STRUCTURAL AND FUNCTIONAL ANALYSIS OF COMPONENT X FROM THE BOVINE HEART PYRUVATE DEHYDROGENASE COMPLEX

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For Mom and Dad

"The truth is seldom pure and never simple"

Oscar Wilde (1856-1990)

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ABBREVIATIONS

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

BCOADC	branched chain 2-oxoacid dehydrogenase complex
CAT	chloramphenicol acetyltransferase
DMSO	dimethylsulphoxide
DTT	dithiothreitol
E3/LDH	lipoamide dehydrogenase
GDC	glycine decarboxylase complex
GdnHCl	guanidine hydrochloride
LDH	lipoamide dehydrogenase
Leupeptin	acetyl-L-leucyl-L-arginal
lipoamide	6,8-thioctic acid amide
2-ME	2-mercaptoethanol
MOPS	3-[N-morpholino]propane-sulphonic acid
M_r	relative molecular mass
MSUD	maple syrup urine disease
NEM	N-ethylmaleimide
OGDC	2-oxoglutarate dehydrogenase complex
PBC	primary biliary cirrhosis
PDC	pyruvate dehydrogenase complex
PDM	N,N-1,2-phenylene-dimaleimide
PEG	polyethylene glycol
PMSF	phenylmethylsulphonylfluoride
PVDF	polyvinylidene difluoride
RBS	rat blood serum

TCA cycle	tricarboxylic acid cycle
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TPP	thiamine pyrophosphate
Tween 20	polyoxyethylenesorbitan monolaureate
TX-100	Triton X-100
X	protein X

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ABSTRACT

A comprehensive investigation of the optimal conditions for reconstitution of bovine heart pyruvate dehydrogenase complex activity (PDC) from dissociated E2/X core and E1/E3 fractions is presented, and was employed in the elucidation of the disputed involvement of the lipoyl domains of protein X in overall complex catalysis.

Selective proteolysis of the lipoyl and linker regions of protein X by Arg C, in isolated E2/X core or intact PDC, resulted in a dramatic reduction in E3 binding potential and hence, complex activity (with the former, following reconstitution with dissociated components, E1 and E3), when compared to control complex activities. It is proposed that owing to the proximity of the cleavage site to the E3 binding domain of protein X, loss of E3 binding affinity arises from conformational changes in the aforementioned domain and/or, the specific binding site within. Partial recovery of reconstitution was achieved in the presence of excess porcine E3, and increased with increasing protein X degradation suggestive of an additional, non-specific and hence, low affinity E3 binding site -- presumably the subunit binding domain of E2.

Following removal of the lipoyl and linker regions of either component E2 or X, N-ethyl[2,3-^{14}C]maleimide labelling studies confirmed previous observations of a residual (i.e., approx. 10%) complex activity, indicating the ability of either component to substitute for the lipoyl domains of the other. The physiological implications of this are discussed in relation to the unique diacetylation properties of mammalian PDC. However, as attempts to isolate and sequence the extreme C-

terminal portion of protein X following the recent discovery of a dihydrolipoamide acetyltransferase active site-like motif by J.C. Neagle (University of Glasgow, unpublished observations), remained in the initial stages, it is unclear whether deacetylation of the lipoyl domains of protein X services an acetyltransferase active site on E2 or X.

Precise stoichiometric determinations employing: comparisons of ^{14}C-acetylation in E2 and X; and, densitometric scan analysis of highly purified protein X, E2 and bovine heart PDC samples resolved by SDS-PAGE and silver stained, indicated the presence of 12mol protein X/mol PDC, and of 60mol E2/mol PDC, respectively. Structural studies involving covalent N,N'-1,2-phenylene-dimaleimide (PDM), crosslinking of the lipoyl domains of protein X in E2-lipoyl domain depleted complex, revealed dimeric organisation. Presented together, this evidence is in close agreement with the 1:1 (E3:E1) binding relationship of bovine heart OGDC, and is strongly suggestive of the involvement of six protein X dimers in the binding of six dimers of E3 to the E2/X core in bovine heart PDC. Furthermore, the presence of 60 E2 subunits supports previous proposals for a non-integrated, external protein X core positioning.

CHAPTER 1: INTRODUCTION

1.0 INTRODUCTION

1.1 The 2-oxoacid dehydrogenase complex family

1.1.1 A role in metabolism

The pyruvate dehydrogenase complex (PDC) is a member of the thiamine pyrophosphate-requiring 2-oxoacid dehydrogenase complex family which also comprises the 2-oxoglutarate dehydrogenase complex (OGDC), and the branched chain 2-oxoacid dehydrogenase complex (BCOADC). Together, these self-assembling multienzyme complexes with calculated M_r values in the range of 4-10 x 10^6 (Bates *et al.*, 1977; Reed, 1974), represent classic examples of macromolecular structure and organisation. As such, they have been the subject of considerable interest since the initial purification and characterisation of PDC from *Escherichia coli* (Nawa *et al.*, 1960; Koike *et al.*, 1963; Reed & Oliver, 1968; Reed & Mukherjee, 1969) and later, eukaryotic sources (Linn *et al.*, 1969, 1972; Wieland & von Jagow-Westermann, 1969) began in the late 1950s with the work of Lester J. Reed and others.

Located in the mitochondrial matrix in eukaryotes and probably associated with the inner mitochondrial membrane (Yeaman, 1986), all three complexes occupy key positions in metabolism and are instrumental in the regulation of substrate flux to, from and within the TCA cycle.

Each is composed of multiple copies of three individual enzyme components known as E1, E2 and E3, which catalyse the sequential partial reactions in the oxidative decarboxylation of 2-oxoacids to their acyl CoA derivatives with the concomitant production of CO_2. Thus, whilst PDC controls the committed step in carbohydrate metabolism with the conversion of pyruvate to acetyl CoA for subsequent oxidation by the TCA cycle or, utilisation in fatty acid or ketone body synthesis, OGDC catalyses the oxidative decarboxylation of 2-oxoglutarate to succinyl CoA and thereby regulates flow through the TCA cycle itself. Furthermore, BCOADC is responsible for the irreversible oxidation of the 2-oxoacids derived from the essential branched chain amino acids, leucine, isoleucine and valine and, of 2-oxobutyrate and 4-methylthio-2-oxobutyrate (the products of threonine and methionine metabolism) which, following further oxidation may enter the TCA cycle as acetyl- or succinyl CoA.

Reflected in the characteristically narrow (PDC & OGDC), or broad (BCOADC), substrate-specificities of the complexes are the corresponding catalytic preferences of the complex-specific E1 and E2 components known as: pyruvate dehydrogenase (E1, EC 1.2.4.1) and dihydrolipoamide acetyltransferase (E2, EC 2.3.1.12) of PDC; 2-oxoglutarate dehydrogenase (E1, EC 1.2.4.2) and dihydrolipoamide succinyltransferase (E2, EC 2.3.1.61) of OGDC; and the branched chain α-ketoacid decarboxylase (E1, EC 1.2.4.4) and dihydrolipoamide acyltransferase of BCOADC. In contrast, the highly conserved lipoamide dehydrogenase (E3, EC 1.8.1.4) or LDH, is common to all three complexes and also appears as the "L" protein of the glycine decarboxylase system of bacteria and mammals (Sokatch & Burns, 1984). Notably, structural similarities also exist between the E2 component and the lipoic acid-containing "H" protein of the glycine decarboxylase complex (GDC) -- see Fujiwara *et al.* (1986, 1991).

In addition, LDH is also a member of the disulphide oxidoreductase family which comprises trypanothione, mercuric and glutathione reductases -- to the latter of which *Azotobacter vinelandii* E3 has been shown recently, through high resolution X-ray crystallography, to share common structural features although, amino acid homologies between the different members of this group range from only 20-30% identity (Mattevi *et al.*, 1991). Whilst apparently the product of a single LDH gene in mammalian and (possibly) plant mitochondrial 2-oxoacid dehydrogenase complexes (Bourguignon *et al.*, 1992; Turner *et al.*, 1992; and references within), the situation is complicated by the characterisation of complex-specific LDH genes in *Pseudomonas putida* (Palmer *et al.*, 1991a,b), *Pseudomonas aeruginosa* (McCullet *et al.*, 1986), *E. coli* (Richarme, 1989) and, by the discovery of several isoforms in rat liver and potato mitochondria (Carothers *et al.*, 1987; R.E. Millar, University of Glasgow, unpublished observations).

In *Pseudomonas*, two genetically distinct forms designated, LDH-Val and LDH-Glc, have been characterised -- the former specific for BCOADC (Burns *et al.*, 1989a), and the latter, for OGDC, GDC and possibly, PDC (Palmer *et al.*, 1991a). Furthermore, a third lipoamide dehydrogenase of unknown function, LDH-3, isolated from *P. putida* (Burns *et al.*, 1989b), appears capable of substituting, at least in part, for LDH-Glc in OGDC and PDC. Notably, the close similarity of the LDH-3 encoding gene to that of eukaryotic LDH (Palmer *et al.*, 1991b) may provide further insight into the origins of E3. In parallel, two forms of E3 exist in *E. coli*, one encoded by the *lpd* gene and specific for PDC and OGDC; and, a second exhibiting similarities to LDH-Val of *P. putida* but of unknown function. However, the identification of lipoamide dehydrogenases from *Trypanosoma brucei*, *Clostidium sporogens* and archaebacterium, *Halobacterium halobium*, which lack the 2-oxoacid dehydrogenase complexes (Danson *et al.*, 1987) coupled, with evidence for the involvement of E3 in galactose and maltose transport in *E. coli*

(Richarme & Heine, 1986), has led to proposals for an additional role in sugar transport, for E3 from bacteria .

1.1.2 General reaction scheme

As summarised in the reaction scheme in Fig. 1.1.2.1, E1 catalyses the initial steps in the rate-limiting and irreversible decarboxylation of the appropriate 2-oxoacid substrate to an hydroxyacyl-TPP intermediate via the transient ionisation of the thiazole ring of co-factor, TPP, to which the negatively charged carboxyl group of the 2-oxoacid binds and is stabilised prior to the extraction of CO_2. The oxidation to an acyl group and corresponding reductive acylation of the S^8-thiol of the E2-linked lipoic acid co-factor, also catalysed by E1, results in the regeneration of the carbanion of TPP. Transfer of the acyl group to a reduced CoA molecule is then facilitated by E2, leaving the lipoic acid S^6,S^8-thiols in a reduced state -- ready for reoxidation and regeneration of the disulphide by E3 following the successive reduction and oxidation of co-factors FAD and NAD^+.

1.1.3 Macromolecular structure and organisation

Central to the structure and function of the 2-oxoacid dehydrogenase complexes are the dihydrolipoamide acyltransferase (E2) subunits. Forming the structural core about which all other components arrange, non-covalent aggregates of E2 polypeptides provide a framework for the coordination of the partial reactions involved in complex catalysis. In mammalian OGDC, BCOADC and PDC from

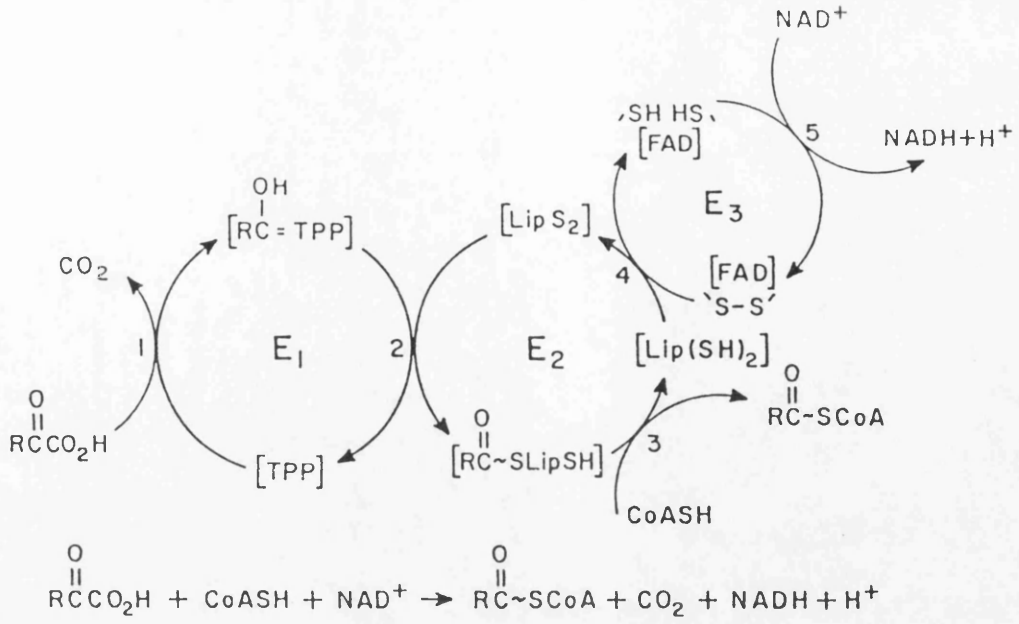


Fig.1.1.2.1 Reaction scheme for the oxidative decarboxylation of 2-oxoacids to acyl CoA derivatives.

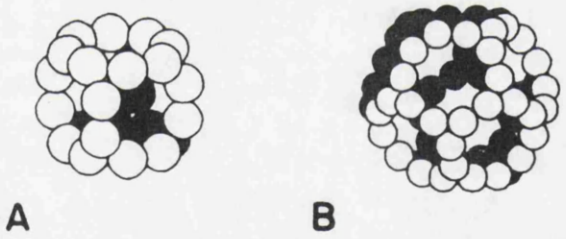


Fig. 1.1.3.1 Interpretive models of E2 quaternary structure.

Model A: 24 E2 molecules arranged with octahedral symmetry.
Model B: 60 E2 molecules arranged with icosahedral symmetry.

Gram negative bacteria such as *E. coli* and *A. vinelandii*, the core consists of 24 identical copies arranged with octahedral (432) symmetry which gives a cubic appearance when viewed by electron microscopy (Oliver & Reed, 1982). In contrast, PDC from Gram positive bacteria, *Bacillus stearothermophilus*, *Bacillus subtilis* and *Streptococcus faecalis*, and from mammalian, avian and fungal sources, exhibit dodecahedral cores comprising 60 E2 components arranged with icosahedral (532) symmetry (see Perham, 1991 for review; see Fig. 1.1.3.1). Thus, the 2-oxoacid dehydrogenase complexes appear species-specific and furthermore, growing evidence from plant sources suggests organelle-specificity in some species (Taylor *et al.*, 1992).

Information from negative stain electron microscopy images of PDC (Oliver & Reed, 1982), and low resolution X-ray crystallography of E2/E3 subcomplexes (Fuller *et al.*, 1979), led to a model where dimers of E1 and E3 are thought to associate along the edges and faces of the E2 core, respectively. Whilst six E3 homodimers of total M_r 110000 appear common to all 2-oxoacid dehydrogenase complexes, the number, M_r values and subunit configuration of E1 varies from the 12 homodimers of M_r 220000 in *E. coli* to the 20-30 split-chain $\alpha_2\beta_2$ heterotetramers of icosahedral PDC and mammalian BCOADC which, in bovine heart PDC, comprise a total M_r of 154000 with subunit M_r values of 42000 (α) and 36000 (β). It has been suggested that reactions 1 and 2 of the overall reaction scheme (see Fig. 1.1.2.1) are catalysed by E1α and E1β, respectively -- although this remains somewhat controversial (see Reed & Yeaman, 1987; Reed & Hackert, 1990 for reviews). In addition, a fourth component known as protein X was identified in PDC from mammalian and yeast sources (De Marcucci & Lindsay, 1985; De Marcucci *et al.*, 1985; Jilka *et al.*, 1986; Behal *et al.*, 1989), and found to exhibit close structural/functional identity, tight association and copurification with E2, although present at considerably lower levels (i.e., 6-12 molecules/complex).

Considerable information concerning the 2-oxoacid dehydrogenase complexes has come to light from the cloning and sequencing of the genes encoding individual components. Initial research involved elucidation of the *ace E* (E1, PDC), *ace F* (E2, PDC), *suc A* (E1, OGDC), *suc B* (E2, OGDC) and, *lpd* (E3) genes from *E. coli*, led by the work of J.R. Guest & coworkers (Guest *et al.*, 1989). However, the genes for E1, E2 and E3 of PDC and BCOADC, from various eukaryotic sources including human, and additional prokaryotes, have since been isolated and sequenced (reviewed in Yeaman, 1989; Thekkumkara *et al.*, 1989). In addition, the full length cDNA sequence for protein X from *S. cerevisiae* (PDX 1) is also known (Behal *et al.*, 1989). Sequence alignments have revealed surprisingly little homology between the E1 components of PDC and OGDC, suggestive of early divergence during evolution. In contrast, both the E2 and E3 components exhibit close sequence identity across species and complex type (approx. 40% identity for E3) -- the former, also possessing remote similarities to chloramphenicol acetyltransferases and the latter component, to related enzymes of the disulphide oxidoreductase family.

Furthermore, a small number of the complex-specific regulatory enzymes, kinase and phosphatase, have been found tightly and rather more loosely associated with the E2 core, respectively, of mammalian, avian and *Neurospora crassa* PDC and of mammalian BCOADC (Linn *et al.*, 1969; Reed & Yeaman 1987 for review). PDC kinase and phosphatase are also known to be present at low levels in plant mitochondria -- a fact which has precluded a successful purification to date (Randall *et al.*, 1990). In contrast, both enzymes have been purified to homogeneity from bovine kidney and heart mitochondria (Linn *et al.*, 1972; Stepp *et al.*, 1983; Teague *et al.*, 1982), and found to exist as heterodimers of M_r 48000 and 45000 (kinase) and, M_r 50000 and 94000 (phosphatase), of which the former appears to be the catalytic subunit (as with the 33000-M_r subunit of BCOADC phosphatase -- see

Yeaman, 1989). An unsubstantiated regulatory role has been proposed for the 45000-M_r (K_b) subunit of PDC kinase (Stepp *et al.*, 1983), supported by the recent findings of enhanced kinase activity following its removal by Arg C (Rahmatullah *et al.*, 1989a) whereas, the 48000-M_r (K_c) subunit is thought to be involved in the binding of the kinase heterodimer to E2 (Radke *et al.*, 1993). In contrast, both the catalytic involvement of the 94000-M_r FAD-containing subunit of PDC phosphatase and, the subunit composition of the 460000-M_r BCOADC phosphatase, remain to be established. In addition, due to problems encountered with tight core association, which in the isolation of PDC kinase required the employment of denaturing and chaotrophic conditions such as high salt, alkaline pH and β-hydroxymercuriphenyl sulphonate to effect release (Stepp *et al.*, 1983), little is known of the structure of BCOADC kinase.

Recent sizing of both mammalian (Roche *et al.*, 1993) and *E. coli* PDC (Wagenknecht *et al.*, 1992), employing quasielastic light scattering and cryoelectron microscopy, respectively, have resulted in estimations for complex diameters of approximately 50nm -- far exceeding that previously determined by Oliver & Reed (1982). However, as the later studies employed frozen-hydrated complex, in contrast to the earlier methods using dehydrated and fixed samples, it is likely that these lower determinations reflect the collapse of flexible, extended regions.

