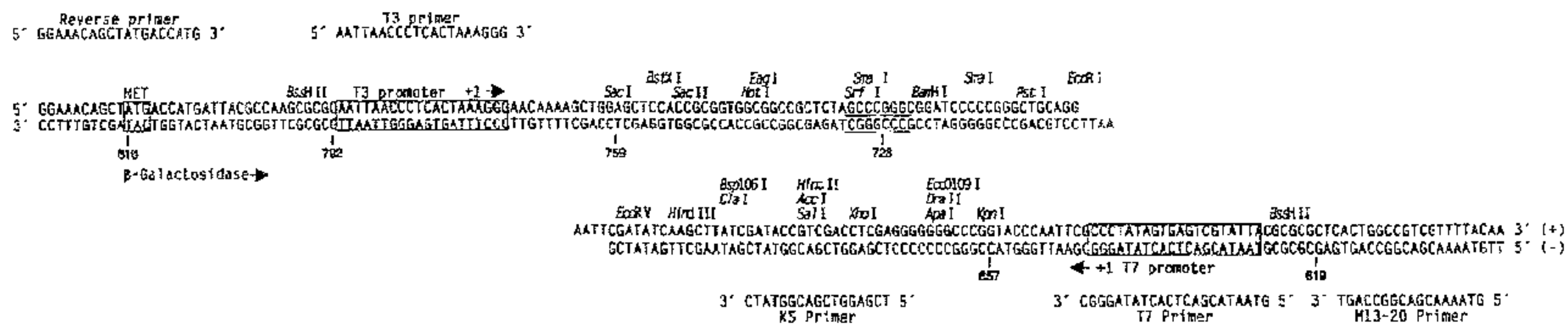
***lacZ* gene** (*lac* promoter: 816–938 bp) This portion of the *lacZ* gene provides α-complementation for blue–white color selection of recombinant plasmids. An inducible *lac* promoter upstream from the *lacZ* gene permits fusion protein expression with the β-galactosidase gene product.

Multiple cloning site (MCS) (657–759 bp) Multiple cloning site flanked by T3 and T7 promoters. (Please see the polylinker sequence below.)

Ampicillin (1975–2832 bp) Ampicillin-resistance gene (Amp^r) for antibiotic selection of the cloning vector.

Please Note The upper strand is designated the (+) strand and the lower strand is designated the (–) strand. The altered nucleotides of the pBluescript SK(+) phagemid are shaded in the polylinker sequence below.





2.3.4 Enzymes and molecular mass ladders

Restriction endonucleases were obtained from Boehringer Mannheim (Lewes, UK) or New England Biolabs (Hitchin, UK). The Expand high fidelity PCR system was purchased from Boehringer Mannheim. The following molecular mass ladders were used in agarose gel electrophoresis:

1) 50 base pair ladder, Gibco BRL

2) Low DNA Mass Ladder, Gibco BRL

3) DNA Molecular Weight Marker III, Boehringer Mannheim

2.3.5 Miscellaneous items

DNA was eluted from the agarose plugs using Gen Elute columns purchased from Supelco (Bellefonte, USA). *In Vitro* transcription/translation was carried out using the single tube protein system Novagen (Abingdon, UK). Wizard plus mini preps, used to purify plasmid DNA, were obtained from Promega Ltd (Southampton, UK).

2.4 Molecular biology methods

2.4.1 DNA electrophoresis

Agarose gels were routinely used to afford separation of DNA fragments (Andrews, 1991). Slab gels were prepared by dissolving agarose in (x0.5) TBE buffer, pH 8.3 (89mM Tris, 89mM boric acid, 2mM EDTA) to a final concentration of 0.7-2.5% (w/v). The set gel was placed in an electrophoresis tank and covered with (x0.5) TBE buffer. DNA samples were prepared in loading buffer (40% (w/v) sucrose, 0.25% (w/v) bromophenol blue, 50mM EDTA) and loaded into the submerged wells in the gel. Electrophoresis was carried out at a constant voltage (2-5 volts/cm gel).

Following electrophoresis, DNA bands in the agarose gels were visualised with an ethidium bromide solution (0.5-1μg/ml in dH_2O) by gentle agitation for 1h at room temperature. Background stain was removed by destaining for 2h with dH_2O. DNA was visualised under 320nm UV light and photographed with a Polaroid DS34 direct screen imaging camera.

2.4.2 Growth of bacteria

The growth media used for this work are detailed over. All solutions were sterilised before use by autoclaving. Alternatively, heat labile solutions were sterilised by passage through a 0.22μm sterile filter.

A) LB medium 1%(w/v) NaCl, 1% (w/v) tryptone, 0.5% (w/v) yeast extract, pH to 7.0 with 5M NaOH

B) LB agar as above except 20g agar per litre LB

C) SOB Medium 2% (w/v) tryptone, 0.5%(w/v) yeast extract, 0.05%(w/v) NaCl. Following autoclaving, 10ml of 1M $MgCl_2$ and 10ml of 1M $MgSO_4$ were added. The solution was then filter sterilised

D) SOC Medium (prepared fresh) 2ml of 20%(w/v) glucose solution added to 100ml SOB and filter sterilised.

Ampicillin was prepared as a 25mg/ml stock in dH_2O; IPTG as a 0.2M stock and X-gal freshly prepared at 10% (w/v) in dimethylformamide (DMF). All solutions were filter sterilised.

Transformed *Escherichia coli* (see section 2.4.8) were selected by plating out on LB-agar plates containing ampicillin (final concentration 50µg/ml) and overnight incubation at 37°C. Colonies were screened for an insert by blue/white (untransformed/transformed) identification employing IPTG/X-gal. *E. coli* bacterial cultures were grown at 37°C in LB media supplemented with ampicillin (final concentration 25µg/ml) overnight in an orbital shaker at 225rpm. Long term storage of cultures was achieved by the addition of glycerol to a final concentration of 15% (w/v) to an aliquot of the culture and storage at -80°C.

2.4.3 Ethanol precipitation of DNA

Sodium acetate, pH 5.5 was added to a final concentration of 0.3M. DNA was precipitated from the aqueous solution by the addition of 2.5vol of 100% (v/v) ethanol and incubation for 30min at -80°C. Pelleted material was recovered by centrifugation at 10 000g for 15 min at 4°C. Subsequently, the pellet was washed with 70% (v/v) ethanol to remove any salt which may have co-precipitated. Finally, after removing the wash supernatant and air drying the pellet, the DNA was resuspended in the appropriate volume of dH_2O or TE buffer (Gannon & Powell, 1991)

2.4.4 Quantification and storage of DNA

DNA content of relatively concentrated samples was determined by UV light absorption at 260nm using a 1ml quartz cuvette. An absorbance unit of 1 corresponds to an approx. concentration of 50µg/ml for double stranded DNA (Sambrook *et al.,* 1989). To obtain an estimation of purity, the absorbance at 280nm was also obtained to allow the construction of a 260/280 ratio for the sample. For dilute DNA samples, agarose gel electrophoresis and comparison with a Low DNA Mass Ladder, (Gibco BRL) was used.

DNA vectors, recombinants and oligonucleotides were routinely stored at -20°C in TE buffer (10mMTris/HCl, pH 8.0, 1mM EDTA). Under these conditions DNA remains stable for several years.

2.4.5 PCR amplification of the nucleotide sequence encoding SP-22 from bovine brain cDNA

Bovine brain cDNA (Clonetech) served as a template for amplification using the Expand PCR System (Boehringer Mannheim). The PCR reaction contained: 1ng of cDNA, 0.2μM of each primer 1 and primer 2; 200μM dNTPs; 1.5mM $MgCl_2$; 2.6U of expand high fidelity PCR system enzyme mix. The PCR cycling conditions were as follows: 1 x 5min at 95°C; 30 x [55°C for 3min; 65°C for 3min; 95°C for 30sec]; 1 x 65°C for 2min; 1 x 72°C for 5min. The products were analysed on a 2% (w/v) agarose gel.

2.4.6 Quantification of recovery and size determination of amplified DNA fragments.

The major product of two PCR amplification reactions with bovine cDNA was excised from an agarose gel using sterile conditions. The DNA was eluted from the agarose plug using Gen Elute columns (Supelco) and precipitated using sodium acetate/ethanol. For quantitative recovery of DNA, the DNA pellets were resuspended overnight in 20μl TE buffer at 4°C. Samples (5μl) of each purification were analysed on a 1.5% (w/v) agarose gel against a mass ladder.

2.4.7 Cloning of PCR fragments into pCR-Script Amp SK (+)

Cloning of the purified SP-22 fragment into pCR-Script Amp SK(+) was achieved as detailed in the instruction manual (Stratagene). Briefly, the Expand generated PCR product was polished in a reaction mixture containing 0.5U of *pfu* polymerase and 2.5mM dNTPs in the appropriate buffer conditions. After gentle mixing, 20 µl mineral oil was overlayed and the polishing reaction allowed to proceed for 30 min at 72°C.

The polished inserts (1-4µl) were incubated in a reaction mixture containing 10ng cloning vector, 5U *srf* I restriction enzyme, 4U T4 DNA ligase, under the appropriate buffer conditions, in a final volume of 10µl. The reaction was mixed and incubated at room temperature for 1h.

2.4.8 Transformation of Epicurian Coli XL1-Blue MRF' Kan supercompetent cells

Transformation of the cloned insert was performed as detailed in the protocol provided with the pCR-Script Amp SK(+) cloning kit (Stratagene). β-mercaptoethanol (final concentration 25mM) was added to a 40µl aliquot of supercompetant cells in a pre-chilled eppendorf tube and incubated on ice for 10min. 2µl cloning reaction was added, swirled gently to mix, and incubated on ice for a further 30min. Following a heat pulse at 42°C for exactly 45sec and a further 2min period on ice, 450µl fresh SOC media was added and the reaction incubated in an orbital shaker (250rpm) at 37°C for 1h. Aliquots (20-190µl) of

each transformation reaction were spread on LB ampicillin agar plates containing X-gal and IPTG.

2.4.9 Plasmid purification

Plasmids were purified using the Wizard Plus Minipreps DNA purification system (Promega) according to the manufacturer's instructions. Briefly, cells from 1.5ml of each overnight culture were pelleted and resuspended in 200ml of Cell Resuspension Solution (50mM Tris/HCl, pH 7.5, 10mM EDTA, 100µg/ml RNase A). Cell lysis was propagated by the addition of 200ml of Cell Lysis Solution (0.2M NaOH, 1% (w/v) SDS) to each tube and neutralised with 200ml of Neutralisation Solution (1.32M potassium acetate). After clarification of the lysate by centrifugation, it was transferred to a fresh tube containing 1ml of Wizard Mini prep resin and mixed. Employing a vacuum, the resin was washed with 2 x 1ml 80mM potassium acetate, 8.3mM Tris-HCl, pH 7.4, 40µM EDTA, 55% (v/v) ethanol and centrifuged to remove all traces of the ethanol. DNA was eluted by the addition of TE Buffer (50µl) and centrifugation.

2.4.10 Bam H1 restriction analysis of the pCR-Script plasmids, potentially containing the coding region of SP-22

4µl plasmid DNA (1.1-2.1µg) was digested in each reaction with 12 Units of Bam H1 (Boehringer Mannheim) in the appropriate buffer (10mM Tris/HCl, pH 8.0, 100 mM NaCl, 5mM $MgCl_2$, 1mM 2-mercaptoethanol) at a final volume of 20µl for 2h at 37°C. Incubations of control (uncut) plasmid took place in the same buffers and under the same conditions in the absence of Bam

H1. After 2h, 5µl sample loading buffer (40% (w/v) sucrose, 0.25% (w/v) bromophenol blue) was added to each reaction and samples subjected to analysis by 0.8% (w/v) agarose gel electrophoresis.

2.4.11 Restriction analysis, employing a Not 1/Xho 1 double digest, of plasmids derived from clones potentially containing SP-22 sequence

3µl plasmid DNA (0.8-1.5µg) was digested in each reaction with 10 Units of Xho 1 and 5 Units of Not 1 (New England Biolabs) in 50mM Tris/HCl, pH 7.9, 100mM NaCl, 10mM $MgCl_2$, 1mM DTT, supplemented with BSA to a final concentration of 100µg/ml. Digestion was in a final volume of 50µl at 37°C for 2h. Subsequently, 10µl electrophoresis sample buffer was added to each reaction tube and the samples subjected to analysis on a 2% (w/v) agarose gel.

2.4.12 SDS-PAGE separation and fluorographic detection of the [^{35}S]-methionine labelled products derived from *in vitro* transcription/translation reactions

In vitro transcription/translation, using plasmids purified from the clones detailed above, was carried out using the Single Tube Protein System 2,T7 (Novagen) according to manufacturer's instructions. The system is based upon transcription with T7 RNA polymerase, followed by translation in an optimised rabbit reticulocyte lysate (Perara & Lingappa, 1985). Protein products were labelled by the inclusion of [^{35}S]-methionine in the translation reaction.

Reactions (5µl each + 20µl Laemmli sample buffer) were subjected to SDS-PAGE on a 10% (w/v) polyacrylamide gel. The gel was fixed in 25% (v/v) isopropanol: 65% (v/v) distilled water: 10% (v/v) acetic acid for 30 min and rinsed with distilled water. The gel was immersed and agitated slowly for 30 min in Amplify (Amersham) to aid fluorographic detection before being dried under vacuum. Products were detected on Hyperfilm ECL (Amersham).

Unless otherwise indicated, data presented in this thesis is representative of a number of experiments.Where statistical analysis is performed, this value is represenleted by n.

Chapter 3

Purification and reconstitution studies on the components of mammalian PDC

3.1 Introduction

Purification of intact PDC to a high degree of purity from bovine heart and kidney was first described by Linn *et al.* (1972). In eukaryotes, the non-covalent nature of inter-component interactions within PDC precludes the use of some conventional protein purification techniques such as anion/cation exchange chromatography. Thus, over the years, purification regimes under non-dissociating conditions have developed and evolved in a number of key papers (Linn *et al.*, 1972; Stanley & Perham, 1980; De Marcucci & Lindsay 1985; Sanderson *et al.*, 1996a). More recent protocols, based upon Stanley & Perham (1980), detail the purification of PDC and also OGDC from mammalian tissues. These have the added advantage that they do not require prior isolation of mitochondria and rely on membrane solubilisation in a suitable non-ionic detergent, usually Triton X-100. Subsequent protein fractionation, in the presence of detergent, is achieved by a series of polyethylene glycol precipitations. It is thus possible, using Triton X-100 as a solubilising agent and PEG fractionation to purify intact forms of PDC and OGDC to near homogeneity and in high yields.

Introduced as a protein fractionation agent over three decades ago (Polson *et al.*, 1964), PEG precipitates proteins in a non denaturing fashion as a result of differing solubilities of individual proteins at specific PEG concentrations. For example, in the most recent methodologies developed for PDC purification (Stanley & Perham, 1980; Sanderson *et al.*, 1996a), the final precipitation step in the purification, separation from OGDC, employs 0.06 vol of 30%(w/v) PEG 6000 solution to precipitate all the OGDC in the sample; leaving pure PDC in the supernatant. Precipitation can be described qualitatively by a steric exclusion mechanism whereby proteins concentrate and eventually precipitate in

the extra polymer space. Precipitation is dependent on factors such as protein size and concentration (for a full discussion, see Ingham, 1990).

In contrast to the associative conditions used to purify the intact 2-oxo acid dehydrogenase complexes, isolation of the individual enzymes requires that dissociative conditions be employed. This is performed routinely after the intact multienzyme complex has been isolated. In the case of mammalian PDC the protein X component is very tightly associated with the E2 core. It is not possible to remove it under conditions in which the proteins remain in a native state. In studies of mammalian PDC, the method of Linn *et al.*, (1972) has continued to be used to dissociate PDC into its E2/X and E1/E3 fractions. This method utilises alkaline (pH 9.0) conditions and high salt (1M NaCl) to dissociate the PDC into its E2/X and E1/E3 fractions for subsequent separation on a Sepharose 4B column. Examination of the papers using this approach (Behal *et al.*, 1994; De Marcucci *et al.*, 1995 and Kuroda *et al.*, 1996) highlight some of the problems associated with the method. One major drawback is that the E2/X core produced is heavily contaminated with undissociated E1α and β subunits. Further examination of E2/X core samples resolved by SDS PAGE in De Marcucci *et al.* (1995) displays the presence of a 55 000 M_r protein, corresponding to the E3 subunit.

One further significant consideration is that PDC dissociated in this fashion, over a prolonged time interval and at alkaline pH, is able to support only minimal levels of reconstitution (15-20%) (Sanderson *et al.*, 1996b). In light of these facts one can conclude that dissociation of PDC based on the method of Linn *et al.* (1972), while suitable for some proteolytic and immunological studies, is not ideal for analysis of component folding, assembly and reconstitution of complex enzymatic activities. Clearly, a separation protocol which maintains the overall structural integrity and provides for a

higher level of recovery of individual components of mammalian PDC in their active forms is desirable.

In this chapter, I detail the development of an improved method for PDC dissociation into an E2/X core and an E1/E3 fraction. The fractions were able to support high levels of PDC reconstitution (60-80%) upon the re-introduction of stoichiometric quantities of E1/E3 to the E2/X core. The improved purity of the E2/X subcomplex permitted the application of electrospray mass spectrometery (ES/MS) to determine the subunit M_r of the individual transacetylase (E2) and protein X components. It also facilitated the detailed study of the folding, assembly and reconstitution of the E2/X core presented in this thesis (see chapter 4). A novel protocol to facilitate the purification of milligram quantities of E1 and E3 was also developed. This provided a valuable source of mammalian E3 for later reconstitution studies (chapter 4) and should permit future folding and assembly studies on these individual enzymes.

3.2 Results

3.2.1 Preparative dissociation of mammalian PDC

In order to study the folding and assembly of the component enzymes of mammalian PDC a suitable dissociation protocol is required. To this end two alternative protocols were investigated. Previously, our laboratory had developed a dissociation method for mammalian PDC, employing NaCl treatment at a neutral pH, which facilitated high levels of overall complex activity upon reconstitution of the E2/X core with E1/E3 (Sanderson *et al.*, 1996b). However, a number of drawbacks remained evident: low levels of PDC activity was still associated with both E2/X and E1/E3 fractions and thus had to be taken into consideration when calculating levels of PDC reconstitution; SDS-PAGE analysis displayed small but significant levels of E1 α and β still associated with the purified E2/X core. One approach to method development was to try to improve this protocol. Another possibility was to scale-up successfully the analytical dissociation of PDC employed by (West *et al.*, 1995). This used intermediate levels of GdnHCl to dissociate PDC, leaving no E1 components associated with the E2/X core. Preliminary studies focused on the effects of these two salts, NaCl and GdnHCl, on purified PDC. In addition, as purified bovine OGDC was also readily available, these studies were also extended to this member of the 2-oxo acid dehydrogenase complex family.

Figure 3.1a illustrates the effect of NaCl and GdnHCl on the overall complex activities of mammalian PDC and OGDC. As the concentration of both salts increase they exert an inhibitory effect on the activities of both multienzyme complexes. GdnHCl concentrations above 300mM cause complete inhibition of

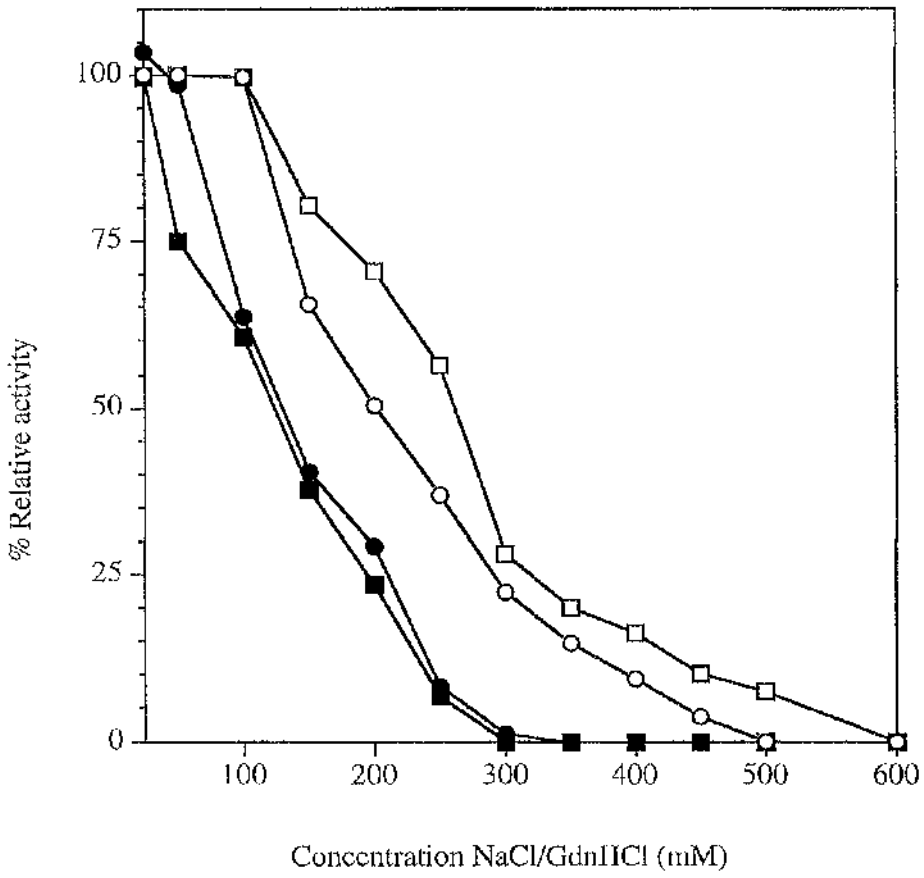


Figure 3.1a Effect of NaCl/GdnHCl on the overall activities of mammalian PDC and OGDC

PDC and OGDC were incubated at 0.5mg/ml in 50mM MOPS/KOH, pH 7.4 containing the stated concentration of NaCl/GdnHCl for 15min at 20°C. Aliquots were removed and activity assays were performed (see Materials and Methods section 2.2.12) in the presence of the stated concentration of salt. All activities are expressed relative to control samples incubated in the absence of NaCl/GdnHCl. PDC NaCl (○); PDC GdnHCl (●); OGDC NaCl (□); OGDC GdnHCl (■).

enzyme activity in both multienzyme complexes. NaCl present at concentrations >500mM exert the same effect. These inhibitory levels of GdnHCl and NaCl represent levels which have shown to have no effect on the gross conformation of the components enzymes of PDC, as measured by circular dichroism (CD) and tryptophan fluorescence (West *et al.,* 1995). It was also observed that levels of GdnHCl below 50mM had a negligible effect on the enzyme activities investigated. This apparent maintenance of conformational integrity, but loss of associated PDC/OGDC activity has been attributed to the highly flexible nature of enzyme active sites, relative to the rest of the protein molecule, and their particular susceptibility to perturbation (Tsou, 1986).

While ion effects on protein activity/solubility are generally non-specific at levels lower than 0.15M, at higher concentrations ion specificity does exist (Franks, 1989). Figure 3.1b illustrates this ion specificity and compares the capacity of PDC and OGDC to reassociate spontaneously, following pretreatment with either the chaotrope GdnHCl or the neutral salt NaCl. In these experiments the levels of both salts were reduced to below 50mM, by dilution prior to assay, as the effects of the two salts on enzymatic activity appear minimal below this concentration (see Figure 3.1a). It is clear that the effects of NaCl treatment on PDC and OGDC activity remain reversible over the range of concentrations investigated (Figure 3.1b). This includes levels of NaCl (>2.0M) in excess of those which are known to promote dissociation of E1/E3 of PDC and E3 of OGDC from their respective cores. Treatment of intact PDC or OGDC with low levels of GdnHCl still allows recovery of maximal activity. In contrast, following pre-exposure to higher levels of this chaotropic salt (>0.5M) it was not possible to recover all the respective complex activity by diluting out the chaotrope.

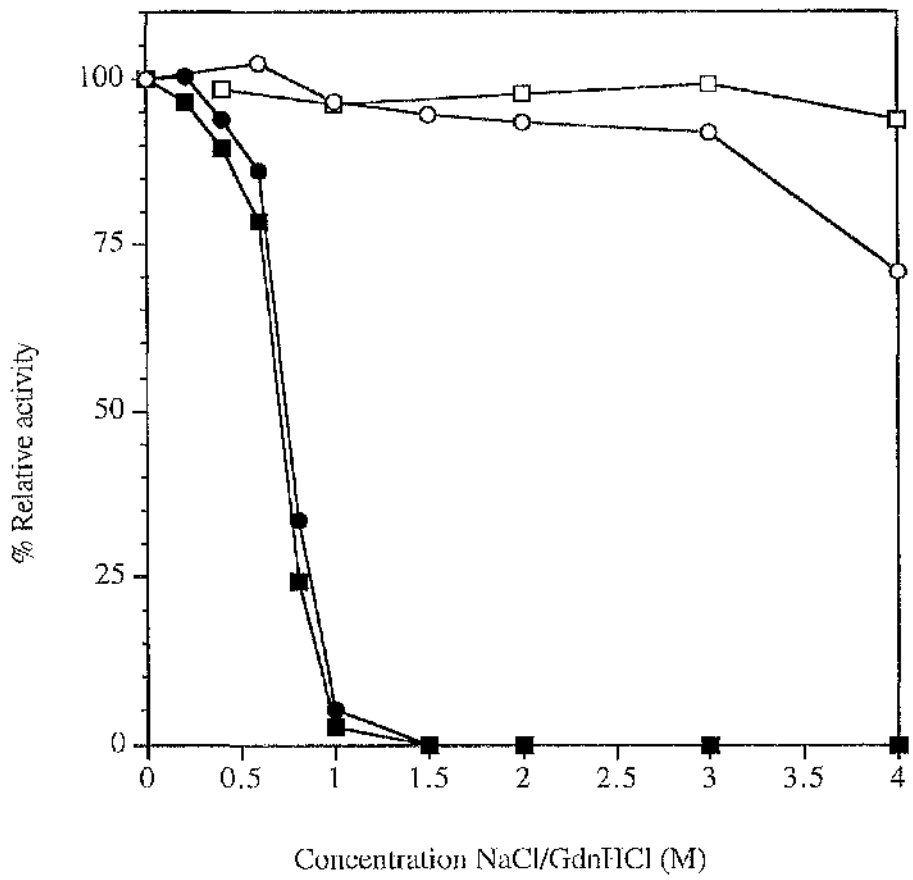


Figure 3.1b Reactivation/renaturation profiles of PDC and OGDC activity following pre-treatment with NaCl/GdnHCl

PDC and OGDC were incubated in NaCl/GdnHCl at the concentration stated for 15min at 20°C. Reactivation /renaturation was initiated by a rapid dilution into the appropriate assay buffer. The final protein concentration in the renaturation mixture was 10-30µg/ml and residual GdnHCl reduced to below 30mM. After a 15 min period at 20°C, activity assays were performed for PDC activity: following NaCl pre-treatment (□); following GdnHCl pre-treatment (■) and OGDC activity following NaCl pre-treatment (○); following GdnHCl pre-treatment (●). All activities are expressed relative to control samples of enzyme incubated in the absence of NaCl/GdnHCl.

Data for mammalian PDC agree with that in a previous study (West *et al.*, 1995) which showed that treatment of PDC with levels of GdnHCl in excess of 0.4-0.5M caused gross conformational changes, as judged by CD and fluorescence measurements. The authors were also unable to regain all PDC activity, by simple dilution, once this threshold level had been exceeded. Denaturant sensitivity of the native complex was found to be superimposable on that of the E1 component enzyme, suggestive that loss of overall PDC activity reflects irreversible conformational changes in its most sensitive component. The effects of individual ions are in accordance with the Hofmeister (lyotrophic) series for reasons that still remain unclear but, in proteins, may relate to the hydration of hydrophobic residues (Franks, 1989).

Following preliminary investigations of the effect of GdnHCl on PDC activity, a scaled-up version of the dissociation protocol detailed in West *et al.* (1995) was performed. Accordingly, the protocol was scaled up from a 25ml Superose 12 column (separation range $1x10^3$-$5x10^5$) to a 100ml prep grade Superose 6 column (separation range $5x10^3$-$5x10^6$). The pre-incubation time (15 min at room temperature in the desired concentration of GdnHCl) and flow rate were kept constant with a view to maintaining the chromatographic characteristics of the successful small scale separation. However, is was not possible to produce E2/X core which showed any real decrease in the levels of associated E1α and β when compared to untreated PDC (results not shown). Furthermore, dissociation at higher levels of GdnHCl (0.6M), where the chaotrope is beginning to cause irreversible conformational changes (Figure 3.1b), did not improve resolution (results not shown).

Another possible solution to the problem of PDC dissociation lay in the refinement of the salt dissociation treatment already employed in the laboratory (Sanderson *et al.*, 1996b). Initial analysis suggested one immediate refinement:

a reduction in the amount of protein being loaded onto the column. Although for GPC (gel permeation chromatography) partition of the protein between the mobile and stationary phase is not concentration dependent, high protein concentrations (> 20mg/ml) can lead to problems associated with their viscosity. Specifically, previous loadings of PDC in excess of 20mg/ml may have resulted in sub-optimal resolution as a result of diffuse leading boundaries in the viscous samples during chromatography (Preneta, 1994). To alleviate this, the maximal loading was decreased to 15mg/ml in a 2ml loading volume.

Another improvement came from a study of the ability of PDC and OGDC to reconstitute whole complex activity after prolonged treatment in 2M NaCl in different buffer systems (Figure 3.2). The impetus for this work came from studies on pyruvate decarboxylase from *Zymonas mobilis* which had highlighted different reactivation profiles for the enzyme following urea pre-treatment in different buffer systems (Pohl *et al.*, 1994). The enzyme was shown to be less stable in the citrate buffer system traditionally used than in others buffers studied. In this context, a series of buffers, with optimal capacity in the pH 7.0-8.0 range, were studied for their ability to promote maximal recovery of PDC and OGDC activity, following pre-treatment with 2M NaCl. Of the buffers examined, only imidazole/HCl buffered solutions showed a marked difference from the Tris/HCl buffered system traditionally used (Figure 3.2). While mammalian OGDC fails to discriminate between the two buffer systems, PDC in the Tris/HCl system reconstitutes activity poorly following 2M NaCl treatment, even at neutral pH, when compared to PDC treated in a similar fashion in the imidazole/HCl buffer system. The decline in both PDC and OGDC activities was obvious in both buffer systems at more alkaline pH. This is related to the documented sensitivity of the E1α component to alkaline conditions (Kiesyl *et al.*, 1980). However, the more pronounced decline in reconstitution of complex activity was observed in the imidazole/HCl buffered

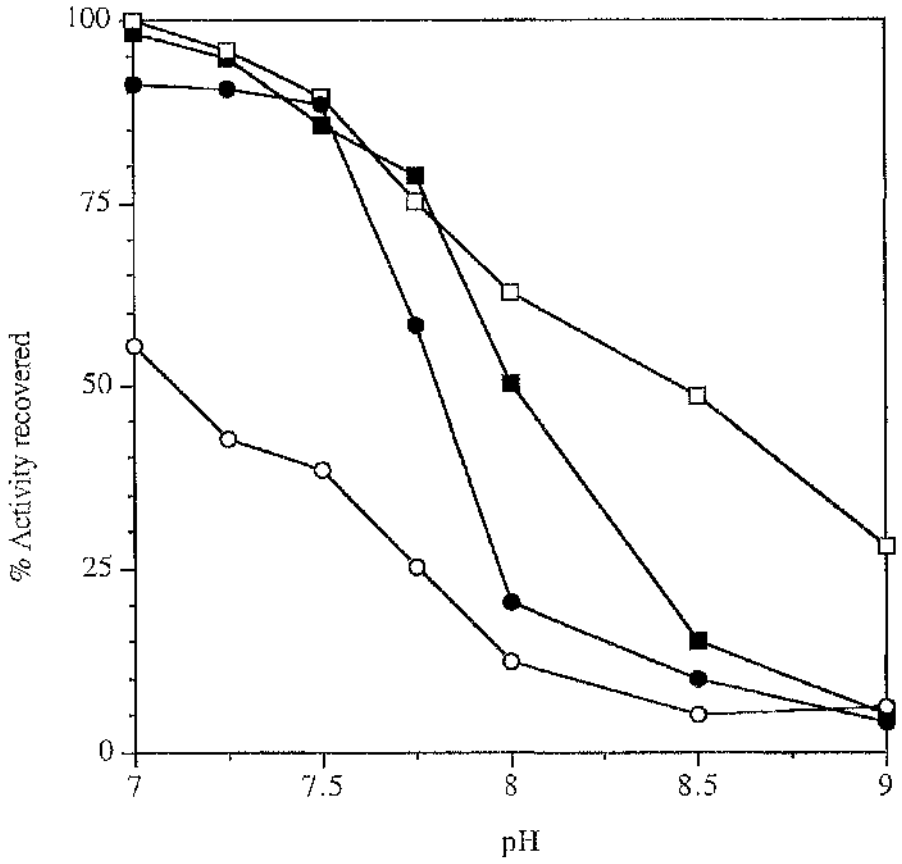


Figure 3.2 Buffer and pH dependence of PDC/OGDC reactivation following treatment with 2M NaCl

PDC and OGDC (2mg/ml) were incubated in the appropriate buffer (50mM) at the pH indicated, in the presence of 2M NaCl, for 15min at 20°C. Aliquots (5µl) were removed and added to the appropriate assay buffer (670µl Buffer A+14µl Buffer B) and allowed to reactivate for 15min at 20°C. Assays were initiated by the addition of the appropriate substrate (pyruvate for PDC, 2-oxoglutarate for OGDC). All activities are expressed relative to control samples of complex incubated in the appropriate buffer and pH in the absence of NaCl. PDC, Tris/HCl buffer (○); PDC, imidazole/HCl buffer (●); OGDC, Tris/HCl buffer (□); OGDC, imidazole/HCl buffer (■).

system at pH values close to 8.0 may be attributable to the loss of buffering capacity of the system at this pH (the pK_a for imidazole =6.95).

The decrease in total PDC loading to 15mg/ml, coupled with the 2M NaCl treatment in the imidazole/HCl buffer system, was integrated into the preparative dissociation of PDC by gel permeation chromatography (for more details see Materials and Methods section 2.2.9). Using these modifications, it was possible to achieve baseline dissociation of PDC into its E2/X and E1/E3 fractions on a preparative scale. Figure 3.3a represents the absorbance at 280nm exhibited by the fractions eluted from the column.The first protein peak to elute from the Superose 6 column, at or near the void volume, corresponds to the E2/X core (V_c=32ml).The second peak corresponds to the E1/E3 fraction of PDC (V_e=60ml). Subsequent SDS-PAGE analysis of the pooled fractions from the preparative dissociation serves to confirm the identity of the peaks (Figure 3.3b). Lane 3 corresponds to the first eluted peak and confirms this to be the E2/X core. Significantly, there was no apparent contamination of the E2 core with E3. The E1 α and β subunits, which stain more heavily with Coomassie blue than the other PDC components (compare Lanes 1 and 4), show a marked reduction in their association with the E2/X core when compared to untreated PDC (lane 1). Enzymatic analysis of the pooled, dialysed fractions confirmed this depletion with less than 5% of the E1 activity still associated with the E2/X sub-complex. Examination of the E1/E3 fraction (lane 4) corresponding to the second peak eluted from the Superose 6 column confirmed the lack of E2/X core contamination. The presence of a small protein (<30 000 M_r),which co-elutes with the E1/E3 fraction, is discussed later (see Chapter 6). This E1/E3 fraction contained all the recovered E3 activity and 95% of the E1 activity (results not shown). Another noticeable improvement was that neither the E2/X core or the E1/E3 fraction alone exibited any overall PDC activity, in contrast to previous studies (De Marcucci *et al.*, 1995; Sanderson *et al.*, 1996b)

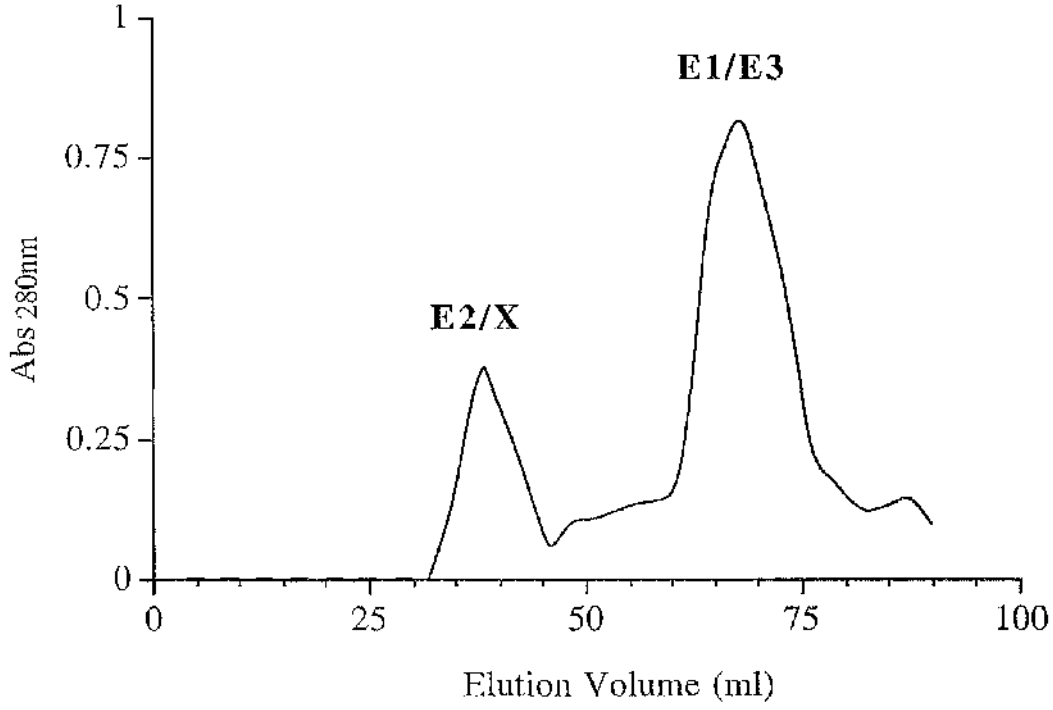


Figure 3.3a Absorbance profile of the preparative dissociation of PDC into active E2/X and E1/E3 fractions

Preparative dissociation of PDC into active E2/X and E1/E3 fractions was performed on an FPLC system (Pharmacia) using a prep grade Superose 6 column (100ml) equilibrated with 50mM imidazole/HCl, pH 7.0, 1M NaCl, 0.01%(v/v)Triton X-100. PDC (14.0mg/ml) in 100mM MOPS/NaOH, pH 6.8, 5mM EDTA, 1%(v/v) Triton X-100 was microfuged at 13000 rpm for 15min before incubation at a 1:1 ratio with 50mM imidazole, pH 7.0, 4M NaCl on ice for 1 h. A 2ml pre-injection of the 4M NaCl buffer was then performed prior to PDC sample loading (2ml) and subsequent elution at a flow rate of 1ml/min. Fractions were pooled on the basis of A_{280} and dialysed extensively, at 4°C, against multiple changes of the appropriate buffer before subsequent concentration, as required, using dry PEG.