1.1.4 Regulation

In all 2-oxoacid dehydrogenase complexes, regulation is facilitated by end-product inhibition, imparted by the increasing concentrations of products, NADH and acyl CoA in relation to the levels of substrates. In addition, changes in the actual amounts of each enzyme complex are facilitated through altered gene expression. However, in PDC from mammals, birds and *N. crassa,* and BCOADC from mammals, a further control mechanism exists involving the regulatory enzymes, kinase and phosphatase. These enzymes, in response to the levels of hormones and other effector molecules, regulate both acute and long-term changes in the active state of the aforementioned complexes via the reversible phosphorylation of the E1 component; in the latter case, also involving the indirect effects of altered kinase/phosphatase gene expression. Notably, this additional regulation is only apparent with complexes exhibiting an $\alpha_2\beta_2$ E1 configuration although, previous unsuccessful attempts to isolate PDC kinase from *S. cerevisiae* indicated one exception to this rule (Kresze & Ronft, 1981a,b). Nevertheless, *S. cerevisiae* retains the potential for *in vitro* regulation by bovine kidney PDC kinase and phosphatase (Uhlinger *et al.*, 1986). Furthermore, recent research from this laboratory has demonstrated significant levels of kinase activity *in vivo*, which were subsequently lost upon complex purification and may therefore, reflect the presence of inactivated or unusually labile forms of the regulatory enzymes (James & Lindsay, 1994).

Inactivation follows the primary phosphorylation of one of three (PDC) or 2 (BCOADC) specific serine residues located within highly homologous sites on the E1α subunits and is accompanied by lowered V_{max} and insensitivity to all allosteric activators, with further phosphorylation of the remaining sites possibly inhibiting

dephosphorylation by phosphatase (Sudgen *et al.*, 1978; Yeaman *et al.*, 1978; Cook *et al.*, 1983). Notably, the site of phosphorylation in *S. cerevisiae* E1 closely resembles the primary phosphorylation sites of E1 from bovine heart and kidney PDC and OGDC (Uhlinger *et al.*, 1986). In PDC, this "on/off" switch is subject to complex catalytic regulation in which both kinase and phosphatase exhibit a Mg^{2+}/Mn^{2+} requirement -- the former enzyme, inhibited by substrates CoA, NAD^+, pyruvate, TPP and activated by products acetyl CoA, NADH; and the latter enzyme, inactivated by NADH, and activated by Ca^{2+} which binds at two sites, one involved in catalysis and the second, in promoting binding to the E2 core (see Reed & Yeaman, 1987; see Fig. 1.1.4.1).

In light of the observed enhancement in E1 phosphorylation following the binding of kinase to the E2 core, Roche and others (Kerbey *et al.*, 1976; Rahmatullah & Roche, 1987; Rahmatullah *et al.*, 1989a), proposed a mechanism for kinase modulation involving the reductive acetylation of the lipoic acid moieties of both components, E2 and X, in response to the relative levels of substrates and products. Although disputed by the findings of Reed *et al.* (1980) which suggested direct allosteric control of the kinase, Roche and co-workers subsequently reported the loss of this core-bound increase in kinase activity, following acetylation of the lipoyls of E2 from bovine kidney PDC, and proposed a critical involvement of one of the latter two moieties in kinase binding (Radke *et al.*, 1993).

PDC is also found in plant mitochondria and chloroplasts, providing in the latter organelle, acetyl CoA for fatty acid and isoprenoid biosynthesis and the only known source of NADH (Camp & Randall, 1985). These two spatially-separated complexes exhibit characteristic substrate specificities, pH optima and Mg^{2+} requirements (Miernyk & Randall, 1987), and are both subject to end-product inhibition. However, whereas plant mitochondrial PDC is additionally regulated by

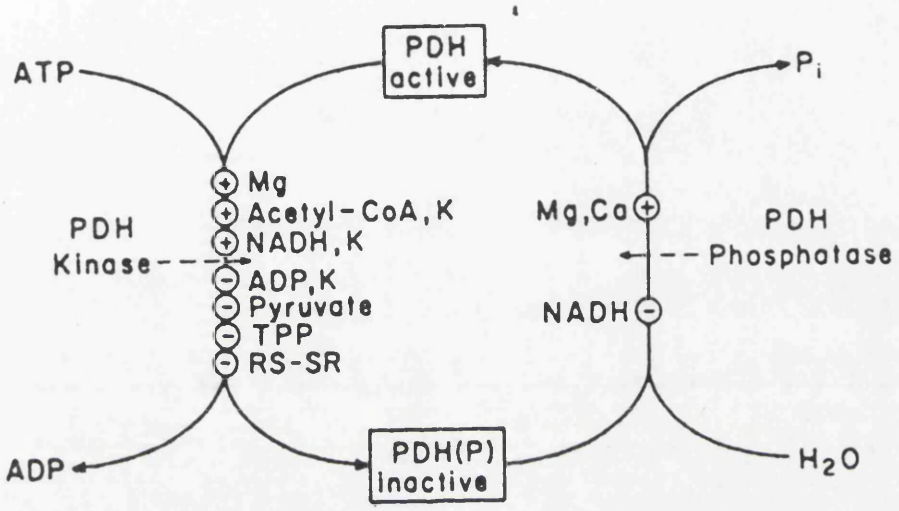


Fig. 1.1.4.1 Schematic representation of PDC regulation via covalent modification and the control elicited by effector molecules.

reversible phosphorylation, chloroplast PDC is controlled by the indirect effects of the light-dark transitions which include, changes in pH and metal ion concentrations (Randall *et al.*, 1990). However, as PDC has only been successfully purified once from broccoli mitochondria (Rubin & Randall, 1977), characterisation of structure, organisation and regulation is still in the initial stages.

1.1.4.1 Acute control

Acute control of BCOADC kinase, unaffected by Ca^{2+}, NAD^+/NADH or CoA/acetyl CoA levels, is facilitated by substrate dependent activation involving all five 2-oxoacid substrates with complex kinetics (see Yeaman, 1989 for review). In contrast, no evidence has come to light for such regulation in either prokaryotic complexes or the octahedral OGDCs. The latter complexes have been shown to exhibit an antagonistic allosteric activation/inactivation by Ca^{2+}/ATP (due to decreasing/increasing K_m values for 2-oxoglutarate), in response to the levels of hormones, adrenaline and vasopressin (Yeaman, 1989).

Acute hormonal Ca^{2+}-mediated control has also been demonstrated for PDC from heart and liver tissue with adrenaline, although clearly not an effect of direct allosteric activation but of PDC phosphatase activation (Denton & McCormack, 1985). However, the classic example of acute hormonal regulation, involving the insulin-directed dephosphorylation of PDC from adipose tissue, also occurs via phosphatase activation and although the mechanism of this activation remains unresolved, it is known not to involve increases in Ca^{2+} ions (Marshall *et al.*, 1984). Less information is available concerning the affects of acute hormonal control on BCOADC, nevertheless rapid inactivation has been observed following

increased levels of glucagon or adrenaline in diaphragm muscle (Palmer *et al.*, 1983).

1.1.4.2 Long term regulation

Owing to the phosphorylated state of heart and muscle tissue BCOADC, almost all active complex resides in the liver, the major site of branched-chain 2-oxoacid oxidation, where an activity state of 90% or higher exists following implementation of a protein-rich diet. Restriction of this diet would then result in the conservation of essential amino acids by a gradual reduction in BCOADC activity over several days in agreement with observed increases in kinase activity. Yet, with another example of long-term control such as, during starvation or alloxan-induced diabetes, BCOADC remains dephosphorylated (Randle *et al.*, 1981). In contrast, the levels of inactive PDC in heart, liver and kidney tissue increase, promoted by enhanced lipid metabolism, resulting in high acetyl CoA/CoA and NADH/NAD^+ ratios and consequent acute kinase activation, coupled with increased kinase production via altered gene expression. This can, however, be accounted for by the necessary utilization of branched-chain amino and 2-oxoacids in glucogenesis and ketogenesis, required for the continual supply of glucose to the brain (see Yeaman, 1989 for review).

1.1.5 Physiological disorders

The regulatory importance of the 2-oxoacid dehydrogenase complexes is also reflected in the severity of related disorders, which include various forms of metabolic acidosis such as, the widely recognised Maple Syrup Urine Disease (MSUD). Arising from a variety of structural aberrations in the branched chain complex and resulting in the accumulation of branched chain amino acids in the urine of sufferers, this autosomal recessive inborn error of metabolism was first described over 30 years ago, characterised by its high prevalence (i.e., 1 in 176 live births), in the Mennonite populations of Philadelphia (Marshall & DiGeorge, 1981). Various phenotypes have been described, ranging from the classic form, characterised by severe mental and physical impairment and early death, to a number of milder conditions with late onset classified as intermediate, intermittent and thiamine-responsive.

A second inborn error of metabolism first documented in 1970 (Blass *et al.*, 1970), involves deficiences of the pyruvate dehydrogenase complex and is associated with a broad spectrum of conditions characterised by low complex activity (typically 10-30% of normal), and accompanied by the presence of primary lactic acidosis and neurological disabilities of varying severity including Leigh's disease -- an autosomal recessive condition affecting normal brain function (Johnston *et al.*, 1984).

Until recently, little was known of the genetic mutations associated with either condition. Since complex activity depends upon the correct translation of 3(-5) components -- E1, E2, E3, (in PDC, E1α, E1β and X); and the regulatory enzymes, phosphatase and kinase, aberrations could arise through mutations in one

or more of 3(-9) gene loci. However, through measurement of individual component activities and immunological screening with component-specific antisera in cultured skin fibroblast samples, together with assessment at the mRNA level employing cDNA clones encoding individual components, recent research has identified several types of specific genetic mutations.

Located predominantly in E1 -- specifically E1α, but also associated with E2, E3, PDC phosphatase and protein X, the situation is clearly one of wide genetic heterogeneity varying from single base pair transitions (Zhang *et al.*, 1989; Fisher *et al.*, 1993), to large sequence deletions resulting in impaired transcription, translation, nuclear processing or catalytic function (Fisher *et al.*, 1991, 1993; Ho *et al.*, 1989; see Robinson *et al.*, 1988 for mini-review; 1990; Marsac *et al.*, 1993). In MSUD, the heterogeneity of E1 related defects is also reflected in the multiple patterns of inheritance, although location of the E1α gene to the X chromosome (Brown *et al.*, 1989) has explained X-linked inheritance in some patients (Bindoff *et al.*, 1989), since E1 is composed of α and β subunits encoded by two separate genes, the mode of inheritance is likely to be more complex. Notably, the rarity of E3-linked deficiencies reflects the severity of the associated condition in which all three 2-oxoacid complex activities are reduced resulting in the accumulation of lactic, 2-oxo- and branched chain, amino acids and their derivatives, in the plasma and urine of sufferers (Liu *et al.*, 1993).

Deficiencies in OGDC activity have been linked to Alzheimer's disease. Here, lowered 2-oxoglutarate/glutamate interconversion, caused by impaired 2-oxoglutarate oxidation, is thought to result in the accumulation of the neurotoxin, glutamate, the potency of which increases where energy metabolism appears suppressed (Beal, 1992). Previous reports of both impaired PDC and OGDC activities in autopsied brain tissue were unable to distinguish between cause and

effect (Butterworth & Bernard, 1990), but a recent study of cultured skin fibroblasts from familial Alzheimer patients revealed both defective OGDC activity and abnormalities of the E2 component, whereas, all other mitochondrial proteins including PDC were normal. The cause remains unclear, although mutations in the gene encoding E2 have been implicated (Sheu *et al.*, 1994).

More recently, six components of the 2-oxoacid dehydrogenase complexes have been identified as autoantigens of the autoimmune condition, primary biliary cirrhosis (PBC). This disease affects the intra-hepatic biliary ductular cells of the liver resulting in end-organ damage, and was first characterised by Walker *et al.* (1965), by the presence of non-organ, non-species specific autoantibodies directed against a group of mitochondrial antigens, later classed as M2 antigens (Berg *et al.*, 1986). Cloning and sequencing of the M2 antigens from λgt11 expression libraries, together with immunological analysis of purified bovine PDC employing patients' sera, initially identified E2 and X of PDC (Yeaman *et al.*, 1988), as the major autoantigens -- characterised by high autoantibody frequency and titre (in greater than 90% of all PBC patients, Palmer *et al.*, 1993); and subsequently, E2 of OGDC, BCOADC, and E1 of PDC (Fussey *et al.*, 1988; 1989).

Considerable research has located the major site of immunoreactivity to the inner lipoyl domain of E2, specifically the lipoate attachment site where the presence of a lipoic acid moiety appears crucial for autoimmunity (Surh *et al.*, 1989; Quinn *et al.*, 1993b), although weak immune responses are also elicited from the outer lipoyl, and peripheral subunit binding domains. Further, the recent generation of human monoclonal antibodies from an Epstein-Barr virus transformed PBC B lymphocyte cell line suggests the involvement of both an E2-specific and a cross-reactive epitope on protein X (Matsui *et al.*, 1993).

The role of M2 autoantigens in the pathogenesis of PBC include proposals for the involvement of : tissue-specific E2 isoforms; bacterial aetiology; or, somatic cell presentation. Prompted by the recent discovery of E2, and a second cross-reactive antigen, on the external surface of affected biliary cells (Joplin *et al.*, 1990; Cha *et al.*, 1993), Van de Water *et al.* (1993) proposed two scenarios for the onset of the disease. The first involves tissue-specific overexpression of the cross-reactive molecule, leading to cell surface presentation by MHC proteins, cell lysis and the resultant release of the 2-oxoacid dehydrogenase complexes arising in the production of autoantigens by the cross-reactive species-primed lymphocytes. Alternatively, the specialised transport of IgA to the lumen of human bile duct epithelial cells may allow contact with E2 during translation or transport to the mitochondrion, thus preventing uptake into the mitochondrion, leading to eventual metabolic disfunction and cell death.

Clearly, further research is necessary to elucidate the mechanism(s) by which intramitochondrially located proteins, distributed throughout the body, can elicite an autoimmune response specific to biliary tissue and prior to cell damage.

1.2 The multidomain structure of E2

In 2-oxoacid dehydrogenase complexes, E2 functions not only as an acyltransferase but also as the structural/functional framework upon which complex assembly and catalysis are dependent. Possessing highly segmented structures consisting of independently folding domains joined by short, flexible regions of polypeptide chain, E2 was first characterised in *E. coli* PDC where limited tryptic degradation resulted in the release of a reductively acetylatable lipoic acid-bearing fragment (M_r 32000 from SDS-PAGE), from a truncated E2 aggregate (composed of M_r 30000 peptides), which continued to exhibit E1/E3 binding potential and E2 activity (Bleile *et al.*, 1979). Extension of the proteolytic studies to mammalian PDC obtained similar results, where electron microscopy and velocity sedimentation revealed maintenance of an apparently native icosahedral core symmetry following release of the lipoyl-bearing domains (Bleile *et al.*, 1981; Kresze *et al.*, 1980).

The discovery of well-aligned sequence homologies between genes encoding E2 from different species and complexes (Thekkumkara *et al.*, 1988; Russell & Guest, 1991), led to the prediction of a multi-domain structure common to all E2 molecules, comprising 1-3 lipoyl domains; a subunit binding domain, involved in E1 and/or E3 binding; and, an inner catalytic domain in which resides the E2 active site, E2 self-assembly sequences and, in some complexes, additional E1 binding sites. Each domain is then separated by conformationally mobile linker regions of variable length and composition (see Fig. 1.2.1). Sequence alignments were also used to plot phylogenetic trees for E2 which suggested early divergence of the three E2 enzymes and therefore, complex types -- apparently prior to mitochondrial symbiosis (Russell & Guest, 1991; Matuda *et al.*, 1992), and the possible

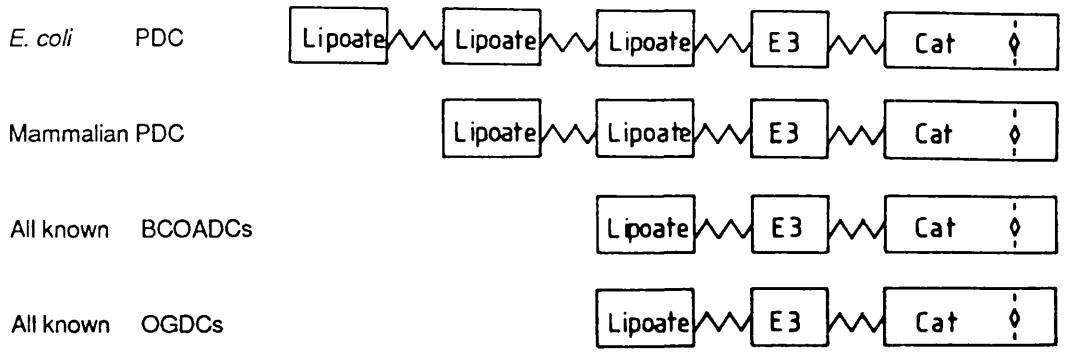


Fig. 1.2.1 Schematic representation of E2 domain structure.

Lipoate indicates lipoyl domains, E3 the peripheral subunit binding domains, and CAT the catalytic, core domain. Linkers are represented by zigzag lines, and active sites by diamonds.

emergence of various types of E2 from an ancestral form possessing one lipoyl domain.

Thus, assembled as an oligomeric core to which E1 and E3 bind, E2 facilitates increased catalytic efficiency through enhanced substrate channelling and stability, via the intimate coupling of the individual active sites by the flexible, so called "swinging arm" of the lipoic acid-lipoyl domain (Reed, 1974).

1.2.1 The lipoyl domains of E2 -- redundancy and active site coupling

Located at the N-terminus of E2 and comprising approximately 80 amino acids in length, lipoyl domains vary in number independently of species, complex or core symmetry type -- in some species, seemingly superfluous to requirement. For example, whilst the octahedral cores of *E. coli* and *A. vinelandii* PDC contain three lipoyl domains per E2 chain, the same core symmmetry in OGDC and BCOADC is associated with only one lipoyl domain. In contrast, the icosahedral core-containing PDH complexes of mammals, and Gram negative bacterium, *Streptococcus faecalis* possess two, whereas PDC of *S. cerevisiae* and both PDC and BCOADC of prokaryotes, *B. subtilus* and *B. stearothermophilus*, have only a single lipoyl domain per E2 subunit (reviewed in Perham, 1991). In addition, recent sizing via immunological screening of SDS-PAGE resolved plant extracts, suggests E2 from pea mitochondria and chloroplasts may only possess one lipoyl domain (Taylor *et al.*, 1992).

Maintenance of complex activity following selective proteolysis/modification of 1-2 of the lipoyl domains of E2 from *E. coli* PDC (Ambrose-Griffin *et al.*, 1980; Stepp *et al.*, 1981), or substitution of the essential lysine with glutamate residues (Graham *et al.*, 1986), revealed the ability of each lipoyl domain to fold independently and support overall complex catalysis. Furthermore, complex activity clearly could not rely upon the serial transfer of acetyl groups between outer and inner domains. These results confirmed previous predictions of a catalytic mechanism, known as active site coupling (Bates *et al.*, 1977; Stanley *et al.*, 1981). This proposed that, through the rapid intramolecular transfer of acyl groups between lipoic acid moieties on different E2 components within an E2 core and, the ability of more than one lipoyl domain to service any given E1 active site, efficient catalysis of a multistep reaction could be facilitated in a complex deviating from a simple 1:1 subunit stoichiometric ratio.

However, as maximum acetylation determinations in *E. coli* PDC have repeatedly reported sub-stoichiometric incorporations of 1.7-2 acetyl groups/E2 chain (Perham, 1991), it remains unclear whether all lipoyl domains are fully lipoylated and therefore, functional, or as proposed recently by Packman *et al.* (1991), upon observing full lipoylation, that previous estimations were subject to systematic error.

1.2.2 Protein-protein interactions of lipoylation and substrate channelling

Little is known of the mechanisms of lipoic acid biosynthesis, and lipoylation which involves the attachment of a lipoic acid moiety to the ε-amino group of conserved lysine residues located within specific cofactor attachment sites on each lipoyl domain (residue 40 in *E. coli* PDC; see Fig. 1.2.2.1). However, two genes, lip A and B, have been identified in *E. coli*, and the former gene product characterised and found to be responsible for the insertion of a suphur atom into the backbone of lipoic acid precursor, octanoate (Hayden *et al.*, 1992; Reed & Cronan, 1993). Further, the inability of this latter enzyme to convert bound octanoyl to bound lipoyl indicates that lipoic acid synthesis precedes that of covalent attachment, discounting the involvement of octanoyl-E2 as a synthetic intermediate (Guest *et al.*, 1991). Comparison of amino acid sequences revealed similarities to a sulphur-insertion enzyme, biotin synthase, suggesting the presence of a common sulphur-donor involved in both biotin and lipoic acid synthesis. Whilst the role of the lip B gene product remains uncertain, findings from lip B gene mutants in *E. coli* point to a possible regulatory function or, following isolation of two lipoyl domain-specific ligases in *E. coli* (Brookfield *et al.*, 1991) and one in *S. faecalis* (Reed *et al.*, 1958), a role in lipoic acid attachment itself (see Fig. 1.2.2.2).

Recent construction and overexpression of a plasmid containing the sub-gene encoding the single lipoyl domain from E2 of *B. stearothermophilus* PDC, in *E. coli*, resulted in the limited production of lipoylated domains compared to the overwhelming production of octanoylated domain (Dardel *et al*, 1990). This result revealed the ability of the relevant ligases in *E. coli* to recognise the lipoyl domains of *B. stearothermophilus* PDC, and to utilise octanoate in the absence of lipoyl

groups, highlighting the potential for mis-modification under conditions of saturation or stress. Similar results were obtained following the first case of *in vivo* lipoylation of a eukaryotic lipoyl domain in *E. coli* (Quinn *et al.*, 1993a). In contrast, expression of the E2 gene from bovine heart BCOADC in *E. coli* resulted in the production of unlipoylated domain, subsequently lipoylated following exposure to bovine heart mitochondrial extracts -- demonstrating some degree of differentiation between ligases of different species/complexes (Griffin & Chuang, 1990). Notably, lipoyl domains exhibit considerable specificity for the E1 component of the same complex and whilst free lipoic acid or lipoamide are substrates for E2 and E3 of *E. coli* PDC (Reed *et al.*, 1958), the lipoic acid cofactor can only be efficiently reductively acetylated by E1 when attached to its protein domain (Graham *et al.*, 1988). Clearly, additional protein recognition processes are involved, conferring specificity on the reductive acetylation of the dithiolane ring by E1, and thereby facilitating a simple but highly effective mechanism for substrate-channelling.

1.2.3 Elucidation of the 3-D structure of lipoyl domains

Owing to the involvement of the lipoyl domains in the specific protein/protein interactions of the lipoylating enzymes and of E1, considerable interest surrounded the elucidation of the 3-D structure. ^{1}H-NMR spectroscopy of both the lipoylated and unlipoylated domains, produced through the overexpression of the the sub-gene encoding the lipoyl domain of E2 from *B. strearothermophilus* PDC, revealed little conformational change associated with such post-translational modification (Dardel *et al.*, 1991). Further analysis then led to the discovery of a flattened eight stranded β-barrel structure, folded around a hydrophobic core with

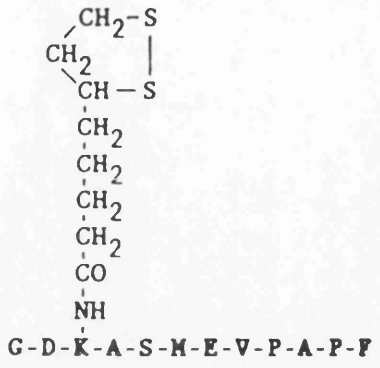


Fig. 1.2.2.1 Structure of the lipoyl peptide.

octanoate $\xrightarrow{lipA}$ 6- or 8-thiooctanoate → lipoate $\xrightarrow{lipB}$ lipoyl-protein

Fig. 1.2.2.2 Lipoic acid biosynthesis.

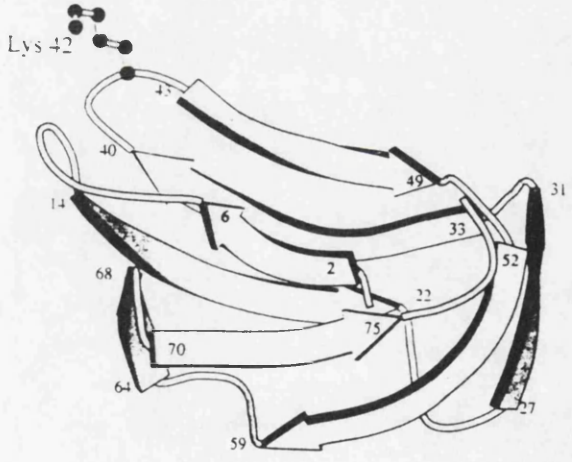


Fig.1.2.3.1 3D-ribbon drawing of the lipoyl domain from *B. stearothermophilus* PDC -- reproduced from Dardel *et al.* (1993).

closely positioned N- and C-termini, and the lipoic acid attachment site, $lysine_{42}$, prominently displayed on an exposed β-turn -- presumably for presentation to E1 (Dardel *et al.*, 1993). Two areas of conservation were located to the hydrophobic core (believed responsible for stabilisation), and to the highly charged, solvent exposed residues presumed to be involved in the specific recognition processes (see Fig. 1.2.3.1).

Sequence alignment and the identification of significant homology between the lipoyl domains from bacterial and eukaroytic sources (Russell & Guest, 1991; Dardel *et al.*, 1993), led to proposals for the existence of a common 3-D fold. This proposal was subsequently extended to include the domain responsible for biotinyl attachment in such biotin containing enzymes as pyruvate carboxylase where, a requirement for lysine and conservation of amino acids at the biotinyl attachment site mirrors that seen in the lipoyl domains of E2. However, research employing mutated forms of the *B. stearothermophilus* lipoyl domain-encoding subgene has very recently discounted a role for the conserved aspartate and alanine residues flanking the lipoyl attachment site in ligase-recognition, pinpointing an important but non-essential participation in E1 recognition (Wallis & Perham, 1994).

1.2.4 The linker regions of E2 -- involvement in active site coupling

In contrast to the compact and folded nature of the functional domains of E2, the linker regions are thought to possess an extended, flexible conformation, central for the formation of the so called "swinging arm" of the lipoyl/lysine moieties on E2.

Early spin-label experiments in *E. coli* PDC demonstrated total rotational freedom of the dithiolane ring of lipoic acid where effectively, this cofactor is in free solution on the surface of the E2 core (Ambrose & Perham, 1976). However, fluorescence energy transfer measurements indicated that the distances between the catalytic sites were too large (i.e., 4.5nm) to be encompassed by the rotation of a single lipoyl/lysine moiety (Shepherd & Hammes, 1977), even though attachment via the ε-amino group of lysine provided a flexible arm of 1.4nm in length. Electron microscopic images revealed a fuzz of low resolution surrounding the E2 core indicating the external positioning of the lipoyl domains of E2, interdigitating between the E1 and E3 components (Bleile *et al.*, 1979; Oliver & Reed, 1982). This provided an explanation for their proteolytic sensitivity and led to the proposal of a "super-arm" in which, additional mobility contributed by the lipoyl domain and linker regions, provides a rotational span wide enough to facilitate active site coupling.

The conformational flexibility of these regions was first demonstrated by ^{1}H-NMR spectroscopy of native PDC from *E. coli* (Perham *et al.*, 1981), and subsequently, with genetically engineered mutants and synthetic peptides representing the amino acid composition of linkers (Radford *et al.*, 1989). Owing to the paucity of aromatic residues, replacement of Glu_{291} by a histidine facilitated

mobility measurements via the detection of two peak resonances generated by the C2 and C4 protons, indicative of placement in a region of high flexibility.

1.2.5 Amino acid composition and flexibility of the linker regions

Occupying segments of poor sequence conservation between the folded domains, considerable variation in the composition of the linkers exists. Most are alanine/proline rich with or without charged amino acids present; fewer, only proline rich -- again with or without charged residues; and a third set, containing an abundance of charged and hydrophilic amino acids. More than one of the above sequence types may also reside within a single linker region (Perham, 1991).

Starting from the N-terminus of E2 from *E. coli* or *A. vinelandii* PDC, the three linkers separating the lipoyl domains from each other and the subunit binding domain are designated, apa 1, 2 and 3 which measure approximately 20-30 amino acids in length, although the absolute lengths remain unclear owing to ill-defined domain boundaries. A shorter fourth linker, separating the subunit binding and inner catalytic domains, is known as apa 4. Deletion of more than 13 residues of the proposed 32 amino acids of apa 3 in a mutant PDH complex from *E. coli* where all but the innermost lipoyl domain of E2 were removed (see Graham *et al.*, 1986 and section 1.2.1 for previous discussion), resulted in a dramatic reduction in complex activity, reductive acetylation and active site coupling, demonstrating the importance of linker length (Miles *et al.*, 1987; 1988). Furthermore, antibodies raised to apa 3 acted like "wedges" preventing active site coupling and complex activity without affecting individual component acitivities, and illustrating the requirement for flexibility in this region (Perham, 1991). While it remains unclear whether apa 1

and 2 can maintain activity following removal of apa 3 from intact PDC, fluorescence anisotropy of *A. vinelandii* PDC has revealed that although apa 1-4 exhibit internal flexibility, movement of the lipoyl domains pivots at apa 3 which the authors proposed as the hinge of the "super arm" (Hanemaaijer *et al.*, 1988). In contrast, deletion of apa 4 indicated little involvement in lipoyl arm flexibility (Schulze *et al.*, 1993), supported by the evidence of similar studies in *E. coli*, where high mobility in this region only followed removal of the lipoyl domains (Radford *et al.*, 1987, 1989; Guest *et al.*, 1989).

Recent circular dichroism and ^{1}H- & ^{13}C-NMR spectroscopic analyses of synthetic peptides with amino acid sequences identical to the linker regions in E2 from *E. coli* PDC, revealed a preponderance of ala-pro peptide bonds in the trans configuration which accounted for the stiff, extended conformation, and the absence of α-helical or random coil structure, associated with cis-bonds. As apa 3 was found to comprise more than 95% trans-peptide linkages, it is thought to be responsible for preventing the lipoyl domains from collapsing into the inner core, maintaining the optimal rotational span for catalysis (Green *et al.*, 1992). This is supported by observations of enhanced catalytic efficiency in an *E. coli* PDC mutant where apa 3 of E2 was replaced by a polyproline linker exhibiting greater extension and inflexibility (Turner *et al.*, 1993), and offers a ready explanation to the presence of three lipoyl (and accompanying linker) regions in *E. coli* and *A. vinelandii* PDC where only one lipoyl/lysine may participate directly in reductive acetylation. Indeed, the tandemly repeated lipoyl domains of this complex, encoded for by three highly homologous but non-identical *lip* gene segments, would be good candidates for recombinational instability and deletion in the absence of a positive selective pressure, such as increased active site coupling attributed to enhanced flexiblity (Graham *et al.*, 1986). In agreement, recent cryoelectron microscopy studies, employing hydrated complex, have revealed a maximal reach of 13nm in *E. coli*

PDC for the outermost lipoyl moiety (Wagenknecht *et al.*, 1992), and ^{1}H-NMR spectroscopy of mutants containing 0 to 9 lipoyl domains per E2 chain in *E. coli* indicated optimal linker mobility and complex catalysis with three lipoyl domains, decreasing in the presence of more (Machado *et al.*, 1992; 1993).

It is also of interest to note that in complexes possessing only one lipoyl domain such as OGDC of *E. coli*, considerable contributions to lipoyl/lysine arm flexibility come from the linker region equivalent to apa 4 of *E. coli* PDC, situated between the subunit binding and inner catalytic domain, and not believed to have much involvement in the latter complex in the "super-arm" mobility (see Perham, 1991 for review).

1.2.6 The subunit binding domain of E2 -- a role in E1/E3 binding

The subunit binding domains comprise one of the most highly conserved regions of E2, spanning 30-50 amino acids and located between the innermost lipoyl and catalytic domains. Common to all E2 components sequenced to date, except apparently that of rat OGDC (Nakano *et al.*, 1991), the peripheral subunit binding properties of this domain were first recognised following limited proteolysis in *B. stearothermophilus* and *E. coli* PDC (Behal *et al.*, 1989; Perham & Packman, 1986).

A relationship between core symmetry and subunit binding has emerged for this domain where, in octahedral cores such as, *E. coli* PDC and OGDC, it is associated primarily with E3 binding (Perham, 1991) and, in icosahedral cores such as, *B. stearothermophilus*, mammalian and *S. cerevisiae* PDC, with both E1 and E3

binding. The situation is complicated, however, by the additional E1 binding affinity of E2 from octahedral *A. vinelandii* PDC (Hanemaaijer *et al.*, 1988), and by the presence of protein X in yeast and mammalian PDC (De Marcucci *et al.*, 1985), which has led to further differentiation of subunit binding roles where E1 associates with E2, and E3 with a similar domain in protein X (Lawson *et al.*, 1991a,b).

Site-directed mutagenesis of the subunit binding domain in *A. vinelandii* PDC located the sites of E3 and E1 binding to the N- and C- termini, respectively, where association of the latter component also involves the N-terminus of the catalytic domain but not, surprisingly, of the linker in between (Schulze *et al.*, 1991c). Furthermore, overexpression of the subgene encoding the catalytic domain of either *A. vinelandii* or *E. coli* PDC in a bacterial host strain of *E. coli*, resulted in native E2 activity and core assembly but no E1 binding, indicating a requirement for the subunit binding domains in both complexes (Schulze *et al.*, 1991b), and contradicting previous observations for E1 binding in *E. coli* (Russell & Guest, 1991).

Similar overexpression in *E. coli* of a subgene encoding a di-domain comprising the linker, lipoyl and subunit binding domain of *B. stearothermophilus* E2 PDC, was followed by E3 binding studies involving both di-domain and the subunit binding domain, proteolytically cleaved from the former (Hipps *et al.*, 1994). These revealed a subunit stoichiometry of one E3 dimer/subunit binding domain suggestive of non-cooperative binding. In agreement, studies in *A. vinelandii* and *Pseudomonas faecalis* PDC have shown that E3 binds when only in a dimeric and therefore, active conformation (Schulze *et al.*, 1991a). Stronger E3 binding affinity was exhibited by the di-domain compared to that of the isolated subunit binding domain which pointed to the involvement of the linker, but not lipoyl regions, in E3 binding (Hipps & Perham, 1992). The varying involvement of

involvement of the linker regions in peripheral subunit binding is subject to growing interest following observations from cryoelectron microscopic images of bovine heart, bovine kidney and *E. coli* PDC of variable E1 and E3 positioning -- thought to reflect flexible tethering (Wagenknecht *et al.*, 1991; 1992). In addition, 1-3nm "gaps" corresponding to areas of poor resolution and therefore, high flexibility were also noted between the surface of the core and the E1 and E3 components. This was then offered further support by the evidence of fluorescence anisotrophy in *A. vinelandii* PDC, which indicated retention of E3 mobility following binding (Schulze *et al.*, 1991a). These results call into question the original face-centred model for E3 binding proposed by Oliver & Reed (1982), whereby each monomer of an E3 dimer spans one face of the E2 core and is bound relatively inflexibly.

1.2.7 Elucidation of the 3-D structure of the subunit binding domain of E2

Recent elucidation of the 3-dimensional solution structure of the subunit binding domains from both an octahedral complex, E2 of *E. coli* OGDC (Robien *et al.*, 1992), and from the icosahedral complex of *B. stearothermophilus* PDC (Kalia *et al.*, 1993) have highlighted close similarities, as previously predicted from the high degree of sequence conservation between different species (Russell & Guest, 1991); but, also significant differences. Synthetic peptides of 51 and 33 residues, representing the amino acid composition of the subunit binding domains of *E. coli* and *B. stearothermophilus,* respectively, were subjected to ^{1}H-NMR spectroscopy, revealing structures composed of two parallel α-helices separated, in *E. coli*, from a disordered loop by a short extended strand and helical turn; and from a highly structured loop of overlapping β-turns in *B. stearothermophilus* by a 3_{10} helix. In

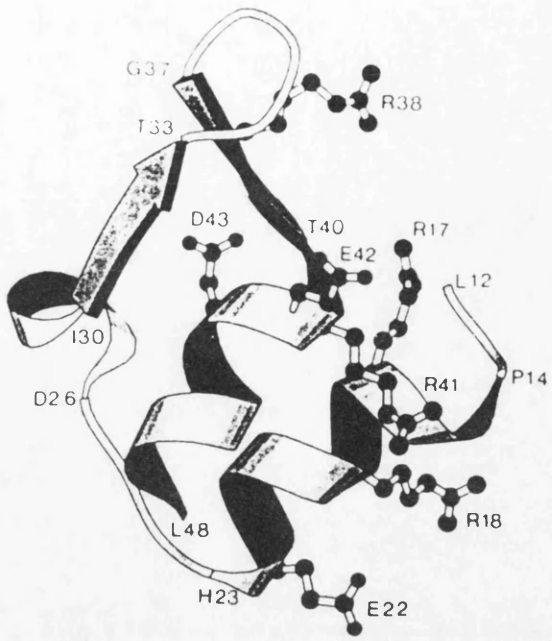


Fig. 1.2.7.1 Schematic ribbon drawing of the E3 binding domain from *E. coli* OGDC -- reproduced from Robien *et al.* (1992).