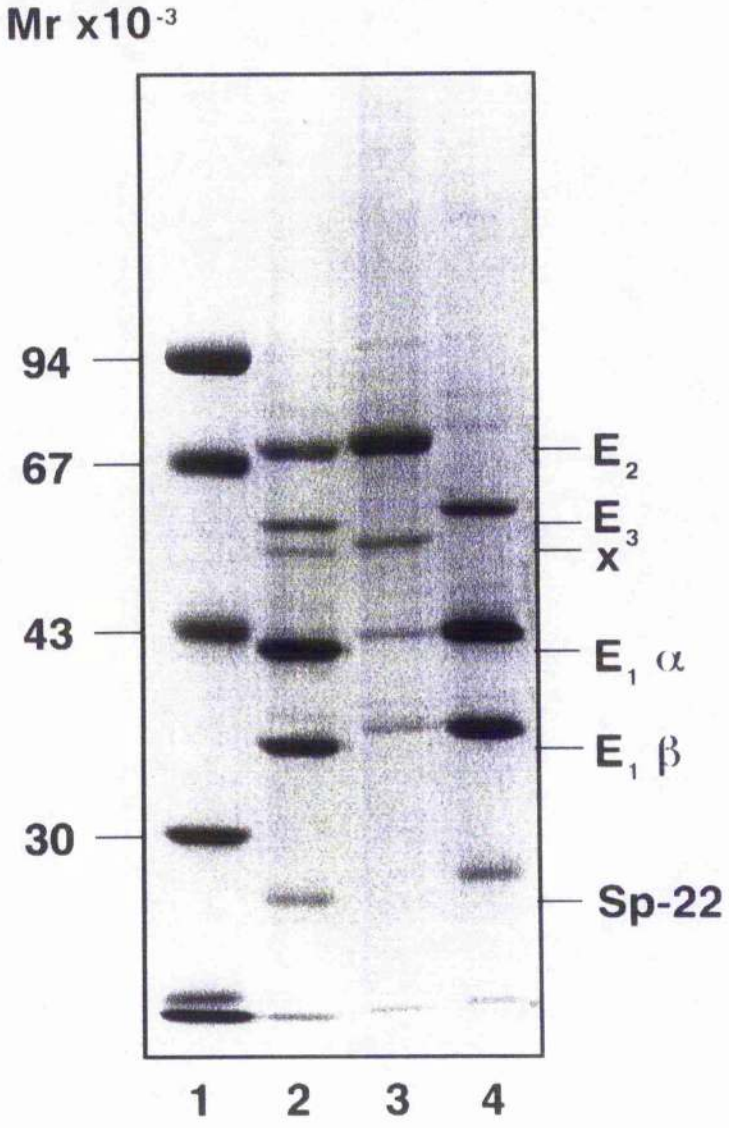


Figure 3.3b Preparative dissociation of PDC into active E2/X and E1/E3 fractions: SDS-PAGE analysis of pooled fractions

Samples were analysed for purity by SDS-PAGE (10%, w/v gel) and stained with Coomassie blue. Lane 1, 10μg low-M_r marker proteins; lane 2, intact PDC (20μg); lane 3, E2/X core (15μg); lane 4, E1/E3 fraction (15μg)

3.2.2 Reconstitution and stability of the E2/X and E1/E3 fractions of mammalian PDC

As shown in the previous section, the modified protocol for dissociating PDC on a preparative scale was successful in producing E2/X and E1/E3 fractions free from significant cross contamination; with both fractions recovered in high yield. A second important criterion upon which the usefulness of the separation was to be judged was that the E2/X and E1/E3 fractions produced were able to re-associate and reconstitute overall PDC activity to high levels, relative to undissociated PDC. To test this, increasing quantities of E1/E3 were titrated into a reaction assay mixture containing a set amount of E2/X. After allowing the components time to re-associate, PDC activity was assayed. The results presented in Figure 3.4a show that it was possible to reconstitute high levels of PDC activity from the dissociated fractions. It is also clear that the two fractions can be combined to reconstitute high levels of PDC activity (typically 60-80%), relative to untreated, native PDC. Significantly, the E2/X:E1/E3 ratio at which one attains maximal levels of reconstitution (1:1.4) is in agreement with the known native stoichiometry of mammalian PDC (i.e. a 60meric E2 core containing 12 molecules of protein X with 30 E1$\alpha_2\beta_2$ tetramers and 6-12 E3 dimers).

The measurements of PDC activity performed in Figure 3.4a were carried out after a 15 min incubation period. This was as a result of the previous observation that time periods less than this resulted in lower levels of PDC reconstitution (S.J. Sanderson, PhD thesis). However, it was unclear if such a lag phase was as a result of the time taken for the E1/E3 component to reassemble onto the E2/X lattice or represented some reactivation/cofactor

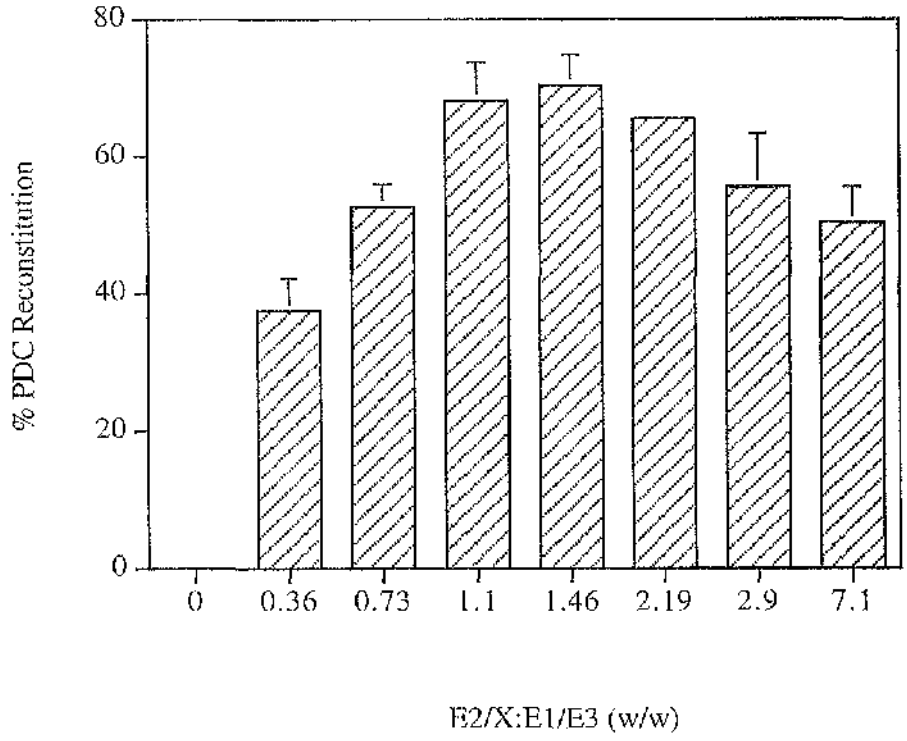


Figure 3.4a Reconstitution of PDC activity from the E2/X subcomplex and E1/E3 fraction: levels of reconstitution and component stoichiometry

Following the dissociation of PDC, the E2/X and E1/E3 fractions were dialysed into 50mM MOPS/KOH, pH 7.4, 1mM EDTA, 50% (v/v) glycerol and stored at 4°C. E2/X and E1/E3 fractions were activated by a 1:1 dilution in 50mM potassium phosphate buffer, pH 7.6, 3mM NAD^+, 2.7 mM cysteine-HCl, 2mM $MgCl_2$, 0.2 mM ThDP, 0.02mM CoASH (buffer AB) and incubated for 15min at 20°C. To initiate reconstitution, increasing quantities of E1/E3 (0-20µg) were added to cuvettes containing E2/X (5µg) and the appropriate assay solutions. The production of NADH was subsequently initiated by the addition of 14µl 100mM pyruvate (Solution C). Activity was monitored at 340nm and activities are expressed relative to the specific activity of undissociated PDC.(n=3)

binding requirement. Specifically, it was possible that the purification and dissociation protocol led to a degree of oxidation of the thiol containing components of PDC; lowering the measured activity upon reconstitution. To discriminate between the two possibilities a 15min incubation in buffer AB containing the reducing agent cysteine-HCl was incorporated into the reconstitution protocol (see Figure legend). The levels of reconstitution of PDC activity observed were compared to those obtained in the absence of such a pre-treatment (Figure 3.4b). It is evident from the data that, following incubation in buffer AB, reconstitution can occur instantaneously to maximal levels. In comparison, untreated E2/X and E1/E3 fractions exhibit a 15-20 min lag period before they are fully active. This would suggest that component assembly is not the reason for the lag phase observed when the two fractions have not been pre-conditioned in buffer AB. Instead, the presence of cysteine in buffer B would appear to be the active component, reversing adventitious oxidation which may have occurred to one or more of the components of PDC during the dissociation.

Following the gel permeation chromatography (GPC) dissociation of PDC, the E2/X and E1/E3 fractions were dialysed immediately into their storage buffer (50mM MOPS/KOH, pH 7.0, 50%(v/v) glycerol) before any reconstitution analysis was performed. A recent study employing sedimentation velocity analysis of the E2/X core of PDC (Behal *et al.*, 1994) employed a different storage buffer (0.1M glycine, pH 9.5, 1M NaCl) which was claimed to maintain the components of E2/X in an active form for up to 1 month at 4°C. To test this, the two buffer systems were compared in the stability study detailed in Figure 3.4c which measured the ability of the E2/X, dialysed into the appropriate buffer system, to reconstitute overall complex activity upon the addition of a stoichiometric quantity of E1/E3. In this study the E1/E3 fraction was always stored in the MOPS/glycerol buffer system, owing to the

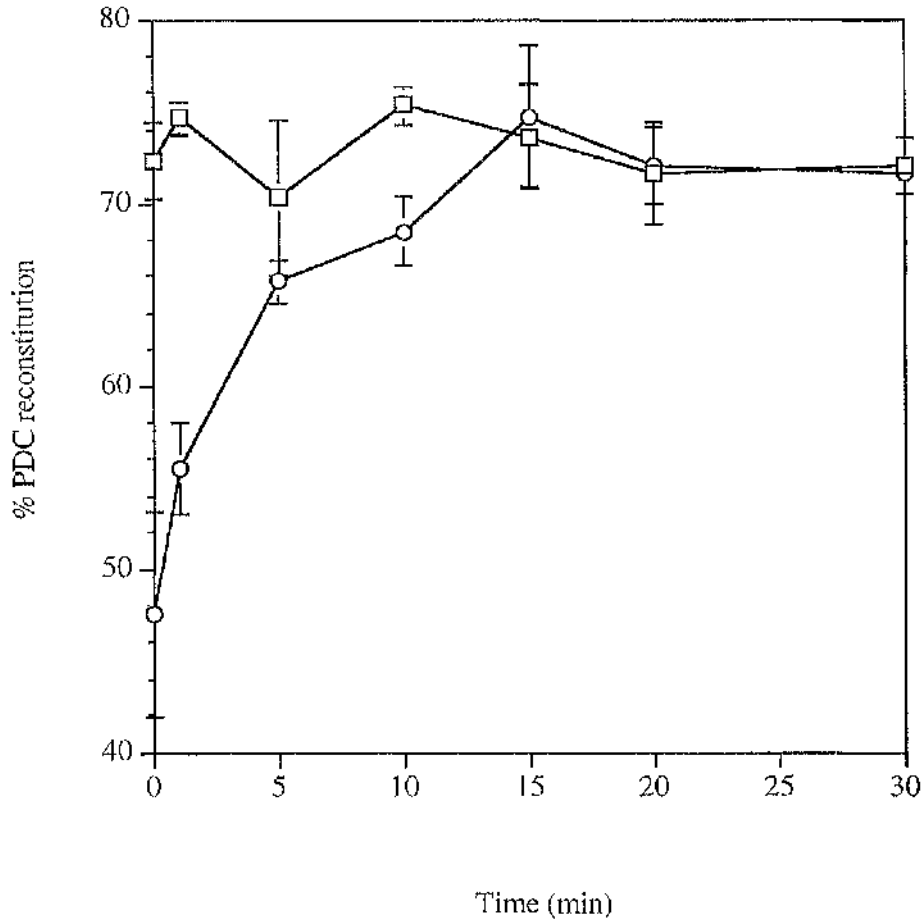


Figure 3.4b Time dependence of reconstitution of PDC activity from the E2/X subcomplex and E1/E3 fraction

Equal volumes of E2/X and E1/E3 were diluted 1:1 in assay solutions A (○) or A plus B (□) to 0.1-0.2mg/ml and were pre-incubated together at 30°C in a cuvette containing solutions A and B without substrate for the times indicated. The assay was initiated by the addition of pyruvate and the production of NADH monitored at 340nm. Reconstitution of PDC activity is expressed relative to undissociated PDC. (n=3)

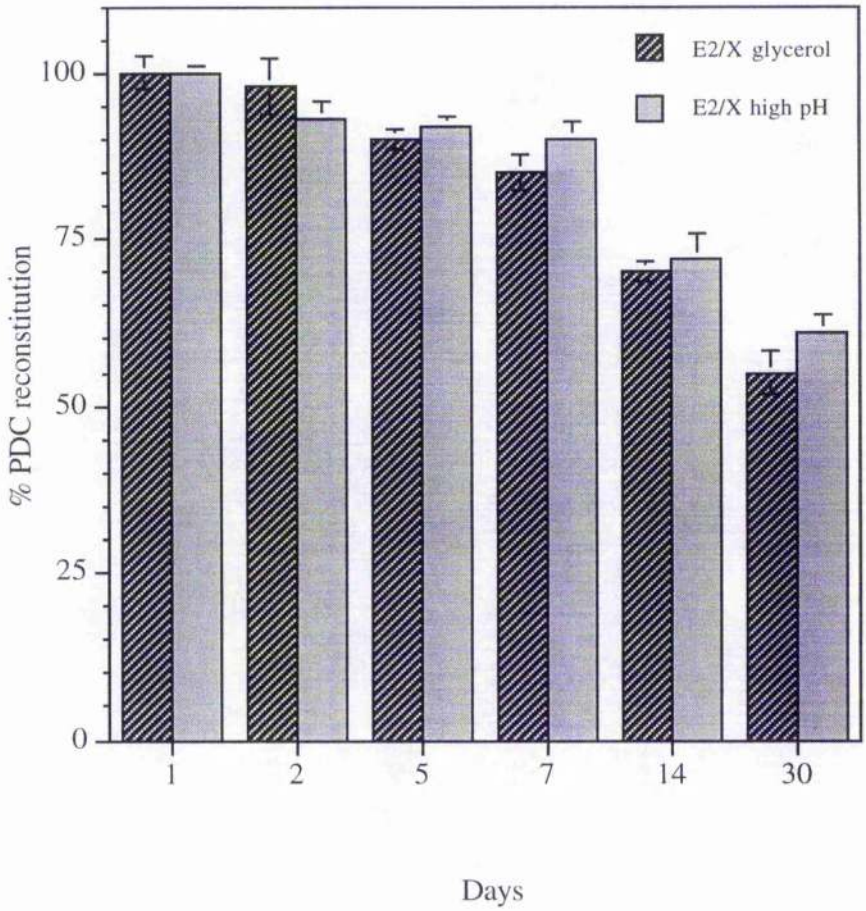


Figure 3.4c Reconstitution of PDC activity from the E2/X subcomplex and E1/E3 fraction: Comparison of E2/X core stability in different storage buffers

Maximal levels of PDC activity was reconstituted with E2/X core stored in 50mM MOPS/KOH, pH 7.4, 1mM EDTA, 50% (v/v) glycerol at 4°C (▨) and E2/X core stored in 0.1M glycine, pH 9.5, 1M NaCl at 4°C (□) at the time points indicated. Activity was expressed as a percentage of the activity obtained following dissociation and dialysis (identical for the two samples).The E1/E3 used for reconstitution was stored in 50mM MOPS/KOH, pH 7.4, 1mM EDTA, 50% (v/v) glycerol at 4°C. (n=1)

documented loss of E1 activity upon prolonged exposure to alkaline conditions. To our surprise, both buffers appeared to be equivalent in terms of their component stability over the time period studied. Thus, there was no apparent difference in in the ability of the two E2/X cores to reconstitute PDC activity. However, the decision was taken to continue to utilise the glycerol storage buffer as this has the added advantage of concentrating the two fractions during the course of the dialysis. This higher concentration of both E2/X and E1/E3 fractions permits their study in denaturation/renaturation studies detailed later (see chapter 4) which depend upon dilution of denaturant to below a threshold level (≤ 30mM for GdnHCl) prior to assay. These studies, incorporate an initial incubation in chaotrope which must take place at a high enough protein concentration so that, following dilution to below the required threshold level of chaotrope, sufficient levels of enzyme are present to permit assay.

3.2.3 Determination of the subunit M_r of E2 and protein X of PDC by electrospray mass spectrometery (ES/MS)

The development of the protocol detailed in section 3.2.1. which produced pure, active E2/X core also facilitated the exact molecular mass determination of both the transacetylase (E2) and protein X components of bovine PDC by electrospray mass spectrometry (ES/MS). (for a review of the application of ES/MS to protein characterisation, see Mann & Wilm, 1995). Previously, this had not been possible, as intact PDC is too complex a polypeptide system (nine individual polypeptides plus multiple phosphorylated forms of E1α) to study by ES/MS directly. By using high salt dissociation to produce the E2/X core the complexity of the polypeptide mixture is reduced sufficiently to allow accurate molecular mass determination (for methodology see figure legends).

Figure 3.5a Electrospray mass spectrum of the E2/X core of bovine PDC: MaxEnt deconvolution of ES/MS of dihydrolipoamide acetyltransferase (E2)

Mass spectrometry was performed on a VG Platform quadruple mass spectrometer (for full details see Materials and Methods section 2.2.7). E2/X core was dissolved in solvent at a concentration of 20pmol/μl, centrifuged at 5000g for 2 min to remove any particulate material, before being injected into the carrier stream. The m/z range 700-1500, containing >95% of the signal intensity if the protein sample, was scanned at least 10 times using a sweep time of 5s. Instrument calibration was carried out, just prior to sample application using horse heart myoglobin. Maximum entropy (MaxEnt) deconvolution procedure was used for quantitative analysis of raw data using 1-1.5Da peak width and 1Da/channel resolution. Routinely, the programme was stopped after 8-10 iterations

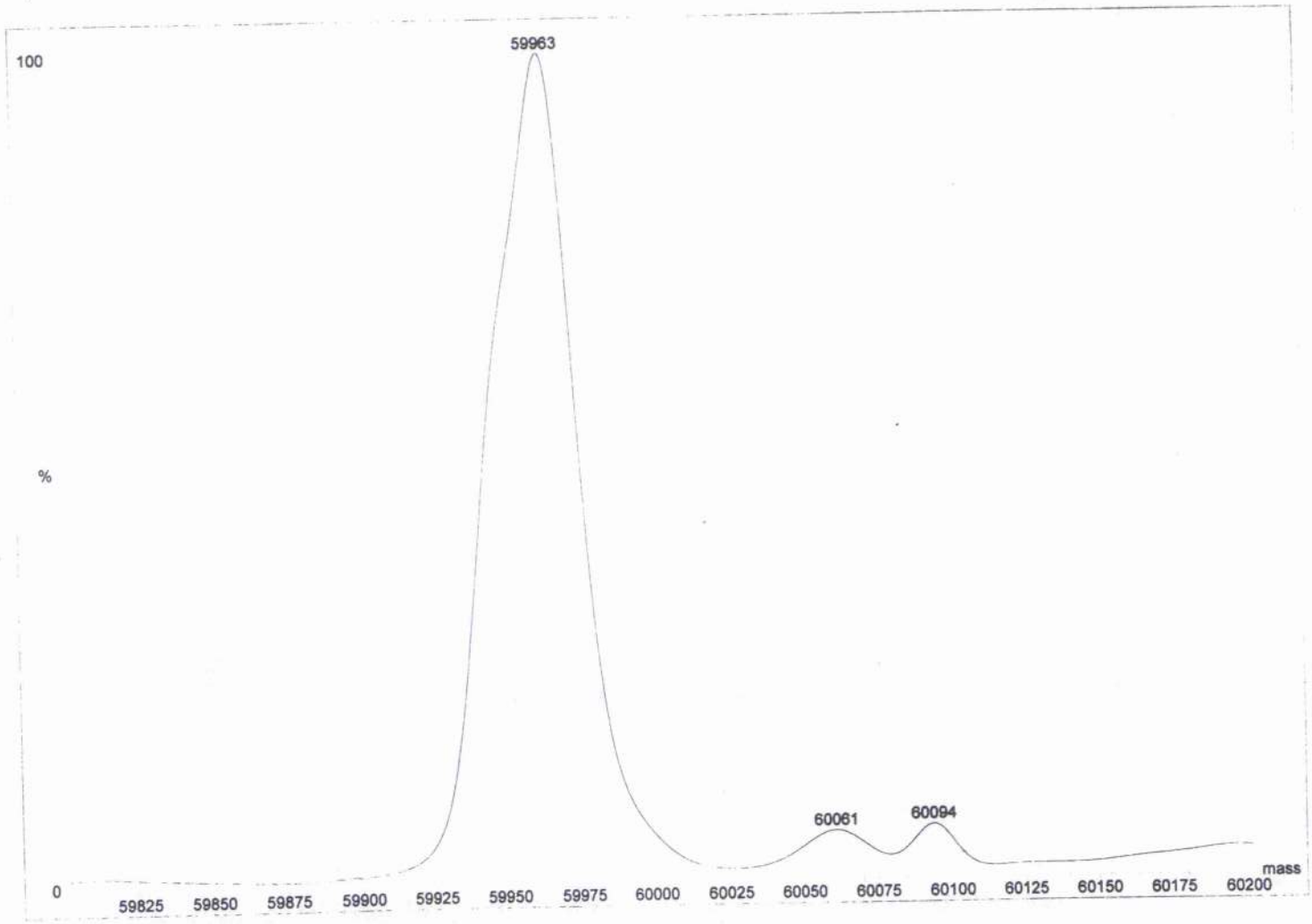

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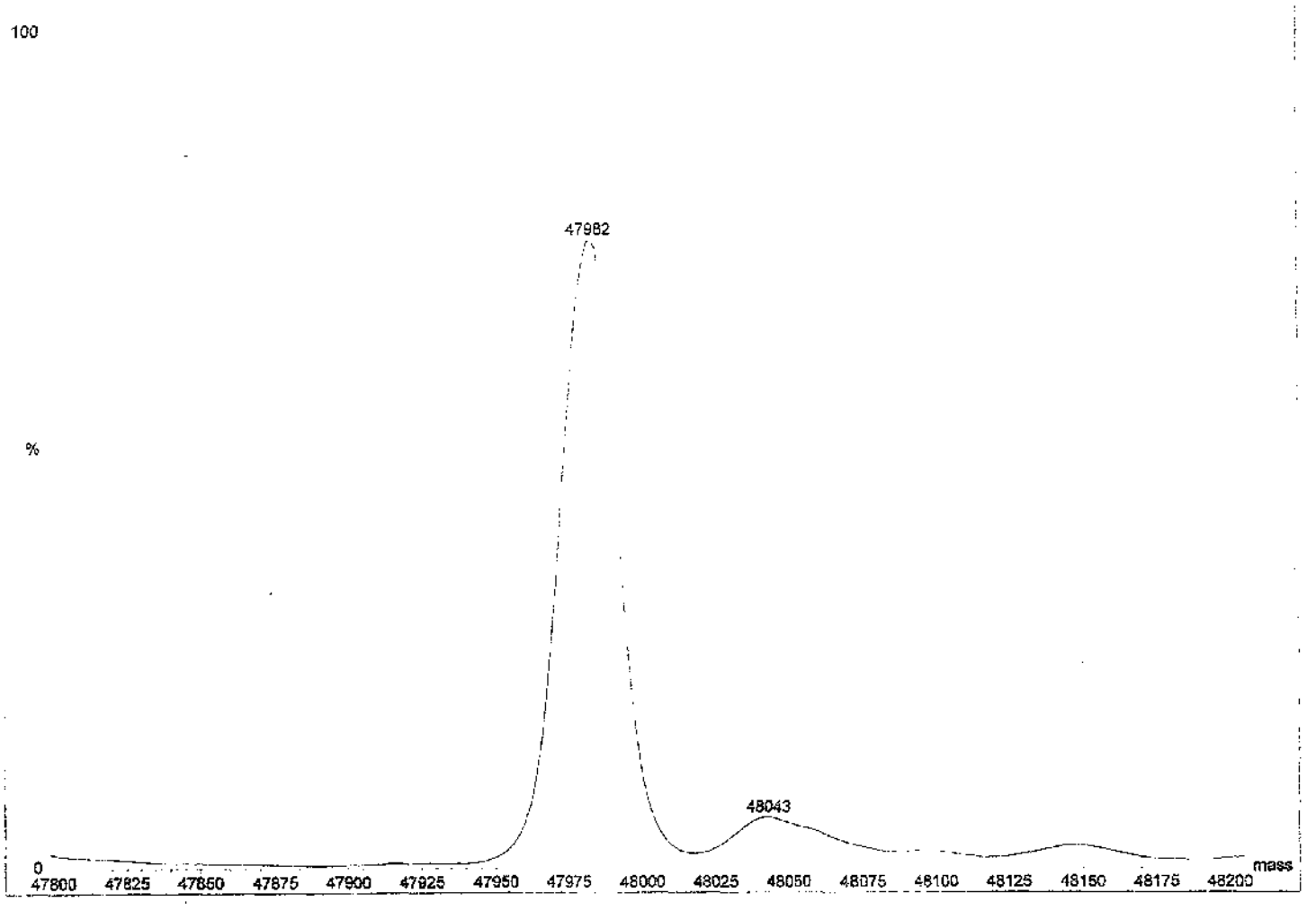
Figure 3.5b Electrospray mass spectrum of the E2/X core of bovine PDC: MaxEnt deconvolution of ES/MS of protein X

Mass spectrometry was performed on a VG Platform quadruple mass spectrometer (for full details see materials and methods section 2.2.7). E2/X core was dissolved in solvent at a concentration of 20pmol/µl, centrifuged at 5000g for 2 min to remove any particulate material, before being injected into the carrier stream. The m/z range 700-1500, containing >95% of the signal intensity if the protein sample, was scanned at least 10 times using a sweep time of 5s. Instrument calibration was carried out, just prior to sample application using horse heart myoglobin. Maximum entropy (MaxEnt) deconvolution procedure was used for quantitative analysis of raw data using 1-1.5Da peak width and 1Da/channel resolution. Routinely, the programme was stopped after 8-10 iterations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It was possible to obtain subunit M_r values for both the E2 and protein X components of bovine PDC (table 3.1). The presence of contaminating levels of the detergent,Triton X-100, prohibited the determination of the molecular masses of the E1 and E3 from bovine PDC. Triton X-100 is used throughout the purification of PDC. It is required for cell membrane solubilisation and is present in the final resuspension buffer used to maintain the solubility of PDC. In the E1/E3 sample, dialysis and multiple rounds of ultrafiltration of the sample into HPLC grade water proved insufficient to remove the detergent, due to the large detergent micelle size. The levels of detergent remained above a level which, in effect, served to obscure any signal from the E1 and E3 components obtained by ES/MS.

Table 3.1 Molecular mass of bovine E2 and protein X by ES/MS

Component	Molecular mass
Dihydrolipoamide acetyltransferase (E2)	59963
Protein X	47982

On-line searching of the Genbank data base of DNA sequences and Swissprot protein database revealed no apparent clones of either the E2 or protein X component of bovine PDC (see Table 3.2). However transacetylase components from other sources, including human E2 (Thekkumkara *et al.*, 1988) were available. In addition, the M_r of the protein X gene cloned from *S.*

Source	Complex	E2 or X	Number of amino acids	M_r	Number of lipoyl domains	Reference
Human	PDC	E2	561 614	59551 65640	2 2	Thekkumkara *et al.* (1988) Coppel *et al.* (1988)
Human	BCDC	E2	421 421	46500 46369	1 1	Lau *et al.* (1992) Danner *et al.* (1989)
Human	OGDC	E2	453	ND	1	Nakano *et al.* (1994)
Rat	OGDC	E2	386	ND	1	Nakano *et al.* (1991)
Rat	PDC	E2	555	58611	2	Matuda *et al.* (1992)
Bovine	BCDC	E2	421	46518	1	Griffin *et al* (1988)
Arabidopsis thaliana	PDC	E2	610	60000	2	Guan *et al.* (1995)
Saccharomyces cerevisiae	PDC	E2	454	48546	1	Nui *et al.* (1988)
Saccharomyces cerevisiae	PDC	X	380	42052	1	Behal *et al.* (1989)
Bacillus subtilis	OGDC	E2	417	45988	1	Carlson & Hederstedt (1987)
Azotobacter vinelandii	PDC	E2	637	64913	3	Hanemaaijer *et al.* (1988)
Azotobacter vinelandii	OGDC	E2	398	41872	1	Westphal & Kok (1990)
Escherichia coli	PDC	E2	629	65959	3	Stephens *et al.* (1983)
Escherichia coli	OGDC	E2	405	43607	1	Spencer *et al.* (1984)
Pseudomonas putida	BCDC	E2	423	45134	1	Burns *et al.* (1988)
Streptococcus faecalis	PDC	E2	539	56466	2	Allen & Perham (1991)
Bacillus stearothermophilus	PDC	E2	427	46265	1	Borges *et al.* (1990)
Human	PDC	X	448	48040	1	Harris *et al* ., (1997)

Table 3.2 Reported M_r values for acyltranferase components of 2-oxoacid dehydrogenase multienzyme complexes

cerevisiae was available (Behal *et al.*, 1989). The molecular masses determined by ES/MS for E2 and protein X were in close agreement with the published masses of the equivalent enzymes found in other organisms (Table 3.2). The molecular mass of bovine protein X is similar to that of its yeast homologue (47982 and 42052, respectively). Both gene products are known to posses one lipoyl domain. The molecular mass of the bovine transacetylase component (59963) agrees with the figure published for other higher eukaryote E2s, notably human E2 (59555), which also contain two lipoyl domains.

3.2.4 Purification of the E1 and E3 components of mammalian PDC

In order to study the folding and assembly of the individual E1 and E3 enzymes and produce pure E3 for further reconstitution studies (see chapter 5) it was necessary to continue purification of the dissociated E1/E3 fraction. Attempts to follow purification protocols employed in the laboratory (Ruth Fullerton PhD thesis), while successful in purifying E3, were unable to recover any significant levels of E1. This failure was suspected to result from non-specific aggregation of E1 prior to, or during, chromatography (results not shown). As a preventative step, a triethanolamine/HCl buffer system supplemented with the detergent CHAPS was developed. This zwitterionic detergent (head groups containing both positive and negative charges) increases protein solubility and does not interfere with the separation characteristics of ion exchange chromatography. For full details of the methodology used to isolate pure E1 and E3 refer to the Materials and Methods section 2.2.10.

The chromatographic separation obtained using this buffer system was successful in separating the E3 component from the E1 component (see Figures 3.6a and 3.6b). It was also possible to recover substantial quantities of both enzymes following chromatography (see Table 3.3). All the E1 activity recovered eluted from the Mono Q column much later in the gradient than E3, suggestive of a higher affinity to the positively charged matrix under the conditions employed. E1 activity eluted as a single peak of activity spread over 12ml. No E3 activity was associated with the peak (Figure 3.6a) and no E3 protein was detectable in the pool by SDS-PAGE (Figure 3.6b, lane 2). In contrast, E3 activity did not elute as a single peak of activity. Instead two peaks of activity, not fully resolved to a baseline level, were eluted (Figure 3.6a). However, there was no evidence of contamination by E1. Instead, SDS-PAGE analysis showed the presence of a small polypeptide, with a molecular mass of less than 30000, associated with E3 in the second peak of activity (Figure 3.6b). Subsequent N-terminal sequence analysis of this small protein (Materials and Methods section 2.2.6) identified it as SP-22, a mitochondrial protein with putative antioxidant properties (for a full discussion of this see chapter 6). This protein, barely visible in the load (lane 3), appears to concentrate in the second E3 peak (lane 5). This may provide preliminary evidence that the role of this antioxidant protein may be more specific than had been envisaged (Watabe *et al.*, 1994). Rather, it may have a role in preventing the adventitious oxidation of the components of mammalian PDC, notably E3.

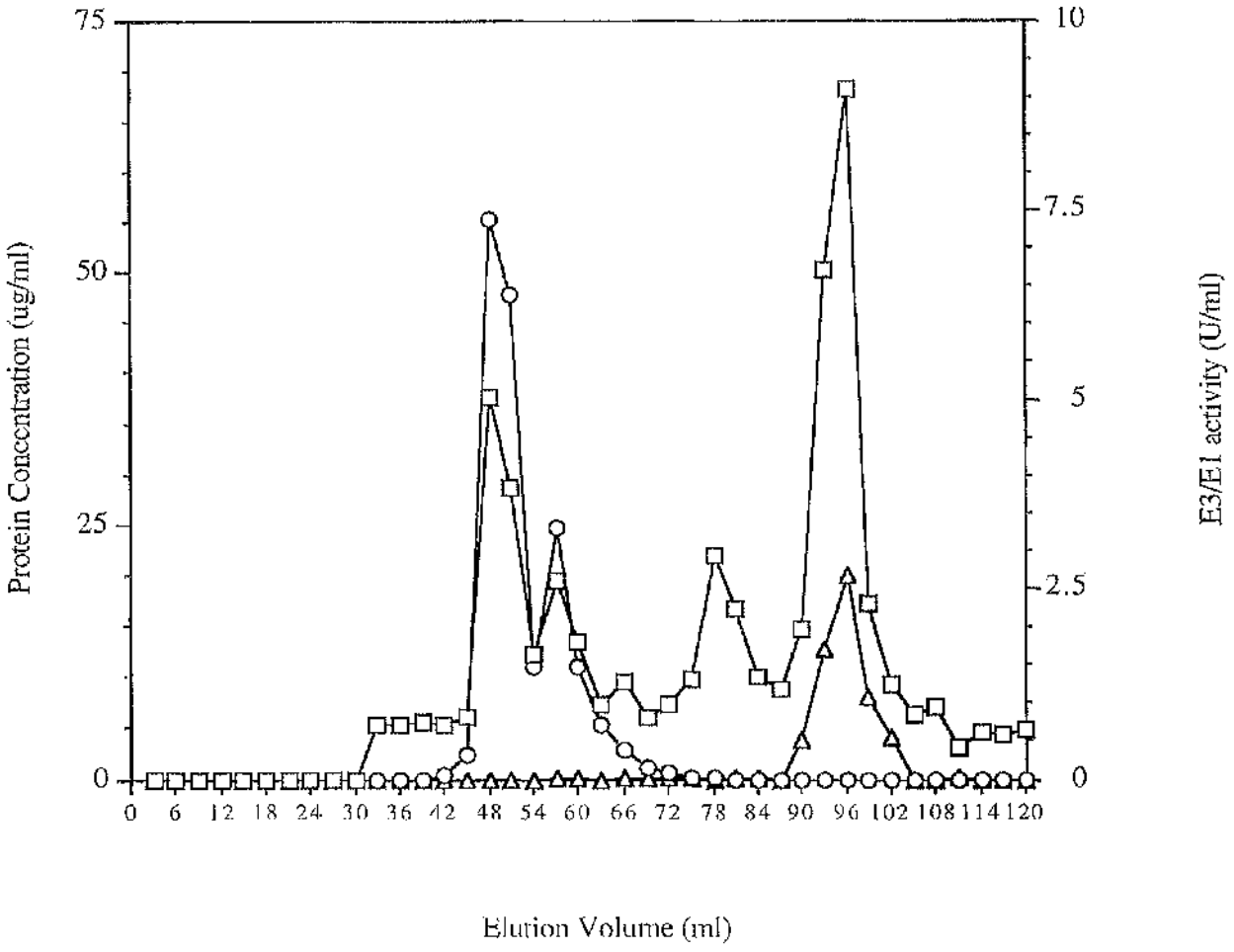


Figure 3.6a Separation of E1 and E3 of PDC by anion exchange chromatography

The E1/E3 fraction, prepared by high salt dissociation of PDC as detailed in the section 3.2.1, underwent buffer exchange into 20mM triethanolamine (TEA), pH 7.4, 10mM NaCl, 1mM CHAPS (buffer A) by extensive dialysis. Typically, 4mg protein was loaded onto an 8ml Mono Q column (Pharmacia) pre-equilibrated with buffer A. The column was washed with 2 column vol of buffer A, before being developed with using a linear gradient of 20mM triethanolamine (TEA), pH 7.4, 500mM NaCl, 1mM CHAPS (buffer B) over 200ml. In addition to monitoring the eluent continuously at 280 nm (□), individual fractions were assayed for E1 (△) and E3 (○) activity.