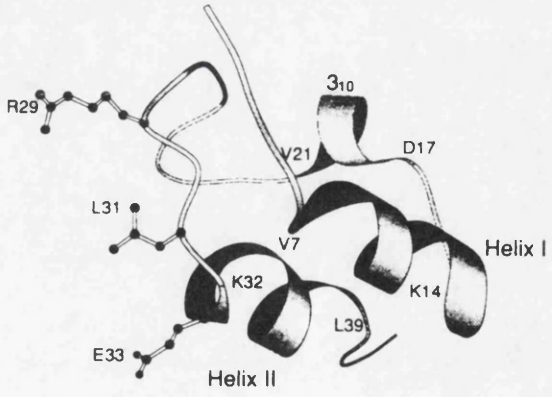


Fig. 1.2.7.2 3D-ribbon drawing of the peripheral subunit binding domain from *B. stearothermophilus* PDC -- reproduced from Kalia *et al.* (1993).

consideration of the extremely small size of these domains (that of *B. stearothermophilus* is the smallest independently folding domain known to date) and, in the absence of stabilising ligands or cofactors, stability is thought to derive from the tight hydrophobic cores and essential aspartate residues buried deep within the loop regions (see Figs. 1.2.7.1-2).

In the absence of X-ray crystallographic images of intact complex, owing to the flexibility of the lipoyl domains, little is known of the E1/E3 binding interface on E2. However, following examination of sequence homologies, Robien *et al.* (1992) proposed that as binding interactions would necessarily involve electrostatic interactions (such as salt bridges, H-bonds) with solvent-accessible residues, the contiguous surface of the two α–helices offered a likely location for binding interactions. In agreement, Russell & Guest (1991) observed that as E1 and E3 dissociation is easily facilitated with high ionic strength/pH and, in light of the highly conserved nature of both E3 and the subunit binding domains, peripheral subunit binding presumably involves a limited number of charged residues such as the conserved glutamate and aspartates found on the α–helices (see Fig. 1.2.7.1).

A highly conserved nucleotide binding motif "Gly Xaa Gly Xaa Xaa Gly $(Xaa)_n$ Lys" is also present within this domain from all species excepting E2 from rat, yeast and *B. subtilis* OGDC, where it has been proposed that TPP, NAD^+, CoA or ATP may bind alongside components E1 and E3 (Matuda *et al.*, 1992).

1.2.8 The inner catalytic core domain of E2 and E2 core self-assembly

The inner catalytic domain of E2 typically comprises 200-250 amino acid residues in which reside : the E2 binding sites involved in the self-assembly of the E2 core; the acyltransferase active site including the highly conserved "Asp His Arg Xaa Xaa Asp Gly" CoA binding site; and, in some complexes, additional E1 binding sites (see section 1.2.6).

Early observations of E2 core and complex self-assembly, and of the maintenance of acyltransferase activity within the core assembly, following removal of a lipoyl-bearing fragment by limited proteolysis in *E. coli* (Koike *et al.*, 1963; Bates *et al.*, 1977; Bleile *et al.*, 1979), were later confirmed via the over-expression of sub-genes encoding the inner catalytic domain of E2 from *S. cerevisiae* PDC (Niu *et al.*, 1990), *A. vinelandii* and *E. coli* PDC (Schulze *et al.*, 1991b) in *E. coli* hosts. Purification and analysis of the 3-D structure by electron microscopy (Stoops *et al.*, 1992), and X-ray crystallography (Mattevi *et al.*, 1992), revealed apparently wild type core structures coupled with fully functional E2 active sites. Although little information is available concerning the mechanisms of self-assembly nor the determining sites involved, high resolution (2.6 Å) X-ray analysis of the individual E2 subunits of the assembled catalytic domains from *A. vinelandii* (Mattevi *et al.*, 1992), resolved a topology identical to that of chloramphenicol acetyltransferases (CAT), confirming a prediction of Guest (1987). This was based upon the discovery of remote but significant homologies between the amino acid sequences of E2 from *E. coli* PDC and OGDC and, of CAT complexes from various species (i.e., 19% identity within the catalytic domains) and followed elucidation of a trimeric organisation in CAT (Leslie *et al.*, 1986).

1.2.9 Structural resemblances between the catalytic domain of E2 and CAT

Fig. 1.2.9.1 illustrates a schematic representation of the trimeric organisation of CAT and of the proposed "building block" of E2 cores (reproduced from Mattevi *et al.*, 1992). Differences in the degree of intratrimeric contact are apparent, where with E2, the N-terminus of one subunit forms an interconnecting arm to a neighbouring subunit within each trimer. In a recent review article, De Rosier (1992) postulated that the enhanced stability afforded by this additional interaction, absent in CAT complexes, may be necessary in the overall process of subunit folding and complex assembly or, to counterbalance instabilities wrought by the flexibility of the lipoyl domains. Indeed, such a requirement for stability is not surprising where the interface between neighbouring subunits forms the catalytic channel of the acyltransferase active site in both 2-oxoacid dehydrogenase and CAT complexes. Thus, in the former complexes the cubic/dodecahedral vertices form a cage-like structure composed of cone-shaped trimers, orientated with flattened bases facing outwards and apices pointing inwards towards a large, solvent filled interior. Measuring 7.6 x 14nm in a core of 22.6nm diameter in *S. cerevisiae* PDC, and comprising 50% of the total core volume, access to this internal cavity (apparently necessary for the internal localisation of the CoA binding cleft), comes from large symmetrical openings across the faces of the core.

Fewer inter-trimeric interactions are involved in the assembly of the E2 cores from the trimer building blocks, although closer interactions have been observed in *S. cerevisiae* PDC (Stoops *et al.*, 1992), compared to that of *A. vinelandii* (Mattevi *et al.*, 1992). Upon E1/E3 binding, the 24 subunit octahedral core of the latter species exhibits a unique dissociation into the smallest known functional PDH

complex of M_r 860000 (Bosma *et al.*, 1984), comprising a tetramer of E2 to which three dimers of E1 and one dimer of E3 are bound. Site-directed mutagenesis experiments have located the sites involved in this coupled dissociation/activation process to the N-terminus of the inner catalytic domain (Schulze *et al.*, 1991c) where clearly, dissociation is dependent upon weaker inter-trimer associations.

Following numerous reports of size heterogeneity in preparations of active PDC from *E. coli* and mammalian sources, Sumegi & co-workers (1987) proposed the existence of smaller active forms *in situ* in equilibrium with larger aggregates. Whilst this could be conveniently explained by the presence of active trimers assembling into cubic then icosahedral cores, no evidence of such a process has been provided by the literature to date.

1.2.10 Mechanistic similarities between the active sites of E2 and CAT

Sequence alignments between the inner catalytic domains of E2 from *E. coli* PDC, OGDC and, that of CAT complexes from various species have pinpointed areas of significant homology centred around the conserved "Pro Xaa Xaa Xaa Gln His His Xaa Xaa Xaa Asp Gly His" active site motif of CAT (Guest, 1987). Mirrored by an "Asp His Arg/Lys Xaa Xaa Asp/Asn Gly" motif located at the equivalent position of all dihydrolipoamide acyltransferases sequenced to date, this observation led to the prediction of similar mechanisms of catalysis in both E2 and CAT. The latter complex catalyses the O-acetylation and inactivation of chloramphenicol in antibiotic resistant bacteria and has been the subject of considerable characterisation.

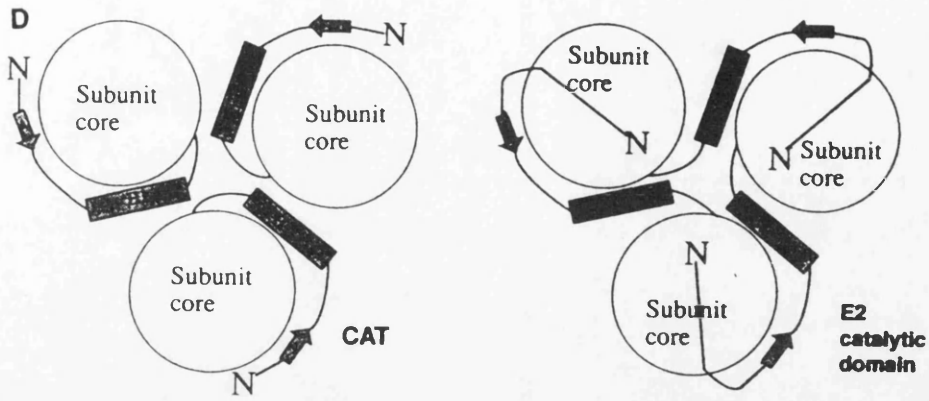


Fig. 1.2.9.1 Schematic representation of CAT and E2 trimers from *A. vinelandii* PDC -- reproduced from Mattevi *et al.* (1992)

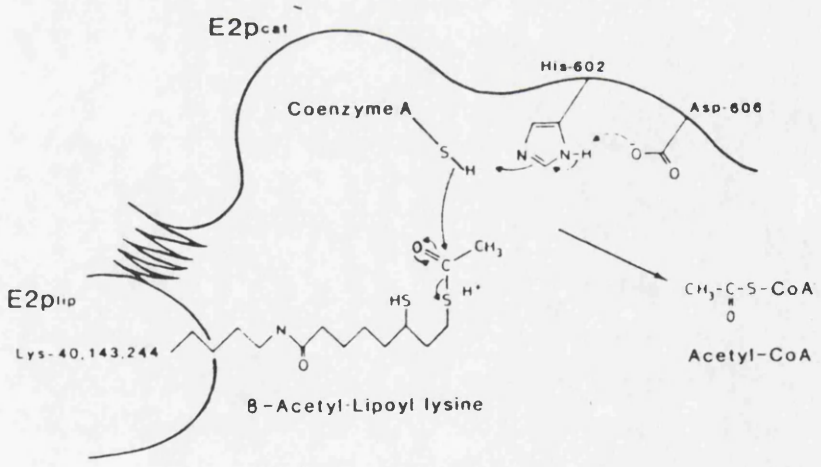


Fig. 1.2.10.1 Schematic representation of the mechanism of catalysis at the active site of E2.

In CAT, the conserved active site His_{195} residue is absolutely essential for catalysis. Acting as a general base, the N-1 protons form an hydrogen bond to the carboxyl of neighbouring Asp_{199} conferring tautomeric stabilisation and thereby, allowing the electron pair of N-3 to abstract a proton from the 3-hydroxyl of substrate, chloramphenicol. This in turn, promotes a nucleophilic attack on the thioester of acetyl CoA, facilitating acetylation of chloramphenicol. Hence, Guest (1987) proposed an analogous mechanism involving proton abstraction from the thiol of CoA and, nucleophilic attack on the 8-acyl lipoamide thioester (Fig. 1.2.10.1). However, considerable variation exists between species concerning the functional requirements of the various active site residues. For example, whilst site-directed mutagenesis in *E. coli* PDC revealed an absolute requirement for His_{602} (reviewed in Russell & Guest, 1991), similar substitutions in *S. cerevisiae* suggested no involvement of the active site histidine residue in acetyltransferase activity (Niu *et al.*, 1990). In contrast, substitution of active site Asp_{431} -- equivalent to Asp_{199} in CAT; Asp_{606} in *E. coli* E2p; and, Asp_{609} in *A. vinelandii* PDC (Mattevi *et al.*, 1992), and involved in salt-bridge formation essential to catalysis, led to loss in complex activity.

Nevertheless, in support of the proposed involvement of histidine, X-ray crystallographic analysis of substrate binding and catalysis in the assembled truncated core domain of E2 from *A. vinelandii* (Mattevi *et al.*, 1993), revealed that the reactive thiol of both substrates, CoA and lipoamide, were within hydrogen bonding distance to the side chain of active site His_{610}. Furthermore, in agreement with the proposed stabilising function of Ser_{148} in CAT, Mattevi & co-workers (1993), characterised an active binding mode for substrate, CoA, involving transition state stabilisation of reaction intermediates by residue, Ser_{558}.

1.3 The active sites of E1 and E3

A similar active site mechanism to that seen in E2 and CAT complexes involving a histidine/glutamate couple, has been proposed for E3 whereby, histidine serves as a proton acceptor during the oxidation of enzyme-bound dihydrolipoamide -- with E3 shuttling between an oxidised and two electron-reduced state (Guest, 1987). The importance of all three active site residues, His_{450}, Pro_{451} and Glu_{455} was later confirmed in *A. vinelandii* E3 by site directed mutagenesis studies (Benen *et al.*, 1991). As a member of the well-characterised flavoprotein disulphide oxidoreductase family, elucidation of the structure/function of E3 has followed refined crystallographic analyses of E3 from *P. putida*, *P. fluorescens*, *S. cerevisiae* and *A. vinelandii*, and comparative studies on the related enzyme, glutathione reductase (Takanaka *et al.*, 1988; Mattevi *et al.*, 1991, 1992b). These have revealed common features such as the involvement of the reactive disulphide bridge in catalysis, and the requirement for dimerisation in the formation of the E3 active site (Schulze *et al.*, 1991a).

In contrast, little is known of the active site mechanisms of E1, although the general chemistry of TPP-dependent catalysis is known (see Perham, 1991 for review). Identification of a proposed TPP binding site motif (Hawkins *et al.*, 1989), following sequence alignments, has suggested the locality of the active site. Spanning 30 residues, this sequence is thought to adopt a βαβ fold and possess essential tryptophan and aspartate residues involved in TPP and possibly, Mg^{2+} binding but not apparently, of pyruvate (Diefenbach *et al.*, 1992). Furthermore, its location on the α-subunit of E1 from icosahedral complexes, has led to the proposal that the active site of this subunit be involved in the formation of the 1-hydroxyethylidene-TPP intermediate, and that of E1β involved in the subsequent

reductive acylation of the lipoic acid moiety of E2 (see Perham, 1991). In addition, following the initial rapid formation of S^8-acetyldihydrolipoamide, intramolecular acetyl transfer between the S^8 and S^6-thiols of the E2-linked lipoic acid cofactor is known to occur in *E. coli* PDC (Yang & Frey, 1986). However, as the isomerisation rate constant ($0.045.s^{-1}$ at pH 6.5) is considerably lower than the turnover number for this complex ($1.6 \times 10^2 .s^{-1}$), the significance of this process in overall complex catalysis remains unclear.

1.4 Protein X -- structural/functional similarities to E2

1.4.1 Identification of a fourth component in yeast and mammalian PDC

Adoption of the neutral Laemmli buffered SDS-PAGE system resulted in improved resolution of PDC and led to the discovery of a fourth component from mammalian and yeast sources, known as protein or component X (De Marcucci *et al.*, 1985).

Previous observations in bovine heart and kidney PDC of an extra band of approximately 50000-M_r migrating with the E3 component, had dismissed X as a proteolytic fragment of E2 or E3 or, as the yet uncharacterised PDC kinase (Stanley & Perham, 1980; Kresze *et al.*, 1980; Bleile *et al.*, 1981). However, immune analysis of whole cell and subcellular preparations from bovine, rat and pig cell lines, under conditions where proteolytic degradation should not occur, revealed the presence of this 50000-M_r band. Further extensive characterisation employing: component specific antisera; one and two dimensional peptide mapping of purified iodinated E2 and X; various ^{14}C-acetylation/deacetylation studies in bovine heart and kidney extracts in this laboratory and elsewhere (De Marcucci & Lindsay, 1985; De Marcucci *et al.*, 1986; Jilka *et al.*, 1986; Hodgson *et al.*, 1986); N-terminal amino acid sequence analysis of E2 and X isolated from bovine heart PDC (Neagle *et al.*, 1989); and, the isolation of a separate gene encoding X in *S. cerevisiae* (Behal *et al.*, 1989), subsequently confirmed the existence of a fourth independent species.

Like E2, protein X possesses a multi-domain structure comprising lipoyl, subunit binding and inner domains, separated by flexible linker regions. In mammalian PDC, the lipoyl domain of X has been shown to be capable of E1-dependent acetylation, CoA dependent deacetylation and NEM modification (Hodgson *et al.*, 1986). Furthermore, protein X exhibits the unique phenomenon of mammalian acetyltransferases of diacetylation whereby, both S^6,S^8-thiols of the dithiolane ring on the lipoic acid cofactor incorporate acetyl groups. Following rapid, initial S^8-acetylation and the exchange of acetyl groups between S^8 and S^6 sulphur atoms, a slow secondary acetylation takes place, leading to an enhanced storage potential for acetyl groups. This is thought to be of physiological importance during mild starvation or diabetes, facilitating the removal of excess levels of NADH and acetyl CoA from the cytosol.

1.4.2 A role for protein X in complex catalysis

In contrast to E1 and E3, protein X remains tightly associated with the E2 core assembly following treatment with high salt or alkaline pH and like the intrinsically core-bound kinase, application of chaotrophic reagents such as, p-hydroxymercurial sulphonate or 5M urea, are necessary for its removal (De Marcucci & Lindsay, 1985; Jilka *et al.*, 1986). This copurification has proved problematic in the determination of a precise role for X in overall complex catalysis. However, the presence of a functional lipoyl domain on X points to some involvement in catalysis.

This is supported by the findings of protein X-mediated activity in both *S. cerevisiae* and bovine kidney PDC following selective gene deletion or,

proteolytic cleavage, of the lipoyl domains of E2 (Lawson *et al.*, 1991b; Rahmatullah *et al.*, 1990). Indeed, in contrast to the active site coupling properties of the lipoyls of E2, proteolytic removal of the protein X lipoyl domains leads to an immediate loss in complex activity, which Gopalakrishnan & co-workers (1989), attributed to disruption in the flow of reducing equivalents from the lipoyls of E2 to E3. Sequence alignment between E2 and X from *S. cerevisiae* and mammalian sources (Behal *et al.*, 1989; Neagle *et al.*, 1989), has revealed 45-50% identity at the N-termini suggestive of a common ancestor and a role for X as an isoenzyme of E2. However, as direct evidence of acetyltransferase activity in preparations of isolated protein X from bovine kidney and yeast sources does not exist (Rahmatullah & Roche, 1987; Lawson *et al.*, 1991a) and, in the absence of an active site motif in the C-terminal domain of yeast X (Behal *et al.*, 1989), such a suggestion seems doubtful. Nevertheless, translation of a 245 base pair cDNA clone encoding the extreme C-terminus of protein X from human PDC, has revealed an active site-like motif of "His Xaa Xaa Xaa Ser Gly" clearly warranting further investigation (J.C. Neagle, University of Glasgow, unpublished observations).

1.4.3 Stoichiometry and organisation of protein X

Little information is available concerning the organisation and subunit stoichiometry of X, again owing to its tight core interactions. Estimates of 6-12 molecules of X per PDH complex have come from : comparisons of ^{14}C-acetylation of the lipoyl moieties of components E2 and X (Jilka *et al.*, 1986); sedimentation coefficients (Lawson *et al.*, 1991a); and, densitometric scan analysis of X band staining in purified complex samples (Jilka *et al.*, 1986).

How these molecules arrange within the E2 core remains unclear. Evidence from covalent crosslinking studies of the lipoyl domains of E2 and X in intact bovine heart PDC (Hodgson *et al.*, 1988), and the proteolytic sensitivity of these regions, suggest that X is arranged like E2 with the extended lipoic acid bearing regions interdigitating between the E1 and E3 components. If an isoenzyme of E2, X would necessarily exist as an integral core component substituting for molecules of E2 and thereby, maintaining icosahedral symmetry. However, E2 core assemblies devoid of X yet exhibiting native symmetry and E2 activity, have been purified from both yeast (Niu *et al.*, 1990), and bovine sources (Powers-Greenwood *et al.*, 1989; Rahmatullah *et al.*, 1989b). These results could be interpreted as an indication of independence from X which could, therefore, exist as an accessory protein bound once core assembly is complete. Notably, a limited binding capacity appears to exist for X, since transformation of protein X-deficient *S. cerevisiae* cells with either single or multi-copy plasmids containing the protein X gene (PDX1), resulted in the same degree of incorporation into the native complex (Lawson *et al.*, 1991a). Nevertheless, such findings do not altogether preclude the possibility of an integrated protein X when E2 core assembly occurs in its presence.

1.4.4 A role for protein X in E3 binding

Following initial observations in bovine kidney PDC of E3 and E1-mediated protection of X and E2, respectively, from proteolytic enzymes, Arg C and trypsin (Rahmatullah *et al.*, 1989b), an E3 binding function was ascribed to protein X. Further examination of peripheral subunit-binding in core preparations of E2, E2/X and E2/X_I -- where the lipoyls of X are selectively removed with Arg C, (Powers-Greenwood *et al.*, 1989; Gopalakrishnan *et al.*, 1989); in protein X-deficient

S. cerevisiae mutants transformed with mutated forms of the PDX1 gene, housing specific domain deletions (Lawson *et al.*, 1991a); and, in E2-deficient *S. cerevisiae* strains transformed with similarily mutated E2 (LAT1) genes (Lawson *et al.*, 1991b), confirmed this prediction. Nevertheless, whilst the latter experiments pin-pointed the E1 binding function to the subunit binding domain of E2, and the E3 binding function to a similar domain on X, the inability of the $E2/X_I$ core to maintain PDC activity following reconstitution with dissociated E1 and E3, indicated a requirement for the lipoyl domains of X in E3 binding. Yet, complete removal of these domains from X has been facilitated in intact bovine heart PDC in the absence of complex activity loss, although E3 affinity was lowered (Neagle & Lindsay, 1991). This result may be explained if, once the complex is assembled and E3 bound, intact protein X is no longer necessary.

It is of interest to note that in the absence of a subunit binding motif in rat E2 from OGDC (Nakano *et al.*, 1991), an E3 binding role has been identified recently for the lipoyl-like sequences at the N-terminus of E1 from bovine heart OGDC (Rice *et al.*, 1992). As with protein X, dissociation of E1 from the E2 core is difficult suggestive of a strong binding interaction, and whilst no lysine-bound lipoic acid moiety resides within the lipoyl domain-like sequence, precluding a catalytic function, other characteristics such as proteolytic sensitivity and immunoreactivity parallel those of functional lipoyl domains. In addition, although protein X has only been identified in yeast and mammalian PDC to date, recent reports of a M_r 67000 and 48000 species from pea mitochondria and chloroplasts, respectively, which exhibits cross-reactivity with anti-X sera, may uncover individual X or X-like sequences on components from plant sources (Taylor *et al.*, 1992).

1.5 Aims of research

1) Conflicting evidence of a role in E3 binding for the lipoyl domain of X from mammalian PDC exists. Re-examination and extension of the work of Roche & coworkers, and of this laboratory, providing a more detailed analysis of reconstitution, should offer an explanation to these discrepancies.

2) Following preliminary unpublished observations of an active site-like motif in mammalian protein X (J.C. Neagle, University of Glasgow), investigations into the nature of the involvement of X in complex catalysis is presented.

3) In light of the scant information available for the subunit stoichiometry and organisation of protein X in mammalian PDC, the final area of research is directed towards the precise quantification and structural positioning of X within the E2 core of bovine heart PDC.

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

The following chemicals were purchased from SIGMA Chemical Company Ltd., Dorset, U.K.:-

silver nitrate, 3-[N-Morpholino]-propane-sulphonic acid (MOPS), imidazole, salicylic acid (2-hydroxybenzoic acid), Coomassie Brilliant Blue R250, Pyronin Y, antifoam A (concentrate), L-cysteine-HCl (anhydrous), β-nicotinamide adenine dinucleotide (oxidised form, β-NAD^+; reduced form, β-NADH), adenosine-5'-triphosphate (ATP; sodium salt), thiamine pyrophosphate chloride (TPP), DL-6,8-thioctic acid amide (DL-lipoamide, oxidised form), benzamidine-HCl, phenylmethylsulphonyl fluoride (PMSF), N-ethylmaleimide (NEM), coenzyme A (lithium salt), acetyl coenzyme A (C2:0) lithium salt, pyruvic acid (sodium salt) and 2-oxoglutaric acid (disodium salt).

The majority of commonly used chemicals were purchased from FISONS FSA laboratory supplies, Leicestershire, U.K. and were of the highest grade available. This included:-

PEG 6000, acrylamide, N,N-methylenebisacrylamide, ammonium persulphate, citric acid, glycine, trichloroacetic acid (TCA), sodium lauryl sulphate and Folin & Ciocalteu's phenol reagent.

Tris (2-amino-2[hydroxymethyl]-1,3 propandiol) base was purchased from BOEHRINGER MANNHEIM, U.K. (Diagnostics and Biochemicals) Ltd., Sussex, U.K.; dithiothreitol (DTT; Cleland's reagent) from CALBIOCHEM Novabiochem, Nottingham, U.K.; and N, N-1,2 phenylene dimaleimide (PDM) from ALDRICH Chemical Company, Dorset, U.K.

2.1.2 Radioisotopes

The following radioactive materials were purchased from AMERSHAM International plc., Buckinghamshire, U.K.:-

N-ethyl[2,3-^{14}C]maleimide (9mCi.mmol^{-1}, 100μCi.ml^{-1}), [1-^{14}C]acetyl coenzyme A (54mCi.mmol^{-1}, 50μCi.ml^{-1}), ^{125}I-labelled Protein A (>30mCi.mg^{-1}).

[2-^{14}C]pyruvic acid, sodium salt (10.4mCi.mmol^{-1}) was purchased from NEN Research Products, DU PONT [U.K.] Ltd., Hertfordshire, U.K.

2.1.3 Enzymes and Proteins

From SIGMA Chemical Company Ltd., Dorset, U.K. the following enzymes and proteins were purchased:-

Trypsin, type XIII (TPCK treated) E.C. 3.4.21.4, from bovine pancreas (12600 U.mg^{-1} solid); collagenase, type VII, E.C. 3.4.24.3, chromatographically

purified from *Clostridium histolyticum* (1700 U.mg^{-1} solid); lipoamide dehydrogenase, type VI, E.C. 1.8.1.4, isolated from bovine intestinal mucosa (170 U.mg^{-1} protein); endoproteinase, Arg C, E.C. 3.4.21.40, HPLC purified from mouse submaxillary gland (sequencing grade); protein A (extracellular), purified from *Staphylococcus aureus*; bovine serum albumin, fraction V of cold alcohol precipitation; leupeptin protease inhibitor.

The majority of endoproteinase, Arg C, used was purchased from BOEHRINGER MANNHEIM, U.K.(Diagnostics and Biochemicals) Ltd., Sussex, U.K. Isolated from mouse submaxillary gland (E.C. 3.4.21.40), the enzyme was of analytical grade (220U.mg^{-1}). Low molecular mass electrophoresis marker proteins were purchased from PHARMACIA LKB Biotechnology, Milton Keynes, U.K.

2.1.4 Photographic materials

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.

High performance Hyperfilm-ECL, AMPLIFY fluor and a Sensitize pre-flash unit were all purchased from AMERSHAM International plc., Buckinghamshire, U.K. Autoradiography cassettes and HIGH SPEED-X intensifying screens were purchased from GENETIC RESEARCH INSTRUMENTATIONS Ltd., Essex, U.K.

2.1.5 Chromatographic materials

Sephadex G25 and Superose 6 and 12 were purchased from PHARMACIA LKB Biotechnology, Milton Keynes, U.K., and the immobilised anhydrotrypsin column purchased from PIERCE (& WARRINER) Ltd., Cheshire, U.K.

2.1.6 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.

2.1.7 Miscellaneous

The Biotrap BT-1000 electroelution kit was purchased from SCHLEICHER & SCHUELL, Dassel, Germany.

HYBOND-C extra supported nitrocellulose (0.45μm pore size) was purchased from AMERSHAM International plc., Buckinghamshire, U.K.; and PVDF protein sequencing membrane purchased from BIO-RAD Laboratories Ltd., Hertfordshire, U.K.

Centricon 10 microconcentrator tubes were purchased from AMICON, Danvers, Ma., U.S.A.

Ecoscint A was purchased from NATIONAL DIAGNOSTICS, Manville, N.J., U.S.A.; and DECON from DECON Ltd., Hove, U.K.

2.2 METHODS

2.2.1 Determination of protein concentration

Protein concentrations were determined using the method of Lowry *et al.*, (1951), modified by Markwell *et al.* (1976). For dilute protein solutions a protein assay modified from Bradford, and the Pierce micro BCA assay were used. In all cases, standard curves were constructed using bovine serum albumin, and absorbances read at 660nm, 750nm or 562nm, respectively.

2.2.2 Concentration of protein samples

Dilute solutions of protein, for subsequent analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), were concentrated by TCA precipitation involving the addition of 10% (w/v) TCA followed by 30min at 4°C, after which protein could be pelleted using a microcentrifuge. Pellets were resuspended in Laemmli sample buffer and the pH adjusted to pH 7 using 2M Tris base.

Column fractions of E2/X and E1/E3 were concentrated, often after overnight dialysis to remove salt, by covering the dialysis tubing with dry poly(ethylene glycol) (PEG 6000) flakes, and stored at 4°C until the required decrease in volume was achieved. Alternatively, E2/X samples could be concentrated by centrifugation at 120000g (r_{av} 9cm), for 16h, with resuspension in

1% (v/v) Triton X-100 buffer of the resultant pellet (see section 2.2.8). Likewise, dilute samples of PDC and OGDC were concentrated by centrifugation at 200000g (r_{av} 9cm), for 2.5h, and pellets resuspended as described above.

Centricon TM tubes were also used to concentrate small volumes of dilute protein where the removal of salt was also required. This involved 20-40min spins at 5000g (r_{av} 8.5cm).

2.2.3 Dialysis of protein samples

Dialysis was carried out at 4°C using Visking Tubing previously boiled for 10min in 2% (w/v) $NaHCO_3$, 1mM EDTA pH 8, rinsed and boiled in distilled water for a further 10min.

2.2.4 Protein analysis by SDS-polyacrylamide gel electrophoresis

Proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), using either the discontinuous Tris/glycine buffered system of Laemmli (1970) or, the continuous sodium phosphate buffered system of Weber & Osborne (1969).

a) Preparation of protein samples

i) **Laemmli system:** Prior to electrophoresis, an equal volume of Laemmli sample buffer (62.5mM Tris/HCl pH 6.8, containing 2% (w/v) SDS, 10% (w/v) sucrose and 0.2% Pyronin Y), and DTT to a final concentration of 100mM, were added to samples which were then boiled for 3-5min.

ii) **Weber & Osborne system:** Protein samples were diluted in sample buffer (5mM sodium phosphate buffer, pH 7 containing 0.5% (w/v) SDS, 5% (v/v) mercaptoethanol (2-ME), 50% (v/v) glycerol, 0.002% (w/v) Bromophenol blue), and boiled for 3-5min.

b) Preparation of analytical SDS-polyacrylamide gels

i) **Laemmli system:** Gels with the dimensions of 170mm x 145mm x 1.5mm were polymerised from the following solutions to give **resolving gels** of 7-15% (w/v) acrylamide (containing 0.375M Tris/HCl buffer pH 8.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate, and 0.08% (v/v) NNN'N'-tetramethylethylene diamine (TEMED); and **stacking gels** of 4.4% (w/v) acrylamide (containing 0.12M Tris/HCl buffer pH 6.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate, and 0.08% (v/v) TEMED).

I) Acrylamide solution	**III) Resolving gel buffer**
29.2% (w/v) acrylamide	0.75M Tris/HCl, pH 8.8
0.8% (w/v) bis-acrylamide	0.2% (w/v) SDS

ii) Stacking gel buffer

0.17M Tris/HCl, pH 6.8

0.14% (w/v) SDS

iv) Ammonium persulphate

0.1g/ml $(NH_4)_2S_2O_8$

The above solutions were degassed prior to polymerisation of the acrylamide, initiated by the addition of TEMED.

ii) Weber & Osborne system: Gels with the dimensions of 170mm x 145mm x 1.5mm were polymerised from the following solutions to give slab gels of 8-10% (w/v) acrylamide (containing 0.1M sodium phosphate buffer pH 7, 0.1% (w/v) SDS, 0.075% (w/v) ammonium persulphate, 0.002% (v/v) TEMED).

i) Acrylamide solution

22.2% (w/v) acrylamide

0.6% (w/v) bis-acrylamide

iii) Ammonium persulphate

15mg/ml $(NH_4)_2S_2O_8$

ii) Gel buffer

0.2M sodium phosphate buffer, pH 7

0.2% (w/v) SDS

c) Conditions for electrophoresis

i) Laemmli system: This employed a constant current of 40-70mA, and electrophoresis 24mM Tris/192mM glycine buffer, pH 8.3 containing 0.1% (w/v)

SDS. Gels were run using vertical gel electrophoresis kits purchased from Bethesda Research Laboratories (BRL).

ii) Weber & Osborne system: Gels were run at a constant current of 100mA, with 1:1 diluted gel buffer as the electrophoresis buffer. Gels were run using BRL vertical gel electrophoresis kits.

d) Conditions for staining and scanning of gels

SDS-polyacrylamide gels were stained overnight to visualise protein bands using either 0.04% (w/v) Coomassie Brilliant Blue R (in 10% (v/v) acetic acid and 25% (v/v) methanol), and destained with 20% (v/v) methanol and 10% (v/v) acetic acid; or, where greater sensitivity was required, with silver stain -- based on the method of Wray *et al.* (1981). This involved overnight pretreatment in 50% (v/v) methanol followed by agitation for 20min in freshly prepared 1% (w/v) silver nitrate (previously a 20% (w/v) solution added dropwise to 0.34% (w/v) NaOH and 0.9M NH_4OH in 20ml, and diluted to give final concentrations of 0.08% (w/v) NaOH and 0.21M NH_4OH). Stringent washings with distilled water over 1h were followed by the addition of freshly prepared developer, containing 0.24mM citric acid and 0.02% (v/v) formaldehyde, until bands were seen. The gel was then treated to further washings with distilled water to remove traces of developer, before scanning with a Shimadzu FDU-3 dual wavelength flying spot scanner at 450nm.

e) Preparation of SDS-polyacrylamide gels for fluorographic analysis

Radiolabelled proteins were resolved following the method described above. However, after electrophoresis the gels were immersed, with agitation, for 1h in fixer (10% (v/v) acetic acid, 25% (v/v) isopropanol), rinsed with distilled water and removed to either: fresh 1M salicylate (solid salicylate was dissolved in 5M NaOH and the pH adjusted to pH 6 using NaOH), and agitated in the dark for a further hour at room temperature; or, to a commercial fluor, AMPLIFY, for 15-30min at room temperature, with agitation. Gels were then dried down under vacuum at 60°C onto Whatman 3MM chromatography paper and exposed to either X-OMAT S film or, high performance Hyperfilm-ECL at -80°C. The film was then developed by hand using commercial developer and fixer.

f) Relative molecular mass (M_r) determination of SDS-polyacrylamide resolved proteins

Using low M_r protein markers to construct a calibration curve relating relative mobility, R_f (see equation below), to log(M_r) for each protein standard, the relative molecular mass of sample proteins from the same gel could be determined. The standards comprised: phosphorylase b (94000); bovine serum albumin (67000); ovalbumin (43000); carbonic anhydrase (30000); soybean trypsin inhibitor (20000); and α-lactalbumin (14000). Relative mobility was calculated as follows:-

$$R_f = \frac{\text{distance migrated by protein}}{\text{distance migrated by tracking dye}}$$

Where protein relative molecular mass determination was required for immunoblot analysis, prestained M_r protein standards with extra protein, myosin (H-chain; 200000), were used for easy visualisation on nitrocellulose.

2.2.5 Preparation of gels for protein sequence analysis

The following modified Laemmli (1970) discontinuous Tris/glycine buffered system was employed in the separation and isolation of protein samples for amino acid sequencing.

a) Preparation of protein samples

Samples were diluted into an equal volume of sample preparation buffer (0.06M Tris/HCl buffer, pH 6.8, containing 10% (v/v) glycerol, 0.5% (v/v) 2-mercaptoethanol, 0.4% (w/v) SDS), boiled for 2min and, 1-3µl of 0.1% (w/v) Bromophenol blue added.

b) Preparation of sequencing SDS-polyacrylamide gels

From the following solutions **10-15% (w/v) resolving gels** (containing 10-15% (w/v) acrylamide, 0.4M Tris/HCl buffer, pH 8.8, 0.1% (w/v) SDS, 0.04% (w/v) ammonium persulphate, 0.001% (v/v) TEMED); and **5% (w/v) stacking**

gels (containing 5% (w/v) acrylamide, 0.13M Tris/HCl buffer, pH 8.8, 0.1% (w/v) SDS, 0.08% (w/v) ammonium persulphate, 0.001% (v/v) TEMED) were polymerised.

i) Acrylamide solution

29.2% (w/v) FLUKA acrylamide

0.8% (w/v) bis-acrylamide

ii) Stacking gel buffer

0.5M Tris/HCl, pH 6.8

4% (w/v) SDS

iii) Resolving gel buffer

1.5M Tris/HCl, pH 8.8

0.8% (w/v) SDS

iv) Ammonium persulphate

0.1g/ml $(NH_4)_2S_2O_8$

All solutions were filtered through 0.2μm MILLIPORE filters, and degassed before the addition of TEMED.

c) Conditions for electrophoresis

Gels were pre-run in electrode buffer (25mM Tris/0.2M glycine buffer, pH 8.3, containing 0.1% (w/v) SDS), at 3mA for 2h, with 250μl of 10mM glutathione added to the upper reservoir. The pre-run buffer was then decanted, and replaced with fresh buffer to which 70μl of 100mM sodium thioglycolate was added. The samples were then run at 60mA for 3h in a BRL vertical electrophoresis kit.

d) Preparation for sequencing

The resolved proteins were then transferred overnight at 40mA to pre-soaked IMMOBILON (100% (v/v) methanol for 5min, followed by transfer buffer, 25mM Tris/0.19M glycine buffer pH 8, containing 0.02% (w/v) SDS, 20% (v/v) methanol), following the method outlined in section 2.2.7. After transfer, the IMMOBILON was washed in distilled water for 5min, stained for 5min with 0.1% (w/v) Coomassie blue stain (in 50% (v/v) methanol), destained with 50% (v/v) methanol for 10min, then rinsed in distilled water before drying. Protein bands were cut out using a scalpel and stored at 4°C in sealed plastic bags before being sent to the SERC Protein Sequencing Facility at Aberdeen University.