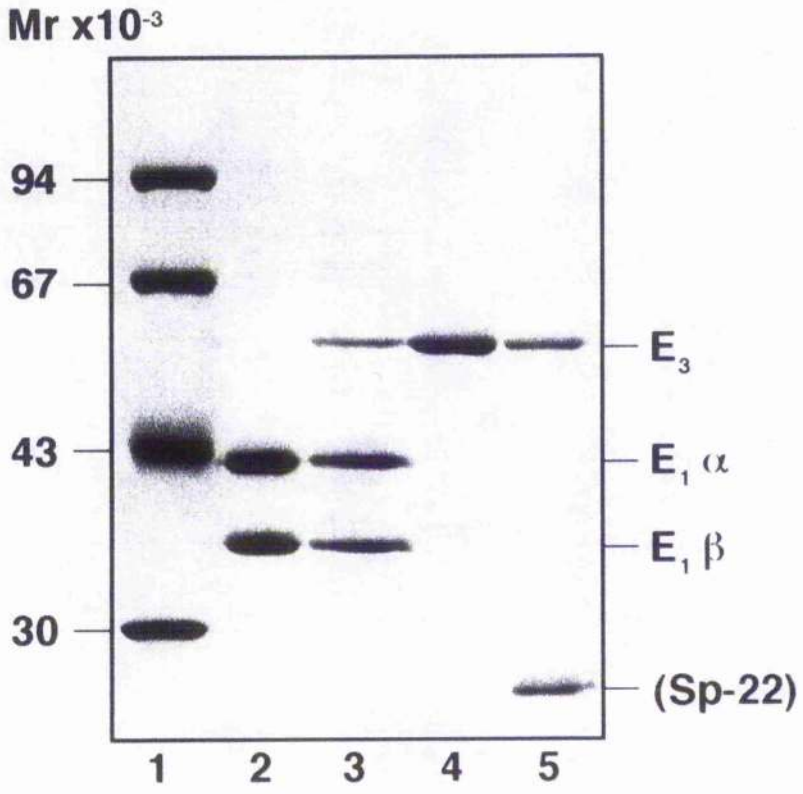


Figure 3.6b SDS-PAGE analysis of pools obtained from anion exchange chromatography on the E1/E3 fraction of mammalian PDC

Samples were analysed for purity by SDS-PAGE (10%, w/v gel) and stained with Coomassie blue. Lane 1, 10μg low-M_r marker proteins; lane 2, E1 fraction (10μg); lane 3, E1/E3 column load (15μg); lane 4, pure E3 fraction (10μg); lane 5, E3 fraction + < 30 000 M_r protein (10μg).

Table 3.3 Purification table for E1/E3 on Mono Q anion exchange chromatography

A) E3 Purification

	Load fraction	**E3 (peak1)pool**
Volume (ml)	4	18
Protein (mg/ml)	0.50	0.02
Total protein (mg)	2.00	0.37
E3 Activity (U/ml)	18.81	3.44
Total E3 activity (U)	94.05	62.08
% E3 recovery	100	66
Specific Activity (U/mg)	46.83	174.33
Purification Factor		3.75

B) E1 Purification

	Load Fraction	**E1 pool***
Volume (ml)	4	5
Protein (mg/ml)	0.50	0.23
Total protein (mg)	2.00	1.02
E1 Activity (U/ml)	2.51	1.16
Total E1 activity (U)	10.04	5.8
% E1 recovery	100	57.7
Specific Activity (U/mg)	5	5.68
Purification Factor		1.136

* Assays on the pooled E1 fractions were carried out after dialysis and concentration into storage buffer (50mM MOPS/KOH, pH 7.4, 50% (v/v) glycerol).

3.3 Discussion

The overall complex activities of OGDC and PDC were shown to be susceptible to inhibition by low levels of NaCl and GdnHCl. Effects of individual ions observed are in accordance with the Hofmeister (lyotrophic) series for reasons that still remain elusive but which may relate to to the hydration state of the individual proteins. At low concentrations, the effects mediated by GdnHCl apparently cause no gross conformational alterations in PDC (West *et al.*, 1995). However, it is easy to envisage that, at low concentrations, perturbations in the spatial geometry of key active site residues would be sufficient to eliminate catalytic activity. Stop flow spectroscopic studies of creatine kinase (Yao *et al.*, 1984) removes the possibility that this effect is similar to reversible inhibition; as the rates of inactivation observed in this study are several orders of magnitude lower than that which would be expected for a reversible inhibitor.

Common denaturants, such as GdnHCl, are thought to exert their effects via general solvent modification. Treatment of mammalian PDC and OGDC complexes with higher levels of GdnHCl (>0.6M) lead to loss of activity which could not be recovered by diluting out the denaturant. Previous data (West *et al.*, 1995) shows this corresponds to GdnHCl concentrations which elicit large-scale alterations in protein structure, as judged by CD and tryptophan fluorescence measurements. Thus, by altering the solvation around the protein,

and in turn altering its stability; one eliminates the small ΔG_{stab} (around 50kJ/mol for most proteins) associated with the native state, leading to component denaturation (Jaenicke, 1995).

Separation of biomolecules using gel permeation chromatography (GPC) depends on molecular shape as well as size with the best resolution usually obtained employing long columns and slow flow rates. A successful high salt dissociation of PDC into its E2/X fraction and E1/E3 fractions was achieved by (i) lowering the protein concentration loaded onto the Superose 6 column and (ii) altering the dissociation and column running buffers. The E2/X core produced contained no trace of the E3 component and minimal levels of E1. No PDC activity was detected in either fraction. In contrast, we were unable to scale-up the GdnHCl dissociation protocol used on an analytical scale in West *et al.* (1995). Possible explanations for this could arise from subtle differences in the chromatographic matrices used (Superose 6 (prep grade) for the scale-up and Superose 12 in the original work). These resins differ not only in their fractionation range, but also more subtly, in terms of their bead and pore size. These differences may have affected separation. Equally, failure of the scale-up may reflect increased band diffusion problems which can be associated with the larger, self packed Superose 6 column, compared to the smaller pre-packed Superose 12 matrix.

Reconstitution studies with the E2/X and E1/E3 fractions achieved the return of high levels of PDC activity (typically 60-80%). Maximal levels of activity were found to be returned by E2/X:E1/E3 ratios at or near to the native stoichiometry of the complex. Previous studies (Rahmatullah *et al.*, 1989; Powers-Greenwood *et al.*, 1989) failed to report the levels of reconstitution obtained, relative to native PDC. This can make the data presented difficult to interpret. For example, it would give cause for concern if conclusions were

drawn from data resulting from only modest levels of PDC reconstitution (10-20%), which, in turn, could be suggestive of critical levels of protein denaturation. Initial reconstitution studies exhibited a 10-15min reactivation period before maximal levels of reconstitution were obtained. The abolition of this lag period was achieved by pre-incubating both fractions in a cysteine containing buffer. This would suggest that adventitious oxidation of the thiol groups on the components of PDC was responsible for the lag period observed before maximal activity was reconstituted in the absence of reducing agent. These data are also indicative of instantaneous assembly of the E1 and E3 components onto the E2/X structural lattice upon addition. Interestingly, an activation lag period was observed in recent work looking at the regulation of *E. coli* PDC activity by its cofactor ThDP (Natalia *et al.,* 1996).The lag period observed was thought to result from the slow binding of a second ThDP molecule to the E1 dimer. However the concentration of the co-factor needed to observe this were at least an order of magnitude lower that employed in our reconstitution assay system and thus represents an unlikely explanation for our data.

The purity of the E2/X core produced, as a result of the refined high salt dissociation, facilitated the determination, for the first time, of the subunit molecular masses of the E2 and protein X components. The masses determined were similar to that of their homologues found in other eukaryotic species. It is also of note that the apparent molecular mass that bovine E2 (72 000) and protein X (51 000) display on SDS-PAGE differ considerably from those determined by ES/MS. The reasons for such a large discrepancy remain unclear, but may have to do with the presence of the covalently attached lipoyl domains inhibiting SDS binding. It has been observed previously that succinylated, maleylated or acidic proteins display reduced electrophoretic mobility (Weber *et*

al., 1971). It is possible that lipoyl domains have a similar ability to retard subunit mobility in SDS-PAGE.

The separation of E1/E3 components of mammalian PDC from each other was achieved using anion exchange chromatography. This produced milligram quantities of both proteins in high purity and provided a source of bovine (native) E3 for reconstitution studies (see chapters 4 and 5). The presence of the zwitterionic detergent CHAPS helped to mitigate the loss of the E1 component during the separation, although it did not completely eliminate this problem. The separation also led to the N-terminal sequencing and subsequent identification of the small (<30 000 M_r) protein associated with part of the E3 fraction as the mitochondrial protein SP-22 (Watabe *et al.,* 1994).The presence of two proteins in a single fraction on a high resolution of anion exchange column, may be indicative of a direct interaction between the dihydrolipoamide dehydrogenase component and SP-22. The possible role of SP-22 in preventing thiol oxidation is the subject of later investigation (see chapter 6).

Chapter 4

Refolding and reconstitution studies on the transacetylase-protein X (E2/X) subcomplex of mammalian pyruvate dehydrogenase complex (PDC):evidence for specific binding of the dihydrolipoamide dehydrogenase component (E3) to sites on reassembled E2.

4.1 Introduction

Located in the mitochondrial matrix, in close association with the inner membrane, the pyruvate dehydrogenase complex (PDC) is one of the largest multienzyme complexes detected to date with an apparent molecular mass of $9\text{-}10 \times 10^6$. This macromolecular assembly is responsible for the oxidative decarboxylation of pyruvate, yielding acetyl CoA, CO_2 and NADH. It thus occupies a key site in flux regulation of two carbon units into the TCA cycle. Central to the catalysis is the consecutive action of its constituent enzymes: a pyruvate decarboxylase, E1; a dihydrolipoamide acetyltransferase, E2 and, a dihydrolipoamide dehydrogenase, E3. PDC from mammalian sources is organised around a 60-meric E2 core with icosahedral (532) symmetry to which are attached a maximum of 30 $\alpha_2\beta_2$ E1 tetramers and 6-12 E3 homodimers (for recent reviews see Behal *et al.*, 1993; Patel & Harris, 1995).

Mammalian PDC contains an additional subunit, protein X, 12 copies of which are very tightly associated with the E2 core assembly. Its major role appears to be that of binding and positioning the E3 component at the correct sites on the surface of the E2 core (Maeng *et al.*, 1994; Sanderson *et al.*, 1996a). In addition, several copies of tightly associated kinase and loosely associated phosphatase are responsible for the regulation of PDC activity by a phosphorylation/dephosphorylation mechanism involving covalent modification of the E1α subunit (Yeaman, 1989).

PDC deficiency is one of the commonest causes of congenital lactic acidosis (Patel *et al.*, 1992). The cases of human pyruvate dehydrogenase deficiency studied to date implicate defects in the E1α component as the major cause, with considerably fewer lesions specifically associated with other enzymes of the complex. However, recent

studies (Marsac *et al.,* 1993; Geoffroy *et al.*, 1996) have identified patients, suffering from chronic lactic acidosis, with reduced levels (15-20%) of PDC activity and an apparent absence of protein X. In these cases, while the levels of E3 appeared normal, no immunologically detectable protein X was present in cultured fibroblast cells from the patients. Further studies have successfully cloned the cDNA for human protein X and localised it to chromosome 11p13 (Aral *et al.,* 1997; Ling *et al.,* 1998). Interestingly, one study identifies a case of patient protein X deficiency resulting from a 4bp deletion in the mitochondrial targeting signal sequence of the polypeptide (Aral *et al.,* 1997). Further cases of deletion mutations in the protein X gene have been identified, in addition to a single case where the patient did not appear to express protein X mRNA (Ling *et al.,* 1998).

Association of the individual enzymes of the complex is achieved solely by non-covalent forces, with the E2 component providing the structural lattice to which the constituent enzymes are attached. Evidence from genetic studies of PDC from *Saccharomyces cerevisae* (Niu *et al.*, 1990; Lawson *et al.,* 1991a, b) indicate that this core must be in a native or near native state for whole complex assembly to occur. More recent reports (Behal *et al.*, 1994; De Marcucci *et al.,* 1995) using denaturation and sedimentation velocity analysis, provide compelling evidence that assembly of the E2/X subcomplex proceeds in an ordered fashion, involving a lower molecular weight (8S) intermediate, as opposed to other possible random or sequential assembly mechanisms. However, the dissociation method employed by these authors has been shown to be suboptimal as the harshness of the dissocation conditions promoted a significant degree of component denaturation and resulted in poor levels of reconstitution (i.e., <20%) of original overall complex activity (Sanderson *et al.*, 1996b).

Recently our laboratory has developed a gentle but effective method of separating the E2/X subcomplex from E1/E3 which has facilitated reconstitution studies. It has

been possible to utilise the improved separation protocol to produce a more detailed picture of the processes involved in PDC folding and assembly (Sanderson *et al.*, 1996b).

In this chapter we demonstrate that dissociation of the E2/X subcomplex, and by implication its assembly, is an ordered, co-operative event. Complete unfolding of mammalian E2/X in guanidine hydrochloride and subsequent removal of the denaturant by either, (i) slow dialysis or (ii) rapid dilution, results in both instances in complete restoration of E2 catalytic activity and reassembly of the multimeric structure. However, compared to the native E2/X core, the reassembled E2 cores exhibit, respectively (i) a reduced level of bound protein X, or (ii) no immunologically detectable protein X. Further, both core preparations can still mediate E3 binding, through proposed low affinity, specific sites on E2, and thus support PDC activity, a property not previously observed. This ability provides *in vitro* evidence in support of previously reported clinical data (Geoffroy *et al.*, 1996) and clarifies data from a previous study (Sanderson *et al.*, 1996b) where low affinity binding to proteolytically cleaved protein X remained a possible explanation.

4.2 Results

4.2.1 Association state analysis of E2/X subcomplex

Analysis of the quaternary structure of the purified E2/X subcomplex involved pretreatment with 0, 2 or 4M GdnHCl prior to gel permeation analysis on a Superose 12 column equilibrated at the same concentration of denaturant (Fig. 4.1). The results provide evidence for an ordered, cooperative decline in the apparent M_r value of the E2/X core produced at discrete increments in the concentration of chaotrope. No initial dissociation of the native core was observed in the presence of 0-1.5M GdnHCl; thereafter, there was a rapid transition to an approx. 200,000 M_r species which was stable in the range 1.8-2.8M GdnHCl. At higher GdnHCl levels, there was a further rapid conversion to a partially or fully-unfolded monomeric form with an apparent M_r value of 82, 000.

Previous attempts (West *et al.*, 1995) to produce GdnHCl-induced dissociation of the E2/X core resulted in the production of non-specific aggregates. In this study, inclusion of 200mM NaCl in the gel permeation buffer appears to stabilise structure and minimise aggregation of E2/X dissociation products. Moreover, the appearance of an intermediate species, stable in 2M GdnHCl, between that of the native core and the monomeric form present at 4M GdnHCl, is consistent with the reversible perturbations in tertiary and/or quaternary structure observed by intrinsic tryptophan fluorescence (West *et al.*, 1995) whereas little or no corresponding disruption of secondary structure (as judged by circular dichroism measurements) was detected under these conditions. This result suggests that assembly of the E2 structural lattice may occur through a lower order intermediate, as opposed to possible random or sequential mechanisms (Behal *et al.*, 1994). Molecular mass estimations produce an apparent M_r

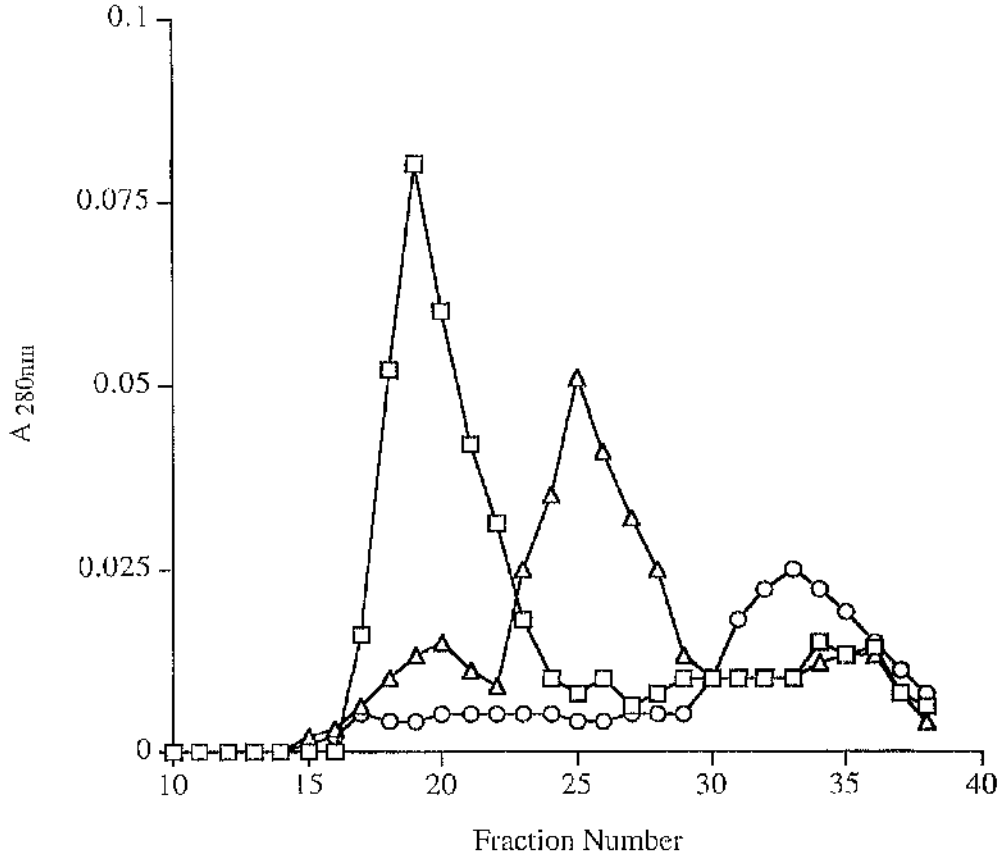


Figure 4.1. Gel permeation analysis of E2/X association

Association state analysis of the E2/X subcomplex was performed on a Pharmacia Superose 12 FPLC column (24ml) equilibrated, at 0.2ml/min, with increasing concentrations of GdnHCl in 50mM imidazole/HCl pH 7.4, 200mM NaCl. E2/X subcomplex (100µg), in column equilibration buffer, was pre-incubated at room temperature for 15 min in the appropriate concentration of GdnHCl and then subjected to analysis on the Superose 12 column. Protein elution was detected at 280nm. Elution profiles in the absence of GdnHCl (□), 2M GdnHCl (Δ) and 4M GdnHCl (O) are shown.

of 200,000 +/- 24,000 (n=4) for the species in 2M GdnHCl and a M_r of 82,000+/- 12,000 (n=4) for that formed in 4M GdnHCl. These M_r values are consistent with formation of E2 trimers at 2M GdnHCl and further dissociation to fully or partially-unfolded monomers when the chaotrope concentration is increased to 4M. Available crystallographic data on the truncated transacetylase core from *Azotobacter vinelandii* argues for strong intra-trimeric interactions between transacetylase monomers as the basic unit for core association (Mattevi *et al.*, 1992a). In addition, the structural and functional similarity of E2 to chloramphenicol acetyltransferase, CAT (Guest, 1987), active as a trimer, led to the suggestion that the 60-meric mammalian E2 core represents an association of twenty trimeric units, an idea which has received experimental support from recent publications (Behal *et al.*, 1994; De Marcucci *et al.*, 1995) and current evidence.

4.2.2 Kinetics of E2 refolding/reassembly

We have reported previously that overall PDC activity is rapidly inhibited at low levels of GdnHCl (0-0.2M) under conditions which have only a limited effect on the catalytic functions of its three constituent enzymes. The susceptibility of the intact complex to GdnHCl results from its case of dissociation into the individual E1, E2/X and E3 components. All three constituent enzymes display similar susceptibilities to the presence of GdnHCl with approx. 50% inhibition occurring by 0.3M and complete inhibition in the range 0.7-1.0M with the E2 component proving slightly more refractory to denaturant treatment. However, in all cases, inhibition occurs at levels of denaturant which are significantly lower than those which produce any major structural impact (West *et al.*, 1995). Since, in multidomain proteins, individual domains can be viewed as separate folding entities, it is probable that each domain displays a particular sensitivity to denaturant. Thus, initial loss of component activity usually reflects minor architectural changes in the most sensitive domain which are readily reversible. It has

been proposed that in a number of proteins the active site is particularly sensitive to denaturant perturbation (Tsou, 1986).

Fig. 4.2 illustrates the profiles of irreversible loss of overall complex activity and that of its individual component enzymes with increasing GdnHCl concentrations as judged by their inability to recover spontaneously following rapid dilution directly into assay buffer. In each case, the concentration of GdnHCl was reduced to below 30mM, given the acute sensitivity to GdnHCl demonstrated previously (West *et al.*, 1995). Irreversible loss of constituent enzyme activities correlates closely with the onset of major structural perturbations as judged by circular dichroism and intrinsic tryptophan fluorescence measurements (West *et al.*, 1995). Recovery of intact complex activity is intrinsically coupled to the sensitivity of the E1 and E3 enzymes which are most susceptible to GdnHCl-induced denaturation. Interestingly, however, major differences were observed in the ability of the E2/X subcomplex to support PDC reconstitution compared to the return of its intrinsic transacetylase activity. Whereas irreversible loss of E2-linked transacetylase activity occurs in the range 3.8-5 M GdnHCl (Fig. 4.2), the ability of the GdnHCl-treated E2/X core to promote overall complex reconstitution declines rapidly at lower levels of GdnHCl (2.8-4M). Extension of the renaturation/ reconstitution period prior to assay had no effect (results not shown). This discrepancy between the ability of the reactivated core assembly to support PDC reconstitution and the return of its transacetylase activity raised the possibility of a renaturation /refolding process dependent on the correct re-integration of protein X into the refolded core (see later).

Refolding by rapid dilution was optimised to maximise the recovery of transacetylase activity from fully unfolded monomers and found to exhibit a marked temperature and protein concentration dependence. The kinetics of refolding/ reassociation of the E2 core at 4°C (the optimal temperature) were monitored by activity assays (Fig. 4.3A). There is a rapid and efficient reconstitution of E2 activity

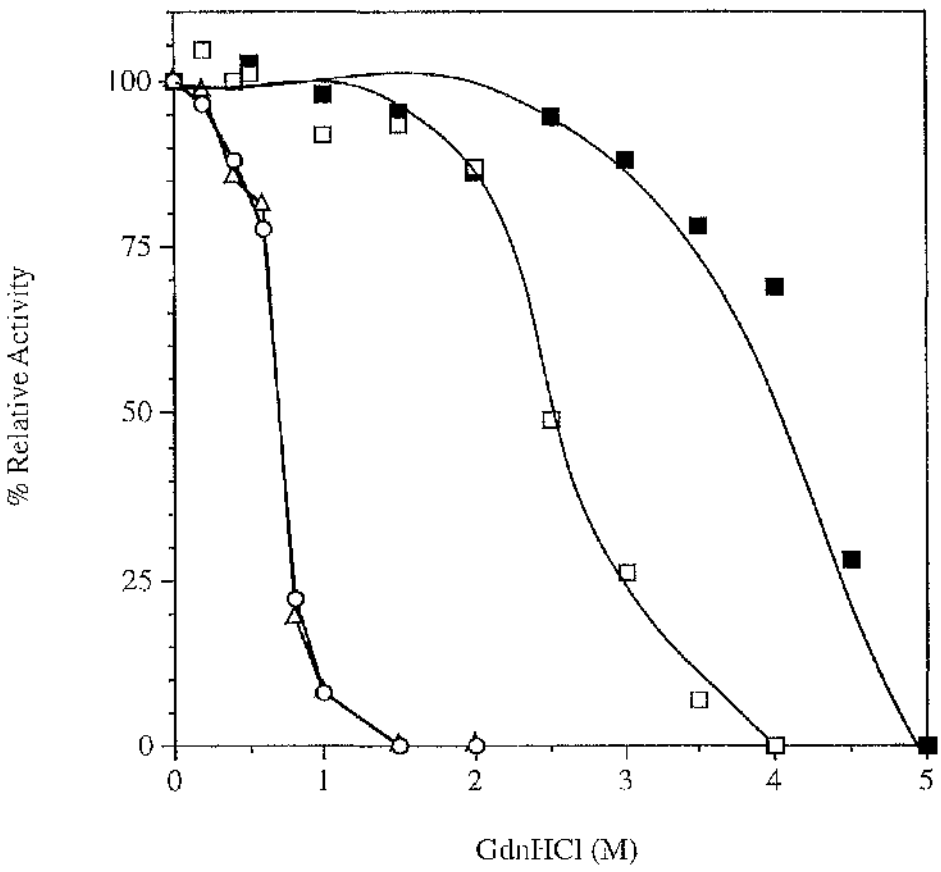


Figure 4.2. Reactivation/renaturation profiles of PDC, E2/X, E1/E3 and E2 (transacetylase) activity following pre-treatment with GdnHCl

Purified PDC, E2/X or E1/E3 preparations were incubated in 50mM MOPS/KOH, pH 7.4 containing the stated concentration of GdnHCl for 15min at 20°C. Renaturation/reactivation was initiated by a rapid dilution into the appropriate assay buffer. The final protein concentration in the renaturation mixture was 10-30µg/ml and residual GdnHCl concentration was less than 30mM. After a 15 min period at 20°C, reconstitution/activity assays were performed (see experimental) for PDC activity on: intact PDC (O); pre-treated E2/X (□); pre-treated E1/E3 (Δ). E2 transacetylase activity was also determined for pre-treated E2/X (■). All activities are expressed relative to control samples incubated in the absence of GdnHCl.

(at 50μg.ml^{-1}) with 60-80% of original transcetylase activity appearing within 30-45 min and complete recovery occurring in 4-6 h.

The biphasic nature of the kinetic profiles is consistent with two populations of folding intermediates. Fast folders are thought to collapse rapidly to the native state whereas slow folders undergo incorrect nucleation and are thus trapped in non-native/misfolded states. These molecules must unfold to some degree before proceeding further along the correct folding/assembly pathway (Todd *et al.*, 1996). Similar biphasic kinetics for E2 recovery, although over a prolonged time scale (25h), have been reported previously (Behal *et al.*, 1994; De Marcucci *et al.*, 1995). The improved rates of E2 core assembly reported here may reflect the milder conditions employed in the initial purification of the E2/X core (Sanderson *et al.*, 1996b) in comparison to the original protocol of Linn *et al.* (1972) routinely used by other investigators which results in significant component denaturation and poor levels (<20%) of overall complex reconstitution (Sanderson *et al.*, 1996b). Attempts to refold at elevated temperatures and protein concentrations resulted in dramatic declines in the yield of active E2, presumably resulting from promotion of competing non-specific aggregation reactions.

Despite the rapid and complete reconstitution of E2-linked catalytic function on dilution from 6M GdnHCl, these reassembled E2 cores were unable to sustain PDC activity on addition of the correct stoichiometric levels of E1/E3. As a result, a slow dialysis protocol was developed which permitted the return of approx. 35% of PDC activity (Fig. 4.3B) upon addition of the appropriate amounts of native bovine E1/E3. Slower removal of the chaotrope appears to minimise incorrect hydrophobic interactions within the E2/X core during folding and allow a more controlled passage through the critical, aggregation-sensitive, midpoint of folding. This observation represents the first report of the recovery of PDC complex activity following complete unfolding of the E2/X core. In addition, renaturation by slow dialysis still allows the

A

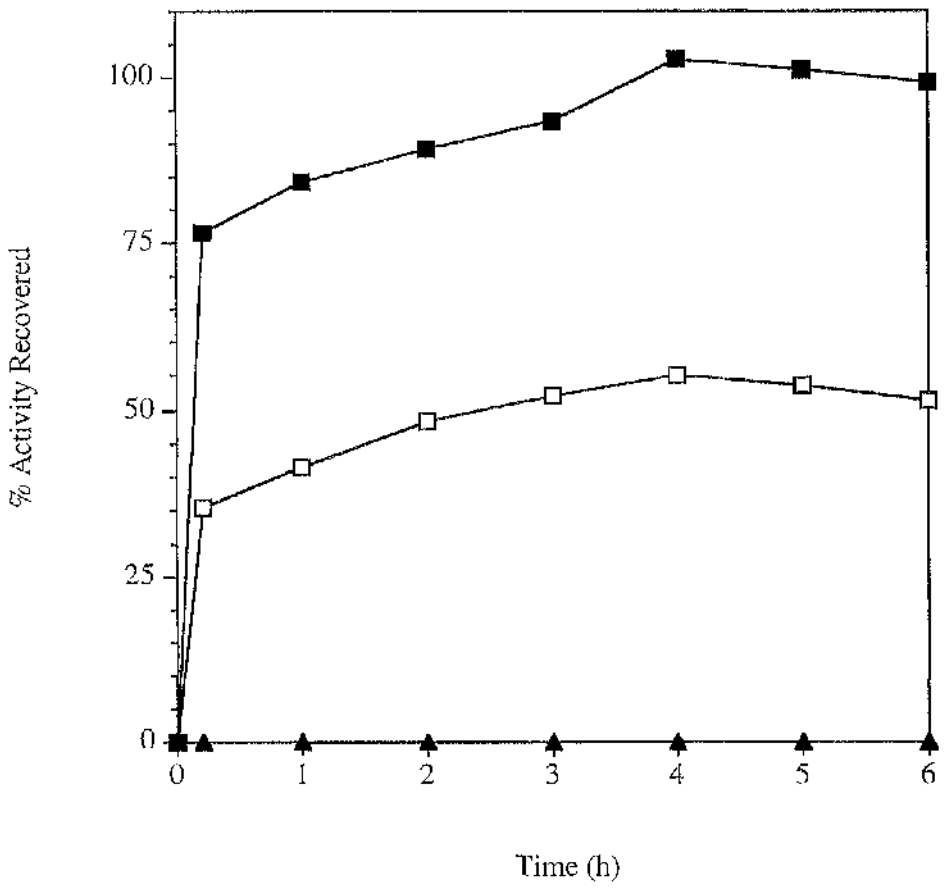


Figure 4.3A. Renaturation of E2/X subcomplex initiated by rapid dilution

Renaturation of E2/X, after complete denaturation in 6M GdnHCl for 2h at 20°C, was initiated by a rapid dilution into renaturation buffer (50mM potassium phosphate, pH 7.0 5mM cysteine-HCl) at 4°C. The protein concentration in the renaturation buffer was 50µg.ml^{-1} or 200µg.ml^{-1}. Aliquots were removed at the time points indicated and assayed for transacetylase (■) 50µg.ml^{-1}, (□) 200µg.ml^{-1} and reconstituted PDC activity (▲) 50µg.ml^{-1} and 200µg.ml^{-1} (Δ). Activities are expressed relative to control samples which underwent the entire protocol in the absence of GdnHCl.

B

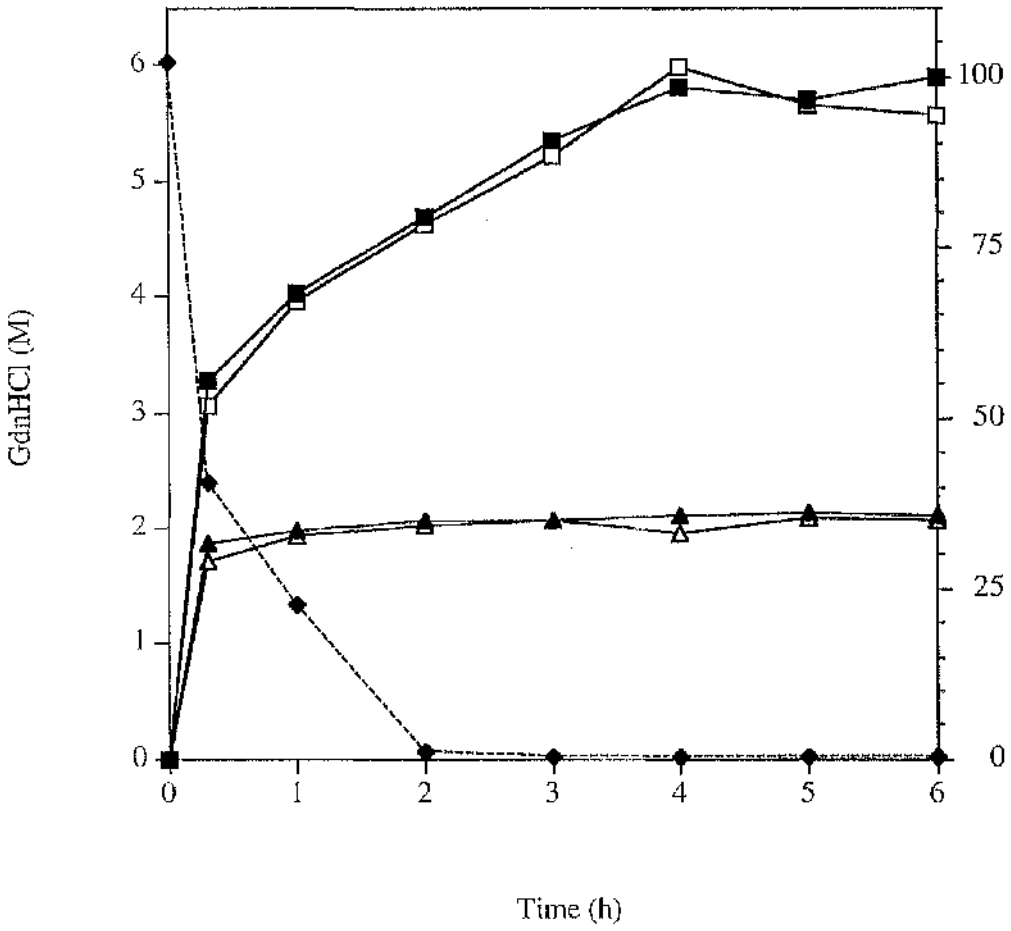


Figure 4.3B. Renaturation of E2/X subcomplex by dialysis

Following complete denaturation of the E2/X subcomplex in 6M GdnHCl for 2h at 20°C, reactivation and reassociation was initiated by dialysis against 4l of 50mM potassium phosphate buffer, pH 7.5, 250mM NaCl, 1mM $MgCl_2$, 0.1mM EDTA, 5mM β-mercaptoethanol at 4°C. At the time points indicated, aliquots were removed and assayed for transacetylase activity: (■) 0.5mg.ml^{-1} (□), 1mg.ml^{-1}, and the ability to reconstitute PDC activity: (▲) 0.5mg.ml^{-1}, (Δ) 1mg.ml^{-1}, upon the addition of the correct stoichiometric quantity of E1/E3. GdnHCl concentration (◆), at the time points indicated, was determined by refractive index measurements. Activities are expressed relative to control protocols carried out without GdnHCl.

complete return of E2 activity within 6h and is effective at protein concentrations 20-50-fold higher than the rapid dilution method with no marked concentration/ aggregation effects. From direct measurement of GdnHCl concentrations during the dialysis step (Fig. 4.3B), it is clear that a rapid decrease in denaturant concentration occurs in the initial stages. A slower stepped dialysis was developed which allowed the removal of GdnHCl at a much reduced rate. However, this did not produce any improvement in the levels of reconstituted PDC activity (results not shown).

4.2.3 Immunological analysis of refolded/assembled E2/X subcomplexes

It was anticipated that the differing efficiencies of the two types of reassembled E2 cores in promoting overall PDC function could be correlated with the extent of re-incorporation of the peripherally-located protein X subunit during *in vitro* assembly. Immunological analysis of the purified, reconstituted cores (Fig. 4.4) confirmed a reduction in bound protein X (to 30-40% of normal levels) from the subcomplex reassembled by slow dialysis (lane 2) and an elimination of protein X from the reassembled core produced by rapid dilution (lane 3) when compared to untreated E2/X core (lane 1). In contrast, identical loadings showed no detectable differences in the levels of the E2 component (lanes 4-6). Thus, loss of protein X does not affect E2 core assembly, as previously demonstrated using a genetic approach in *S. cerevisiae* (Niu *et al.*, 1990). The extent of depletion of protein X (lane 2) correlates closely with the reduced ability (approx. 35%) of this reassembled core to promote PDC reconstitution (Fig. 3B) while the absence of immunologically-detectable protein X (lane 3) provides an explanation for the ineffectiveness of this core in sustaining PDC activity in view of the known participation of protein X in high-affinity E3 binding (Fig. 4.3A).