2.2.6 Electroelution of proteins from SDS-polyacrylamide gels

The following method of electroelution was used in the preparation of isolated E2 and component X for the quantification of X and for the production of component specific antibodies.

a) Separation and visualisation of proteins

In the preparation of isolated components, E2 or X. 2-5mg of highly purified PDC or E2/X core were run on a single-well 8% (w/v) SDS-PAGE gel to give maximum separation of E3 from component X. The gel was then treated with a

non-fixative $CuCl_2$ stain. This involved several washes with distilled water, followed by agitation in 0.3M $CuCl_2$ for 5min after which bands could be visualised against a dark background and excised from the gel before destaining with several changes of 0.25M EDTA in 0.25M Tris/HCl buffer pH 9, followed by re-equilibration in elution buffer (see section 2.2.6b).

b) Electroelution of proteins

Samples of electrolytically resolved proteins (see section 2.2.6a) were electroeluted from 0.5cm x 0.5cm excised gel pieces using a Schleicher and Schuell Biotrap BT 1000 apparatus following the method outlined in the accompanying manual. The gel pieces were placed in a 1cm x 1.2cm mini elution chamber and the apparatus placed into a horizontal electrophoresis tank and both filled with elution buffer (40mM Tris/20mM acetic acid/1mM EDTA buffer containing 0.025% (w/v) SDS, pH 8; or, 25mM Tris/192mM glycine buffer containing 0.025% SDS, pH 8.3) to the level of the submerged gel pieces. A voltage of 100V was applied overnight at 4°C, which promoted the migration of the protein molecules through the microporous membrane wall into a 200-800µl capacity trap whereupon further movement was prevented by a second membrane. The protein concentration of the eluted protein was then determined using the micro-assays outlined in section 2.2.1.

2.2.7 Procedure for Western blotting

Proteins, resolved by SDS-PAGE, were electrolytically transferred to nitrocellulose membranes for subsequent immunological detection following the method of Towbin *et al.* (1979). Gels were overlaid with pre-wetted Hybond-C extra transfer membrane and placed into cassettes, between pre-wetted Whatman 3MM chromatography paper. Cassettes were then placed into a Bio-Rad Transblot^{TM} tank filled with transfer buffer (25mM Tris, 0.19M glycine, 0.02% (w/v) SDS, 20% (v/v) methanol) and a current of 40mA applied overnight. A non-fixative stain, Ponceau S, was used to check that proteins had transferred successfully onto the nitrocellulose and this was then rinsed off with wash buffer (20mM Tris/HCl buffer, pH 7.2, containing 0.15M NaCl, 0.5% (v/v) Tween 20). The nitrocellulose was agitated in wash buffer for 1h at room temperature, before addition of 1:100 diluted antiserum, and 1:20 diluted heat-inactivated donkey serum in 50ml fresh wash buffer, for a further 90min. Antisera was prepared employing the method of Hunter & Lindsay (1986), except where the electroelution technique listed in section 2.2.6 was used in the isolation of protein from SDS-PAGE gels. Unbound antiserum was removed using repeated washings with buffer over 1h, before addition of ^{125}I-labelled Protein A (3 x 10^6 cpm, in 50ml wash buffer), for 1h at room temperature. Again, unbound label was removed using several changes of wash buffer over 1h, the nitrocellulose air-dried, and placed into an autoradiography cassette for exposure at -80°C to X-OMAT S film.

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

The isolation of PDC and OGDC from bovine heart tissue was carried out at 4°C following the method of Stanley & Perham (1980) with the modifications stated below.

Fresh ox heart, trimmed of fat and connective tissue, was diced and used directly or stored at -80°C and thawed overnight before use. To 600g, 500ml of 3% (v/v) Triton X-100 buffer (50mM MOPS, 2.7mM EDTA, 3% (v/v) Triton X-100, 0.1mM DTT, 1mM PMSF, 1mM benzamidine, 0.2% (v/v) anti-foam, adjusted to pH 7 with 6M NaOH) was added, before blending for 5min, and the final volume adjusted to 2 litres with buffer. The homogenate was then clarified at 10000g (r_{av} 8.5cm) for 20min, the pellets discarded and the supernatant adjusted to pH 6.45 with 10% (v/v) acetic acid. The complexes were precipitated by the addition of 0.12vol. of 35% (w/v) poly(ethylene glycol), stirred for 30min, and pelleted at 18000g (r_{av} 8.5cm) for 15min. The pellets were resuspended in 400ml, 1% (v/v) Triton X-100 buffer (50mM MOPS, 2.7mM EDTA, 1% (v/v) Triton X-100, 1mM PMSF, 1mM benzamidine, 1.5μM leupeptin, 0.2mM DTT, adjusted to pH 6.8 with 6M NaOH) by homogenisation with a loose fitting glass-teflon homogenizer, before clarification at 25000g (r_{av} 8.5cm) for 40min.

The supernatant was filtered through muslin to remove fat and 0.013vol. of 1M $MgCl_2$, and 0.05vol. of 1M sodium phosphate buffer, pH 6.3 were added together with 0.5M NaOH to maintain pH 6.8. After adjusting the pH to 6.45 with acetic acid, a second 0.12vol. of 35% (w/v) poly(ethylene glycol) was added and the

supernatant stirred for 30min at 4°C. The complexes were pelleted at 25000g (r_{av} 8.5cm) for 10min, resuspended by homogenisation in 160ml of 1% (v/v) TX-100 buffer, pH 6.8, and fresh solutions of 1mM PMSF, benzamidine, leupeptin and 0.5% (v/v) rat serum added, and left overnight at 4°C.

Re-homogenisation was followed by a clarifying spin at 25000g (r_{av} 8.5cm) for 1h, and the supernatant adjusted to pH 6.45 before the addition of 0.04-0.06vol. of 35% (w/v) poly(ethylene glycol) for 30min with stirring. This differential cut enables the separation of OGDC, subsequently pelleted at 25000g (r_{av} 8.5) for 10min, from PDC which remains in the supernatant and is pelleted thereafter, either: by addition of 0.14vol. of 35% (w/v) poly(ethylene glycol) for 30min followed by a 10min 25000g (r_{av} 8.5cm) spin; or, by an ultracentrifugal 200000g (r_{av} 9cm) spin, for 2.5h. In a successful separation, 95% of the OGDC activity is found in the pellets leaving 90% of PDC activity in the supernatant. Complex activities are assayed by monitoring NADH production at 340nm, as outlined in section 2.2.11.

The pelleted complexes were subsequently resuspended in 1% (v/v) TX-100 buffer, pH 6.8 (no protease inhibitors), and were further purified employing a 100cm x 3cm Sepharose CL-2B gel filtration column, equilibrated overnight at 4°C with 50mM sodium phosphate buffer pH 7, containing 2.7mM EDTA and 1% (v/v) Triton X-100, at a flow rate of 24ml.min^{-1}. Approximately 100mg of protein were routinely resolved, 12ml fractions collected and assayed for PDC and OGDC activities, and pooled accordingly. The individually purified complexes were then pelleted at 200000g (r_{av} 9cm) over 2.5h, and resuspended by hand homogenisation in 1% (v/v) TX-100 buffer.

2.2.9 Preparation of isolated E2/X core

Purified PDC (30-50mg) treated 1:1 with 4M NaCl (in 50mM Tris/HCl, pH 9) for 1h at 4°C, or 20min at room temperature, was dissociated into E2/X and E1/E3 fractions after resolution on a 100ml (100cm x 1cm) Superose 6 gel filtration column, previously equilibrated with 50mM Tris/HCl buffer, pH 9, containing 1M NaCl and 0.01% (v/v) Triton X-100, at a flow rate of $1ml.min^{-1}$ and with a pre-injection of 4M NaCl. Protein was detected by absorbance at 280nm and fractions containing E2/X pooled, dialysed overnight against 50mM imidazole/HCl buffer, pH 7.4 containing 0.1mM EDTA, to remove salt, and poly(ethylene glycol) concentrated as described in section 2.2.2. The purity was assessed by SDS-PAGE and if residual E1 and E3 remained attached to the core, salt treatment was repeated, and the isolated E2/X core pelleted at 130000g (r_{av} 9cm) over 16h, leaving dissociated E1/E3 in the supernatant fraction.

2.2.10 Preparation of reconstituted PDC

a) Dissociation of PDC into enzymatically active E2/X and E1/E3 fractions

Purified PDC (30mg) treated 1:1 with 4M NaCl (in 50mM Tris/HCl buffer, pH 7) for 1h at 4°C was dissociated into E2/X and E1/E3 fractions on a 100ml (100cm x 1cm) Superose 6 gel filtration column, at neutral pH, and the E2/X and E1/E3 fractions pooled, dialysed and concentrated as described in section 2.2.9.

b) Reconstitution of PDC activity

The protein concentration and residual PDC activity of each fraction was determined and the optimum ratio (w/w) for reconstitution determined -- normally 1: 0.7-0.8 (E2/X:E1/E3). Typically, 10-20µl samples of either fraction were pre-incubated, for 10min at 33°C, in assay buffers A and B (see section 2.2.11i) to a final volume of 50-60µl, and 5µl of the reconstituted PDC removed to assay activity. The extent of reconstitution was then determined as a percentage of the original specific activity of PDC determined before salt treatment.

2.2.11 Enzyme assays

i) 2-oxoacid dehydrogenase complexes

The overall activities of PDC and OGDC were assayed spectrophotometrically at 340nm, as the rate of formation of NADH, at 30°C (Danson & Perham, 1978). Approximately 2-5µg of complex were assayed in the presence of 670µl **solution A** (50mM potassium phosphate buffer, pH 7.6, containing 3mM NAD^+, 2mM $MgCl_2$, 0.2mM TPP), 14µl **solution B** (0.13M cysteine HCl, 0.13mM Li_2CoASH), and 14µl **solution C** (100mM pyruvic acid (PDC), or 2-oxoglutarate (OGDC)). The reaction was initiated by the addition of enzyme to the above solutions, preincubated at 30°C in a quartz cuvette, and activity expressed in nkatals (the amount of enzyme which is required to produce 1nmole of $NADH.s^{-1}$ at 30°C).

ii) Lipoamide dehydrogenase (E3)

The activity of lipoamide dehydrogenase was determined spectrophotometrically at 340nm as the rate of formation of NADH, at 30°C, from the conversion of dihydrolipoamide to lipoamide.

a) Preparation of dihydrolipoamide

Dihydrolipoamide was prepared from the oxidised form of DL-6,8-thioctic acid amide (DL-lipoamide) following the method of Kochi & Kikuchi (1976), whereby 60mg of DL-lipoamide was dissolved in 1.2ml of 50% (v/v) ethanol in 1M potassium phosphate buffer pH 8, to which 2.4ml of freshly prepared 5% (w/v) $NaBH_4$ (in 10mM NaOH), was added for 10min, followed by neutralisation with 1.2ml of 3M HCl. The dihydrolipoamide was then extracted, after mixing, into the upper solvent layer by the addition of 3 x 3ml volumes of toluene. Evaporation of the toluene under N_2 left crystalline dihydrolipoamide which was stored at -20°C until use, whereupon 20.7mg was dissolved in 1ml of 70% (v/v) ethanol.

b) Conditions for assay

Approximately 2-5µg of complex or isolated E3 were added to 670µl of **solution A** (50mM potassium phosphate buffer, pH 7.6, containing 3mM NAD^+, 2mM $MgCl_2$, 0.2mM TPP), and 10-20µl fresh **dihydrolipoamide** (preincubated at 30°C in a quartz cuvette), and the resultant activity expressed as nkatals (the amount of enzyme required to produce 1nmole of $NADH.s^{-1}$ at 30°C).

iii) Pyruvate dehydrogenase (E1)

E1 activity was measured spectrophotometrically at 600nm as a colour change, due to the reduction of coloured 2,6-dichlorophenolindophenol (DCPIP), to a colourless form. Various amounts of 2-oxoacid dehydrogenase complex or isolated E1 were added to 670µl **solution A** (50mM potassium phosphate buffer, pH 7.6, containing 3mM NAD^+, 2mM $MgCl_2$, 0.2mM TPP), 14µl **solution C** (100mM pyruvic acid (PDC), or 2-oxoglutarate (OGDC)), and 14µl **DCPIP** ($1mg.ml^{-1}$), preincubated at 30°C in the assay cuvette, and the resultant activity expressed as the amount of enzyme required to reduce 1µmole of $DCPIP.min^{-1}$ at 30°C.

2.2.12 Crosslinking of N-ethylmaleimide (NEM) pretreated 2-oxoacid-dehydrogenase complexes

This method was used in the elucidation of the structural organisation of component X in bovine heart PDC. This involved the covalent crosslinking of adjacent lipoyl groups followed by Western blot analysis. Prior to the crosslinking of the reduced lipoyls of E2 (OGDC), or X (PDC) with N,N-1,2-phenylene-dimaleimide (PDM), PDC and OGDC were pretreated with cold NEM to block non-specific crosslinking to reduced sulphydryl groups. The unbound NEM was then removed by centrifugal gel filtration (Penefsky, 1977).

a) Pretreatment with NEM

1mg of complex, diluted in 100mM Tris/HCl buffer, pH 7.5 to a final concentration of $2\mu g.\mu l^{-1}$, was incubated at 4°C for 1h in the presence of 1mM TPP, 1mM $MgCl_2$, 0.5mM NEM and 0.25% (v/v) Triton X-100. Immediately following this, unbound NEM was removed using centrifugal gel filtration (see section 2.2.12b).

b) Removal of unbound NEM via centrifugal gel filtration

Mini (0.4mm x 7mm) gel filtration columns, made from 1ml syringes fitted with glass wool filters, were packed with Sephadex G-25 gel (medium grade) which had been pre-swollen in 100mM Tris/HCl buffer, pH 7.5 overnight at 4°C, and warmed to room temperature prior to use. The gel matrix was packed by several 2min spins at 800g (r_{av} 15cm) in a TJ-6 Benchtop centrifuge, equilibrated twice by the addition of 200µl column buffer, followed each time with a 800g (r_{av} 15cm) spin, before the addition of the protein sample (maximum: 100µg in 50-100µl), which was eluted into an Eppendorf by a 2min 800g (r_{av} 15cm) spin. The % recovery was estimated by assaying complex activity (see section 2.2.11i), and traces of NEM detected by virtue of an NADH-induced inhibition of complex activity, whereby lipoyl groups are reductively labelled with NEM in the presence of 0.2mM NADH over 30min, thereby causing inactivation.

c) Crosslinking with N,N-1,2-phenylene-dimaleimide (PDM)

The lipoyl domains of E2 from PDC were proteolytically cleaved by treatment with 10% (w/v) collagenase in 50mM Tris/HCl buffer, pH 7.5, containing 0.5mM $MgCl_2$, 0.2mM TPP and 2mM $CaCl_2$ at 30°C, leaving the lipoyls of component X intact for subsequent treatment with crosslinker, PDM. Digestion was monitored through direct assay of complex activity, and by the removal of aliquots to Laemmli sample buffer for subsequent immunoblot analysis, until only 20% of the original activity remained (i.e., after 60min), upon which, the reaction was arrested by the addition of 4mM EGTA at 4°C. Control samples were also included to monitor non-specific proteolysis.

Prior to crosslinking, stock solutions of 4mM NAD^+, 4mM NADH, 50mM substrate and 0.1mM PDM were freshly prepared in 100mM Tris/HCl buffer pH 7.5 -- in the latter case, by the serial dilution of a 10mM stock (i.e., 0.0135g PDM in 5ml DMSO).

The lipoyls of E2 (OGDC) and component X (PDC) were then reductively crosslinked by the addition of 0.2mM NADH or, 2mM pyruvate/2-oxoglutarate and 1mM NAD^+, to 0.5$\mu g.\mu l^{-1}$ concentrations of complex in the presence 100mM Tris/HCl buffer, pH 7.5 containing 5mM $MgCl_2$, 1mM TPP, and 0.25% (v/v) Triton X-100. A range of crosslinker concentrations were used (typically 5-10μM), and the reaction initiated by the addition of crosslinker immediately after addition of NADH or the appropriate substrate, and the removal of 20μl and 5μl samples to 20mM 2-ME for subsequent immunological and complex activity analysis. Control crosslinking was also carried out in the presence of 0.2mM NAD^+. Incubation was carried out at 30°C for 10-30min during which time, 5μg aliquots were removed to

20mM 2-ME at 4°C and complex activity assayed. Once 80-90% inhibition was achieved, the reaction was terminated by addition of 20mM 2-ME after which samples were resolved on 8% (w/v) SDS-polyacrylamide gel electrophoresis prior to immunological detection of the crosslinked species with a variety of component specific antisera.

2.2.13 Measurement of incorporation of radioactive precursors

The measurement of incorporation of [1-^{14}C]acetyl CoA and [2-^{14}C]pyruvic acid onto the lipoyls of E2 and X of PDC was used in the quantification of component X, as follows.

a) Incorporation of radioactive precursors, [2-^{14}C]pyruvic acid and [1-^{14}C]acetyl CoA

Samples of PDC were diluted to 1-2mg.ml^{-1} in **solution A** (see section 2.2.11i) and pre-incubated for a few minutes at room temperature prior to the initiation of acetylation by the addition of 0.25-1mM [2-^{14}C]pyruvic acid (10.4mCi.mmol^{-1}; 50-150μCi/tube), followed by a further 30min incubation at room temperature. In the case of 0.5mM [1-^{14}C]acetyl CoA (54μCi.mmol^{-1}; 3μCi/tube), a 10min preincubation with 0.3mM NADH was followed by the addition of [1-^{14}C] acetyl CoA for an additional 60min at room temperature. Control samples lacking 2-oxoacid dehydrogenase complex were treated in a similar manner. The reaction was

terminated by the addition of 45mM 2-ME and each sample diluted in Laemmli buffer and treated at 70°C for 2min prior to resolution by 8% (w/v) SDS-PAGE.

b) Measurement of radioactivity

Following Coomassie blue staining and subsequent destaining, the gels were rinsed in distilled water, then dried onto Whatman 3MM chromatography paper at 60°C under vacuum to facilitate the excision of the resolved E2 and X bands from triplicate loadings of 20μg or 30μg PDC (determination of incorporation from 40, 60 or 80μg PDC involved 2, 3 or 4 x 20μg loadings). Gel pieces were also taken from the equivalent positions of control samples lacking PDC, for the calculation of background incorporation. The excised bands were then removed to scintillation vials, the gel re-hydrated in 0.5ml distilled water and the chromatography paper removed. Vials were then drained and the protein solubilised overnight in 1ml of SOLVABLE NEF-910G at 37°C. ECOSCINT A (4.5ml) was then added and the incorporation of ^{14}C-acetyl groups determined over 10min, in triplicate, in an LKB WALLAC 1209 Rackbeta liquid scintillation counter. The ratio of incorporation into E2 and X was then calculated as a mean value of the triplicate results, differing by <10% and from which the corresponding background count was subtracted.