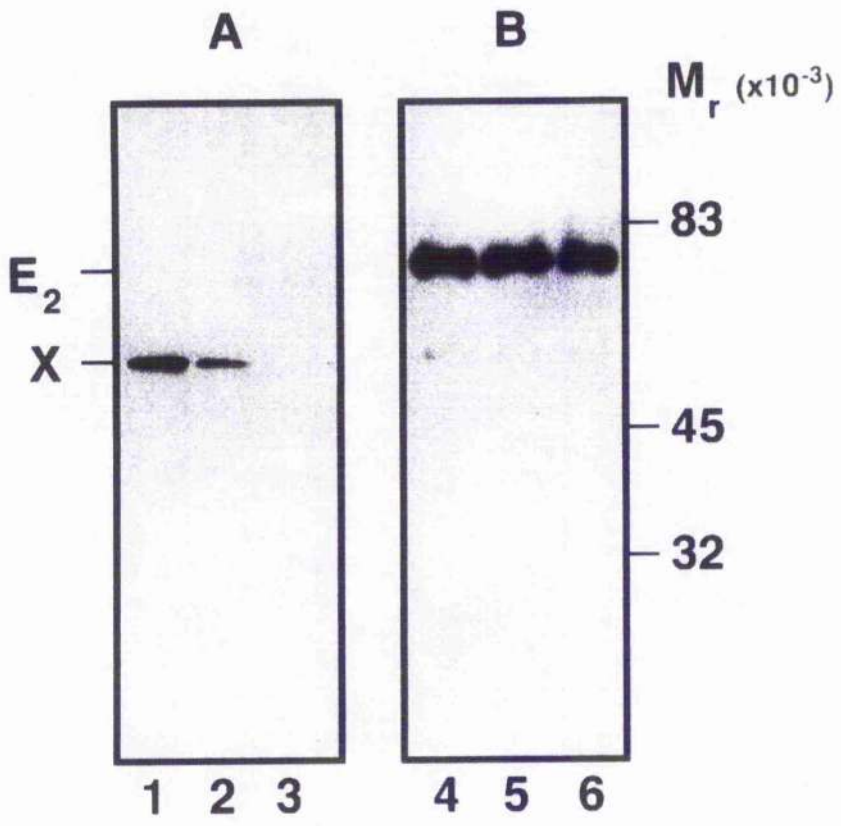


Figure 4.4. Immunological analysis of refolded/reassembled E2/X cores

Samples (500µl) containing renatured transacetylase and native untreated core were pelleted by ultracentrifugation using an optima TL ultracentrifuge (Beckman) for 25min at 100,000g through a 200µl 30%(w/v) sucrose cushion. Following resuspension and protein assay, identical quantities of each sample (5µg) were subjected to SDS-PAGE and subsequent immunological analysis with anti-protein X (lanes 1-3, panel A) or anti-E2 (lanes 4-6, panel B) serum. Lanes 1 and 4 untreated E2/X core (control); Lanes 2 and 5 refolded E2/X core (slow dialysis); Lanes 3 and 6 refolded E2/X core rapid dilution.

4.2.4 Reconstitution studies with E2(X) refolded cores

As indicated in Fig. 4.3, levels of PDC reconstitution, obtained after incubating either reassembled E2 core, devoid of protein X, or X-deficient E2 core with stoichiometric amounts of E1/E3, yielded values of 0% and 30-35%, respectively compared to recoveries with native E2/X. Both types of reconstituted E2 subcomplex were then tested for their ability to promote PDC activity in the presence of an increasing excess of bovine heart E3 (Fig. 4.5). In both cases, this resulted in the restoration of appreciable levels of PDC activity in the reassembled complexes. Thus, E2/X subcomplex deficient in protein X could support 60-65% of control PDC activity, virtually double its basal activity, in the presence of a 100-fold molar excess of E3. Importantly, reassembled E2 core lacking protein X can now be induced to reconstitute significant levels of PDC activity (25-30%) by a similar molar excess of native bovine E3. Thus, it appears that mammalian E2 oligomer can interact physically and functionally with E3, albeit with low affinity. No similar stimulation of PDC activity on addition of excess E3 is evident with native E2/X core which can sustain maximal yields of PDC activity with minimal (stoichiometric) amounts of E1 and E3. In support of these findings, patients with genetic disorders in PDC function, have been described recently which are characterised by the total absence of the protein X subunit. Interestingly, these patients still have measurable, although much reduced (<20%), PDC activity (Geoffroy *et al.*, 1996). Such evidence is also consistent with a limited ability of the E2 core to interact physically and functionally with E3 in the absence of its protein X subunit.

Reconstitution analysis with these refolded E2/X core preparations employing a number of heterologous E3s in vast excess (Fig. 4.6), confirm the specificity of the E3-induced stimulation of PDC activity, indicating that it is not the result of non-

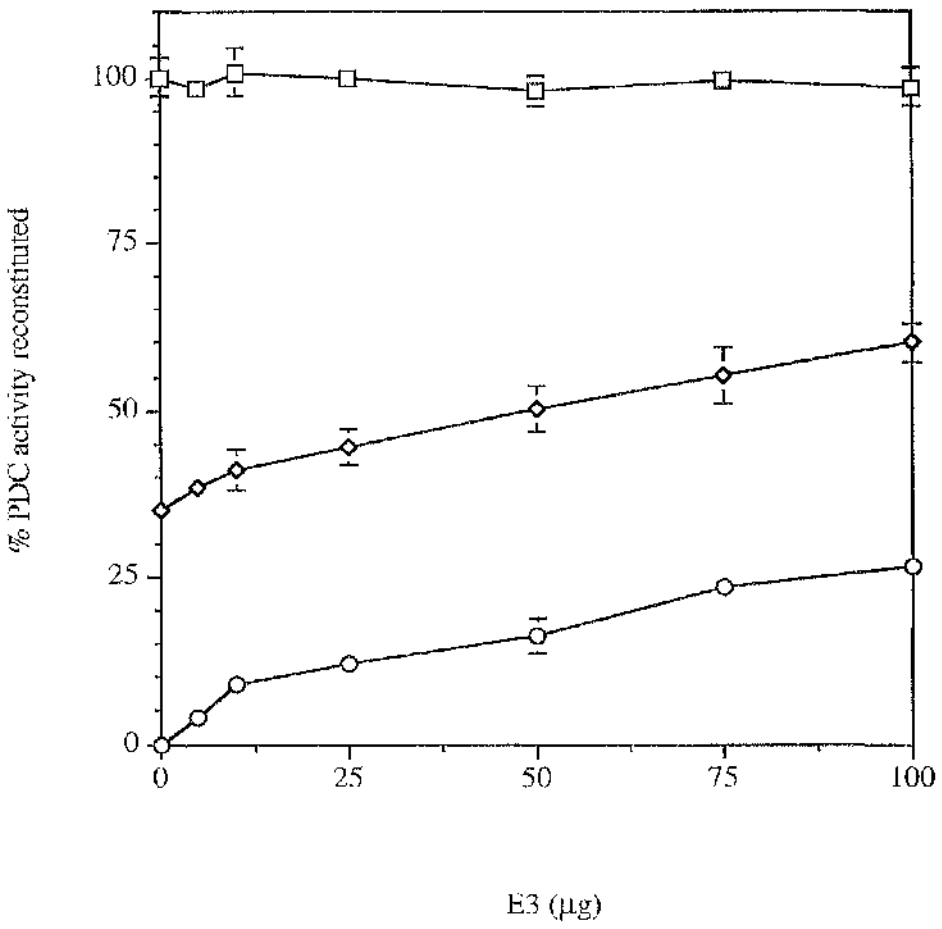


Figure 4.5. Reconstitution of PDC activity of refolded E2/X cores in the presence of excess parent E3

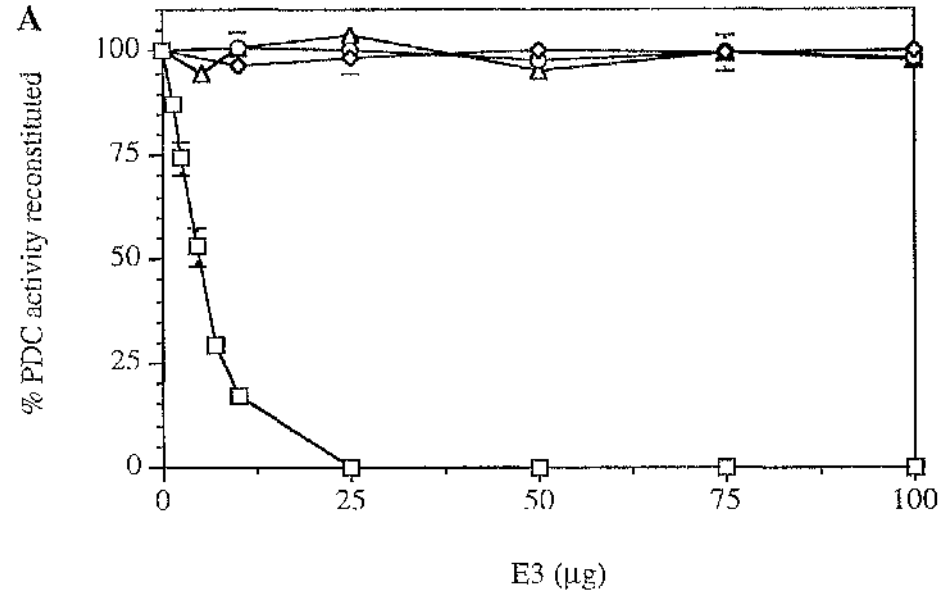
Renatured/reassembled E2/X cores (5µg) were reconstituted with the correct stoichiometric quantity of E1/E3 in assay mixture without substrate. Additional E3 was added at the amounts shown and allowed to associate prior to addition of pyruvate and assay of PDC activity: native E2/X core (□); refolded E2(X) core, slow dialysis (◇); refolded E2(X) core, rapid dilution (O). Values expressed as +/- S.E.M (n=2).

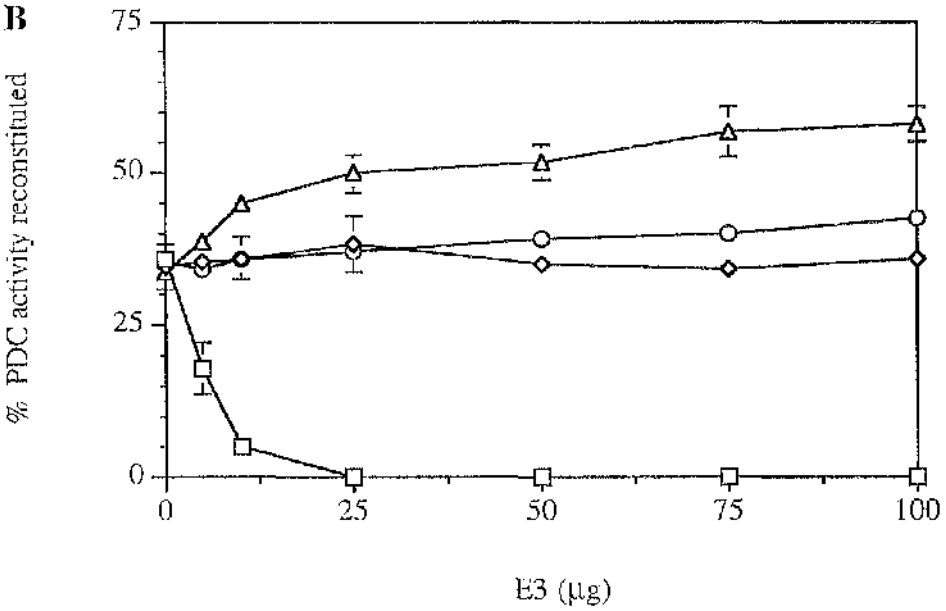
specific interaction or random collision of E3s with the reconstituted E1/E2 subcomplex. As control, Fig. 4.6A illustrates that bovine, bacterial and porcine E3, when added in excess over and above stoichiometric amounts of parent E1/E3, have no additional stimulatory effect on reconstituted PDC employing native (untreated) E2/X core; however, addition of yeast E3 produces a marked inhibition of PDC activity. This phenomenon has been observed previously with protease argC-treated E2/X core and native E1/E2 core from OGDC. It apparently reflects tight and specific binding of yeast E3 to these cores in an orientation which is unable to support complex catalysis (Sanderson *et al.*, 1996b). This marked inhibition is also observed when yeast E3 is added to the E2/X core produced by slow dialysis (Fig. 4.6B). In this instance, while bacterial E3 has no detectable effect, presumably because it is unable to bind, porcine E3 does produce a small stimulation. However, it is much less pronounced, representing only 25% of that observed with native bovine E3.

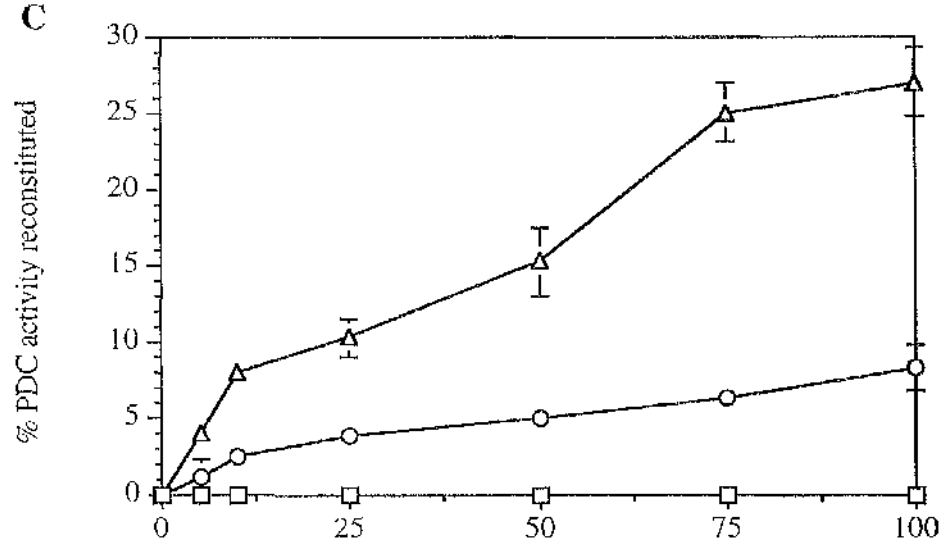
The specificity of the E3 response is also observed with E2 core totally lacking protein X (Fig. 4.6C). A small but significant level of stimulation is obtained with porcine E3 (again around 25% that of bovine heart E3) whereas bacterial and yeast E3s are completely ineffective. This ability of porcine E3 to promote the final step in PDC catalysis reflects the high degree of conservation between bovine and porcine E3s. However, usage of porcine E3 is clearly suboptimal in reconstitution studies and may explain why previous investigators were unsuccessful in detecting any PDC reconstitution after refolding of the denatured E2/X core (De Marcucci *et al.*, 1995).

Figure 4.6. Reconstitution of PDC activity of refolded E2/X cores with excess heterologous E3

Renatured/reassembled E2/X cores (5μg) were reconstituted with the correct stoichiometric quantity of E1/E3 in assay mixture without substrate. Additional E3 was added at the amounts shown and allowed to associate prior to addition of pyruvate and assay of PDC activity. (**A**) native (untreated) E2/X core; (**B**) refolded E2/X core produced by slow dialysis; (**C**) refolded E2/X core produced by rapid dilution. Parent E3 (Δ); yeast E3 (□); bacterial E3 (◇); porcine E3 (O). No results are presented for bacterial E3 in panel C as it also gave zero reconstitution. Values expressed as +/- S.E.M (n=2).


A
% PDC activity reconstituted
100
75
50
25
0
0
25
50
75
100
E3 (μg)


B
% PDC activity reconstituted
75
50
25
0
0
25
50
75
100
E3 (μg)


C
% PDC activity reconstituted
30
25
20
15
10
5
0
0
25
50
75
100

4.3 Discussion

Gel permeation analysis of mammalian E2/X core indicates that it can be dissociated into discrete intermediates corresponding to trimers and subsequently fully-unfolded monomers as judged by M_r analysis in specific ranges of GdnHCl concentration. Thus, the 200, 000 +/- 24,000 M_r intermediate is close to the expected value for a trimeric species since bovine monomeric E2 has an M_r value of 59,963 as determined by electrospray mass spectrometry . Although the monomeric species has an apparent M_r value of 82, 000 +/- 12,000 on gel filtration, this is compatible with monomeric E2 existing in a loosely or fully-unfolded state at high denaturant concentrations which would result in its more rapid elution from the gel permeation matrix relative to a globular intermediate of equivalent size. Interestingly, a stable trimeric species of bovine branched-chain 2-oxoacid dehydrogenase complex (BCOADC) E2 has been isolated after dissociation in GdnHCl (Wynn *et al.*, 1994). Moreover, previous sedimentation velocity analysis of chaotrope-treated E2/X subcomplexes of PDC from bovine kidney in sucrose gradients produced data consistent with an ordered assembly of this subcomplex (Behal *et al.*, 1994). Notably a range of subcomplex species were detected intermediate to the purported trimer and native 60-mer, thus arguing for a non-cooperative transition. In contrast no evidence of such species were detectable during our study.

In this context, the recent discovery that sucrose and other polyhydric compounds oppose the effect of denaturants on water structure, thus increasing the concentration of denaturant required to unfold an enzyme is of considerable interest (Taylor *et al.*, 1995). In addition to influencing the state of aggregation of E2 dissociation products

located at different levels in the sucrose gradient, this could explain discrepancies between the critical chaotrope concentrations required for trimer formation and complete unfolding of monomers stated in (Behal *et al.*, 1994) in relation to those determined in this publication and in other studies (West *et al.*, 1995).

The rapid and successful chaperone-independent refolding and functional maturation of the mammalian E2(X) core in high yield *in vitro* is somewhat surprising, given its complex multidomain organisation and quaternary structure. Elevated ionic strength (200mM) has been suggested as a key factor which enables kinetically trapped intermediates to return to productive folding by lowering the activation energy barrier between the trap and its normal folding pathway (Todd *et al.*, 1996). For example, ribulose bisphosphate carboxylase (RUBISCO) does not fold successfully at low ionic strength but behaves as a slow folder in elevated ionic strength buffers (Schmidt *et al.*, 1994). Previous successful folding and assembly of a truncated 24-meric core of BCOADC could only be achieved with the inclusion of molecular chaperones (Wynn *et al.*, 1994). It is possible that the absence of the N-terminal region of this polypeptide prevents the establishment of critical transient associations which are essential for promoting ordered folding of the E2-BCOADC oligomer. In this study, although functional re-assembly of the E2 oligomer in high yield could be achieved by rapid dilution or slow dialysis, partial re-integration of the protein X subunit was critically dependent on both the controlled removal of denaturant and the presence of 200mM NaCl (Fig. 4.3B). These findings on E2/X core formation of PDC, however, do highlight its high intrinsic capacity for self-assembly and provide further evidence in support of the hypothesis (currently under investigation in our laboratory) that the extended presequences on the cytosolic precursor forms of the various complex-specific E2s may be involved in preventing their premature association prior to mitochondrial import (Hunter and Lindsay, 1986; De Marcucci *et al.*, 1988; Clarkson and Lindsay, 1991).

Whereas regeneration of active E2 cores displaying complete restoration of dihydrolipoamide acetyltransferase (E2) activity, occurs rapidly in high yield on removal of GdnHCl by either slow dialysis or rapid dilution, these reassembled structures have distinctive properties, reflecting their differing protein X content which influences their ability to support overall complex reconstitution on addition of stoichiometric amounts of E1 and E3. Thus, reconstitution by rapid dilution is incompatible with re-incorporation of the protein X subunit into the re-assembled E2 cores whereas 30-40% of expected protein X levels can be functionally re-integrated by controlled removal of denaturant. As expected, no significant reconstitution of PDC can be obtained with the E2 oligomer lacking protein X whereas corresponding levels of reconstitution (30-40%) are readily achieved with the X-depleted core in the presence of stoichiometric levels of E1 and E3. The possibility of achieving complete functional integration of wild-type levels of protein X into reassembled E2 oligomers in the presence of molecular chaperones is currently under investigation.

Interestingly, maximal levels of PDC reconstitution (30-35%) are restored within 30-45 min using re-assembled E2(X) cores formed by slow dialysis whereas only 50-60% of intrinsic acetyltransferase activity has re-appeared at this stage with complete re-activation of E2 occurring over 4-6h. Such data suggest that initial formation of E2 oligomers and subsequent incorporation of protein X occurs on a relatively rapid time scale whereas further more subtle rearrangements of enzymatically-inactive E2 polypeptides incorporated into the repolymerised core structures (slow folders) are necessary to effect the complete recovery of acetyltransferase activity. These observations are compatible with the observation that E2 does not catalyse the rate-limiting step in the overall reaction; moreover, as the lipoyl domains of individual E2 molecules form a complex interacting network within the core assembly, it has been shown previously that removal of a substantial fraction of E2 lipoyl domains can be accommodated without significant effects on overall complex activity (Ambrose-Griffin *et al.*, 1980; Stanley *et al.*, 1981).

In yeast, sequence similarities between protein X and transacetylase E2 components have been documented previously (Behal *et al.,* 1989). This extends over most of the N-terminal region and also includes putative peripheral subunit (specifically E1/E3) binding domains. The major differences appear in the C-terminal regions which, in the E2 component, are known to be involved in catalysis and also self-association. The presence of equivalent putative subunit binding domains in both mammalian and yeast E2 and X components displaying significant homology may suggest that E2 oligomers have retained a residual affinity for E3 (Sanderson *et al.*, 1996b; Maeng *et al.,* 1996).

Evidence reported here demonstrates for the first time that reconstituted PDC, deficient in or totally devoid of its protein X subunit can, in the presence of a large excess of its E3 component, still support overall complex activity via specific low affinity E3 interactions with the putative peripheral subunit binding domains on the E2 component. Thus, E2 cores containing 30-40% of normal levels of protein X are able to support an equivalent level of reconstitution with stoichiometric amounts of E1 and E3 in contrast to E2 core totally stripped of protein X which is totally ineffective under similar conditions. Thereafter, both types of core can sustain an additional 25-30% of control PDC activity in the presence of a 100-fold excess of parent E3, consistent with the involvement of protein X in mediating high affinity E3 binding and a residual capacity of E2 to promote low affinity E3 interactions.

In support of this hypothesis, a group of patients with genetic defects in PDC function have been discovered who contain no immunologically-detectable protein X subunit (Geoffroy *et al.*, 1996). These patients display residual levels of PDC activity (10-20% of controls) which can now be accounted for in terms of low affinity E2-mediated interactions with the E3 component. Current studies also represent a significant advance on previous analysis of the role of protein X in E3 binding

employing selective proteolysis of this subunit with protease arg C (Sanderson *et al.*, 1996b). In this investigation, the single arg C cleavage site on protein X was located at the N-terminal boundary of its putative E3 binding domain and the truncated 35,000 M_r fragment remained tightly-associated with the E2 core. Consequently, reconstitution of complex activity in the presence of excess E3 could have been mediated directly via the E2 component or reflect low affinity interaction with a partially-disrupted E3 binding domain on the truncated protein X component.

In summary, these results demonstrate unequivocally for the first time that the presence of the protein X subunit of mammalian PDC is not absolutely essential for maintaining partial complex function *in vitro* and probably also *in vivo*. Thus, significant levels of complex reconstitution can be attained in the presence of an excess of the E3 component using the purified constituent enzymes of PDC in dilute solution. Partial PDC activity also appears to be maintained *in vivo* where protein concentrations are in order of 200mg.ml^{-1} in the mitochondrial compartment. These observations highlight the distinctive but overlapping roles of the E2 and protein X subunits of mammalian PDC. Whilst the dihydrolipoamide acetyltransferase (E2) component has a prominent role in complex catalysis, specifically the acetylation reactions, in the absence of E2-lipoyl domains, a degree of complex activity (approx. 20%) can be mediated exclusively via lipoyl groups on protein X (Sanderson *et al.*, 1996b). Similarly, as shown here, the primary involvement of protein X in mediating optimal high affinity binding of dihydrolipoamide dehydrogenase (E3) homodimers can be replaced, at least partially, by direct E3 association with equivalent regions situated on the E2 oligomer. Access to a catalytically-active form of PDC, lacking protein X, will permit more detailed investigation of the properties of the modified PDC and facilitate elucidation of possible additional catalytic functions of the protein X subunit.

Chapter 5

The 2-oxoglutarate dehydrogenase (E1) component of mammalian 2-oxoglutarate dehydrogenase complex (OGDC) is responsible for binding the dihydrolipoamide dehydrogenase (E3) component to the multienzyme complex

5.1 Introduction

The 2-oxoglutarate dehydrogenase complex (OGDC), along with the pyruvate dehydrogenase complex (PDC) and the branched chain 2-oxoacid dehydrogenase complex (BCOADC), constitute the family of mammalian 2-oxoacid dehydrogenase multienzyme complexes important in carbon flux into and around the citric acid cycle. Located in the mitochondrial matrix, these complexes catalyse the irreversible oxidative decarboxylation of their respective 2-oxoacid substrates; yielding acylCoA derivatives (succinyl CoA in the case of OGDC), NADH and CO_2 as the final reaction products. Central to this catalysis is the consecutive action of three catalytic components, present in multiple copies: 2-oxoacid dehydrogenase (E1); dihydrolipoamide acyltransferase (E2), and dihydrolipoamide dehydrogenase (E3) (see Patel & Harris, 1995; Behal *et al.,* 1993 for recent reviews).

2-oxoacid dehydrogenase complexes are composed of multiple copies of the three constituent enzymes, assembled in a tight but noncovalent fashion. Their multi-component and multi-copy nature results in the formation of very large complexes (M_r, 5-10 x10^6).These structures are composed around 24/60meric E2 cores (dependent on the specific multienzyme complex and source organism), exhibiting octahedral/icosahedral symmetry respectively. In prokaryotes, in conjunction with its catalytic role, the E2 component is responsible for binding and orientating both the E1 and E3 components within the multienzyme complexes (for reviews of structure-function relationships, see Reed & Hackert 1990; Mattevi *et al.,* 1992a; Mande *et al.,* 1996). In higher eukaryotes the identification of an additional component of PDC, protein X, with similarities in domain structure to E2, suggested a possible devolution of

subunit function (De Marcucci and Lindsay 1985; Jilka *et al.*, 1986). Subsequent studies of protein X (also termed E3BP) have shown that it is responsible for mediating the binding of the E3 component to the PDC multienzyme complex in these organisms (Powers-Greenwood *et al.*, 1989; Neagle & Lindsay 1991; Lawson *et al.*, 1991a,b; Maeng *et al.*, 1994).

Furthermore, a study of mammalian OGDC in this laboratory, employing specific proteolysis and N-terminal sequence analysis, identified a "lipoyl like" region of the E1 component with significant sequence similarity to protein X and E2 components of mammalian PDC (Rice *et al.*, 1992). This similarity suggested that the E1 component may perform the function of E3 binding in the case of mammalian OGDC. Further evidence, in support of this putative additional role for E1 of OGDC, also came when the genes for rat and human OGDC E2 were cloned (Nakano *et al.*, 1991 and 1994). Analysis of the amino acid sequences deduced from the genes failed to locate any apparent E1/E3 binding motifs. Such sequences have been located in OGDC E2 genes cloned from other organisms, notably *Escherichia coli* (Spencer *et al.*, 1984) and *Azotobacter vinelandii* (Westphal & de Kok 1990).

Previous studies of OGDC have successfully purified the intact multienzyme complex to homogeneity (Stanley & Perham 1980; De Marcucci *et al.*, 1985). However, no method for the dissociation of OGDC into functionally active E2 and E1/E3 fractions has been reported. This has prevented the study of any potential E1/E3 interaction. In this chapter we report the development of a method for the successful dissociation of mammalian OGDC into its E2 and E1/E3 fractions. Subsequent reconstitution studies with the E2 core recovered appreciable levels (30%) of OGDC activity, upon the addition of E1 and E3. Gel permeation analysis of the E1/E3 fraction of OGDC, under associative conditions, demonstrated that the two components interact with each other to

form a subcomplex with an apparent M_r consistent with an E1/E3 subunit interaction in a 1:1 ratio. Immunological analysis, employing antisera raised specifically to the E1 subunit of OGDC identified distinct cross-reactivity with E2 of mammalian PDC, indicative of similarity between the two proteins. The cross-reactivity of E1 with antisera specific for mammalian protein X is also presented. These studies provide biochemical evidence that the E1 of mammalian OGDC is responsible for binding the E3 component to the multienzyme complex.

5.2 Results

5.2.1 Preparative dissociation of bovine heart OGDC

Proteolytic studies and N-terminal sequence analysis (Rice *et al.*, 1992) indicated that the E1 component of OGDC contained protein X/E2 like sequences which had the potential to direct the binding of the E3 component to the E1. Attempts within this laboratory to purify the native E1/E3 fraction away from the E2 core remained elusive. The development of such a protocol remained highly desirable as it would facilitate the study of the putative E3 binding properties of E1 by direct methods. Figure 5.1 details preliminary studies on the combined effect of treatment of OGDC with 1M $MgCl_2$ at a variety of pH conditions. The data suggest that the high salt conditions have a minimal effect on enzyme activity at pH 6.5 and 7.0 (neutral/slightly acidic conditions). In contrast, treatment of OGDC in more alkaline conditions (pH 8.0) caused an immediate inactivation of enzyme activity (i.e. 70% loss at t_0) with a further decreases over the time course investigated; leading to almost complete extinction of OGDC activity after 5h. The treatment with $MgCl_2$ at pH 7.6 (mildly alkaline conditions) caused some inactivation of the enzyme, but more than 50% of the enzyme remained functional after a 1-2h period. Alkali treatment on its own had no effect (results not shown). It was probable that the salt treatment at pH 7.6 was having a dissociative effect on the multienzyme complex and was integrated into a dissociation protocol for OGDC (see below).

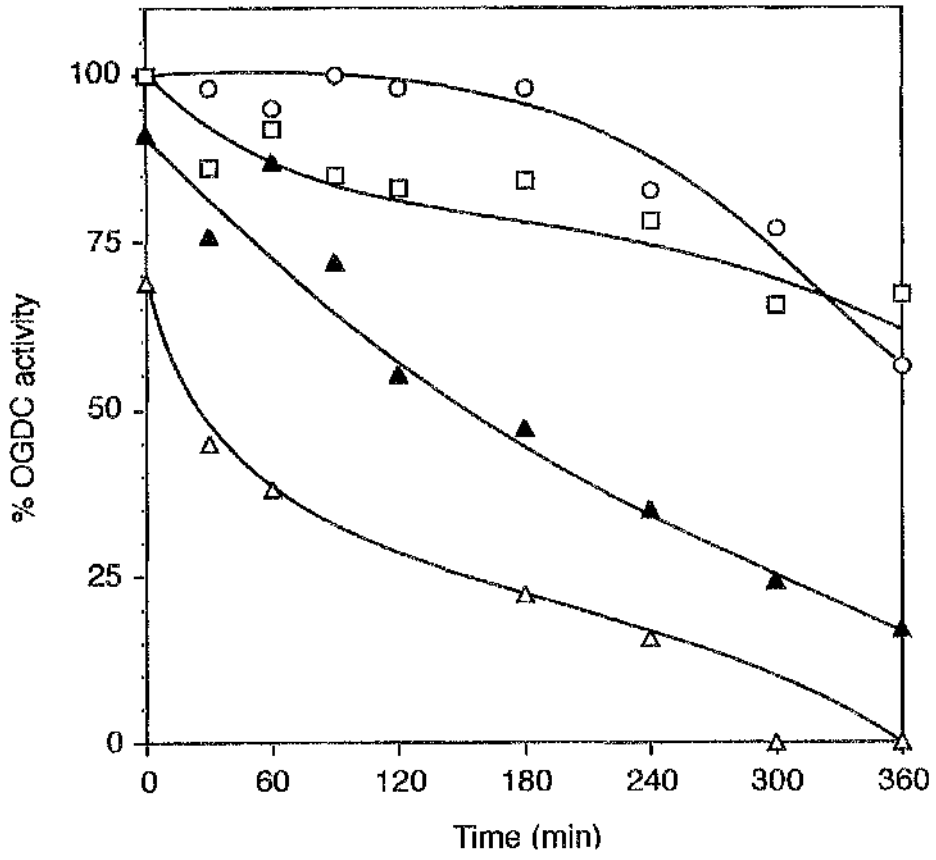


Figure 5.1 Reactivation profiles mammalian OGDC activity following pre-treatment with 1M $MgCl_2$ over the pH range 6.5-8.2.

OGDC was incubated in 1M $MgCl_2$ at the pH stated at 20°C. During the time course, samples were removed and reactivation /renaturation was initiated by a rapid dilution into the appropriate assay buffer. The final protein concentration in the renaturation mixture was 10-30µg/ml and residual $MgCl_2$ reduced to below 30mM to prevent enzyme inhibition. Samples were assayed for OGDC activity. All activities are expressed relative to control samples of enzyme incubated in the absence of $MgCl_2$. pH 6.5, O; pH 7.0 □ ; pH 7.6 ▲; pH 8.2, Δ. (Experiment performed by Mrs H. Lindsay)

Preparative dissociation of OGDC was performed on a Superose 6 prep grade column (see Materials and Methods section 2.2.16). The elution profile obtained from measuring the absorbance at 280nm suggested that the protocol had indeed achieved some degree of separation of OGDC, with apparently two peaks eluting from the column (Figure 5.2a). The initial peak of absorbance, corresponding to the high M_r E2 core, eluted at or near the void volume of the column (V_e, 30-32ml). The second peak, subsequently shown to contain the E1 and E3 enzymes, eluted in the later column fractions (V_e 60-66ml). The nature and extent of the dissociation achieved was evident from SDS PAGE analysis of the column fractions (Figure 5.2b). Clearly, the $MgCl_2$ treatment and column chromatography had been successful in removing E1 and E3 from the E2 core of OGDC. Lanes corresponding to the E2 core (column fractions 14-16) show no apparent E3 still core associated, and minimal levels of E1 still present. The elution profile obtained appears consistent with elution from the Superose 6 column on the basis of size: thus, the oligomeric E2 component elutes first, close to the void volume of the column, followed by the E1 and finally the E3 component. The E1 and E3 components appear as a single unresolved peak in the absorbance 280nm trace. However, the SDS-PAGE analysis reveals that, in 1M $MgCl_2$ elution buffer, E1 elutes before E3 (lanes corresponding to column fractions 18-32), although there is a degree of overlap. This is also suggestive that, under the column conditions, that the E1 and E3 components are not associated as a single complex.

5.2.2 Reconstitution studies with bovine OGDC

While the protocol detailed above appeared successful in promoting the dissociation from the E2 core of the E1 and E3 components of OGDC, the effect of the dissociation on the three enzyme activities remained unclear. For OGDC it

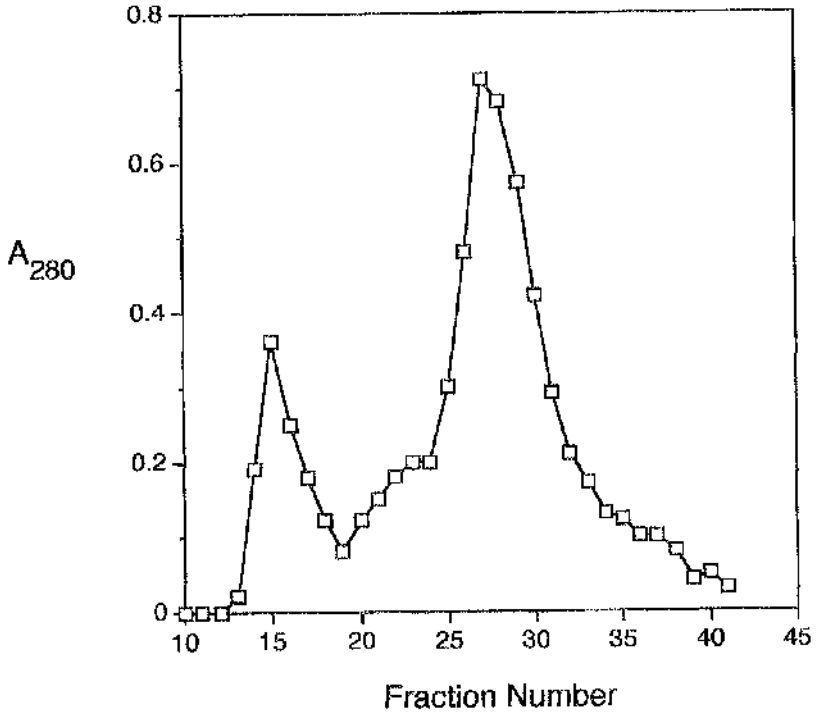


Figure 5.2a Elution profile of the preparative dissociation of bovine heart OGDC

Preparative dissociation of OGDC into active E2 and E1/E3 fractions was performed on an FPLC system (Pharmacia) using a prep grade Superose 6 column (100ml) equilibrated with 50mM MOPS/KOH, pH 7.6, 1M $MgCl_2$, 0.1% (v/v) Triton X-100 (see Materials and Method section 2.2.16). Fractions were pooled on the basis of A_{280} and dialysed extensively, at 4°C, against multiple changes of the 20mM MOPS/KOH, pH 7.0, 10mM KCl, 0.1% (v/v) Triton X-100.