2.2.14 Labelling of individual enzyme components with [2,3-^{14}C] NEM

This method was used to analyse diacetylation in both Arg C/collagenase treated and untreated intact/reconstituted PDC.

b) Pretreatment with Arg C/collagenase

In some cases, prior to labelling, the intact complex was digested in **solution A** (see section 2.2.11i) with either, 3% (w/v) Arg C for 90min at 33°C (with an additional 3% (w/v) at 60min); or, 10% (w/v) collagenase for 60min at 33°C (in 50mM Tris/HCl buffer, pH 7.5, containing 2mM $CaCl_2$, 3mM NAD^+, 0.5mM $MgCl_2$, 0.2mM TPP), and the decrease in complex activity assayed at 340nm at regular time intervals (see section 2.2.11i). With reconstituted complex, protease treatment was carried out on the isolated E2/X core before reconstitution with E1/E3 (see section 2.2.10b). In either case, the reaction was stopped by the addition of appropriate inhibitors (Arg C -- 5mM benzamidine; collagenase -- 5mM EDTA). Undigested complex treated under the same conditions was used as a control.

b) Conditions for [2,3-^{14}C]NEM labelling

Five conditions were set up in 1ml Eppendorf tubes for both control and digested samples with 100-200μg of sample (1-2mg.ml^{-1}) per tube. All five conditions were run simultaneously as follows:-

1) 0.85mM[2,3-^{14}C]NEM for 10min.

2) 0.85mM[2,3-^{14}C]NEM added following 4mM pyruvate -- incubated for 10min.

3) 10min preincubation with 0.2mM NADH, followed by 0.85mM[2,3-^{14}C] NEM for 10min.

4) 10min preincubation with 0.2mM NADH, 1min with 0.4mM acetyl CoA, followed by 0.85mM[2,3-^{14}C]NEM for 10min.

5) 10min preincubation with 0.2mM NADH, 60min with 0.4mM acetyl CoA, followed by 0.85mM[2,3-^{14}C]NEM for 10min.

The labelling reaction was terminated by the addition of 45mM 2-ME, the samples diluted in Laemmli sample buffer lacking DTT and warmed to 70°C for 2min before analysis by SDS-polyacrylamide gel electrophoresis (see section 2.2.4). Radioactive incorporation was visualised by fluorographic analysis (see section 2.2.4e).

2.2.15 Preparation for C-terminal peptide sequence analysis

This method was employed in the preliminary isolation of the C-terminal tryptically derived peptides of components E2 and X prior to the determination of the N-terminal amino acid sequence and therefore, the possible presence of an acetyltransferase active site-like sequence.

Anhydrotrypsin is a catalytically inert derivative of trypsin in which the active site residue, Ser-195, has been converted to a dehydroalanine residue which selectively binds, under weakly acidic conditions, peptides that have arginine, lysine or S-amino-ethylcysteine at the C-terminus. Thus, assuming no arginine or lysine residues at the C-terminus of the protein of interest, following complete tryptic digestion, isolation of the C-terminal peptide is facilitated by virtue of its non-adsorption to the anhydrotypsin column. The method used was as described in the accompanying manual but with the following modifications.

Prior to column treatment, 100-200µl of a 2-4mg.ml^{-1} sample of purified E2/X core (see section 2.2.9), denatured by treatment at 65-100°C for 5min and diluted in 0.1M NH_4HCO_3 buffer, pH 8 containing 0.1mM $CaCl_2$ (to inhibit autolysis), was incubated with 1% (w/v) trypsin overnight at 37°C, with a second 1% (w/v) addition for a further 2h. Samples (5µg) were removed before and after tryptic degradation and following the subsequent dilution and termination of proteolysis in binding buffer (0.05M sodium acetate buffer, pH 5, containing 0.02M $CaCl_2$, 0.05% (w/v) sodium azide) at a 1:10 (v/v) dilution, and precipitation of insoluble protein in an Eppendorf microfuge. The pH of the sample was then checked and adjusted to pH 5 by addition of acetic acid, and all the following procedures carried out at 4°C.

A 500µl aliquot was then layered onto a 1ml (0.5cm x 2cm) column (pre-equilibrated with 20ml of binding buffer; $0.5ml.min^{-1}$), and allowed to adsorb to the column matrix before addition of 20ml binding buffer and subsequent elution of unbound protein. Elution of the adsorbed peptides employed an alteration in pH to 2.5, with the addition of 10ml elution buffer (0.1M formic acid), and the column regenerated with 20ml binding buffer. Fractions (1ml) were collected and an elution profile constructed from A_{280} readings and silver stained gel samples.

RESULTS CHAPTER 3:

Investigation of the E3 binding function of protein X in reconstituted and intact PDC employing selective proteolysis.

3.0 INTRODUCTION

In general, binding of the peripheral components E1 and E3 to the core of the 2-oxoacid dehydrogenase complexes involves a highly conserved sequence of amino acids situated between the innermost lipoyl domain and the inner catalytic domain of the E2 component, termed the subunit binding domain. This function varies in acyltransferases between complex type and species; for example, whereas this domain of E2 from *E. coli* PDC and OGDC is thought to bind only E3, acetyltransferases from *A. vinelandii* and *B. stearothermophilus* bind both E1 and E3 with the possible specific additional involvement, in E1 binding, of the N-terminus of the inner catalytic domain (Schulze *et al.*, 1991c). Furthermore, sequence analysis of a cDNA clone of the rat OGDC E2 gene recently revealed the absence of this subunit binding domain motif (Nakano *et al.*, 1991), a finding made more interesting by the discovery of E3 binding properties associated with the N-terminus of bovine heart OGDC E1 (Rice *et al.*, 1992). In addition, whilst antisera raised against protein X detected species of M_r 67000 and 48000 from pea mitochondrial and chloroplast sources, respectively, it is unclear whether for the former this represents a separate protein X molecule or, the presence of X-like sequences on the 67000-M_r E3 component from the same organelle. Hence, the recent implication of an E3 binding function in protein X, which to date has only been found in yeast and mammalian PDC, simply adds to an already complex picture of peripheral subunit binding in the 2-oxoacid dehydrogenase complexes.

The involvement of component X in the binding of E3 to the acetyltransferase core was first proposed from studies on bovine heart and kidney PDC by Rahmatullah *et al.* (1989b). They noted that the proteolytic degradation of component X in an isolated E2/X/kinase core fraction, by either Arg C or trypsin,

resulting in the release of the lipoyl domain, was prevented by the addition of exogenous E3. In addition, prior incubation of the core fraction with a specific IgG raised against component X, led to an 85% reduction in overall PDC activity upon reconstitution with E1 and E3. This inhibition was greatly reduced by the addition of E3 to the core prior to IgG incubation and subsequent reconstitution. In contrast, E1 was only found to protect E2, and N-terminal amino acid sequence analysis of the truncated E2 core revealed that in the presence of E1, trypsin generated a larger lipoyl fragment with the additional presence of the subunit binding domain.

From these findings, Rahmatullah and co-workers (1989b), proposed that component X was essential for E3 binding and although E2 may contribute, the latter component appeared to be involved primarily in E1 binding interactions.

In support of this conclusion, Gopalakrishnan *et al.* (1989) employing microtitre-plate binding assays, found that bovine kidney PDC-derived core preparations devoid of X, or with truncated protein X_I present (the lipoyl domain released by Arg C) bound 50% less E3, yet the equivalent amount of E1 as the intact E2/X/kinase core isolate. In addition, a protein X-enriched E2/X core fraction bound considerably more E3 but less E1 whereas, an isolated component X/kinase fraction (with minor E3 contamination) bound equivalent amounts of E3 to the intact E2/X/kinase core fraction, yet no E1. These results suggested that an intact component X was essential for maximal E3 binding and that a full complement of E2 was likewise required for maximum E1 binding. Gopalakrishnan and co-workers (1989). postulated that an E3 binding site might be formed from the lipoyl of protein X and the subunit binding domain of E2.

In addition, Powers-Greenwood *et al.* (1989) using 3-step sucrose gradient centrifugation to study the binding affinities of various mammalian PDC-derived

core fractions for E1 and E3, also found that E2 core or E2/X_I core (treated with Arg C to remove the lipoyl of X) bound less E3, but the equivalent amount of E1 as intact E2/X/kinase core. Furthermore, reduced PDC activity was achieved upon reconstitution with E1 and E3 compared with the intact E2/X/kinase core, underlining the importance of an intact protein X for overall complex activity.

Gopalakrishnan *et al.* (1989) related removal of the lipoyl domain of protein X, by Arg C, in isolated E2/X/kinase core to the resultant decrease in reconstituted PDC activity, and noted that the rate of loss of intact X initially exceeded the rate of loss of reconstituted activity. These results suggest that the lipoyl of protein X is essential but not rate-limiting to the activity of PDC; however, it should be noted that residual E3 present in the core sample could afford some degree of protection and hence, alter the pattern of protein X degradation. Notably, once 90% of component X had been degraded only 10% of PDC activity remained.

In direct contrast, Neagle & Lindsay (1991), presented evidence suggesting that complete removal of the lipoyl domain of protein X, by Arg C, is accompanied by only a slight decrease (i.e., 15%) in complex activity. They criticised the methods used in the production of the various core fractions which, in some instances, involved high salt in combination with 5M urea, with the addition of mercurial reagents in the isolation of the protein X/kinase fraction. Under such conditions, proteins could be much more easily denatured leading to altered binding affinities. Furthermore, they argued that the loss in activity reconstituted was a result of non-specific Arg C degradation of residual core-bound E1α rather than the cleavage of the lipoyl domain of protein X, and demonstrated that in the presence of cofactors, TPP and Mg^{2+}, full protection of E1α, and hence complex activity, was possible. However, lowered core E3 affinity and increased salt sensitivity, leading to rapid complex inactivation, was noted by Neagle & Lindsay (1991) in the absence

of intact protein X, indicating that the combination of both Arg C and high salt led to a great reduction in E3 affinity.

In support of these findings, Lawson & coworkers (1991a), produced viable *S. cerevisiae* cells in which the gene for component X (PDX1) was disrupted, resulting in an inactive PDC devoid of both protein X and E3, yet exhibiting full wild type E2 activity and core structure. Transformation of these cells with a single copy plasmid containing either the entire PDX1 gene or, with the lipoyl encoding region deleted, resulted in both cases in active complexes possessing both protein X and E3. In contrast, cells transformed with the PDX1 gene minus a region located between the lipoyl and C-terminal domains, exhibited no PDC activity and, although the truncated protein X appeared to be associated with the E2 core, no E3 was present. This region, through comparison of the recently sequenced *S. cerevisiae* protein X gene (Behal *et al.*, 1989), with known acetyltransferase gene sequences, has been identified as a putative subunit binding domain.

Similar studies in *S. cerevisiae* involving transformation of disrupted E2 gene (LAT1) mutant cells with deleted forms of the LAT1 gene indicated an E1, but not E3, binding role for the subunit binding domain of E2 (Lawson *et al.*, 1991b). Hence, from these and the above findings, it would appear that the similar domain in protein X functions in the binding of E3 to the E2/X core.

Nevertheless, the slight reduction in intact complex activity observed by Neagle & Lindsay (1991) upon the removal of the lipoyl domain of protein X, was mirrored by a similar (30%) reduction in complex activity exhibited by the mutant *S. cerevisiae* cells transformed with the PDX1 gene minus the lipoyl encoding sequence. This may represent some loss of stability in the truncated E2/X core whereas, the complete loss exhibited by the reconstituted complex may be due, in

part, to non-specific E1α degradation by Arg C or, increased instability resulting from the denaturing conditions used in the preparation of the core samples.

The importance in gaining a full understanding of the precise nature of the E3 binding function of protein X in bovine heart PDC necessitated the investigation of the plausibility of this explanation to the discrepancies described above. This involved further proteolytic studies of protein X, employing Arg C, and encompassing the points raised by Neagle & Lindsay (1991) -- specifically, the protection of E1α, and the importance of non-denaturing conditions for core preparation. In addition, the lack of quantification provided by previous authors concerning the precise conditions used in reconstitution, prompted a more detailed analysis herein. Furthermore, the accompanying alterations in E3 binding affinity were also studied in both intact and reconstituted complex.

3.1 METHODS

3.1.1 Dissociation of bovine heart PDC into isolated E2/X core and E1/E3 fractions

Crucial to the study of the E3 binding properties of protein X in isolated E2/X core was the dissociation of bovine heart PDC into native E2/X and E1/E3 fractions, which upon reconstitution, give reproducibly high recovery of initial complex specific activity. This was achieved through the modification of a standard method used in the isolation of kinase which employs 2M NaCl treatment of PDC for 20min at room temperature, followed by resolution on a 100ml Superose 6 gel filtration column in 50mM Tris/HCl buffer, pH 9, containing 1M NaCl, 1mM DTT and 0.025% (v/v) Triton X-100 (modified from Linn *et al.*, 1972 and Stepp *et al.*, 1983). The resulting fractions exhibit only 20% reconstitution of complex activity indicating possible denaturation of the individual components.

Thus, an initial study of intact complex activity incubated at room temperature in high salt and at different pHs was carried out. Fig. 3.1.1.1 illustrates that at neutral pH complex activity is only marginally affected by high salt whereas, at a more alkaline pH, such as that used in the original core preparation, a dramatic loss in PDC activity, amounting to a 70% reduction over 30min at room temperature, is noted. This can probably be attributed to the E1α component which is known to exhibit enhanced instability at alkaline pH (Kresze *et al.*, 1980). In addition, greater maintenance of complex activity was achieved if salt treatment was carried out at 4°C (results not shown).

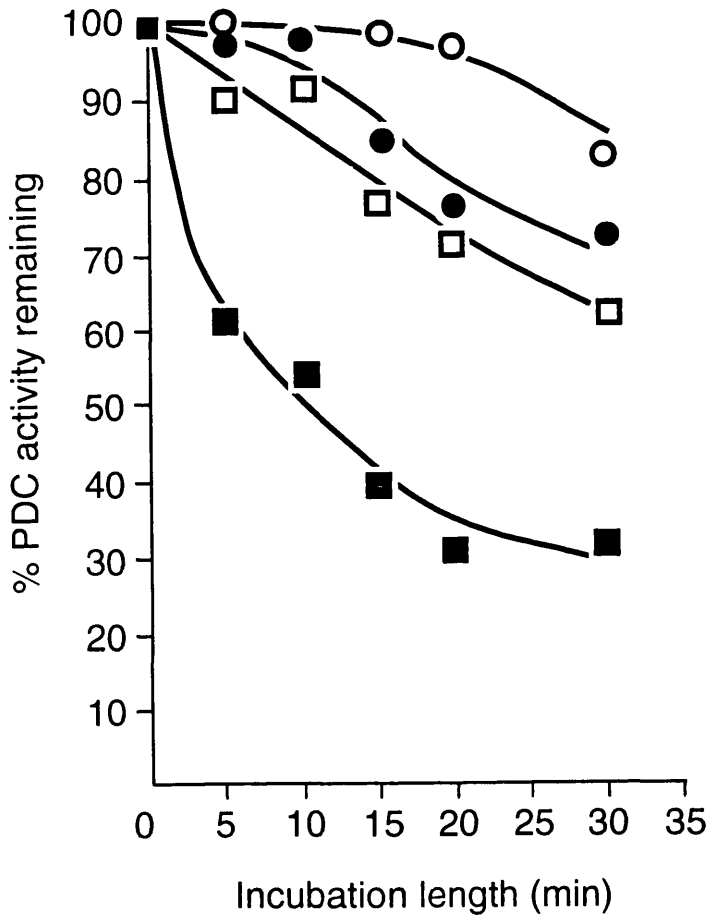


Figure 3.1.1.1 Loss in complex activity with incubation in high salt at pH 7.4-9

Samples of PDC (1mg.ml^{-1}) were incubated in column buffer (50mM Tris/HCl, 1M NaCl, 1mM DTT, 0.025% (v/v) Triton X-100), at different pHs over 30min at room temperature. At the intervals shown, 2-5µl duplicate aliquots were removed to assay for intact PDC activity (see 2.2.11), and the resultant decrease, expressed as a % of the original activity (each time point is an average of duplicate results differing by less than 10%).
(o—o) pH 7.4; (•—•) pH 8; (□—□) pH 8.5; (■—■) pH 9.

A preliminary dissociation of bovine heart PDC with 2M NaCl at pH 7.5 over 1h at 4°C resulted in significantly increased reconstitution (i.e., 50-60%), and subsequent analysis of the isolated E2/X core and E1/E3 fractions by SDS-PAGE revealed no loss in resolution. Following success in reproducibility, the modified salt treatment was adopted (see section 2.2.10 for full method). Figs. 3.1.1.2 and 3.1.1.3 illustrate a typical elution profile after resolution by gel filtration, and the resulting E2/X and E1/E3 fractions analysed by 10% (w/v) SDS-PAGE, with subsequent immunoblotting using E3 and protein X-specific sera. Clearly good separation was achieved where only traces of undissociated E3 remained attached to the E2/X core, with E2/X virtually undetectable in the E1/E3 fraction. Notably, such good resolution was not achieved by Rahmatullah *et al.* (1989) or Gopalakrishnan *et al.* (1989), whilst employing the more stringent conditions listed therein.