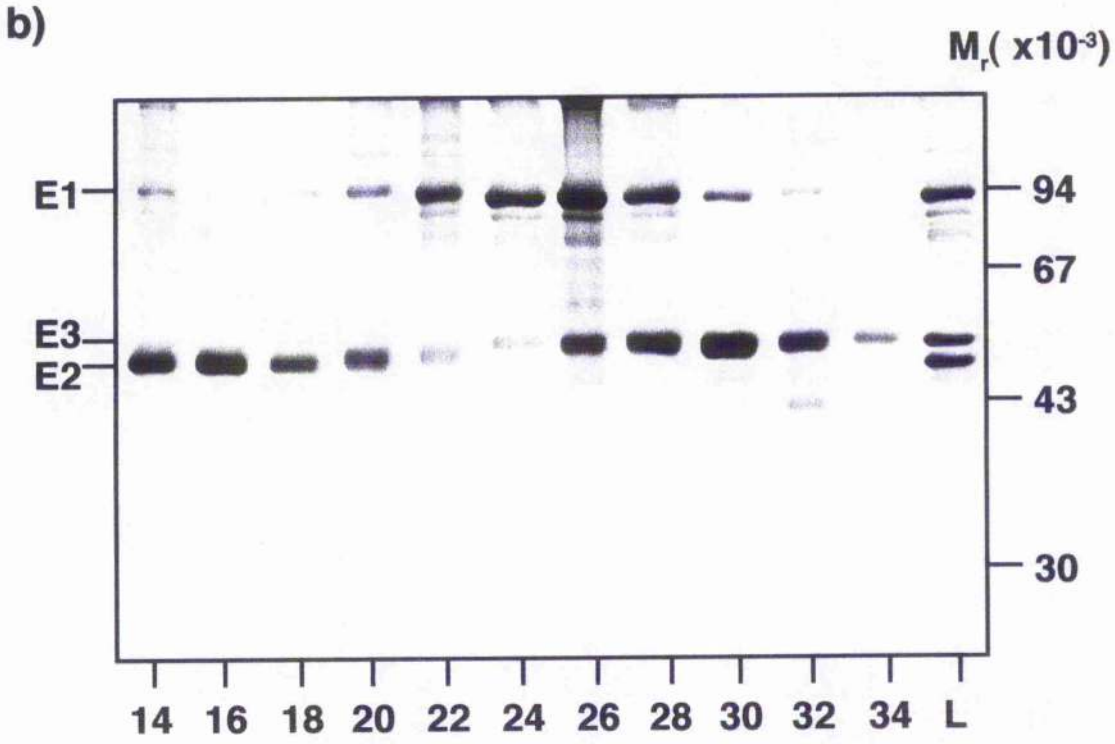


Figure 5.2b SDS-PAGE analysis of resolved OGDC fractions after dissociation on a Superose 6 column

Samples (1ml) of the eluent fractions were subjected to TCA precipitation prior to protein analysis by SDS-PAGE (10% w/v gel). Protein was stained with Coomassie blue. Lane L, OGDC column load (15µg); Lanes 14-34, TCA-precipitated samples from column fractions.

is possible to examine all three enzyme activities at once by adding amounts of the E1/E3 pool back to the E2 core, reconstituting overall complex activity (see experimental). The levels of reconstitution obtained can be expressed as a percentage of the activity of undissociated OGDC and thus provide an index of the degree to which the dissociation protocol has maintained the structural integrity of the components. SDS-PAGE analysis indicated, that the pooled, dialysed fractions were representative and exhibit no apparent cross contamination (Figure 5.3b). The data in Figure 5.3a illustrate the ability of the E2 and E1/E3 components to reconstitute OGDC activity up to 0.8 U/ml. This figure corresponds to recovery of approximately 30% OGDC complex activity, when compared to native untreated complex. Hence the components have retained their ability to bind each other and produce a native/near native OGDC capable of catalysis. Neither the E2 core nor the E1/E3 fraction were able to support OGDC activity by themselves, indicative of good fraction resolution during the dissociation and illustrating clearly that the recovery of OGDC activity measured results only from reconstitution of the complex (results not shown). Maximal levels of reconstitution were obtained with E2:E1/E3 ratios above 1:2. No increase in the levels of OGDC activity was obtained by adding additional quantities of E1/E3. In the case of OGDC reconstitution, maximal levels of overall complex activity are regained with sub-stoichiometric levels of E1/E3 (the native ratio being E2:E1/E3=1:3). The reason for this remains unclear, but may indicate the functional inactivation of a fraction of the E2 acyltransferase component in the preparation. Alternatively, this reconstitution of maximal OGDC activity with sub-stoichiometric quantities of E1/E3 may reflect the situation in the native enzyme where the ratio of 1:2 is indeed sufficient for maximal rates of catalysis. Optimal levels of reconstitution of mammalian PDC were previously obtained employing sub-stoichiometric levels of E1/E3 (Sanderson *et al.*, 1996b).

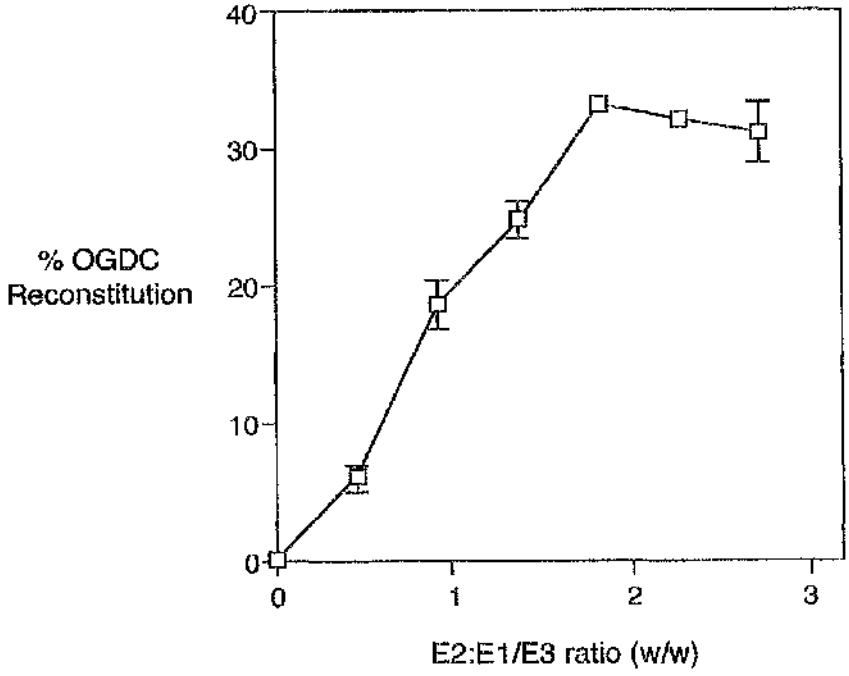


Figure 5.3a Reconstitution of overall OGDC activity from E2 core and E1/E3 fractions: levels of reconstitution and component stoichiometry

Following the dissociation of OGDC, the E2 and E1/E3 fractions were dialysed into 50mM MOPS/KOH, pH 7.4, 1mM EDTA, 50% (v/v) glycerol and stored at 4°C. E2 and E1/E3 fractions were pre-conditioned by a 1:1 dilution in 50mM potassium phosphate buffer, pH 7.6, 3mM NAD^+, 2.7 mM cysteine-HCl, 2mM $MgCl_2$, 0.2 mM ThDP, 0.02mM CoASH and incubated for 15min at 20°C. To initiate reconstitution, increasing quantities of E1/E3 (0-40μg) were added to cuvettes containing E2/X (10μg) and the appropriate assay solutions. The production of NADH was subsequently initiated by the addition of 14μl of 100mM 2-oxoglutarate. Activities are expressed as Units/ml.

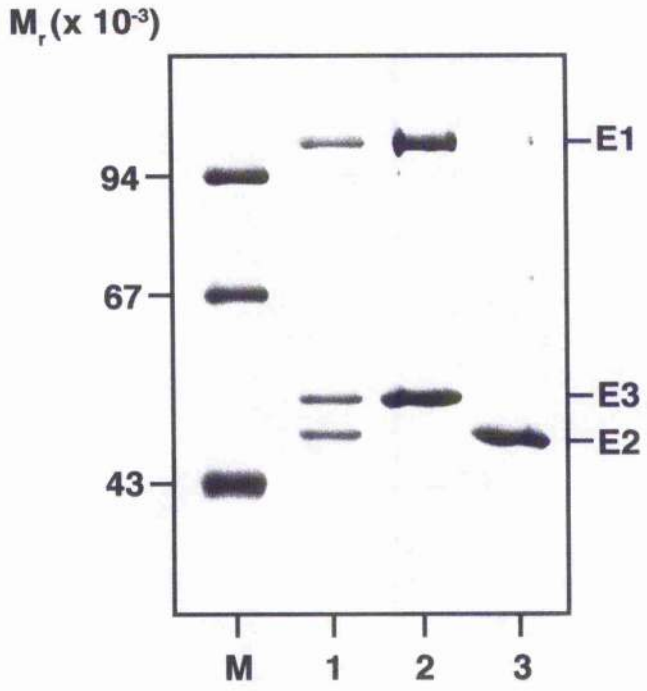


Figure 5.3b SDS-PAGE analysis of the pooled E2 core and E1/E3 components

Samples were analysed for purity by SDS-PAGE (10% w/v gel) and stained with Coomassie Blue. Lane M, 10μg low-M_r marker proteins; lane 1 intact OGDC (10μg; lane 2 E1/E3 fraction (15μg); lane 3 E2 core (15μg).

5.2.3 Association state analysis of the E1 and E3 components of mammalian OGDC

The levels of reconstitution of OGDC activity obtained were considered sufficient to validate any study of the direct interaction of the E1 and E3 components by gel permeation analysis. Following dissociation from the E2 core as detailed above, the E1/E3 pool was rapidly dialysed, employing multiple changes of buffer, into a low salt buffer (20mM MOPS/KOH, pH 7.0, 10mM KCl, 0.1% (v/v) Triton X-100) and 500µl of the pool applied to a Superose 12 column (24ml) already equilibrated in the low salt buffer (for details see Materials and Methods section 2.2.18). The monitoring of the column eluent at 280nm revealed the presence of two peaks of absorbance, not fully resolved to baseline levels (Figure 4a). Assays of the fractions collected from the column showed that the two absorbance peaks coincided with two eluted peaks of E3 activity (Figure 5.4a). The protein present in the two peak fractions was precipitated by TCA and subjected to SDS-PAGE analysis (Figure 5.4b). This revealed the presence of both E1 and E3 in the higher molecular mass peak (P1) and the presence of free E3 in the lower molecular weight peak (P2). The Superose 12 column was calibrated with a range of marker proteins and the column ran under identical conditions as detailed in figure 4. The elution profiles obtained were used to construct a calibration curve for the determination of the apparent M_r of the E1/E3 complex and free E3 peak eluted under the low salt associative conditions. The equation of the line of best fit was used to determine the apparent molecular masses for the two peaks (Table 5.1).

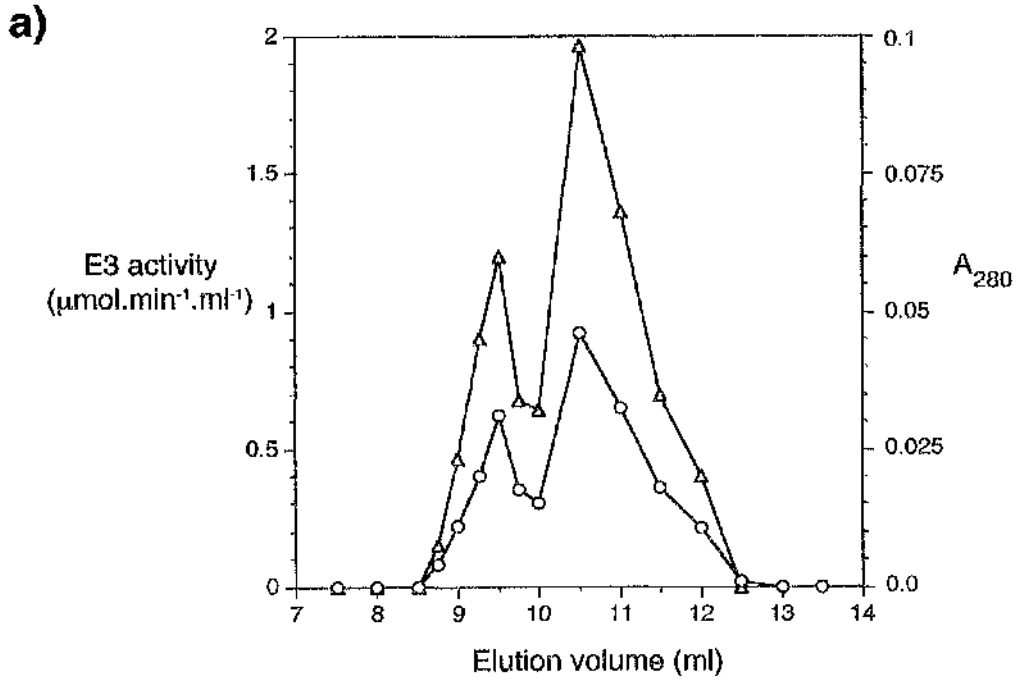


Figure 5.4a Elution profile of gel permeation chromatography analysis of the E1 and E3 components of mammalian OGDC

Association state analysis of the E1/E3 pool of OGDC was carried out using a Superose 12 column (for column conditions, see experimental). Protein elution was detected at 280nm (Δ) and assays for E3 activity (o) performed on the column fractions.

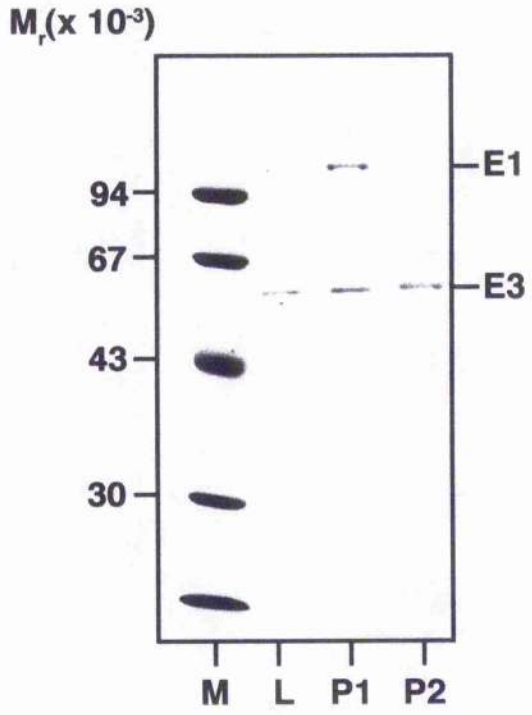


Figure 5.4b SDS-PAGE of peak fractions eluted from Superose 12 column

Peak fractions from the column were subjected to TCA precipitation and subsequent SDS PAGE analysis Lane M, low M_r markers; Lane L, E1/E3 load fraction; Lane P1, peak 1 fraction (E1/E3); Lane P2, peak 2 fraction (E3 only)

Table 5.1 Association state analysis of the E1/E3 components of mammalian OGDC

The Superose 12 column was equilibrated, at 0.3ml/min, in 20mM MOPS/KOH, pH 7.0, 10mM KCl, 0.1%(v/v) Triton Tx-100. E1/E3 samples were clarified for 15min at 10,000g in a benchtop centrifuge prior to loading onto the column. The column was calibrated with molecular weight marker proteins (Sigma) under identical conditions. The apparent M_r of E1 and E3 species eluted from the column were obtained by interpolation.

Peak	**Apparent M_r**	**SEM**	**n**
E1/E3	303,000	7500	3
E3	126,000	13500	3

Since E3 is a homodimer with a subunit M_r corresponding to 55,000, the figure of 126,000 obtained from the data above is in broad agreement with the expected value 110,000 , given that elution volume is dependent upon hydrodynamic volume as well as size. For the larger molecular mass peak, an apparent M_r value of 303,000 is in good agreement with the expected M_r of an E1/E3 complex of 302,000; representing one E3 dimer (110,000) bound specifically to one E1 dimer (192,000). The presence of this higher M_r complex, containing both E1 and E3 in apparent 1:1 ratio. represents the first direct biochemical evidence that the E1 and E3 components of mammalian OGDC physically interact with each other.

5.2.4 Immunological analysis of E1 antisera cross-reactivity

The evidence from the association state analysis details the interaction between E1 and E3 of mammalian OGDC. It would appear that E1 is responsible for E3 binding to the multienzyme complex. In this respect it is functionally equivalent to protein X in mammalian PDC and E2 in prokaryotes. Immunological analysis employing E1 anti-sera, clearly demonstrates a cross reactivity with the E2 component of mammalian PDC; as well as a strong specific response to the E1 component of OGDC (Figure 5.5, panel a). In addition to this it is possible, employing mammalian protein X antisera, to demonstrate a specific cross-reactivity with the E1 component of mammalian OGDC (Figure 5.5, panel b). Sequence similarities between E1 of OGDC and mammalian E2 and protein X have already been documented (Rice *et al.*, 1992). This immunological evidence indicates that such similarity extends beyond primary structure and that the three proteins share related antigenic epitope(s).

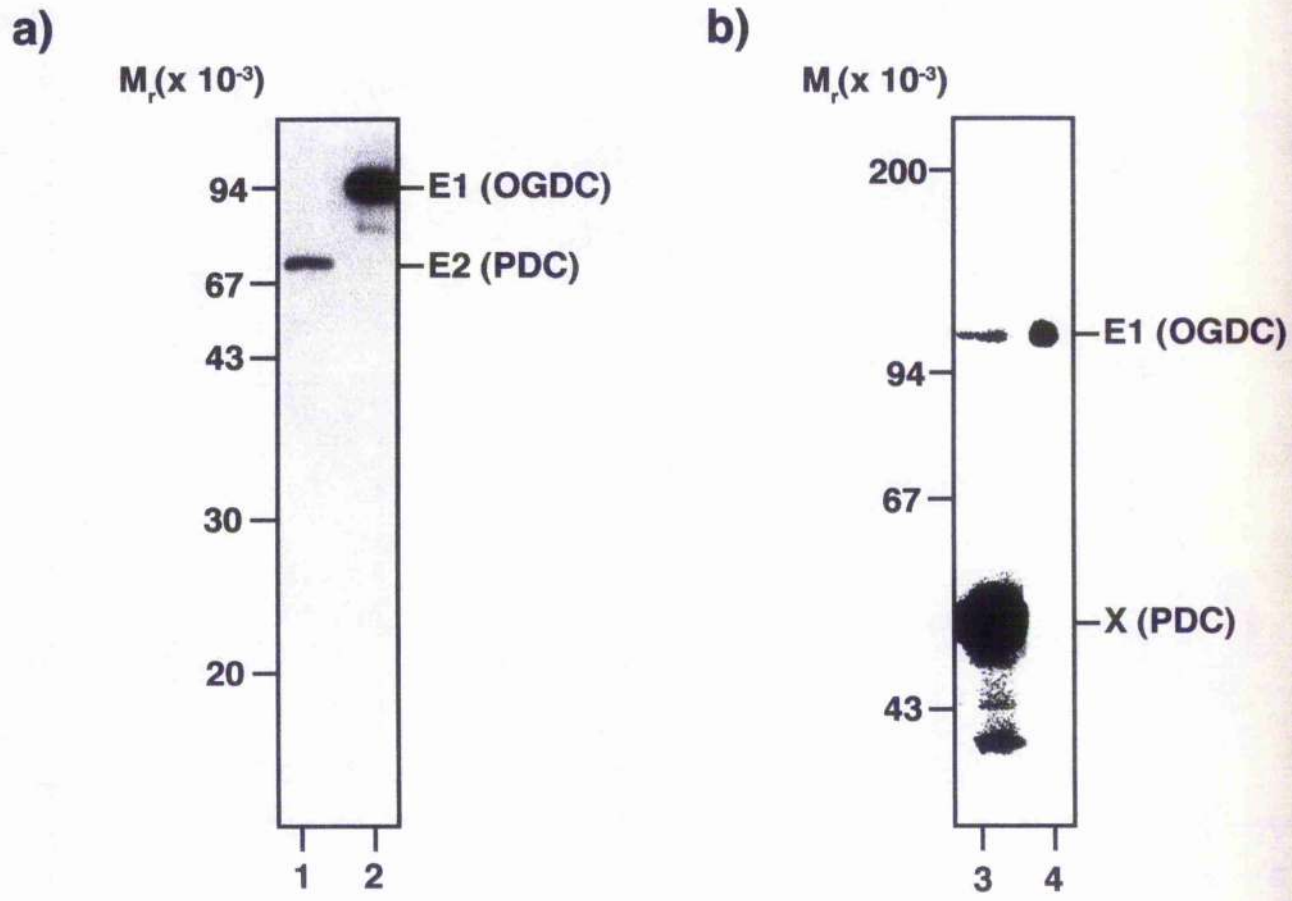


Figure 5.5 Immunological analysis of OGDC E1 cross-reactivity

Purified PDC and OGDC were subjected to SDS-PAGE and subsequent immunological analysis with anti-E1 (lanes 1-2, panel A) or anti-protein X (lanes 3-4, panel B) serum. identical quantities of each multienzyme complex was loaded (5µg). Lanes 1 and 3 mammalian PDC. Lanes 2 and 4 mammalian OGDC. (Performed by Dr S.J. Sanderson)

5.3 Discussion

Previous proteolytic studies and subsequent N-terminal sequencing located a lipoyl-like domain within the N-terminal region of E1 of mammalian OGDC. Selective proteolysis of E1 with trypsin led to the release of an N-terminal peptide (M_r 10 000) and abolition of E3 binding; indicative that the E3 binding capacity of mammalian OGDC rests within the E1 component (Rice *et al.*, 1992).

Initial protocol development to dissociate OGDC into E2 and E1/E3 fractions focused on the adaptation of the high salt dissociation protocol designed for the dissociation of PDC into its E2/X and E1/E3 fractions. While this met with some success in isolating the E3 component away from OGDC, the E1 component remained tightly associated with the E2 core (results not shown). Attempts to use low nondenaturing levels of GdnHCl were also unsuccessful. However, reactivation studies carried out on intact OGDC indicated that there was some effect on multienzyme structure following treatment with 1M $MgCl_2$ combined with mildly alkaline conditions (pH 7.6). Moreover, in contrast to more alkaline conditions, this did not abolish all of the OGDC activity. The successful dissociation, reported here, of mammalian OGDC into its E2 and E1/E3 fractions uses the combination of high levels of $MgCl_2$ and slightly alkaline pH to achieve optimum resolution. Under the dissociative conditions employed it is clear that the E1 and E3 components are not interacting, presumably as a result of the high ionic strength of the column running buffer.

An important criterion, on which the success of the OGDC dissociation was judged, was that it produced the components which maintained their native structure and were thus functionally active. Reconstitution analysis provides a good indication if this is achieved. The observed levels of reconstitution achieved (30%) are appreciably lower than those achieved with the E2/X and E1/E3 fractions of PDC of 60-80% (Sanderson *et al.,* 1996b). However given the functional overlap between OGDC E1 and PDC protein X the two reconstitution protocols are not equivalent, with considerably harsher treatment required to remove the E1 from the E2 core of OGDC. No method for removing protein X from the E2 core and maintaining its activity has been found. Given the possible functional overlap between the two proteins it is interesting to note that both E1 of OGDC and protein X of mammalian PDC are very tightly associated with their respective E2 cores. Recent structural studies of the E2/X core of *Saccharomyces cerevisiae* position the protein X binding sites at 12 large openings in the E2 core structure (Stoops *et al.,* 1997). Similar studies on mammalian OGDC may be employed to shed light on whether the E1 homodimers are positioned in a similar fashion.

Structurally, mammalian OGDC is built upon a structural E2 lattice which forms 24meric core exhibiting octahedral symmetry. The absolute number of E1 and E3 homodimers associated with the E2 core has not been determined in mammalian systems. However, studies of *E. coli* OGDC, which also has an octahedral 24meric E2 core, report a chain ratio E1:E2:E3 of 0.5:1.0:0.5 (Reed 1974). This suggests that the E1 and E3 may be present in equimolar ratio with 6 homodimers of each component associated with the E2 core of mammalian OGDC. Gel permeation analysis of the E1/E3 fraction, under low salt associative conditions, was successful in isolating a higher molecular mass complex (M_r 303 000) corresponding to a E1:E3 complex exhibiting 1:1 stoichiometry. The presence of an apparent excess of E3 may reflect loss of

some of the E1 component during the course of the protocol. Analysis of the rat and human succinyltranferase (E2) genes indicate that the domain typically involved in E3 binding is absent from the sequences.(Nakano *et al.*, (1991) and (1993). In human BCOADC E2, the enzyme with which the OGDC E2 genes exhibit a very high degree of similarity, the domain responsible for E3 binding is coded within a single exon (Lau *et al.*, 1992). It is likely that the loss of this exon, along with the surrounding introns occurred during the evolution of mammalian OGDC E2. However, the evidence that E1 can physically interact with E3 is indicative that this E3 binding role now rests within the 2-oxoglutarate component in mammals.

Subsequent immunological analysis confirmed that antisera, specific for the E1 component of mammalian OGDC, also recognises antigenic epitope(s) on mammalian E2 of PDC. Protein X antisera was also able to specifically detect OGDC E1. Digestion of OGDC, with the protease trypsin, has been shown to produce a very specific cleavage of the E1 component into an enzymatically active C-terminal E1' fragment (M_r 100, 000) and a stable peptide (M_r 10, 000). Interestingly, the peptide was responsible for the major proportion of the immunogenic response elicited; owing to its lipoyl-like structure (it is not however lipoylated) (Rice *et al.*, 1992). Indeed, high immunogenicity is a characteristic of lipoyl domains which are implicated in the auto-immune disease, primary billiary chirrosis (for a review, see O'Donohue & Williams 1996). Thus, the cross-reactivity observed, between the E1 OGDC antisera and the E2 of PDC, is indicative of some sequence similarity. Since the E3 (dihydrolipoamide dehydrogenase) component is common to all mammalian multienzyme complexes, it is not surprising that similar sequence motifs/domain structures appear to be present on the subunits involved in its binding.

In mammalian OGDC the binding of E3 to the complex is not mediated by the E2 component, as in other 2-oxoacid dehydrogenase complexes, since the protein lacks the domain involved in E3 binding. In contrast to PDC from higher eukaryotes, the E3 binding role in OGDC has not been transferred to a separate gene product (protein X). Instead, it would appear that the E1 of mammalian OGDC is a multifunctional enzyme; responsible for binding the E3 component to the complex and carrying out the oxidative decrboxylation of 2-oxoglutarate. Recent identification of the *odhA* gene from *Corynebacterium glutamicum* , coding for the E1 component of OGDC, revealed an N-terminal extension with sequence homology to E2s from PDC and OGDC, albeit to their C-termini (Usuda *et al.,* 1996). The authors intimate that this may represent a degree of bifunctionality in this E1 protein. Thus, the catalytic role of mammalian OGDC E1 in the oxidative decarboxylation of 2-oxoglutarate is supplemented by a second functional one of binding the E3 component to the multienzyme complex.

Chapter 6

Identification and cloning of bovine SP-22, a putative thiol specific antioxidant protein associated with mammalian PDC

6.1 Introduction

Since most organisms exist in an aerobic environment, there has been a need to develop mechanisms to prevent or limit damage to cellular components resulting from reactive oxygen species. To combat this, a variety of enzymatic and non enzymatic antioxidant systems have been developed. The list of molecules with antioxidant properties include glutathione (GSH), superoxide-dismutase, catalase, glutathione peroxidase. These and others, depending on their specific function and localisation, are involved in protection, prevention, interception and repair. Reactive oxygen species are generated naturally within the cell during respiration. However, conditions in which the levels of oxidants strain the capacity of the antioxidants to deal with them has been termed oxidative stress (Sies, 1997).

Although all the cellular compartments have to defend themselves against the deleterious effects of reactive oxygen, it is the mitochondrion more than any other organelle which is particularly vulnerable. This susceptibility primarily results from its central role in respiration. Indeed over 90% of the molecular oxygen utilised by the organelle is reduced to water (Stacpoole, 1997). During these reactions a number of potentially damaging molecular species are formed. The species formed as a result of the incomplete reduction of oxygen include: hydrogen peroxide (H_2O_2); the superoxide anion radical ($O_2\cdot^-$); the hydroxyl radical ($HO\cdot$) and hydroxyl anion (HO^-). There are also other ways to produce reactive oxygen including, light, UV radiation, redox-cycling drugs and the stimulation of host phagocytes (Chae *et al.,* 1994). Of further consideration is

that cellular processes also generate reactive sulphur species (RS, $RSSR^-$, RSOO) from thiol containing compounds capable of damaging cells.

The reactive oxygen and sulphur species damage all the major classes of biological molecules. Periods of oxidative stress can lead to DNA damage, both in terms of base modifications and strand breaks. Mitochondrial DNA has been shown to be particularly vulnerable, probably as it lacks the protection potentially afforded by histone proteins to the nuclear genome (Wallace, 1992). It is also possible to cause membrane disruption, lipid peroxidation, and protein oxidation. Of particular interest, are conditions which lead to the oxidation of proteins which have been implicated in a number of disease states, including Alzheimer's disease, Parkinson's syndrome, cancer and athrosclerosis (see Stacpoole, 1997 for review). What remains unclear is whether the protein oxidation observed in these pathological states represents a causative effect, or merely symptomatic. It is also not understood if such disease states arise from an increase in the levels of reactive species or by lesion(s) in the cellular defence mechanisms.

Another medically important condition, in which reactive oxygen species play a pathological role, involves tissue injuries which lead to deprivation of oxygen and other nutrients. This condition is termed ischaemia. Perversely, reperfusion of such tissues, notably brain and cardiac muscle, generates reactive oxygen species which contribute to the cycle of mitochondrial membrane/protein damage, leading to further free radical generation and more component damage (Halliwell & Gutteridge 1990; Bolli, 1988). In particular, PDC activity has been shown to be severly compromised following reperfusion, and sulphydryl oxidation has been implicated as the major cause of inactivation (Tabatabaie *et al.*, 1996).

The pyruvate dehydrogenase complex catalyses a key step in oxidative glucose metabolism. The product, acetyl CoA, can be utilised to drive a complete circuit of the tricarboxylic acid cycle, or used in other metabolic processes, notably fatty acid synthesis and, in neurological tissues, acetylcholine synthesis. PDC and PDC kinase have both previously been shown to have their thiodisulphide status altered by free radical agents (Crane *et al.*, 1983; Paetzke-Brunner & Wieland, 1980 and Petit *et al.*, 1982). General free radical scavengers do exist within mitochondria and indeed, roles have been demonstrated for glutathione (Reed, 1986) and superoxide-dismutase (Sies & Moss, 1978). However, potential requirement of additional thiol specific antioxidant systems within this compartment can be easily reconciled with the known sulphydryl group sensitivity af many mitochondrial enzymes, including the family of 2-oxoacid dehydrogenase multienzyme complexes.

In this chapter we identify SP-22, a recently discovered mitochondrial protein (Watabe *et al.*, 1994), and detail preliminary evidence for its association with component(s) of bovine PDC. The protein has been shown to protect free radical sensitive enzymes such as glutamine synthase and tryptophan hydroxylase *in vitro* (Watabe *et al.*, 1995). Recently the protein was proposed to be a thioredoxin-dependent peroxide reductase (Watabe *et al.*, 1997). However, to date limited evidence exists for any specific interaction. Also reported is the cloning of the protein from full length bovine brain cDNA, employing a PCR protocol and primers specific to the database sequence which has recently been published (Hiroi *et al.*, 1996). The possibility that this protein may be involved in the specific protection of components of PDC from adventitious oxidation is discussed.

6.2 Results

6.2.1 Identification of a 22kDa protein associated with mammalian PDC

Purification of PDC from the mitochondrial matrix is performed under associative conditions to ensure that the noncovalent assembly of individual enzymes remains intact (See Materials and Methods section 2.2.8). SDS-PAGE analysis of the purified complex, after high speed centrifugation and subsequent resuspension of the pelleted PDC, revealed the presence of a polypeptide (M_r 22 000) still associated with the multienzyme complex. This was somewhat surprising, given that the ultracentifugation step should only pellet large M_r complexes. Furthermore, preparative dissociation of PDC into its E2/X and E1/E3 fractions saw the partitioning of the polypeptide (M_r 22 000) with the E1/E3 fraction (for evidence of this and the association with PDC, see chapter 3 Figure 3.3b).

As a result of this, N-terminal amino acid sequence analysis was performed on the polypeptide (as detailed in Material and Methods section 2.2.6) in an attempt to identify it. N-terminal sequence analysis identified the first 24 residues of the polypeptide and an alignment was found to the N-terminus of the mature form of a protein previously sequenced at the amino acid level, bovine SP-22 (Watabe *et al.,* 1994). The sequence obtained for the unknown polypeptide exhibited a 86% identity in a 24 amino acid overlap (see Figure 6.1). The discrepancies between the two sequences occur among the penultimate residues in the N-terminal sequence for the unknown protein. This

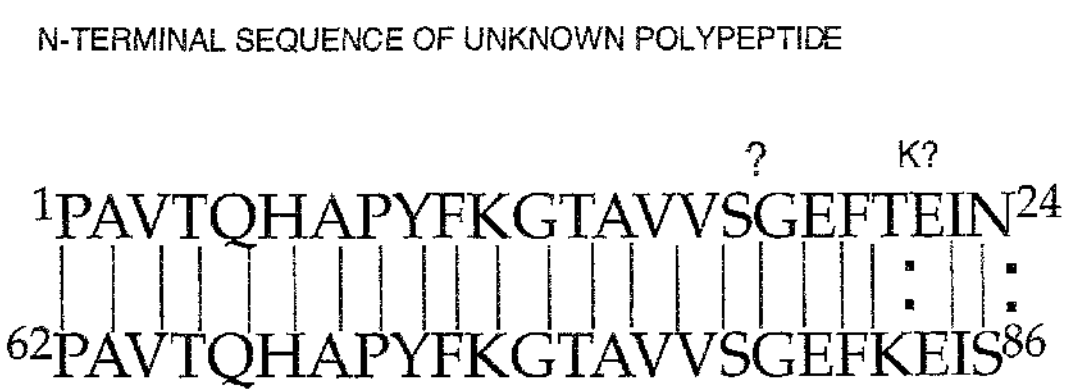


Figure 6.1 Alignment of N-terminal amino acid sequence of unidentified polypeptide with bovine SP-22

Matched amino acids are shown as joined by vertical black lines. Uncertain/alternative assignments are indicated with a questionmark and,where possible, the alternative residue indicated. The unknown polypeptide displayed an 87% identity to SP-22 in a 24 amino acid overlap.

probably results from difficulties in ascribing residues, either as a result of sample quantity, or build up of contaminants. However, the level of identity between the polypeptide (M_r 22 000) and bovine SP-22 was felt sufficient to make a positive identification.

SP-22 had been initially identified as a substrate for an ATP dependant protease (Watabe *et al.,* 1994). The study used the strategy of proteolytic digestion and amino acid analysis to produce a polypeptide sequence. However, a novel cysteine derivative, cysteine sulphonic acid (S-O_2H), was identified by FAB (fast atom bombardment) mass spectrometry. This led to speculation that the protein may perform a redox role.

Further details of the potential interaction between the polypeptide, now identified as SP-22, with components of the PDC complex came from the anion exchange protocol developed to separate the E1 and E3 enzymes (see Materials and Methods section 2.2.10). It has already been stated that SP-22 co-elutes with the E1/E3 fraction during high salt gel permeation chromatography. Subsequent anion exchange chromatography and SDS PAGE analysis of the fractions/pools demonstrate that the polypeptide remains associated with a fraction of the E3 eluted from the Mono Q column (See chapter 3, Figure 3.6b). The presence of SP-22, still associated with E3 of PDC, suggests that the two components interact. It may also indicate a possible thiol protection role for SP-22.

Sequence alignment of the amino acid sequence of SP-22 with proteins present in the data base enabled the definition of key conserved regions around the active cysteines, and indicated that SP-22 belonged to a family of antioxidant proteins already discovered and related to the mouse MER5 gene (Chae *et al.,* 1994 and Tsuji *et al.,* 1995). Figure 6.2 details the levels of identity exhibited

by the family/superfamily members with the MER5 gene product. It is clear that SP-22 has one of the highest levels of identity (86%) which extends over the entire polypeptide. Interestingly, SP-22 and MER5 are mitochondrially located proteins; in contrast to the majority of other family members. Recent alignment data (Hiroi *et al.*, 1996) shows that both the MER5 and SP-22 presequences have the propensity to form amphiphilic α helical structures of the sort readily associated with mitochondrial import and signal peptide cleavage (von Heijne *et al.*, 1989, and Rusch & Kendall, 1995). Another mitochondrial protein, Aop1, as well as showing a high degree of similarity to MER5, displays 88% identity to SP-22. This would indicate that the Aop1 protein represents the human homologue of SP-22. Expression of the Aop1 protein in a mutant *E. coli* strain, deficient in the alkylhydroperoxide reductase (ahpC), was sufficient to rescue resistance to alkylhydroperoxide (Tsuji *et al.*, 1995).

Figure 6.2 also demonstrates the conserved nature of the region surrounding the two potentially catalytically important cysteines. Region 1, consisting of seven amino acids around cys 47 in the mature form of SP-22, appears to be absolutely conserved, except for a single amino acid variation in the Arch protein in *M. thermoautotrophicum*. This residue has been shown to be a sulphonic acid derivative (Cys-S-O_2H) in SP-22 by fast atom bombardment and mass spectrometery of the appropriate peptide fragment (Watabe *et al.*, 1994). Recent speculation (Watabe *et al.*, 1995) is that this represents an active Cys-S-OH two electron redox centre *in vivo* . Although the level of amino acid conservation around region 2 is lower, it remains nonetheless significant and may indicate a second active thiol centre within the polypeptide.

Gene	Organism	%Indentity	Region1	Region2	Ref
C22	*S. typhimurium*	38	TFVCPTE	GEVCPAK	Jacobson *et al.*, 1989
26kDa	*H. pylori*	49	TFVCPTE	GEVCPAG	O'Toole *et al.*, 1991
ORF3	*C. pasteurianum*	54	TFVCPTE	GGMCALD	Mathieu *et al.*, 1992
TSAS. *cerevisiae*		58	TFVCPTE	VTVLPCN	Chae *et al.*, 1993
29kDa	*E. histolytica*	47	TFVCPTE	GAVCPLN	Torian *et al.*, 1990
Arch	*M. thermoautotrophicum*	30	TFVCTTE	GVAAPAN	Takao et al., 1990
Aop1	*H. sapiens*	86	TFVCPTE	GEVCPAN	Tsuji *et al.*, 1995
Msp 23	M. musculus	64	TFVCPTE	GEVCPAG	Ishi *et al.*, 1993
PAG	*H. sapiens*	65	TFVCPTE	GEVCPAG	Prosperi *et al.*, 1993
SP-22	*B. taurus*	85	TFVCPTE	GEVCPAN	Hiroi *et al.*, 1996

Figure 6.2 Comparison of two conserved regions around key cysteine residues in the MER5 family

The alignment was carried out using BLAST. Identity values between MER5 and the other gene products represent the best matched alignment. Seven residues from around conserved cysteines are shown. (Adapted and updated from Tsui *et al.*, 1995)

6.2.2 The cloning of bovine SP-22: PCR amplification and product purification

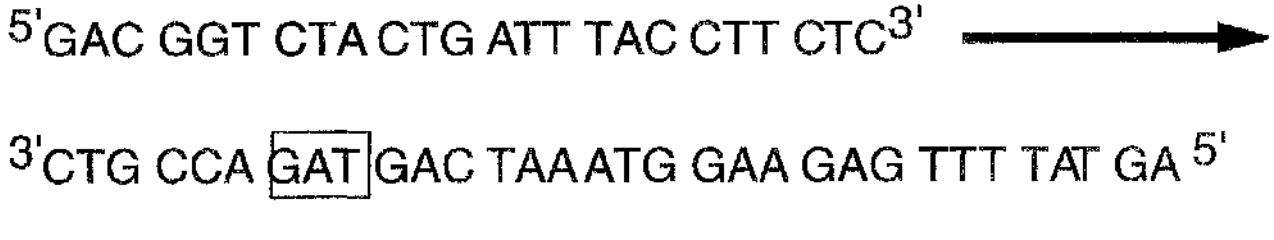
In order to further study the SP-22 protein it was felt necessary to obtain a clone. The protein had been previously purified from bovine adrenal cortex (Watabe *et al.,* 1994), although the same study had detected its expression in other tissues, including liver and heart, by radioimmunoassay. Since the protein was expressed at high levels in bovine brain, full length bovine brain cDNA was chosen as a suitable substrate for the specific amplification of the SP-22 coding sequence. This was commercially available (Clonetech) and removed the need to prepare cDNA by mRNA isolation and cDNA synthesis. A search of nucleotide sequence databases was successful in locating the cDNA sequence coding for the protein, despite the absence of any such sequence in the published literature. This enabled the design of specific primers. Figure 6.3 details the primers which were designed to the 5' region upstream of the ATG start site and to the 3' region of the coding sequence including the translation stop codon of SP-22. The primers were designed to have similar dissociation temperatures and GC contents in the recommended 40-60% region. The primer sequences were also checked for both inter and intra primer complementarity using Gene Jockey software. In particular the 3' regions were compared to reduce the possibility of primer-dimers. In order to minimise the tolerance of mismatch neither primer had a T at its 3' terminus.

The PCR reactions were performed with the Expand High Fidelity PCR system (Boehringer Mannheim) in an attempt to minimise base misincorporation. The system contains, as well as *Taq* polymerase, *Pwo* DNA polymerase which exhibits 3'-5' exonuclease proof-reading activity. Mixtures

SP-22 primer 1

SP-22 cDNA

SP-22 primer 2



SP-22 cDNA

Primer	length (bp)	%GC	Tm (°C)
Primer 1	20	55	60
Primer 2	24	46	60

Figure 6.3 Specific primers designed to amplify the coding region of SP-22 (820bp) from bovine cDNA.

Primers were designed using Genejockey software to amplify the entire SP-22 coding region by PCR. Included in the primer pair are translation start and stop codons (indicated as boxed regions in the sense cDNA).

of the two thermostable enzymes have been shown to reduce the error rate at least three fold; while maintaining high yields of PCR product. PCR amplification was performed using the bovine brain cDNA and the primer pair detailed in Figure 6.3 (details of the PCR protocol are given in Materials and Methods section 2.4.5). Figure 6.4 illustrates the product obtained by PCR (Lane 3) migrating on a 2%(w/v) agarose gel. The absence of reaction component contamination was confirmed by the lack of amplified product in the control reaction without any template DNA (Lane 2). However, the PCR reaction components were functional; i.e. capable of generating the expected 200bp fragment, using specific primers, from the pBluescript plasmid (Lane 1). The PCR reaction with bovine cDNA template resulted in the production of a number of DNA products of varying sizes (apparent sizes 270, 800 and 820bp. Lane 3). No primer-dimers were detected. The major product had an apparent size of 820bp, identical to that predicted from the database cDNA sequence for SP-22 (Figure 6.4). The presence of smaller amplified fragments may result from mispriming events during early cycles. Alternatively, amplification may reflect the presence within the template DNA of other sites, possibly coding for homologues of SP-22, for which the primers have the ability to anneal under the conditions of the PCR.

The large 820bp fragment was excised from an agarose gel, under UV, using a sterile scalpel . The DNA was then purified by means of a GenElute agarose spin column (Supelco) and subsequent ethanol/sodium acetate precipitation (for full details refer to Materials and Methods sections 2.4.3 and 2.4.6). An aliquot of the resuspended DNA was subjected to electrophoresis on a 1.5% (w/v) agarose gel to determine the quantity and quality of PCR product recovered. Figure 6.5 shows that the PCR product appears as a single band, running slightly bigger than the 800bp marker and that no contamination with other DNA fragments is apparent. A low M_r ladder (Gibco) was used to

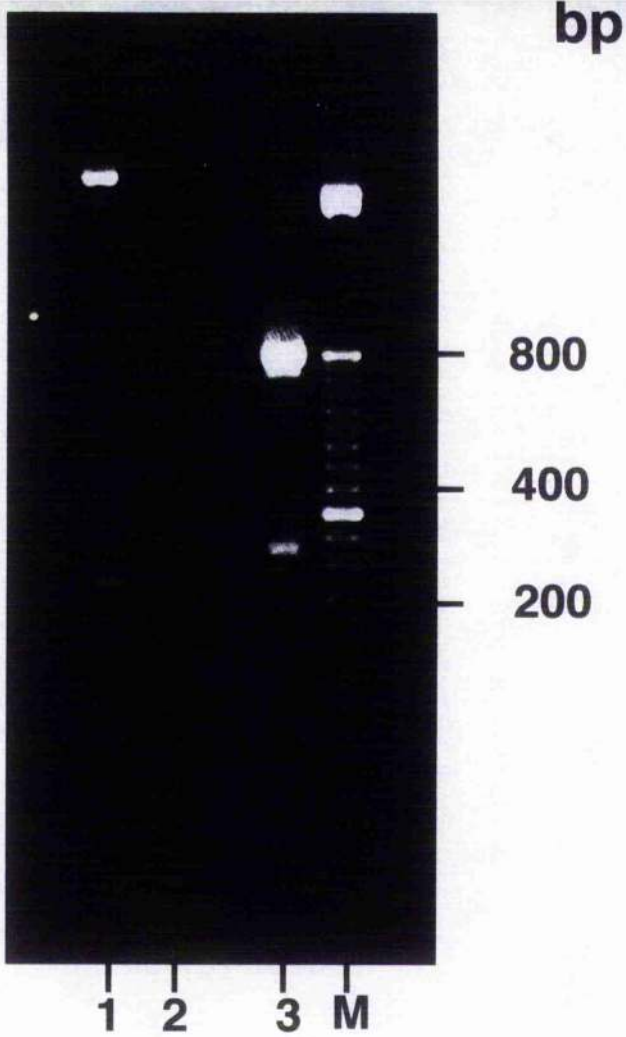


Figure 6.4 PCR amplification of the nucleotide sequence encoding SP-22 from bovine brain cDNA

Bovine brain cDNA (Clonetech) served as a template for amplification using the Expand PCR System (Boehringer Mannheim). The PCR reaction contained: 1ng of cDNA, 0.2µM of each primer 1 and primer 2; 200µM dNTPs; 1.5mM $MgCl_2$; 2.6U of expand high fidelity PCR system enzyme mix. The PCR cycling conditions were as follows: 1 x 5min at 95°C; 30 x [55°C for 3min; 65°C for 3min; 95°C for 30sec]; 1 x 65°C for 2min; 1 x 72°C for 5min. The products were analysed on a 2% (w/v) agarose gel.

1: Positive DNA control (pBluescript + M13 forward and reverse primers), 2: Negative DNA control (template DNA omitted), 3: Bovine brain cDNA template, M: 50 base pair ladder.

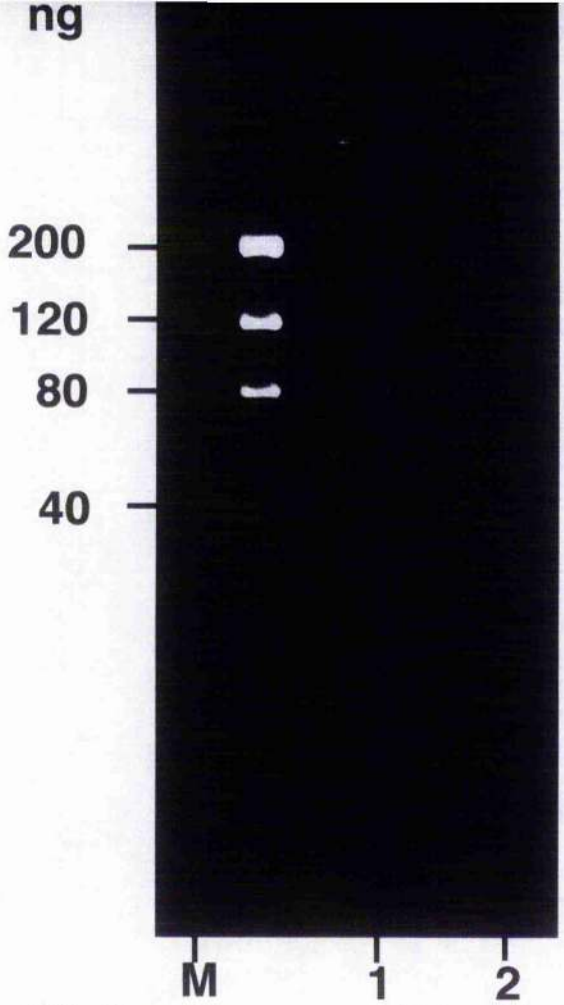


Figure 6.5 Quantification of recovery and size determination of amplified DNA fragment

The major product of two PCR amplification reactions with bovine cDNA (as detailed in Figure 6.4) were excised from an agarose gel using sterile conditions. The DNA was eluted from the agarose plug using Gen Elute columns (Supelco) and precipitated using sodium acetate/ethanol. For quantitative recovery of DNA, the DNA pellets were resuspended overnight in 20µl of TE buffer at 4°C. Samples (5µl) of each purification were analysed on a 1.5%(w/v) agarose gel.

M: Mass ladder 200, 400, 800, 1200, 2000 base pairs (corresponding to 20,40, 80, 120 and 200ng of DNA, lanes 1+ 2 Duplicate samples of DNA (5µl) recovered and prepared as detailed above.

estimate that an approximate yield of 40ng of DNA in the 5µl of resuspended DNA loaded from two purification procedures. Thus the total recovery of DNA in 40µl was 320ng.

6.2.3 The cloning of bovine SP-22: product ligation, bacterial transformation and transformant identification

DNA fragments amplified using *Taq* polymerase typically exhibit single deoxyadenosine (A) overhangs at their 3' termini. While this facilitates cloning of PCR fragments into plasmid vectors which can be cleaved to generate 3' T overhangs (TA cloning kit Invitrogen), the cloning of such fragments in a blunt ended fashion requires a prior polishing step. To this end, the pCR-Script Cloning Kit (Stratagene) was chosen as a suitable vector system to ligate in the purified PCR fragment containing the coding region of SP-22 (Figure 6.6). Clones which contained the vector could be selected for by plating out on agar plates which contained the antibiotic ampicillin. Detection of positive clones which contained an insert was afforded by blue/white screening on an IPTG/X-Gal background. The vector also contained transcription initiation sites (T7 and T3). These would allow the detection of clones which were capable of producing a protein product in an *in vitro* transcription/translation system (see later).

Due to the inefficient nature of blunt end ligation reactions it is important that the insert:vector ratio is high (typically between 40:1 and 100:1). Unfortunately, in this instance the insert DNA was too dilute to deliver such ratios in the ligation reaction; to permit adherance to the volume limits of the polishing step. Therefore, the ligation reactions were performed with

insert:vector ratios of 5:1 and 10:1. This represented the only change with the manufacturer's protocol (see Materials and Methods, section 2.4.7).

Following fragment polishing, ligation into pCR-Script and transformation into Epicurian Coli XL1-Blue MRF' Kan supercompetent cells was performed. Subsequently, bacteria were plated out on LB/agar plates containing ampicillin (50μg/ml), X-gal (20μl of 10% (w/v) stock) and IPTG (20μl of 0.2M stock). The plates were left overnight at 37°C to allow for colony development. Examination of the plates revealed the presence of bacterial colonies, with >90% of the colonies appearing white (transformed) under the conditions of the IPTG/X-Gal detection system . Twelve clones (C1-C12) were picked from a suitable plate and grown in 5ml LB/ampicillin (25μg/ml) suspension cultures overnight. Following this, plasmid DNA from the twelve overnight cultures was prepared using the Wizard Mini plasmid prep system (Promega).

Analysis of yield and plasmid purity is detailed in Figure 6.6. Typically, values of 1.8 to 2.0 for 260/280nm ratios represent relatively pure preparations of DNA/RNA. The plasmid samples prepared using the Wizard mini preps give variable values ranging between 1.2-3.0. This probably represents contamination of the plasmid preparations with protein as a result of the alkaline lysis based protocol. Phenol, which also absorbs at 280nm, is not used in the protocol so possible sample contamination with this can be eliminated. However, the yields of plasmid DNA are generally very high (15-20μg) Indeed, this may be part of the problem with the purity levels observed. The yields of plasmid DNA are known to depend on the plasmid copy number and the host strain. The high copy number of pCR-script (which contains the *colE1* origin of replication) probably contributes to the high yields, but compromised purity, of the plasmid DNA samples.

Clone	Abs 260/280nm	DNA concentration (µg/µl)	DNA recovered (µg)
C1	1.35	0.38	17.0
C2	1.48	0.36	16.3
C3	1.45	0.41	18.3
C4	1.50	0.54	24.4
C5	1.46	0.46	20.7
C6	1.30	0.27	12.2
C7	1.30	0.42	19.1
C8	1.34	0.35	15.8
C9	2.60	0.26	11.8
C10	1.84	0.42	18.0
C11	1.16	0.38	17.1
C12	1.30	0.47	21.2

Figure 6.6 Wizard minipreps of plasmid DNA from overnight cultures grown from selected transformant colonies

Plasmid DNA prepared according to manufacturer's instructions

It was also observed that two of the overnight cultures (C9 and C6) did not grow to the same density as the other cultures. This may explain the relatively low plasmid yields obtained from these two cultures. Glycerol stocks were prepared from all the overnight cultures and stored at -80°C. The plasmid preparations were also stored at this temperature.

6.2.4 Identification of clones containing the correct insert: plasmid single digest.

Having purified plasmid from potential SP-22 transformants, as determined by blue/white selection on IPTG/X-gal containig media, a strategy was developed which would identify those clones which contained an insert. Since the control plasmid (pBluescript Stratagene) exhibits a size (2960bp) almost identical to the vector into which the purified PCR product was ligated pCR-script (2961bp), the presence of larger linearised plasmids should indicate that these clones contain inserts. A suitable restriction enzyme for this would: be efficient at cleaving supercoiled plasmids; have a unique restriction site within the plasmid; would lack a restriction site within the cloned insert. The restriction enzyme Bam H1 was identified by restriction analysis of the plasmid and insert sequences, using the programme Gene jockey, as fulfilling the criteria outlined above. It was used to digest samples of control plasmid and the purified plasmid from clones 1-12 (C1-C12). The products of the digestion and samples of uncut plasmid were analysed by electrophoresis on a 0.8% (w/v) agarose gel (Figure 6.7 panels 1-3).

The assay utilises the fact that a supercoiled plasmid usually migrates more rapidly through the agarose gel matrix than its linearised counterpart. In Figure 6.7 (panel 1) all the plasmids appear to be cleaved by Bam H1; the majority of

the linear versions (lanes 6, 7, 10, and 11) exhibit the same size as the cut control pBluescript (lane C). However, it is evident that the supercoiled version of the C3 clone (lane 4) migrates to the same position as the linearised control plasmid and that cleavage of C3 produces a linearised product much larger than the control (lane 9). The presence of salt in the digest samples probably results in the poor resolution of the DNA markers, thus preventing a more quantitative determination of the plasmid sizes. In addition the cleaved plasmid product from the C4 clone produces two products (lane 8), one of which is larger than the linearised control vector. It may be that, in this clone, there are two populations of plasmid, one of which contains an insert.

The restriction digest used to examine clones1-6 (C1-C6) was also employed to analyse the remaining clones 7-12 (C7-C12). Figure 6.7 (panel 2) details the electrophoretic analysis of the assay. Of the plasmids analysed in Gel 2 only the C7 (lanes 5 and 6) clone contains a linearised plasmid which is bigger than the control. There is also some evidence that the digestion of the plasmid purified from clone C8 (lanes 3 and 4) did not go to completion. There also appears to be very little plasmid present in the preparation from clone C9 (lanes 1 and 2). This concurs with the data for a low plasmid yield (0.26µg/µl) from the clone presented in Figure 6.6. Analysis of plasmid digests in Figure 6.7 (panel 3) reveals the presence of another clone C10 (lanes 5 and 6) which may contain an insert. However it does not appear to be as large as the other insets detected.

Figure 6.7 BamH1 restriction analysis of the pCR-Script plasmids, potentially containg the coding region of SP-22, purified from clones C1-C12

4µl plasmid DNA (1.1-2.1µg) was digested in each reaction with 12U BamH1 (Boehringer Mannheim) in the appropriate buffer (10mM Tris/HCl, pH 8.0, 100 mM NaCl, 5mM $MgCl_2$, 1mM 2-mercaptoethanol) at a final volume of 20µl for 2h at 37°C. Incubations of control (uncut) plasmid took place in the same buffers and under the same conditions in the absence of BamH1. After 2h, 5µl sample loading buffer (40% (w/v) sucrose, 0.25% (w/v) bromophenol blue) was added to each reaction and samples subjected to analysis by 0.8% (w/v) agarose gel electrophoreisis.

Panel 1

1: C6 undigested, 2: C5 undigested, 3: C4 undigested, 4: C3 undigested, 5: C2 undigested C: control (pBluescript digested), 6: C6 digested, 7: C5 digested, 8: C4 digested, 9: C3 digested, 10: C1 digested, 11: C2 digested, 12: C1 undigested, M: Hind III digest of phage λ DNA

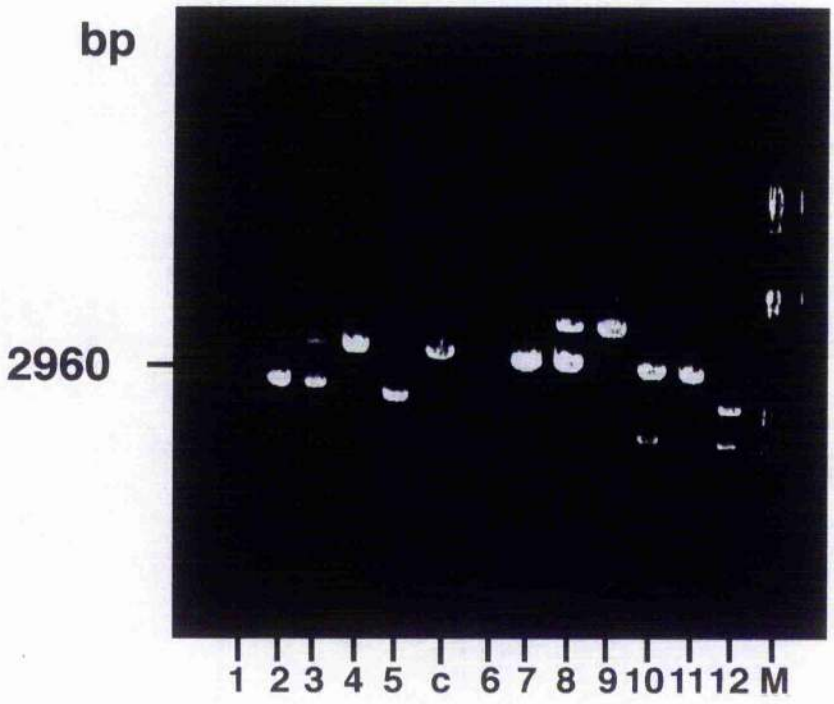
Panel 2

C: control (pBluescript digested), 1: C9 digested, 2: C9 undigested, 3: C8 digested, 4: C8 undigested, 5: C7 digested, 6: C7 undigested, M: Hind III digest of phage λ DNA

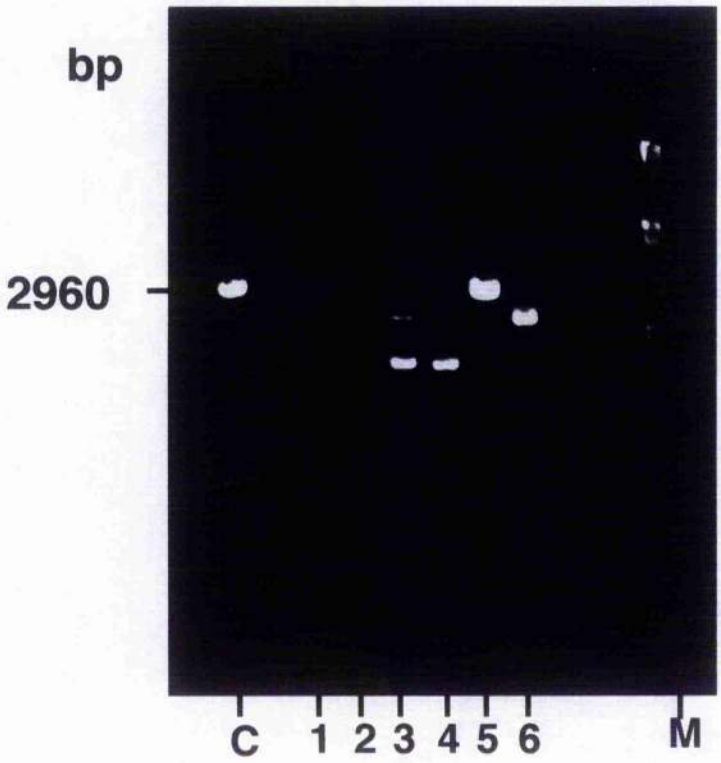
Panel 3

C: control (pBluescript digested), 1: C12 digested, 2: C12 undigested, 3: C11 digested, 4: C11 undigested, 5: C10 digested, 6: C10 undigested, M: Hind III digest of phage λ DNA

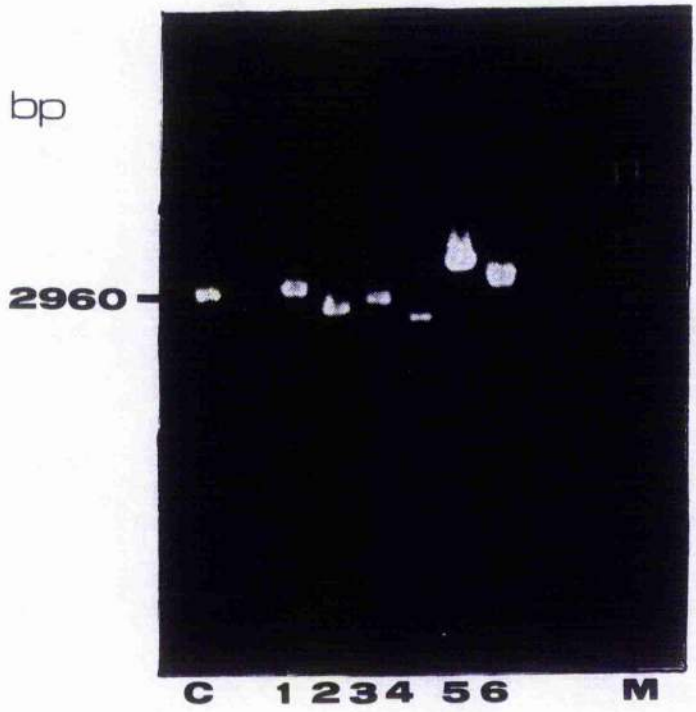
Panel 1



Panel 2



Panel 3



6.2.5 Identification of clones containing the correct insert: excision of insert by double digestion

While the single plasmid digest assay had been successful in the possible identification of clones which contained an insert, the results remained inconclusive. In order to identify clones containing an insert of the expected size it is possible to reamplify the fragment inserted within pCR-Script using PCR and the original SP-22 primers. Two disadvantages of this method are (i) possible contamination of the template DNA with salts/ions which could inhibit the PCR (ii) the need to re-optimise the PCR conditions. A simpler and quicker approach employs excision of the insert using two restriction enzymes selected for unique clevage sites at the immediate 5' and 3' loci of the insert. These enzymes, if no cleavage sites are present within the insert DNA, should release the insert fragment and allow its size to be determined. The restriction enzymes Not1 and Xho1 were chosen as suitable partners for a double digest. They exhibit their endonuclease activity in similar ionic strength and are able to cut supercoiled plasmid DNA efficiently. Both enzymes cleave within the multiple cloning site of pCR-script (Figure 6.6); specifically Not 1 clcavagc is 5' with respect to insert DNA and Xho1 cleavage is 3'. The nucleotide sequence for the coding region displays no suitable cleavage sites for either enzyme.

Double digest restriction analysis of purified plasmid from clones C1-C6 is presented in Figure 6.8a. The inclusion of a linearised pBluescript control (lane 10) illustrates that all digests have been successful in producing linear versions of the plasmids purified from the six clones. The double digest released an insert from plasmids derived from the C3 and C4 clones. The size of this product would appear to be approx. 840bp, as determined by its migration on a

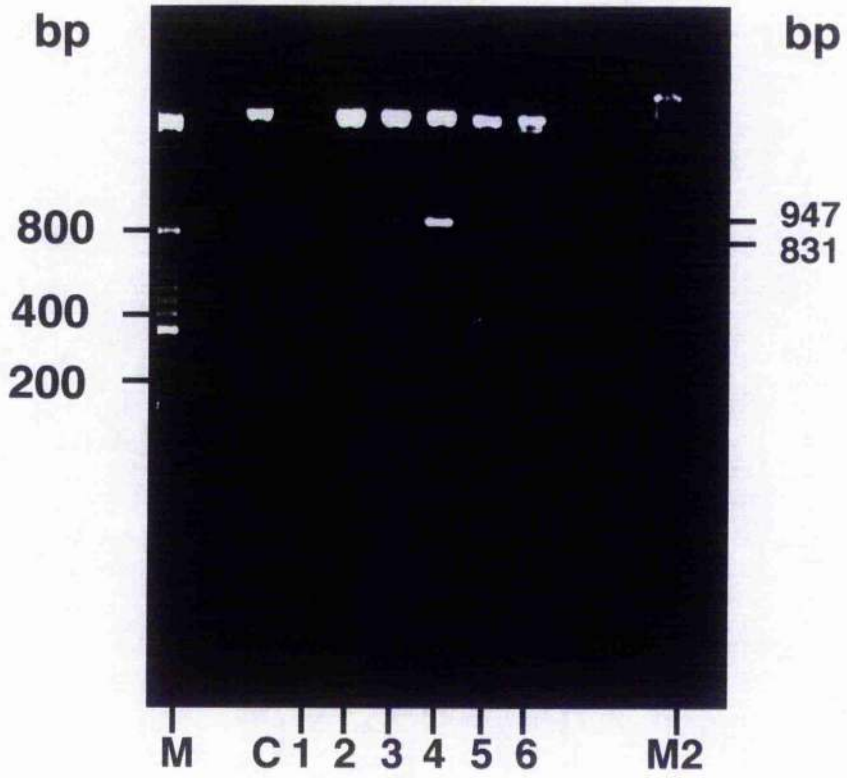


Figure 6.8a Restriction analysis, employing a Not 1/Xho 1 double digest, of plasmids derived from clones C1-6

3µl of plasmid DNA (0.8-1.5µg) was digested in each reaction with 10U Xho 1 and 5U Not 1 (New England Biolabs) in 50mM Tris/HCl, pH 7.9, 100mM NaCl, 10mM $MgCl_2$, 1mM DTT, supplemented with BSA to a final concentration of 100µg/ml. Digestion was in a final volume of 50µl at 37°C for 2h. Subsequently, 10µl agarose sample buffer was added to each reaction tube and the samples subjected to analysis on a 2% (w/v) agarose gel.

M1: 50 base pair ladder, C: Control (pBluescript digested), 1: C6, 2: C5, 3: C4 , 4: C3, 5: C2, 6 C1, M2: DNA III molecular mass markers (Boehringer Mannheim).

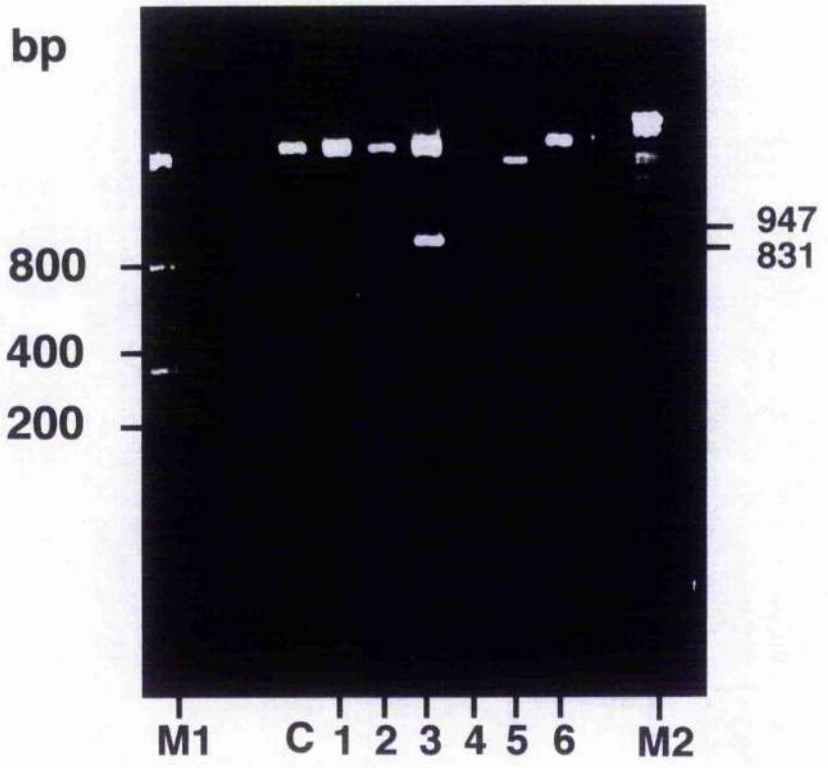


Figure 6.8b Restriction analysis, employing a Not 1/Xho 1 double digest, of plasmids derived from clones C7-12

3µl of plasmid DNA (0.8-1.5µg) was digested in each reaction with 10U Xho 1 and 5U of Not 1 (New England Biolabs) in 50mM Tris/HCl, pH 7.9, 100mM NaCl, 10mM $MgCl_2$, 1mM DTT, supplemented with BSA to a final concentration of 100µg/ml. Digestion was in a final volume of 50µl at 37°C for 2h. Subsequently, 10µl agarose sample buffer was added to each reaction tube and the samples subjected to analysis on a 2% (w/v) agarose gel.

M1: 50 base pair ladder, C: Control (pBluescript digested), 1: C12, 2: C11, 3: C10 , 4: C9, 5: C8, 6 C7, M2: DNA III molecular mass markers (Boehringer Mannheim).

2% (w/v) agarose gel. The inclusion of some of the plasmid linker region DNA on the fragment excised provides an explanation for the apparent increase in insert size over the expected 820bp product. It is noticeable that considerably less of the fragment is produced in the C4 digest (lane 3) in comparison to the C3 one (lane 4). The low quantity of plasmid DNA derived from C6 derives from its poor growth in overnight culture and subsequent low plasmid yield (Figure 6.6)

Identical double restriction digest analysis was performed on plasmids purified away from the remaining six clones (C7-C12) (Figure 6.8b) . In this instance, a further single clone, C10 (lane 3) was identified as containing an insert of between 831 and 947bp. These results taken together with the single plasmid digests performed earlier strongly indicate that Clones C3, C4, and C10 all contain the desired insert, coding for the entire amino acid sequence of the antioxidant protein SP-22.

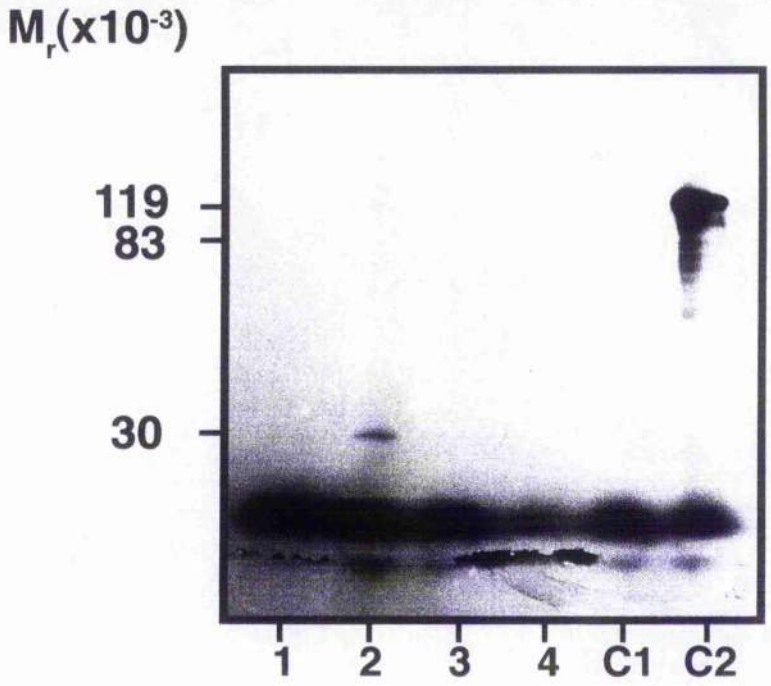
6.2.6 *In Vitro* expression of cloned SP-22:Insert orientation analysis

The results from the restriction analysis of the clones C3, C4 and C10 has identified these clones as containing the appropriate insert. However, as the cloning of the PCR product into the pCR-script vector was achieved by blunt end ligation, the orientation of the clones within the plasmid remains uncertain. One method to define the orientation involves a series of restriction enzyme double digests, using at least one enzyme which cuts within the SP-22 nucleotide sequence. The analysis of the results of the various enzyme permutations would allow determination of the insert orientation.

A more elegant and clear-cut alternative to insert orientation is the use of a transcription/translation reaction system, specific for one of the two transcription initiation sequences which flank the insert site. An additional advantage is this would also allow the identification of clones which contain the correct translation start and stop codons and are capable of producing a protein product. To this end the single tube protein system (Novagen) was employed. The system can be used for the direct synthesis *in vitro* of protein products from supercoiled plasmids. Transcription is dependent upon the presence of a bacteriophage T7 RNA promoter. The pCR-Script vector contains this promoter 3' relative to the insert site.

The *in vitro* transcription/translation reaction was carried out (see Materials and Methods section 2.4.12) on four of the clones C2, C3, C4, C10. The first control reaction was an *in vitro* transcription/translation reaction carried out in the absence of any DNA template. Also included, was the control reaction supplied by the manufacturers; STP2 T7 control DNA which produces a 119 000 M_r product. The products of the reactions were labelled by the incorporation of [^{35}S] methionine into the protein product. Detection of the radiolabelled products was achieved, following resolution by SDS PAGE, by fluorography.

Figure 6.9 illustrates the production of a protein band, slightly less than 30 000 M_r , in the reaction which included plasmid DNA from C3 clone as a template (Lane 2). It has already been stated that the mature form of the SP-22 protein, appears as a 22 000 M_r band on a 10% (w/v) SDS-PAGE gel. However, the sequence cloned includes the nucleotide sequence which codes for the mitochondrial import pre-sequence. This adds a further 60 amino acids to the protein product , giving an expected mass of 28 000 M_r. This is consistent with the protein product formed from C3 plasmid DNA (lane 2). No other protein



6.9 SDS-PAGE separation and fluorographic detection of the [^{35}S]-methionine labelled products derived from *in vitro* transcription/translation reactions, employing plasmids purified from clones C2, C3, C4 and C10 as template DNA.

In vitro transcription/translation using plasmids from the clones detailed above was carried out using the single tube protein system (Novagen) according to manufacturer's instructions (see Materials and Methods section 2.4.12). Protein products were labelled by the inclusion of [^{35}S]-methionine in the translation reaction. Reactions (5µl each + 20µl Laemmli sample buffer) were subjected to SDS PAGE on a 10% (w/v) gel. The gel was fixed in 25% (v/v) isopropanol: 65% (v/v) distilled water: 10% (v/v) acetic acid for 30 min and rinsed with distilled water. The gel was immersed and agitated slowly for 30 min in Amplify (Amersham Life science) to aid fluorographic detection before being dried under vacuum. Products were detected on Hyperfilm ECL (Amersham).

1: C2, 2: C3, 3: C4, 4: C10, C1: Control reaction 1 (no template DNA), C2: Control reaction 2 (STP2 T7 control DNA)

products were detected in the reactions derived from plasmid DNA purified from the putative clones. While clone C2 (lane 1) was included only as a negative control (previous analysis had indicated that it did not contain any insert), the absence of any product corresponding to clones C4 (lane 3) and C10 (lane 4) may provide evidence that the inserts present in these clones do not function from the T7 promoter. Instead, it is probable that they are inserted in the reverse fashion.

The control reaction in the absence of template DNA (lane 5) did not produce any protein product of the correct size. This demonstrates that there are no DNA substrates endogenous to the reaction system capable of producing the product produced from the C3 clone. Thus, only the insert cloned into the plasmid derived from the C3 clone has been shown to produce produce mRNA possessing the necessary start and stop codons to generate the desired protein product of the correct mass, as determined by SDS-PAGE.

Subsequent work in the laboratory has introduced the SP-22 sequence into the pGEX-5X-1 expression vector (Pharmacia).The system allows for the expression of the SP-22 protein as a GST fusion and facilitates a single step purification on a Glutatione Sepharose 4B column. Following purification, it is possible to cleave SP-22 from its GST fusion partner using factor Xa, a site specific protease. Gel permeation analysis of the SP-22/GST fusion in a crude extract of E. coli JM109 expressing the reveals a shift in GST activity to an apparent higher M_r (U Sattler, personal communication). This is probably a reflection of the higher M_r of the SP-22/GST fusion when compared to GST alone. Further work aims to examine the assembly state of SP-22 and determine the nature and specificity of its interactions with the component enzymes of the mammalian 2-oxoacid dehydrogenase multienzyme complexes.

6.3 Discussion

The presence of a 22 000 M_r polypeptide associated with preparations of mammalian PDC has been observed by other investigators (V. Bunick personal communication). Until recently, it may have been considered a contaminant, despite the fact that the protein remains associated with the large multienzyme complex during the final step in PDC purification, involving ultracentrifugation of the PDC/PEG supernatant at 200, 000g for 2.5h. In addition, evidence for a more specific PDC component interaction with the 22 000 M_r polypeptide came from its ability to remain associated with the constituent E3 enzyme during anion exchange chromatography (chapter 3, Figure 3.6b).

The significance of the association is underlined by the subsequent identification of the polypeptide as SP-22. This protein has, like PDC, been located in the mitochondrial matrix (Watabe *et al.*, 1994) and a putative general antioxidant role ascribed (Watabe *et al.*, 1995). A recent report that the protein is a thioredoxin-dependent peroxide reductase (Watabe *et al.*, 1997) provided evidence that SP-22, along two other protein components, mitochondrial thioredoxin and Nbs2 reductase, had the ability to protect oxyhaemoglobin against ascorbate-induced damage. It is believed that the cysteine-sulphonic acid residue may act as a redox centre in such reactions.The E3 (dihydrolipoamide dehydrogenase) component is a flavoprotein belonging to the disulphide oxidoreductase family, which also includes glutathione reductase. These enzymes employ an active site chemistry which involves reactive disulphide bridge catalysis (for a review see Perham *et al.*, 1995). This may leave the E3 component vulnerable to thiol oxidation. It is interesting to speculate that SP-22 has a more specific role in protecting this and/or other components of mammalian PDC from adventitious oxidation.

PDC is responsible for the production of acetyl-CoA, thus priming the tricarboxylic acid cycle with substrate and providing reducing equivalents which can be used by the respiratory chain to synthesise ATP. It represents a vulnerable site for free radical attack and thiol oxidation has been observed in other studies (Crane *et al.,* (1983); Paetzke-Brunner & Wicland (1980);Petit *et al.,* 1982). In addition, other possible metabolic fates for the acetyl-CoA generated, notably the synthesis of the neurotransmitter acetylcholine in neuronal cells and fatty acid synthesis in chloroplasts can increase its importance. The central role of PDC in carbon metabolism could be used to argue for a specific free-radical quenching mechanism. This is a role which in future may be definitively ascribed to SP-22.

The presence of the nucleotide sequence of the full length cDNA of SP-22 in nucleotide data banks facilitated design of specific primers. This sequence was subsequently published (Hiroi *et al.,* 1996). Using these primers, it was possible to amplify an 820bp fragment, consistent with the size of the known nucleotide sequence, by PCR, utilising full length double stranded bovine brain cDNA as a template. Other amplified bands may reflect mispriming events in the first few PCR cycles and subsequent propagation of these products. Alternatively, sequences in cDNA template upon which the SP-22 specific primers were capable of annealing may be indicative of the presence of SP-22 homologues within that cDNA population.

The 820bp fragment was subsequently purified away from other amplified products, contaminating primers, salts, nucleotides and polymerases. The isolated product was polished, to remove any 3' deoxyadenosine overhangs, and ligated, in a blunt end fashion, into the vector pCR-script. Recombinant clones were identified using blue/white IPTG/X-Gal screening. Plasmids were

purified from overnight cultures of 12 such clones and subject to restriction enzyme analysis. This restriction analysis, utilising both single and double digest strategies, was successful in identifying only three clones C3, C4 and C10 which appeared to contain the correct 820bp fragment. This is probably a result of the low insert:vector ratio used compared to that recommended by the manufacturers. Alternatively, the high levels of false positive clones may reflect the inability of the restriction endonuclease Srf I, used to prevent pCR-Script from re-circularising. This however does not explain the failure of the X-gal/IPTG screen to discriminate between re-circularised plasmid and those which contain inserts. It is possible that very small inserts are not sufficient to disrupt the lacZ gene but no such inserts were detected in the clones by restriction analysis.

The orientation of the three inserts was investigated using an *in vitro* transcription/translation system. The plasmid derived from clone C3 was shown to contain an insert capable of producing a 28 000 M_r protein product. This size of protein product corresponds to that expected of the preprotein form of SP-22 which includes a 62 amino acid mitochondrial import sequence.

Further work will require the sequencing of the insert, to confirm its identity. The overexpression of the polypeptide as GST fusion protein will permit the study of potential protein-protein interactions. These GST pull-down experiments and subsequent SDS PAGE analysis could be carried out with cell homogenates, or purified candidate proteins such as the individual components of mammalian PDC. Furthermore, the purified SP-22 could be used to raise specific antisera which may provide a tool to determine the levels of expression of this antioxidant protein in mammalian cell lines in response to a number of stimuli (heat shock, oxidative/heavy metal stress etc.).

Chapter 7

General Discussion

7 General Discussion

7.1 The E2/X core of mammalian PDC

The incubation of mammalian PDC with 2M NaCl and subsequent gel permeation chromatography on a Superose 6 column, equilibrated in 1M NaCl, produced large quantities of both E2/X and E1/E3 fractions (5-10mg of each). SDS-PAGE and activity assays confirmed that the levels of cross contamination were minimal, with less than 5% of the E1 component still associated with the E2/X core. The ability of the components to reconstitute PDC activity to high levels, typically > 70% compared to native PDC, indicated that the dissociation protocol had not caused high levels of component denaturation.

The production of an E2/X core, containing minimal levels of contamination from other PDC components, allowed for further study of the folding and assembly of this subcomplex. Initial studies, utilising GdnHCl treatment, in conjunction with GPC, was successful in probing the dissociation states of this large oligomeric core. Evidence for the co-operative dissociation of the E2/X core into two smaller oligomeric states, at discrete concentrations of chaotrope, was produced. By calibrating the Superose 12 column with standard proteins, the M_r of the oligomeric species observed could be estimated. These corresponded to trimeric and monomeric forms of the E2 component (Wynn *et al.*, 1994; Behal *et al.*, 1994).

Further studies with GdnHCl on the E2/X core employed both (i) an assay for E2 transacetylase activity and (ii) a PDC reconstitution assay. These

revealed key differences in the ability of a GdnHCl treated E2/X core to recover these activities; at higher levels of denaturant, while transacetylase activity could be recovered, PDC activity could not be reconstituted. Subsequent work optimised refolding conditions to produce two distinct refolded E2 core types (i) produced by rapid dilution (as above), which contained no protein X and (ii) produced by a slow dialysis protocol, in which approximately 30% of protein X could be re-integrated into the refolded core. The refolded E2 core which contained no protein X, as judged by immunological analysis and reconstitution studies, was used to investigate the potential latent ability of the E2 component to bind E3; the possibility of which had been suggested by previous work in the laboratory (Sanderson *et al.,* 1996b).

Reconstitution studies, which used an excess of native E3, purified by a protocol developed and detailed in chapter 3, in addition to stoichiometric levels of PDC E1, demonstrated the ability of E2 cores, devoid of protein X, to reconstitute PDC activity under these conditions. Further experiments, with E3 components from a range of sources, clearly indicated that the E2 interaction with native E3 observed was a specific one.These data offered a biochemical explanation for the findings of clinical studies which had identified patients suffering from congenital lactic acidosis, resulting from a PDC deficiency, specifically an absence of protein X, but who, despite this, displayed significant (20%) levels of PDC activity (Geoffroy *et al.,* 1996).

The development of an *in vitro* system of E2/X reassembly should permit further elucidation, of what may prove to be, a complex folding and association pathway(s). Recent reports which have discussed the ability of each E2 component to bind protein X (Maeng *et al.,* 1996) and the apparent cavities in each face of the E2 core structure which permit protein X binding (Stoops *et al.,* 1997). However, the mechanism and timing of protein X insertion into E2

core structures remains to be studied, particularly in view of the oligomeric stoichiometery of 5:1 in the E2/X core assembly (Maeng *et al.,* 1994; Sanderson *et al.,* 1996a). Given that an homology between E2 and CAT has been reported (Guest *et al.,* 1987), the isolation of a species with the M_r equivalent to that of a trimeric E2 is significant. It serves to underline the similarities between the two proteins and suggests a possible role for the trimeric unit as a functional entity within the E2 core. Indeed, crystal structure determination of E2 cores from a variety of sources has already disclosed the structural importance of strong intra-trimeric interactions (Berg and de Kok, 1997).

Further work on these refolded E2 cores intended to focus on the potential catalytic role of protein X within the complex. The recent cloning of the protein X gene (Harris *et al.,* 1997), while confirming the M_r obtained for the protein by ES/MS in Chapter 3, appeared to eliminate any catalytic function for the protein. Protein X sequence analysis indicated that the protein lacked a crucial serine residue normally required for acyltransferase activity. However, it may be that protein X may have another, as yet undisclosed, catalytic function. Future work could concentrate on the proposed role of protein X in the association and function of tissue specific PDC phosphatase and PDC kinase isoforms (Popov *et al.,* 1994)

7.2 Reconstitution and association studies of mammalian OGDC

Previous protocols had not been successful in dissociating appreciable quantities of the E1 and E3 components of mammalian OGDC from the E2 core while maintaining their native (active) states. The combination of $MgCl_2$ and alkaline pH conditions did promote such a dissociation, the components being

separated by GPC. Reconstitution studies, with the E1/E3 fraction and the E2 core, were successful in recovering significant levels of OGDC activity; albeit at lower levels than had been routinely obtained for mammalian PDC reconstitution. This is probably a reflection of the harsher conditions required to dissociate OGDC and their effect on component activity. The dissociation protocol did produce sufficient quantities of E1 and E3 components to carry out association state analysis on these two components on a Superose 12 column previously calibrated with standard marker proteins to allow M_r estimation. The elution profile obtained, activity assays and SDS-PAGE analysis of the fractions obtained confirmed that the E1 and E3 components did interact and form a stable complex with an apparent M_r corresponding to single copies of the two homodimeric enzymes. This provides evidence to confirm the inference drawn from previous proteolysis studies (Rice *et al.*, 1992) that the E1 of OGDC, in the absence of an E3 binding capacity on the E2 core assembly (Nakano *et al.*, 1991), is responsible for the binding of this component to the multienzyme complex.

7.3 Identification and cloning of the SP-22 protein

N-terminal sequence analysis was used to identify a small protein, which appeared to associate with mammalian PDC, specifically the E3 component, as SP-22. This protein has been characterised as a thiol specific antioxidant protein present in mammalian mitochondria (Watabe *et al.*, 1995). Preliminary work centred on cloning the sequence coding for the protein from a bovine brain cDNA, a tissue where it is known to be expressed at high levels (Watabe *et al.*, 1994). Oligonucleotide primers, specific to 5' and 3' regions of the published SP-22 sequence (Hiroi *et al.*, 1996), were designed and the polymerase chain reaction (PCR) was used to amplify the product (820bp) from

bovine brain cDNA. The PCR product was purified and cloned into pCR-Script vector. Following identification of possible recombinant colonies, *in vitro* transcription/translation analysis of the clones obtained was used to identify one which was capable of producing a protein product with an apparent M_r (28, 000). consistent with that expected for the SP-22 preprotein (Hiroi *et al.,* 1996; Watabe *et al.,* 1997).

The significance and detail of the putative interaction of SP-22 with mammalian PDC remains to be studied. Clearly, oxidation /inactivation of significant levels of PDC and other 2-oxo acid dehydrogenase complexes would have deleterious effect on energy metabolism within the cell. It is interesting to speculate that, given the E3 component is common to all three multienzyme complexes, protection of this component, against oxidative damage, may provide the most effective use of cellular resources. It is also worth noting that lipoate, which serves as a substrate for the E3 component, has thiol species which may be susceptible to oxidative attack. However, an alternative hypothesis, the association of SP-22 can be seen in the context of E3 membership of the family of mitochondrial pyridine disulphide oxido-reductases. Thus, the interaction may represent a more general one, between SP-22 and the members of this family.

Subsequent work in the laboratory as been successful in expressing SP-22 as a GST-fusion protein in *E. coli* and further plans involve the production of knockout mice to investigate what effect, if any, this has on the phenotype displayed. To complement this approach, *in vitro* functional analysis of the ability of the protein to protect against the agents of oxidative damage can be performed. Furthermore, it should be possible to study the interaction of SP-22 with 2-oxo acid multienzyme components by signal plasmon resonance (SPR). Such studies could provide quantitative data on the affinity of the individual

component enzymes for the SP-22 protein and the kinetics of any such interactions.

Within in the field of medical research the field of oxidative damage has become an area of increased focus. Antioxidant defence mechanisms are required to maintain a wide range of cellular functions essential for cell integrity and viability. For mammalian PDC, the identification and putative association with this multienzyme complex of mitochondrial antioxidant protein SP-22 represents a novel observation. The ability to overexpress and purify the protein will facilitate the investigation of multimeric nature and its biochemical/biophysical properties. It may be that levels of SP-22 expressed within a cell are effected by adverse environmental conditions. With the aid of polyclonal antiserum raised to the purified polypeptide and mammalian cell culture, it will be possible to investigate the precise nature of the stimuli (heat shock/oxidative stress) that are capable of inducing such a response.

The nature, specificity and stoichiometery of the interaction between SP-22 and the components of 2-oxoacid multienzyme complexes requires detailed investigation. The suggested thiol protective effect of SP-22 on the E2 and/or E3 components, in the presence of organic peroxides, or within oxidative systems capable of generating reactive oxygen or sulphur intermediates, remains to be investigated. Indeed, the search for other *in vivo* targets for SP-22 intervention may identify a number of additional candidate proteins and thus argue for the inclusion of SP-22, and its homologues, in the list of general cellular antioxidant responses.

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Subunit Interactions in the Mammalian α-Ketoglutarate Dehydrogenase Complex: Evidence for Direct Association of the α-Ketoglutarate Dehydrogenase (E1) and Dihydrolipoamide Dehydrogenase (E3) Components

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Running Title: Direct Interaction of E3 with E1 of Mammalian OGDC

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Abbreviations:

Pyruvate dehydrogenase complex, PDC; α-ketoglutarate (2-oxoglutarate) dehydrogenase complex, OGDC; branched-chain α–ketoacid (2-oxoacid) dehydrogenase complex, BCOADC; α-ketoglutarate dehydrogenase, E1; dihydrolipoamide succinyltransferase, E2; dihydrolipoamide dehydrogenase, E3; thiamine diphosphate, ThDP; trichloroacetic acid, TCA

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Summary

Selective tryptic proteolysis of the mammalian α-ketoglutarate dehydrogenase complex (OGDC) leads to its rapid inactivation as a result of a single cleavage within the N-terminal region of its α-ketoglutarate dehydrogenase (E1) component which promotes the dissociation of the dihydrolipoamide dehydrogenase (E3) enzyme and also a fully-active E1' fragment. Similarities between the N-terminal region of the E1 enzyme and the dihydrolipoamide acetyltransferase and protein X components of PDC are highlighted by the specific cross-reactivities of subunit-specific antisera. Analysis of the pattern of release of E1 and E1' polypeptides from the complex during tryptic inactivation suggests that both polypeptide chains of individual E1 homodimers must be cleaved to permit the dissociation of the E1 and E3 components.

A new protocol has been devised which promotes E1 dissociation from the oligomeric dihydrolipoamide succinyltransferase (E2) core in an active state. Significant levels of overall OGDC reconstitution could also be achieved by re-mixing the constituent enzymes in stoichiometric amounts. Moreover, a high-affinity interaction has been demonstrated between the homodimeric E1 and E3 components which form a stable subcomplex comprising single copies of these two enzymes.

Introduction

The α-ketoglutarate dehydrogenase complex (OGDC), along with the pyruvate dehydrogenase complex (PDC) and the branched-chain α-ketoacid dehydrogenase complex (BCOADC), constitute the family of α-ketoacid dehydrogenase multienzyme complexes important in controlling carbon flux from carbohydrate precursors and a group of five amino acids into and around the citric acid cycle. Located in the mitochondrial matrix in mammals, these complexes catalyse the irreversible oxidative decarboxylation of their respective α-ketoacid substrates yielding the appropriate acyl CoA derivatives (succinyl CoA in the case of OGDC), NADH and CO_2. Central to this catalysis is the consecutive action of three catalytic components, each present in multiple copies: a substrate-specific α-ketoacid dehydrogenase (E1), a distinct dihydrolipoamide acyltransferase (E2) and a common dihydrolipoamide dehydrogenase (E3) (see 1 and 2 for recent reviews)

Alpha-ketoacid dehydrogenase complexes are assembled in a tight but non covalent fashion. Their multi-component and multi-copy nature results in the formation of massive complexes with M_r values in the range 5-10 $x10^6$ which are organised around 24-meric or 60-meric E2 cores (dependent on the specific multienzyme complex and source organism), exhibiting octahedral and icosahedral symmetry respectively. In prokaryotes, in combination with its catalytic role, the oligomeric E2 core is responsible for tethering and orientating both the E1 and E3 enzymes within the multienzyme complexes via compact, peripheral subunit binding domains (3-5). In higher eukaryotes the identification of an additional component of PDC, protein X, with similarities in domain structure to E2 suggested a possible devolution of subunit function (6,7). Subsequent studies of protein X (also termed E3 binding protein, E3BP) have shown that it is responsible for mediating the high-affinity interaction of the E3 component to this multienzyme complex in eukaryotes (8-12).

Studies on mammalian OGDC in this laboratory, employing specific proteolysis and N-terminal sequence analysis, have identified a 'lipoyl-like' region at the extreme

N-terminus of the E1 component with significant sequence similarity to the protein X and E2 components of mammalian PDC (13). These similarities suggested that the E1 component may perform some functions normally devolved to E2 in the case of mammalian OGDC. Further indirect evidence, in support of putative additional roles for E1 of OGDC came also when the genes for rat and human E2-OGDC were cloned (14, 15). Analysis of their predicted primary structures failed to locate any apparent E1 or E3 binding motifs although such sequences were readily located in E2-OGDC genes from other organisms, notably *Escherichia coli* (16), *Azotobacter vinelandii* (17).

Previous studies of OGDC have successfully purified the intact multienzyme complex to homogeneity (18,19). However, no method for the dissociation of OGDC into functionally active E2 and E1/E3 fractions has been reported to date owing to the high affinity of the E1 enzyme for the core assembly in this complex, a property it shares with the protein X subunit of PDC (20).

In this paper, we report the development of a method for the successful dissociation of mammalian OGDC into its E2 and E1/E3 fractions. Subsequent reconstitution studies with the E2 core recovered appreciable levels (30%) of overall complex activity on addition of the isolated E1 and E3 components. Gel permeation analysis of the E1/E3 fraction of OGDC, under associative conditions, demonstrated that these two enzymes interact with high affinity to form a stable complex with an apparent M_r consistent with a 1:1 stoichiometry. These studies provide the first direct biochemical evidence that uniquely, in the case of mammalian OGDC, its constituent E1 enzyme is responsible for binding the E3 component to the multienzyme complex.

Experimental Procedures

Gel permeation analysis of tryptic digestion products of mammalian OGDC Following purification of OGDC from bovine heart as detailed previously (19), samples of OGDC were digested with 0.01% (w/w) trypsin at 30°C in 50 mM potassium phosphate buffer, pH 7.6, 3mM NAD^+, 2mM $MgCl_2$, 0.2 mM ThDP until the complex was 50% or 90% inactivated. A control OGDC sample was incubated similarly in the absence of trypsin. Digestion was stopped by the addition of soya bean trypsin inhibitor and the OGDC immediately loaded onto a Sephacryl S 300 gel permeation column (bed volume-120ml) equilibrated in 50mM potassium phosphate buffer, pH 7.6, 10mM NaCl. The column was eluted at a flow rate of 25ml.h^{-1} and the fractions assayed for OGDC activity (21), E3 activity (22) and E1 activity (23).

Preparative dissociation of mammalian OGDC OGDC was dissociated into its E2 core and E1/E3 fractions by the following method: OGDC (30mg.ml^{-1}) was incubated at a 1:1(v/v) ratio with dissociation buffer (50mM MOPS/KOH, 7.6, 2M $MgCl_2$, 20mM DTT, 0.2%(v/v) Triton X-100) for 1.5 h on ice. The sample was spun at 10,000g in a bench top centrifuge for 15 min to remove any particulate material. The E2 core of OGDC was separated from the E1/E3 fraction on an FPLC system (Pharmacia) using a 100ml bed volume prep grade Superose 6 column (1.6x50 cm) equilibrated with 50mM MOPS/KOH, pH 7.6, 1M $MgCl_2$, 0.1%(v/v) Triton X-100. A pre-injection of the dissociation buffer (2ml) was performed prior to OGDC sample loading (2ml) and subsequent column elution at a flow rate of 1ml.min^{-1}. The eluent was monitored on-line at 280nm. Pooled fractions were dialysed extensively, at 4°C, against multiple changes of buffer, into 20mM MOPS/KOH, pH 7.0, 10mM KCl, 0.1% (v/v) Triton X-100.

Reconstitution of OGDC activity from E2 core and E1/E3 fractions The method used to reconstitute OGDC activity was derived from the one optimised previously for PDC

(24). Reconstitution was started by adding quantities of E1/E3 (0-30μg) to cuvettes containing E2 (10μg) and the appropriate assay solutions. Following a 15 min incubation at 20°C, the production of NADH was initiated by the addition of 14μl 100mM α-ketoglutarate. NADH production was monitored at 340nm and activities are expressed either as $\mu mol.min^{-1}.ml^{-1}$ or a percentage relative to the specific activity of undissociated OGDC.

Association state analysis of E1 and E3 Gel permeation analysis of E1/E3 quaternary structure was performed on a Superose 12 column (24ml; 1x30cm) attached to an FPLC system (Pharmacia). The column was calibrated with gel filtration molecular mass markers (Sigma) for the construction of a calibration curve of V_e/V_o versus log M_r after initial equilibration, at $0.3ml.min^{-1}$, in 25mM MOPS/KOH, pH 7.0, 10mM KCl, 0.1%(v/v) Triton X-100. E1/E3 samples were clarified for 15 min at 10,000g in a benchtop centrifuge prior to loading onto the column (500μl sample). Elution profiles obtained were from on-line protein detection at 280nm and from E3 activity assays on the eluted fractions.

Immunological analysis Denaturing gel electrophoresis and Western Blot analysis were performed as detailed previously (20), except that immunocomplexes were detected with Amersham ECL detection reagents according to the manufacturer's instructions.

Results

Immunological analysis of similarities between the E1 component of OGDC and the E2 and protein X subunits of PDC

Previous biochemical studies on mammalian OGDC detected significant sequence similarities between the N-terminus of its E1 component and that of the E2 and protein X subunits of eukaryotic PDCs corresponding to the N-terminal regions of their peripherally-located, conformationally-mobile lipoyl domains (13). Similar investigation of the N-terminus of the E1' fragment indicated that these sequence relationships extended into the interior of the the E1 polypeptide and that the tryptic cleavage site was situated at a putative subunit binding domain. In this respect it also resembles protein X of PDC which contains a highly susceptible arg C cleavage site on the N-terminal boundary of its E3 binding domain (24).

These similarities are confirmed in Fig. 1 which demonstrates significant immunological cross-reaction between these three components. Anti-E1 (OGDC) serum elicits a clear cross-reaction with the E2 component of mammalian PDC as well as a strong and specific response to the E1 component of OGDC (panel A). In addition, as depicted in panel B, anti-protein X (PDC) serum also recognises the E1 component of mammalian OGDC. As indicated, sequence similarities between E1 of OGDC and mammalian E2 and protein X subunits have been documented previously (13); this immunological evidence confirms that these three polypeptides possess shared antigenic determinants and are thus likely to contain equivalent domains with common or overlapping functions.

Fig. 1

In Fig. 2 (insert) the specificity of tryptic digestion of intact OGDC is illustrated by SDS-PAGE analysis following treatment resulting in 0% (lane 1), 50% (lane 2) and 90% (lane 3) loss of overall complex function. It is apparent that there is a close correlation between the extent of OGDC inactivation and the percentage conversion of the 110,000 M_r E1 subunit to a stable 100,000 M_r E1' fragment with no detectable

degradation of the E2 and E3 components. Resolution of the partially inactivated complex (50%) on a Sephacryl S 300 gel filtration column (Fig. 2) shows that the constituent E1 and E3 activities are distributed approx. equally between two peaks corresponding to the complex-associated and free forms of these enzymes which elute at the void volume along with residual OGDC activity and as individual homodimeric enzymes with M_r values of 200,000 and 110,000 respectively. Similar profile analysis of intact OGDC indicates that all the constituent enzymes elute at the void volume whereas, after 90% tryptic inactivation, the vast majority of the E1' and E3 activities are no longer associated with the complex (not shown). Interestingly also, there is no evidence for any interaction between the E1' and E3 species which are partially separated by gel filtration as expected on the basis of their differing M_r values.

Fig. 2.

Fig. 3 (panel A) compares the percentage of OGDC inactivation induced by trypsin with time to the extent of E1 conversion to E1' and the corresponding amount of E3 release from the complex. It is apparent during the early stages of tryptic degradation of OGDC that the rate of E1 proteolysis exceeds the rate of complex inactivation or the extent of E3 dissociation. A possible explanation for this phenomenon is that both subunits of the E1 polypeptide must be cleaved prior to complex dissociation and that the heterodimeric E1/E1' species which will be produced preferentially in the initial stages of the digestion (assuming a random cleavage mechanism) is able to maintain the structural integrity and full catalytic activity of the complex. This idea is supported by assessment of the distribution of E1 and E1' species in the complex-bound and free forms of the enzyme (Fig. 3B) isolated by gel filtration after 50% inactivation of OGDC with trypsin as shown in Fig. 2. It is clear from this SDS-PAGE analysis that the complex-associated E1 enzyme contains a mixture of E1 and E1' polypeptides whereas only E1' fragments are located in the peak of free E1 activity. In contrast, if only a single cleavage were required to effect E1 dissociation, it would be expected that the complex-bound E1 should contain exclusively intact E1 polypeptide chains whereas the peak of free E1 activity should contain a mixture of full-length E1 subunits and E1' fragments.

Fig. 3

Dissociation of bovine heart OGDC

The promotion of the complete dissociation of OGDC into its constituent E2 and E3 enzymes and an active, stable E1' species by a single proteolytic cleavage event within the N-terminal region of the E1 component underlines the importance of this region in maintaining subunit interactions which are critical to the structural integrity of the complex. Earlier sequence comparisons have also suggested that the site of proteolytic cleavage may lie within a putative subunit binding domain (13), implying the potential for direct interaction between the E1 and E3 components in this complex. It has not been possible to test this hypothesis to date owing to the tight association of E1 component with the oligomeric E2 core in mammalian OGDC as no methods have been reported which permit release of the E1 component from the E1/E2 subcomplex in active form.

Preliminary studies in Moscow (Dr. Victoria Bunik, personal communication) indicated that high concentrations of divalent cations, in particular Mg^{2+} ions, were capable of disrupting the high affinity interaction between the E1 and E2 enzymes of OGDC whilst also promoting release of the E3 component under mildly alkaline conditions.

Fig. 4 demonstrates the ability of bovine heart OGDC to be reconstituted successfully after pre-treatment with 1M $MgCl_2$ at a variety of pH values. The data suggest that high Mg^{2+} concentrations have a minimal effect on overall complex activity at pH 6.5 and 7.0 (neutral/slightly acidic conditions). In contrast, treatment of OGDC in more alkaline conditions (pH 8.0 or above) caused rapid inactivation of the complex with a further decrease over the time course investigated leading to almost complete disappearance of OGDC activity after 5 h. Incubation with $MgCl_2$ at pH 7.6 (mildly alkaline conditions) caused a slow inactivation of the complex although 50-80% of the original OGDC activity could be restored after a 1-2 h incubation period. Further control studies (not shown) indicated (a) that the intact complex was stable to pH treatment alone in the range 6.5- 9.0 indicating that the marked pH sensitivity was

Fig. 4

induced by the dissociating conditions provided by the presence of divalent cations and (b) that the increased stability to 1M $MgCl_2$ at acidic or neutral pH values partly reflected incomplete dissociation of the E1 component under these conditions. Accordingly, it was considered that the optimal pH for OGDC disassembly, consistent with maintaining maximum stability of the E1 component, was 7.6.

Preparative dissociation of OGDC was performed by Superose 6 gel permeation chromatography (see Methods section). The elution profile suggested initially that dissociation of the high M_r OGDC had occurred with two peaks of UV absorbance eluting from the column (Fig. 5A). The initial peak, corresponding to the high M_r E2 core, eluted at or near the void volume of the column (V_e, 30-32ml). The second peak, containing the E1 and E3 enzymes, eluted in the later column fractions (V_e, 60-70 ml). Fig. 5 The nature and extent of the dissociation achieved was evident from SDS-PAGE analysis of the column fractions (Fig. 5B). Clearly, $MgCl_2$ treatment and column chromatography had been successful in removing E1 and E3 from the E2 core of OGDC. Lanes corresponding to the E2 core (column fractions 15-17) show no significant contamination with associated E1 or E3 enzymes although a small amount of E1 could be detected at the void volume (fractions, 13 and 14) which may have contained some aggregated material. The observed elution profile is consistent with appearance of the individual enzymes of OGDC from the Superose 6 column on the basis of size; the 24-meric E2 core elutes first, close to the void volume of the column, followed by the homodimeric E1 and E3 components. The E1 and E3 components appear as a single unresolved peak in the absorbance trace; however, SDS-PAGE analysis reveals that in 1M $MgCl_2$ elution buffer, E1 (220,000 M_r) elutes before E3 (110,000 M_r) although there is a degree of overlap as expected; however, under these conditions, the E1 and E3 components are clearly not associated as a single complex.

Reconstitution studies with bovine OGDC

While the protocol detailed above was successful in promoting the dissociation of the E1 and E3 components of OGDC from its E2 core, the effect of this dissociation

on the three constituent enzyme activities remained unclear. For PDC, it is possible to examine all three enzyme activities in concert by mixing stoichiometric amounts of the E1/E3 pool with the E2 core, reconstituting overall complex activity in high yield (60-80%; 24). A similar approach was adopted for OGDC in which the levels of reconstitution are expressed as a percentage of the activity of undissociated OGDC, thus providing an index of the degree to which the dissociation protocol has maintained the structural integrity of the constituent enzymes. Fig. 6 (panel A) illustrates the ability of the E2 and E1/E3 components to reconstitute overall OGDC activity up to approx. 30% of the levels compared to native untreated complex; hence the components have retained the capacity to re-assemble and produce a native or near native OGDC capable of significant rates of catalysis. Neither the purified E2 core or E1/E3 fractions were able to support OGDC activity by themselves, indicative of good fraction resolution during the dissociation (Fig. 6B), whilst also providing definitive proof that the recovery of OGDC activity resulted from genuine reconstitution of the complex. Maximal levels of reconstitution were obtained with E2:E1/E3 ratios above 1:2 on a weight for weight basis. No increase in the levels of OGDC activity were obtained by adding extra quantities of E1/E3. In the case of OGDC reconstitution, maximal levels of overall complex activity are expected in this range as the ratio of E2:E1/E3 in the native complex is approx. 1:2 which is in the range for the expected stoichiometry of the native complex assuming there are 6 copies of the E1 and E3 enzymes present per E2 core.

Fig. 6.

Optimal levels of reconstitution of mammalian PDC were previously obtained also employing sub-stoichiometric levels of E1/E3 (24). While this may also be the case for OGDC, the reason for this remains unclear, but may reflect the situation in the native complex where maximal occupancy of all potential E1 and E3 binding sites is not essential for maximum rates of catalysis. Alternatively, it was noted that the relative recoveries of the E1 component in the isolated E1/E3 fraction were only 30-50% of those achieved for E3, presumably reflecting its inherent instability and/or a degree of preferential selection during the pooling procedure.

Association state analysis of the E1 and E3 components of mammalian OGDC

The measured levels of reconstitution of OGDC activity were considered sufficient to permit detection of a direct interaction between the E1 and E3 components by gel permeation analysis. Following dissociation from the E2 core as detailed above, the E1/E3 pool was rapidly dialysed, employing multiple changes of buffer, into a low salt buffer (20mM MOPS/KOH, pH 7.0, 10mM KCl, 0.1% (v/v) Triton X-100) and a 0.5 ml sample applied to a Superose 12 column (24ml) already equilibrated in the low salt buffer (see Methods section). The monitoring of the column eluate at 280nm revealed the presence of two peaks of absorbance, not fully resolved to baseline levels (Fig.7A). Assays of the collected fractions demonstrated that the two absorbance peaks coincided with two eluted peaks of E3 activity. The protein present in the two peak fractions was precipitated with TCA and subjected to SDS-PAGE analysis (Fig. 7B). Both E1 and E3 enzymes were present in the higher molecular mass peak (P1) whereas only free E3 was detected in the lower molecular mass peak (P2).

Fig 7.

Estimation of the M_r values of the two resolved peaks of E3 activity was performed after calibration of the column with standard marker proteins. Since E3 is a homodimer with a subunit M_r corresponding to 55,000, the average value of 126,000+/-13,000 (n=3) is in close agreement with the expected value of 110,000. For the larger molecular mass peak, its measured M_r value of 303,000+/- 8,000 (n=3) correlates well with the expected M_r for an E1/E3 complex comprising one E3 homodimer (110,000) bound specifically to one E1 homodimer (220,000). The presence of this distinctive subcomplex represents the first direct biochemical evidence that the E1 and E3 components of mammalian OGDC interact physically and functionally with each other. The additional peak of free E3 activity representing 60-70% of total E3 is as expected from the reduced recoveries of the E1 enzyme relative to its companion E3 component as described previously.

Discussion

Selective proteolysis of OGDC

Previous proteolytic studies and subsequent N-terminal sequencing have located a 'lipoyl-like' domain at the N-terminus of the E1 component of mammalian OGDC. Specific degradation of E1 with trypsin results in a single cleavage of its constituent polypeptide resulting in the production of an N-terminal peptide (subunit M_r 10 000), abolition of E3 binding and dissociation of an active E1' species (subunit M_r 100,000). Complete disassembly of the OGDC following this single cleavage event indicates that the subunit binding potential of mammalian OGDC is likely to reside in this segment of the E1 component where a putative peripheral subunit binding domain has been identified at the site of tryptic attack (13). It is now apparent that this limited proteolysis also promotes release of the E1 component implying that sequences critical for its high affinity interaction with the E2 core also reside in this N-terminal region or that a conformational change occurs on generation of the E1' species which drastically reduces its affinity for the E2 core.

Interestingly, a single cleavage within an E1 homodimer generating an E1/E1' intermediate appears insufficient to promote dissociation of the complex. This evidence suggests that a single intact E1 polypeptide chain within a E1/E1' heterodimer is capable of supporting overall complex activity. The biphasic degradation of E1 by trypsin (Fig. 3A) may also indicate that one site on the E1 homodimer is more accessible than the equivalent site on its companion subunit. Such an observation is compatible with a recent, elegant X-ray structural determination of E3 interaction with its E2-derived subunit binding domain from *Bacillus stearothermophilus* PDC (5) which demonstrates that a single peripheral subunit binding domain mediates the binding an E3 homodimer via a site located near to its twofold axis of symmetry, thereby preventing access to a second identical binding site on the adjacent E3 subunit. A degree of protection of the protein X subunit of PDC from cleavage by protease arg C in the presence of the bound E3 enzyme has been observed previously (24) and

similar data have been obtained for arg C-mediated proteolysis of E1-OGDC (not shown).

Dissociation of mammalian OGDC

Initial protocol development to dissociate OGDC into E2 and E1/E3 fractions focused on the adaptation of the standard high salt (NaCl) dissociation procedure designed for the disassembly of PDC into its E2/X and E1/E3 fractions. Although NaCl (1-2M) could be employed successfully for releasing the E3 component from native OGDC, the E1/E2 subcomplex was stable to treatment with 4M NaCl (results not shown). Attempts to use low (non denaturing) levels of GdnHCl also proved fruitless. The successful disassembly of mammalian OGDC into active E2 and E1/E3 fractions, reported here for the first time, employs a combination of high levels of $MgCl_2$ and slightly alkaline pH to achieve optimal dissociation with minimal denaturation of the individual constituent enzymes. Under the dissociative conditions employed in this separation (Fig. 5), it is clear that the E1 and E3 components are not interacting, as would be expected owing to the high level of divalent cations in the column running buffer.

An important criterion, upon which the success of the OGDC dissociation was judged, was that it produced components which maintained their native structure and were thus functionally active. Reconstitution analysis provided a good indication if this had been obtained. The extent of reconstitution (30%) was appreciably lower than that achieved with the E2/X and E1/E3 fractions of PDC of 60-80% (24). However, it was also noted that dissociated E1 was quite unstable and lost 50%-70% of its original activity within 12h at 4°C even when dialysed as rapidly as possible into buffers at pH 6.5-7.0. As this represents the minimum time required to prepare this enzyme for reconstitution and binding studies, further work is required to improve its stability during the isolation procedure although it can then be stored for up to 2-3 weeks in 50% (v/v) glycerol at -20°C without further loss of activity.

Parallels with protein X function

It is of interest to note the physical and functional parallels between E1 of OGDC and protein X of PDC (25). Although it was not possible to obtain as high levels of reconstitution of OGDC (30%) compared to PDC (60-80%), the two dissociation and reconstitution protocols are not strictly equivalent, with harsher treatment required to remove the E1 from the E2 core of OGDC than is the case for PDC. Moreover, for PDC, no comparable method exists for removing protein X from the E2 core and maintaining its activity. In view of the possible functional overlap between the two proteins i.e. participation in E3 binding, it is also interesting that both E1 of OGDC and protein X of mammalian PDC are tightly, but peripherally, associated with their respective E2 cores. Recent structural studies of the E2/X core of *Saccharomyces cerevisiae* PDC position the protein X binding sites at 12 large openings in the E2 core structure (26). Similar studies on mammalian OGDC will be necessary to shed light on whether its E1 homodimers are positioned in a similar fashion although mammalian OGDC is built upon a structural E2 lattice which forms a 24-meric core exhibiting octahedral symmetry. If this proves to be the case, it would be predicted that the mammalian OGDC would be composed of 6 E1 homodimers attached to the surfaces of its cubic (24-meric) E2 core mediating the association of an equivalent number of E3 dimers. The absolute number of E1 and E3 homodimers associated with the E2-OGDC core has not been determined unequivocally in mammalian systems. However, studies of *E. coli* OGDC, which also has an octahedral 24-meric E2 core, in this case supporting the binding of both peripherally-associated enzymes, report a chain ratio E1:E2:E3 of 0.5:1.0:0.5 (27). These values suggest the presence of 6 E1 and E3 dimers bound to the E2 core in *E. coli* OGDC.

Gel permeation analysis of the isolated E1/E3 fraction from bovine heart OGDC, under low salt associative conditions, was successful in detecting the formation of a higher molecular mass complex (303,000 M_r) corresponding to an E1:E3 subcomplex exhibiting 1:1 stoichiometry. The presence of an apparent excess of E3 reflects the relatively greater losses of E1 incurred during the isolation procedure. The direct

participation of the E1 component in E3 binding is borne out by analysis of cDNAs encoding the rat and human dihydrolipoamide succinyltranferase (E2) genes which indicates that the domain typically involved in E3 binding is absent from these sequences (14,15). In the human gene for E2 of BCOADC which exhibits a high degree of similarity to the corresponding OGDC gene, the domain responsible for E1/E3 binding is encoded within a single exon (28). It is likely that loss of this exon, has occurred during the evolution of mammalian E2-OGDC. Moreover, the evidence that E1 can physically interact with E3 in this complex is indicative that this E3 binding role now resides within the α-ketoglutarate dehydrogenase component in mammals.

Subsequent immunological analysis confirmed that antisera, specific for the E1 component of mammalian OGDC, also recognises antigenic epitope(s) on mammalian E2 of PDC. Anti-protein X serum was also able to detect E1-OGDC specifically, emphasising the close structural and functional relationship between segments of these polypeptides. Selective digestion of OGDC with trypsin, has been shown to produce a very specific cleavage of the E1 component into an enzymatically active E1' fragment (subunit M_r 100, 000) and a stable peptide (subunit M_r 10, 000). Interestingly, the peptide was responsible for the major proportion of the immune response elicited due to its lipoyl-like structure (13). Indeed, high immunogenicity is a characteristic of lipoyl domains which are implicated in the autoimmune disease, primary biliary cirrhosis (29). Thus, the observed antigenic cross-reactivity between these three polypeptides undoubtedly reflects the presence of sequences usually found in lipoyl domains and other common sequence elements. Since the E3 (dihydrolipoamide dehydrogenase) enzyme is common to all mammalian α-ketoacid dehydrogenase complexes, it is not surprising that similar sequence motifs/domain structures appear to be present on the enzymes involved in its binding and catalytic function.

In mammalian OGDC the binding of E3 to the complex is not mediated by the E2 component, as in other α-ketoacid dehydrogenase complexes, since this protein lacks the domain involved in E3 interactions. In contrast to PDC from higher eukaryotes, the E3 binding role in OGDC has not been transferred to a separate gene product (protein

X). Instead, it would appear that the E1 of mammalian OGDC has acquired an additional structural role in this respect which is normally performed by the E2 or protein X components in other prokaryotic and eukaryotic complexes. Recent identification of the odhA gene from *Corynebacterium glutamicum* encoding the E1 component of OGDC, has revealed an N-terminal extension with sequence homology to E2s from PDC and OGDC, albeit to their C-termini (30). The authors intimate that this may represent a degree of bifunctionality in this E1 protein also. In our case, the catalytic role of mammalian E1-OGDC in the oxidative decarboxylation of 2-oxoglutarate is supplemented by a second structural one, namely mediating the integration of the E3 component into the multienzyme complex in optimal orientation to promote the required interaction with conformationally-mobile lipoyl domains located on the oligomeric E2 core.

Current research involves overexpressing the native E1 enzyme of OGDC and various subfragments in *E. coli* with a view to mapping its domain organisation more precisely, in particular those regions involved in maintaining critical subunit contacts.

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Figure legends

Fig. 1 Immunological analysis of subunit cross-reactivity in mammalian OGDC and PDC

Purified samples of PDC and OGDC (5μg) were subjected to 10% (w/v) SDS-PAGE and subsequent immunological analysis with anti (OGDC)-E1 (lanes 1-2, panel A) or anti-protein X (lanes 3-4, panel B) serum. Lane 1 and 3, bovine heart PDC; lanes 2 and 4, bovine heart OGDC. M_r markers, PDC and OGDC samples were run in adjacent lanes and stained with Coomassie blue to aid subunit identification.

Fig. 2 Gel permeation analysis of OGDC partially inactivated by limited treatment with trypsin

OGDC (15 $mg.ml^{-1}$) was digested with 0.01% (w/w) trypsin at 30°C in 50 mM potassium phosphate buffer, pH 7.6, 3mM NAD^+, 2mM $MgCl_2$, 0.2 mM ThDP until it retained 50% of its original activity. Following termination of digestion by addition of a 10-fold molar excess of soybean trypsin inhibitor, the OGDC was loaded onto a Sephacryl S 300 gel permeation column (bed volume-120ml) equilibrated in 50mM potassium phosphate buffer, pH 7.6, 10mM NaCl at a flow rate of $25ml.h^{-1}$. Fractions (1ml) were collected and assayed for OGDC (●—●), E1 (○···○) and E3 (▲—▲) activity.

The insert depicts the specificity of tryptic digestion of OGDC illustrating the extent of E1 degradation to E1' as detected by 10% (w/v) SDS-PAGE and Coomassie blue staining. Lane 1, control OGDC (no trypsin); lane 2, after 50% inactivation with trypsin; lane 3, after 90% inactivation; lane M, M_r marker proteins

Fig. 3 Analysis of rate of decline of OGDC activity as compared to E1' appearance and E3 release during trypsin-induced inactivation of intact OGDC.

OGDC was degraded with trypsin as described in Fig. 2 and, at the times indicated, samples were taken for assay of residual overall complex activity (●—●), densitometric determination of E1 to E1' conversion (□—□) following 10% (w/v) SDS-PAGE (13) and for assay of E3 release (△—△) after ultracentrifugation of the complex at 130,000g for 5h to pellet intact OGDC (Panel A). All results are expressed as a percentage of initial activity (OGDC) remaining or percentage of E1' and E3 release into the supernatant fraction. Panel B shows a similar SDS-PAGE analysis of the distribution of E1 and E1' species between the complex-bound (lane 3, 5μg) and free states (lane 4, 5μg) harvested from partially-inactivated OGDC (50%) which had been separated by FPLC-based gel permeation chromatography as shown in Fig. 2; lane 1, intact OGDC (15μg); lane 2, OGDC (15μg) after 50% inactivation with trypsin

Fig. 4 Reactivation profiles for bovine heart OGDC following treatment with 1M $MgCl_2$ at a variety of pH values

OGDC (2.5mg.ml^{-1}) was incubated on ice for various times in 50mM MOPS/KOH buffer, 20mM DTT, 0.1%(v/v) Triton X-100 buffer at the stated pH values in the presence of 1M $MgCl_2$. During the time course, samples (10-30μg) were removed and reactivation /renaturation was initiated by a rapid dilution into the appropriate assay buffer. The final protein concentration in the renaturation mixture was 10-30μg/ml and residual $MgCl_2$ reduced to below 30mM to prevent enzyme inhibition. Samples were assayed for OGDC activity after a 3 min pre-incubation. All activities are expressed relative to control samples of enzyme incubated in the absence of $MgCl_2$. pH 6.5, ○—○ ; pH 7.0, □—□; pH 7.6, ▲—▲; pH 8.2, △—△ .

Fig. 5 Dissociation and separation of the constituent enzymes of bovine heart OGDC

Preparative dissociation of OGDC into active E2 and E1/E3 fractions was performed on an FPLC system (Pharmacia) using a Superose 6 column (100ml) equilibrated with 50mM MOPS/KOH, pH 7.6, 1M $MgCl_2$, 0.1% (v/v)Triton X-100 (see Methods section). Fractions were pooled on the basis of A_{280} peaks (Panel A) and dialysed extensively, at 4°C, against multiple changes of the 20mM MOPS/KOH, pH 7.0, 10mM KCl, 0.1% (v/v) Triton X-100.

Aliquots (1ml) of the eluent fractions were subjected to TCA precipitation (Panel B) and separated by 10% (w/v) SDS-PAGE prior to staining with Coomassie blue. Lane L, OGDC column load (15μg); lanes 14-34, TCA-precipitated samples from column fractions showing resolution of the constituent enzymes.

Fig. 6 Reconstitution of overall OGDC complex activity from purified E2 core and E1/E3 fractions: levels of reconstitution and component stoichiometry

Following the dissociation of OGDC as described in Fig. 5, the E2 and E1/E3 fractions were dialysed into 50mM MOPS/KOH, pH 7.4, 1mM EDTA, 50% (v/v) glycerol and stored at 4°C. E2 and E1/E3 fractions were pre-conditioned by a 1:1 dilution in 50mM potassium phosphate buffer, pH 7.6, 3mM NAD^+, 2.7 mM cysteine-HCl, 2mM $MgCl_2$, 0.2 mM ThDP, 0.02mM CoASH and incubated for 15min at 20°C. To initiate reconstitution, increasing quantities of E1/E3 (0-40μg) were added to cuvettes containing E2/X (10μg) under the appropriate assay solutions. The production of NADH was subsequently initiated by the addition of 14μl 100mM 2-oxoglutarate. Activities are expressed as percentage reconstitution relative to the activity of the native OGDC complex before dissociation (Panel A). The purity of the pooled fractions employed for this reconstitution study was determined by 10% (w/v) SDS-PAGE

analysis and Coomassie blue staining. Lane M, M_r marker proteins; lane 1, intact OGDC (10μg; lane 2, E1/E3 fraction (15μg); lane 3, E2 core (5μg).

Fig. 7 Association state analysis of the purified E1 and E3 components

Detection of formation of a stable E1/E3 subcomplex was performed by resolving the purified E1/E3 pool on a Superose 12 column under non dissociating conditions (see Methods section). Protein elution was detected at 280nm (Δ—Δ) and assays for E3 activity (○—○) performed on the column fractions (Panel A). Peak fractions from the column were subjected to TCA precipitation and subsequent 10% (w/v) SDS-PAGE analysis prior to staining with Coomassie blue (Panel B). Lane M, M_r markers; lane L, E1/E3 load fraction (3μg); lane P1, peak 1 fraction (2μg) ; lane P2, peak 2 fraction (2μg) from Panel A.

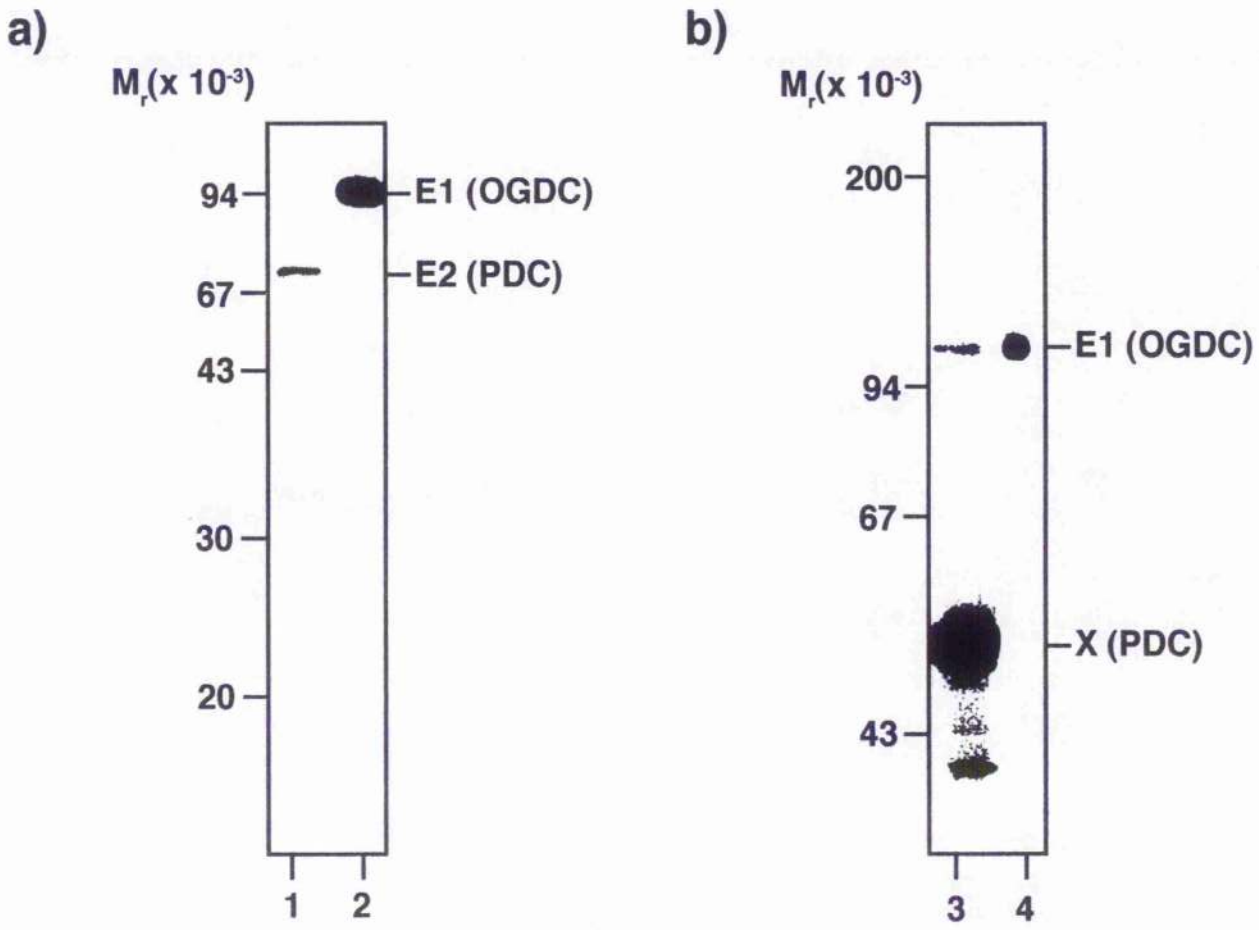
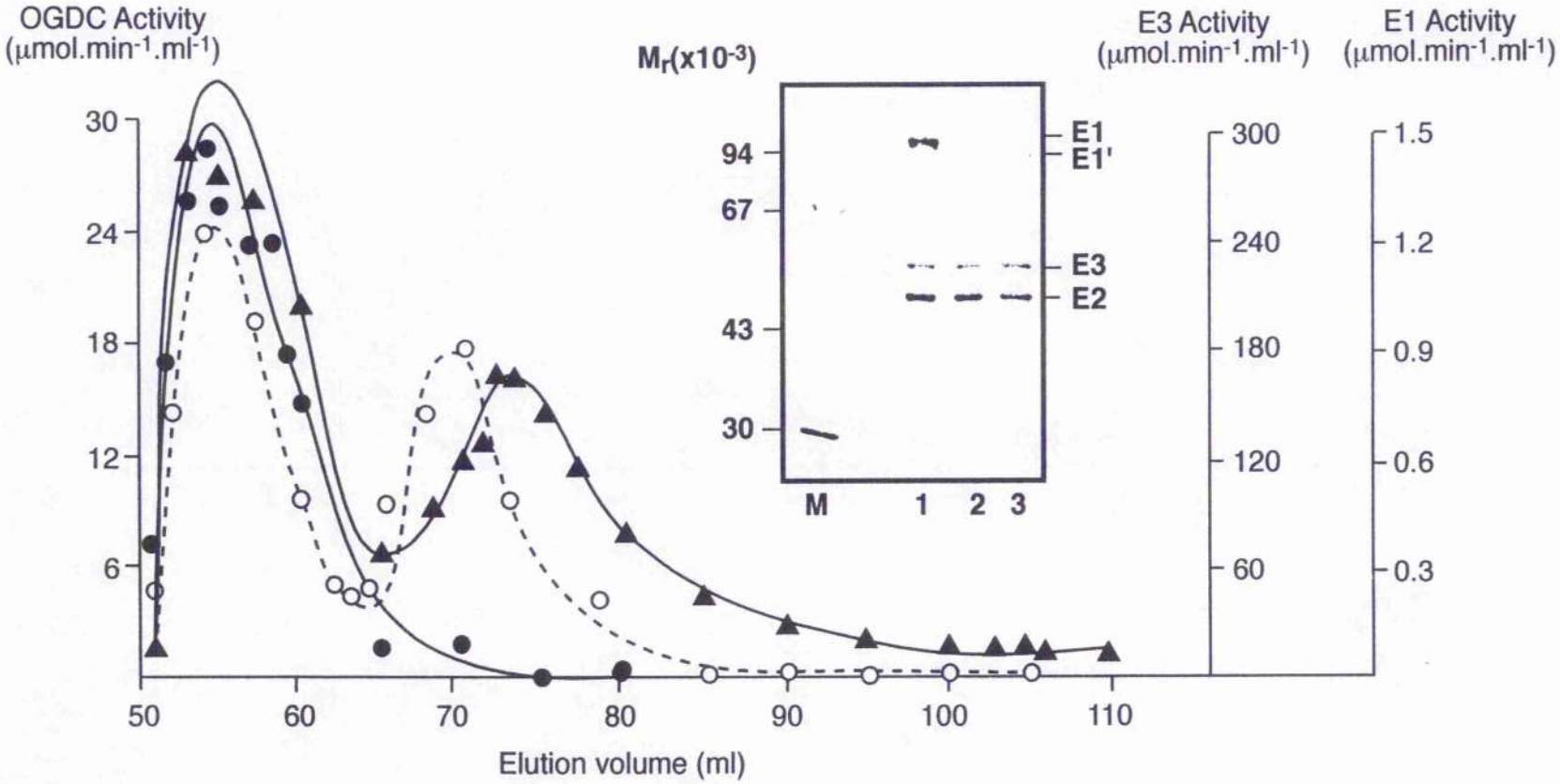
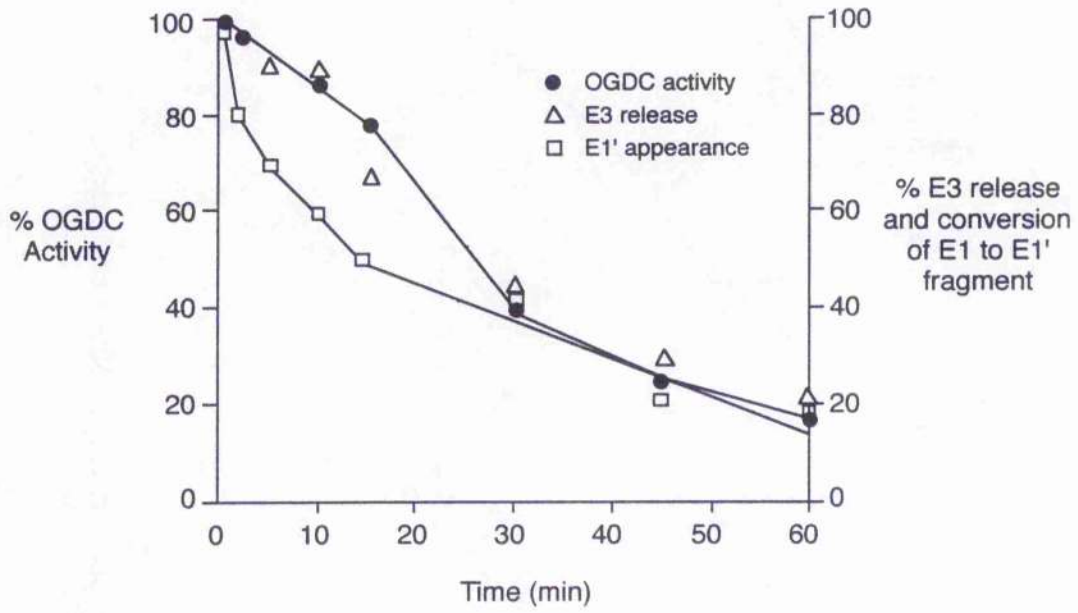

a)
M_r(x 10^{-3})
94
67
43
30
20
E1 (OGDC)
E2 (PDC)
1
2
b)
M_r(x 10^{-3})
200
94
67
43
E1 (OGDC)
X (PDC)
3
4

Figure ①


OGDC Activity
(μmol.min-1.ml-1)
30
24
18
12
6
50
60
70
80
90
100
110
Elution volume (ml)
Mr(x10-3)
94
67
43
30
M
1
2
3
E1
E1'
E3
E2
E3 Activity
(μmol.min-1.ml-1)
300
240
180
120
60
E1 Activity
(μmol.min-1.ml-1)
1.5
1.2
0.9
0.6
0.3

Fisure 2

a)


100
80
60
40
20
0
% OGDC Activity
OGDC activity
E3 release
E1' appearance
% E3 release and conversion of E1 to E1' fragment
0
10
20
30
40
50
60
Time (min)

b)

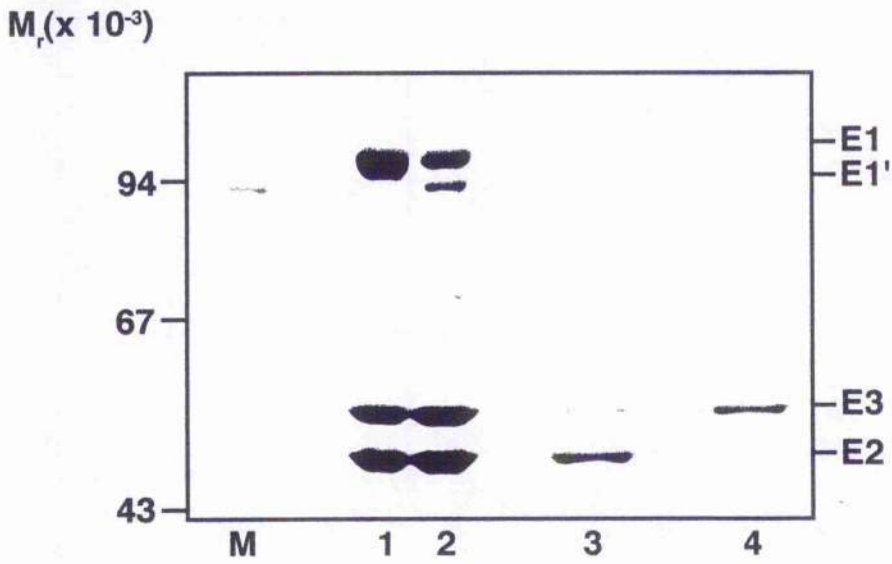

M_r(x 10^-3)
94
67
43
E1
E1'
E3
E2
M
1
2
3
4

Figure 3

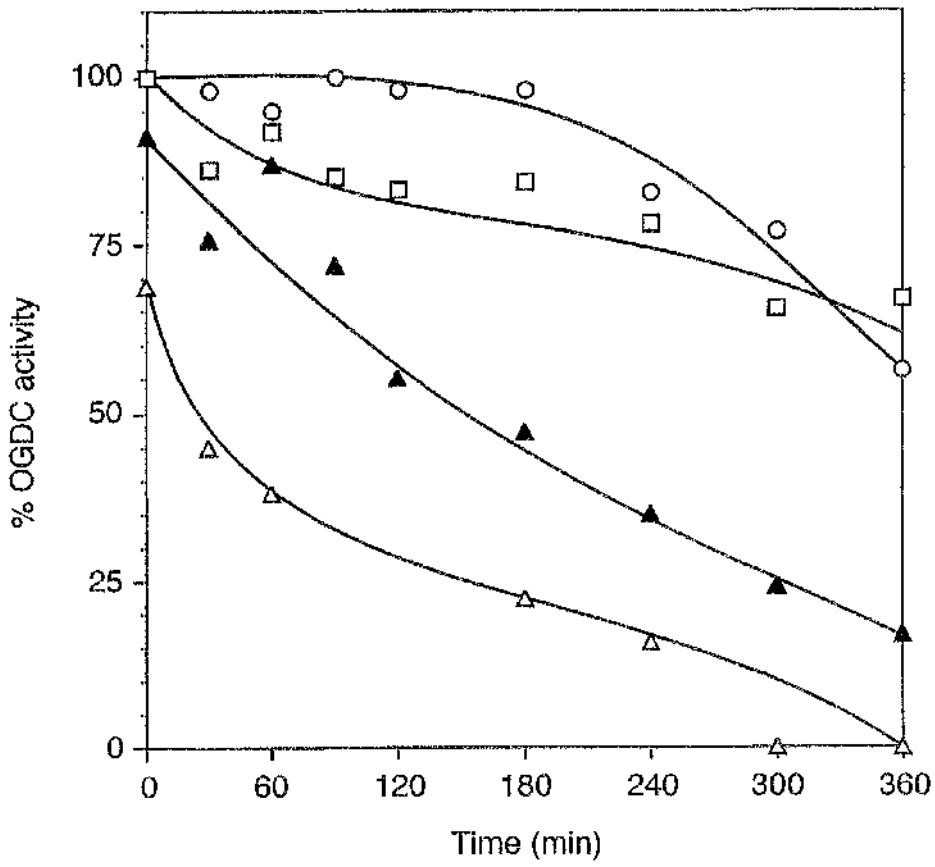
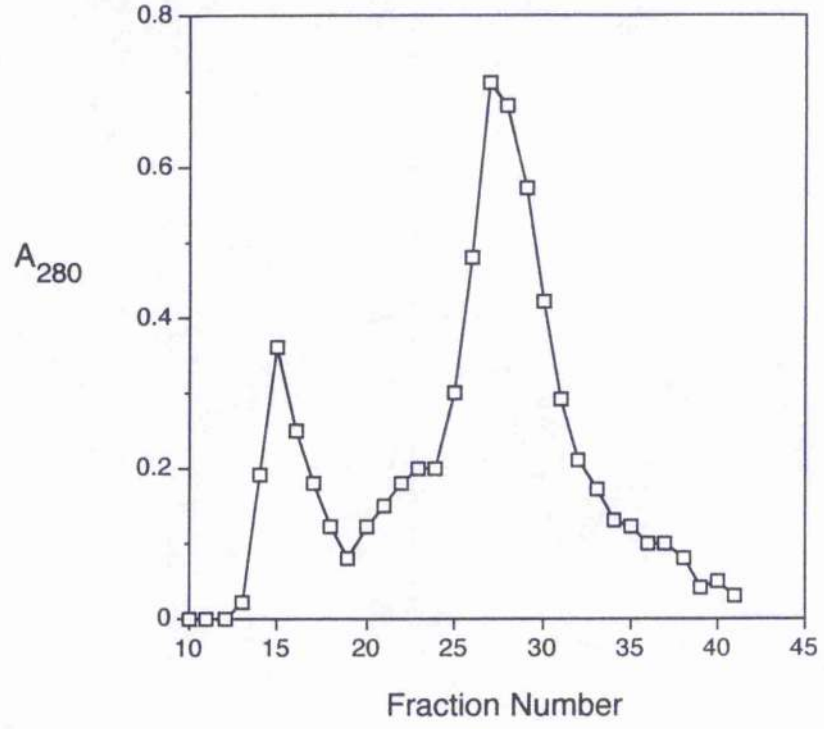


Figure 4

a)


0.8
0.6
0.4
0.2
0
A280
10
15
20
25
30
35
40
45
Fraction Number

b)

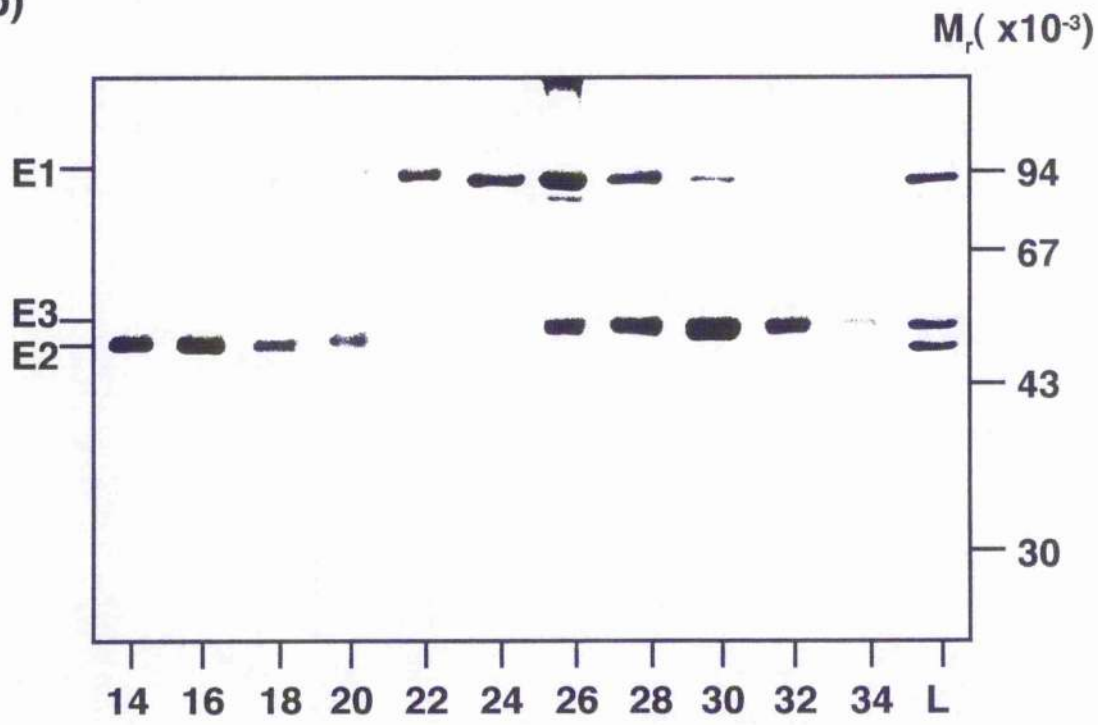
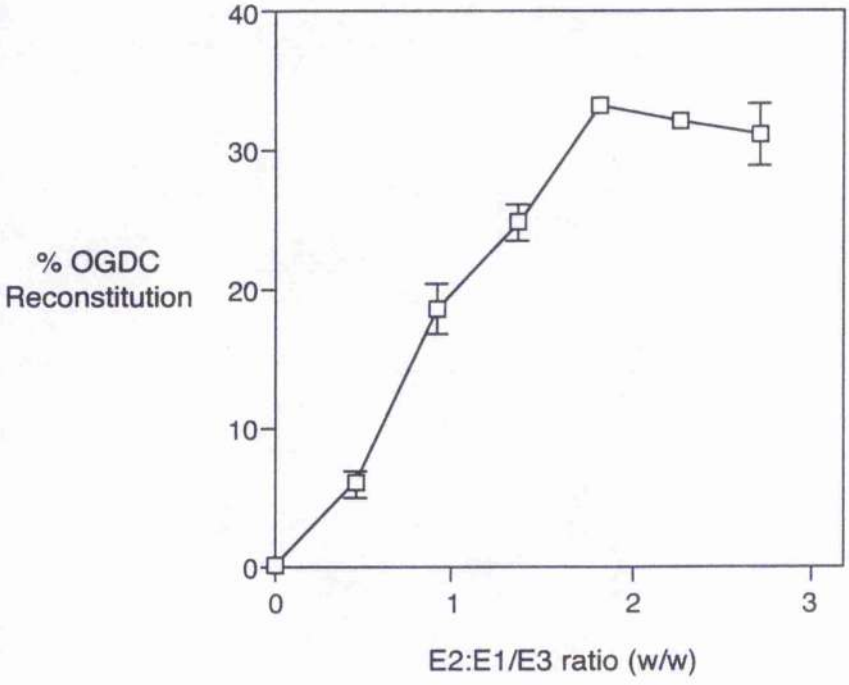

Mr(x10-3)
E1
E3
E2
94
67
43
30
14
16
18
20
22
24
26
28
30
32
34
L

Figure 5

a)


40
30
20
10
0
% OGDC
Reconstitution
0
1
2
3
E2:E1/E3 ratio (w/w)

b)

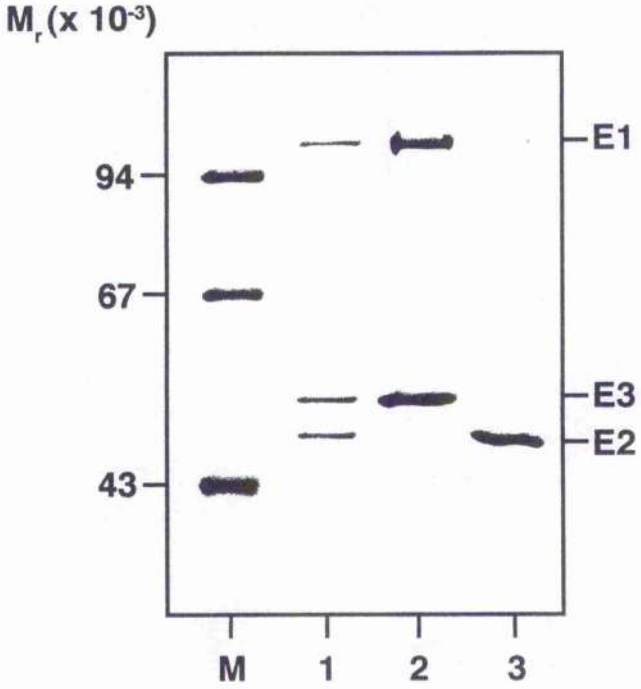
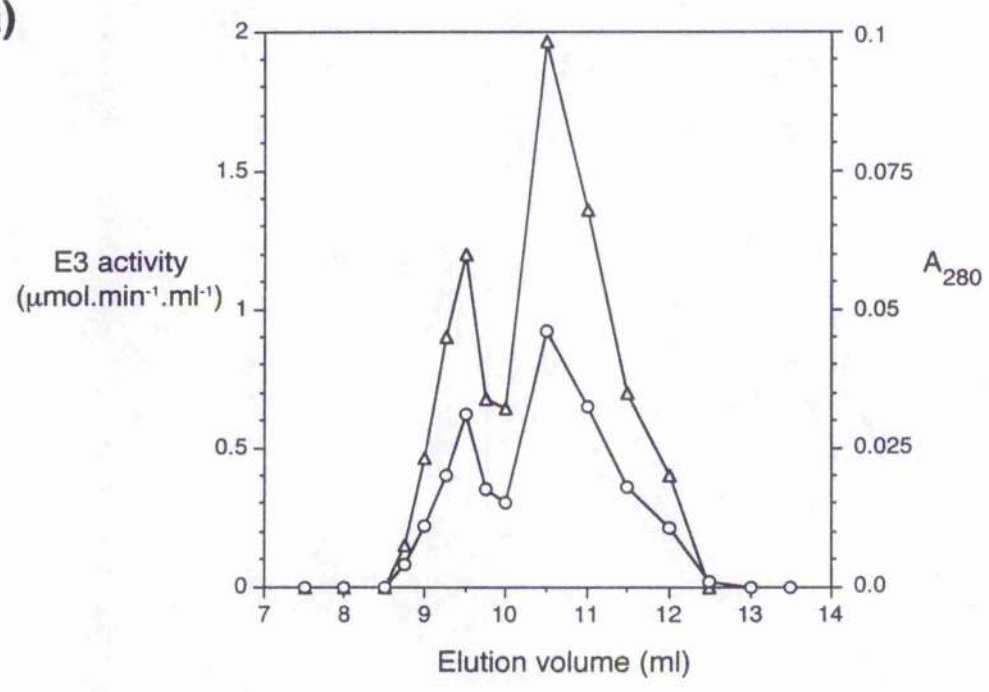

M_r (x 10^-3)
94
67
43
E1
E3
E2
M
1
2
3

Figure 6

a)



b)

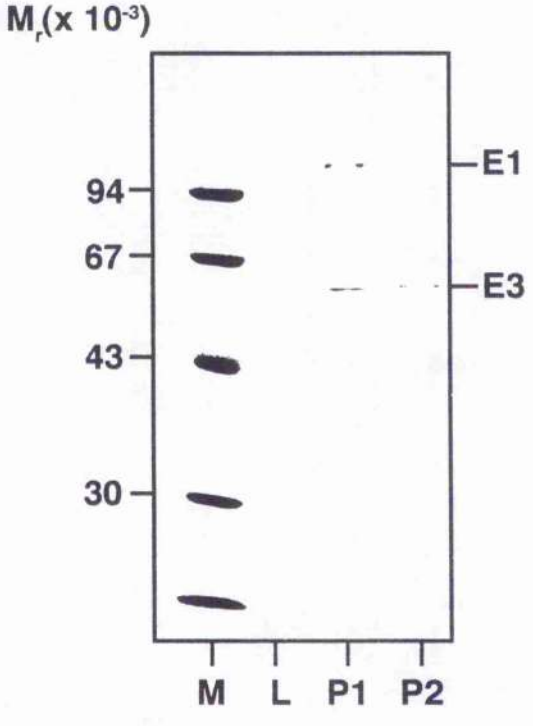


Figure 7