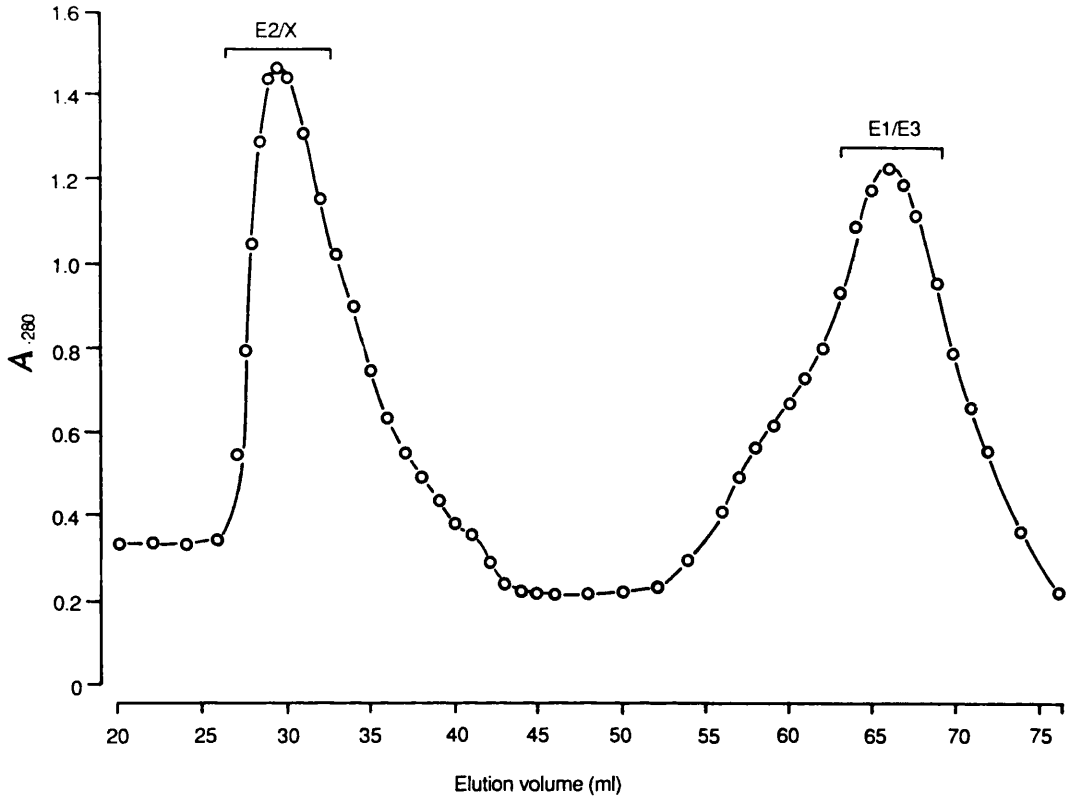


Figure 3.1.1.2 Typical elution profile of dissociated E2/X and E1/E3 fractions from a Superose 6 gel filtration column

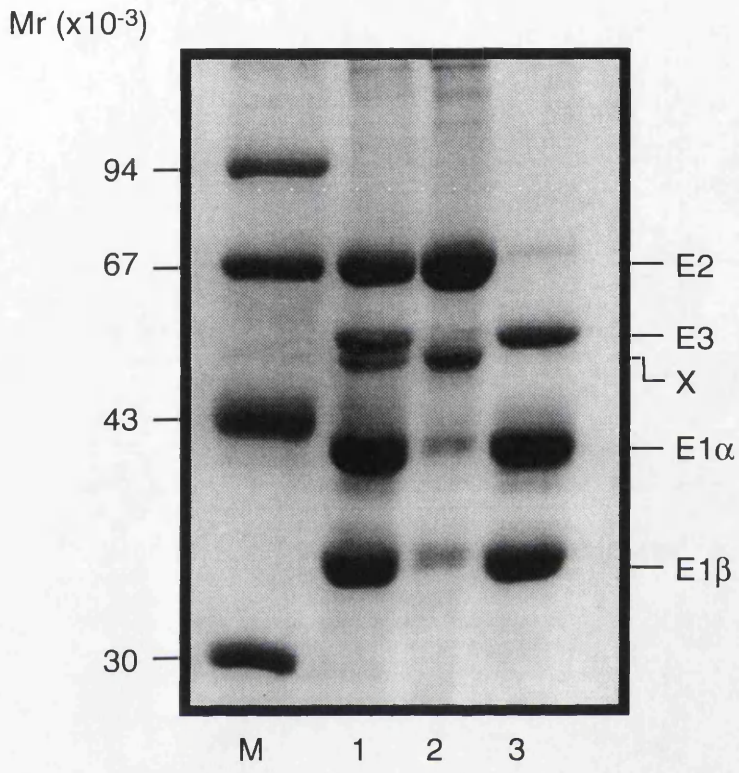
15-30mg of PDC, treated in high salt as described in section 2.2.11, were resolved on a 100ml Superose 6 gel filtration column resulting in two clearly dissociated peaks. The protein concentrations of each fraction (2ml) were analysed at 280nm.

Figure 3.1.1.3 Coomassie and immunological analysis of the E2/X core and E1/E3 peak fractions

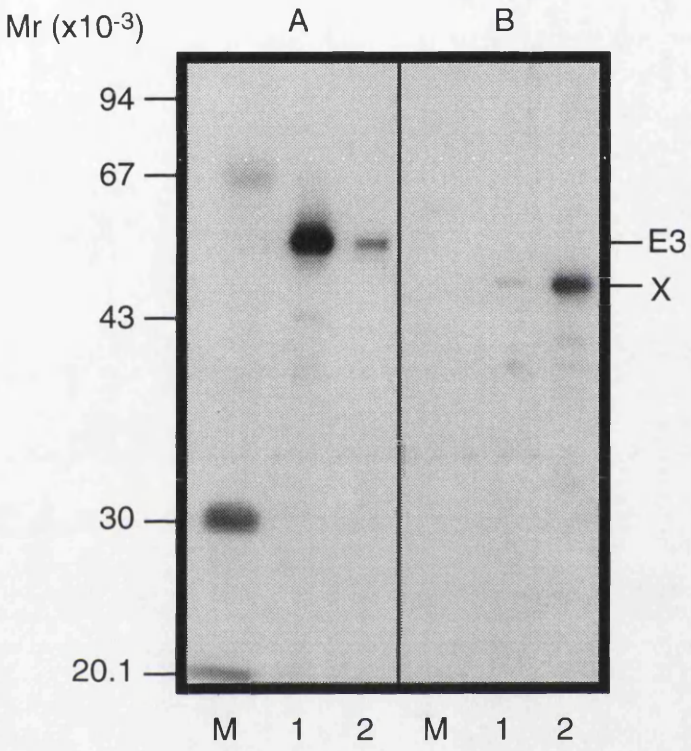
The purity of the peak fractions obtained in Fig. 3.1.1.2 was assessed following dialysis and PEG concentration by 10% (w/v) SDS-PAGE where samples were a) Coomassie blue stained; b) transferred to nitrocellulose and screened with protein X (panel B) and E3 (panel A) specific sera.

a) Lane M, 10μg of low M_r marker proteins; lane 1, 20μg of purified bovine heart PDC; lane 2, 15μg of isolated E2/X core; lane 3, 15μg of dissociated E1/E3.

b) Lane M, 10μg of low M_r markers proteins; lane 1, 10μg E1/E3; lane 2, 10μg E2/X.



a)



b)

3.2 RESULTS

3.2.1 Establishing optimal conditions for maximum reconstitution of complex activity

3.2.1.1 Determination of an optimal preincubation length for reconstitution

Following the preparation of the isolated E2/X core and E1/E3 fractions as detailed in section 3.1.1, the residual PDC activity of each fraction was determined by direct assay. Protein concentrations were also determined employing both Lowry and BCA protein assays, and the resultant specific activity expressed in terms of % residual PDC reconstituted (see section 2.2.10).

The effect of preincubation at 33°C on the residual activities was then examined and Fig. 3.2.1.1.1 illustrates both the typical residual activity of either fraction, and the affect on these of increased incubation at 33°C. Clearly there is little change with time, suggesting that the components in either fraction are in a native and fully functional conformation or, that any refolding and reactivation occurs within the first 2min following addition to the assay buffers.

With reconstitution, equal volumes (10-30μl) of both fractions were preincubated together for varying times in the assay cuvette at 33°C before the addition of substrate. From Fig. 3.2.1.1.2, it would appear that reassembly of the complex from the dissociated fractions occurred very rapidly (within 2min),

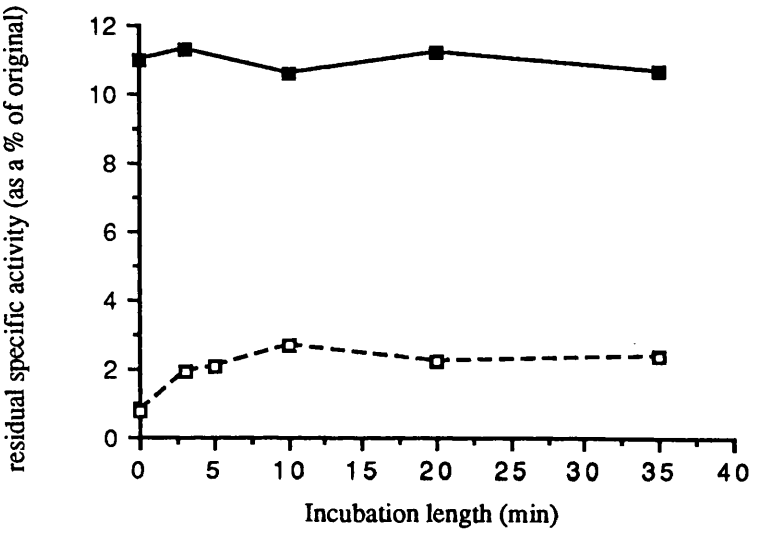


Figure 3.2.1.1.1 Effect of time of preincubation on the residual complex activity of the purified E2/X and E1/E3 fractions

Residual complex activity was determined by the direct addition of 10-30µl of both fractions to the assay cuvette (containing 670µl **Soln A** and 14µl **Soln B**), with preincubation at 33°C for the times shown, before the initiation of catalysis by the addition of 14µl of substrate (see 2.2.11). Specific activity for each time point was determined from duplicate results differing by less than 10%, and expresed as a % of the original intact complex specific activity (see 2.2.10). (□- -□) E2/X; (■—■) E1/E3.

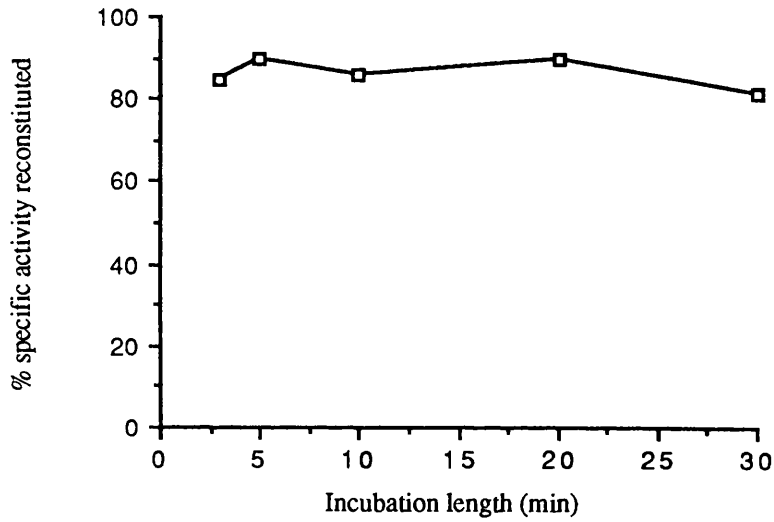


Figure 3.2.1.1.2 Profile of reconstitution with increased lengths of preincubation

Equal volumes (10-30µl) of E2/X (0.1mg.ml^{-1}) and E1/E3 (0.2mg.ml^{-1}) fractions (1:10 in **Soln A**), were preincubated at 33°C in the assay cuvette (containing 670µl **Soln A**, and 14µl **Soln B**), for the times shown, before addition of 14µl of substrate. Specific activity reconstituted was determined from duplicate results differing by less than 10%, and expressed as a % of the original intact complex specific activity.

presumably reflecting the time taken for subunit interactions involved in complex reassembly or possibly, cofactor TPP binding. Although, the % reconstitution at t_0 is not illustrated in Fig. 3.2.1.1.2, additional results (not shown) suggested, at most, a 20% stimulation in reconstitution over the first 2min of preincubation. Hence, a standard 10min preincubation was used throughout the following experiments in agreement with the 10-15min preincubation employed by Schulze *et al.* (1991c), in similar reconstitution experiments involving *A. vinelandii* PDC, and of Kresze *et al.* (1980) and Rahmatullah *et al.* (1990), employing bovine kidney PDC.

3.2.1.2 Identification of the optimal stoichiometric ratio of E2/X:E1/E3 for reconstitution

Although in intact complex the ratio, according to subunit stoichiometry and molecular mass, of E2/X core to E1/E3 is approximately 1:1.3, following high salt treatment and column purification variable yields of either fraction were possible; hence, a range of ratios of E2/X:E1/E3 were examined for maximum reconstitution (see Fig. 3.2.1.2.1). The % reconstitution for each ratio was calculated by the division of the activity obtained upon reconstitution (minus residual activities) by the total fractional protein present in the assay cuvette, and the resulting specific activity expressed as a % of the original complex specific activity (see section 2.2.10).

Notably, maximum reconstitution was achieved at a reproducible E2/X:E1/E3 ratio of 1:0.7-1.3 (w/w), which is within the range of normal complex stoichiometry. Clearly, the lower ratio of 1:0.7 may be attributed to minor inactivation, proteolytic degradation or aggregation of the E2/X core following pre-

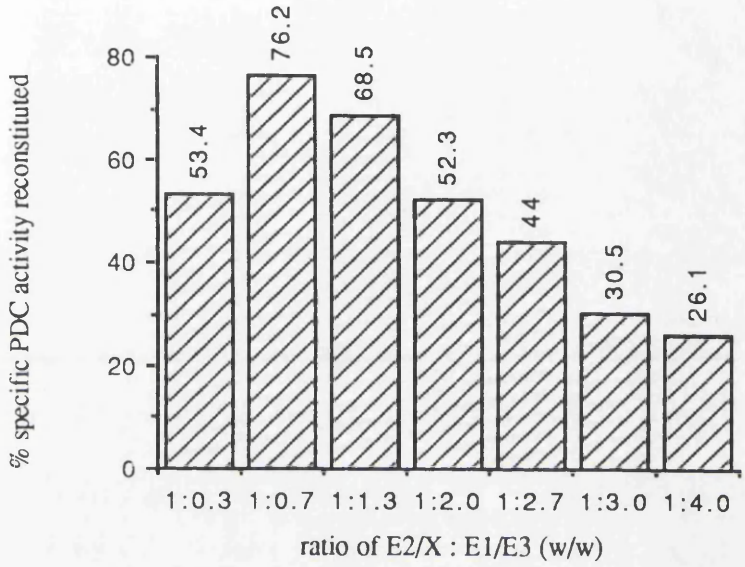


Figure 3.2.1.2.1 Profile of reconstitution at different stoichiometric ratios of E2/X : E1/E3

3.5-60µl of E2/X core (0.1mg.ml^{-1}) were added in the assay cuvette to a constant 15µl of E1/E3 (0.2mg.ml^{-1}) -- diluted as in Fig. 3.2.1.1.2, and preincubated for 10min at 33°C prior to the initiation of catalysis by the addition of substrate. The % complex activity reconstituted was determined as before (see 2.2.10), from duplicate values differing by less than 10%.

longed high salt treatment and column purification -- to which, the dissociated E1 and E3 components appear less susceptible. Alternatively, this could reflect the ability of PDC to function normally in the absence of a full complement of E1 and E3.

As increases from a ratio of 1:0.3 to 1:4.0 (E2/X:E1/E3),were implemented by reducing the amount of E2/X core in relation to a constant amount of E1/E3, it is likely that both the decline in specific activity and net activity reconstituted (latter not shown), from a ratio of 1:2 onwards, resulted from limitations in the E2/X core either as a consequence of partial inactivation or arising from competition between the E1 and E3 components for one or both of the subunit binding domains of E2 and X. However, such competition has only been documented for the E1 binding sites of E2 from *A. vinelandii* PDC (Schulze *et al.*, 1991c), and this is discussed further in sections 3.2.2.1-3. Alternatively, such a decline could reflect decreasing concentrations of E2/X in the assay cuvette from 9$\mu g.ml^{-1}$ to 0.5$\mu g.ml^{-1}$ (a dilution effect varying from 1 in 100 to 1 in 1500), which was also observed where reconstitution involved a constant amount of E2/X and varying amounts of E1/E3. The effect of component dilution on reconstitution was therefore investigated.

3.2.1.3 Effect of protein concentration on the rate and extent of reconstitution of overall PDC activity

In addition to a range of ratios of E2/X:E1/E3, and in light of the results of section 3.2.1.2, reconstitution was also studied under conditions of significantly higher component concentration to assess whether the extent of reconstitution within the limited preincubation period is further dependent upon the relative intimacy of the

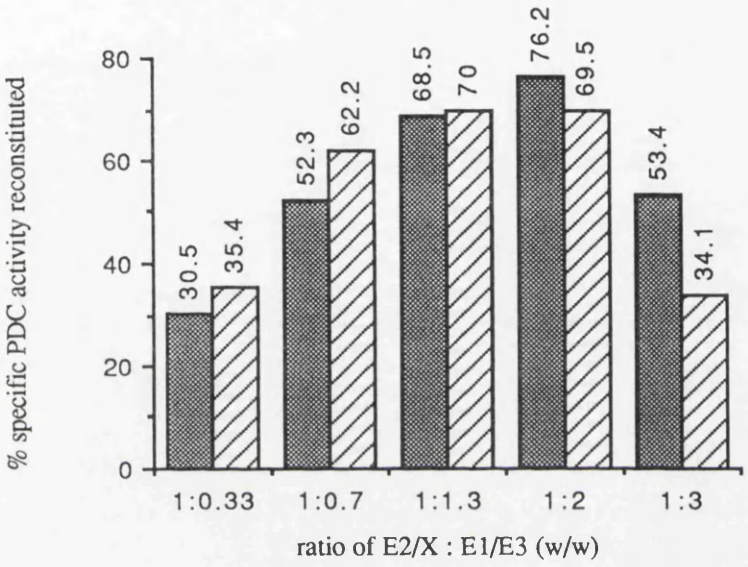


Figure 3.2.1.3.1 Reconstitution at high and low component concentration

5-60µl of E2/X core sample were preincubated with a constant volume of E1/E3, in the presence of **Soln A** and **B** for 10min at 33°C, at high concentration (final volume 100µl), and 5-10µl aliquots removed for assaying; or, low concentration whereby, 5-60µl of E2/X were preincubated with a constant volume of E1/E3 (both 1:10 with **Soln A**), in the assay cuvette containing 700µl of **Soln A** & **B**, prior to the addition of substrate. Resultant activities were expressed as % reconstitution and represent an average of duplicate results differing by less than 10% (see 2.2.10). Where (▨) low concentration; (■) high concentration.

dissociated components. Samples were preincubated at the usual low concentration of 0.7-1.9μg.ml^{-1} E2/X, 4μg.ml^{-1} E1/E3 -- whereby reconstitution was carried out in the assay cuvette; or, at a 70-fold higher concentration of 0.05-0.6mg.ml^{-1} E2/X, 0.3mg.ml^{-1} E1/E3 and aliquots removed to cuvettes for assaying.

From the results (Fig. 3.2.1.3.1), it would appear that component concentration has little effect on the extent of reconstitution over the 10min preincubation period, since similar values were obtained at high and low concentrations. In addition, little difference was noted in the net activity reconstituted for either condition (results not shown). It is possible that at higher concentrations reassembly of the complex occurs at a faster rate, as would be expected for conditions of greater intimacy. However, if as previous results suggest, this occurs rapidly -- possibly within seconds, over the relatively long preincubation period this would not become apparent. Since similar values of reconstitution were obtained with a constant 70-fold difference in dilution, the results suggest that the decrease in reconstitution observed, with increasing ratios (by weight), of E2/X:E1/E3 in Fig. 3.2.1.2.1, was not an effect of increasing component dilution.

3.2.1.4 Effect of storage of purified E2/X and E1/E3 fractions on reconstitution

The ability of the dissociated E2/X core and E1/E3 fractions to maintain reconstitution after storage in 50mM imidazole/HCl buffer, pH 7.5, containing 1mM EDTA, 0.5mM benzamidine and 50% (v/v) glycerol at either 4°C or -20°C over 34 days was studied and the results presented in Fig. 3.2.1.4.1.

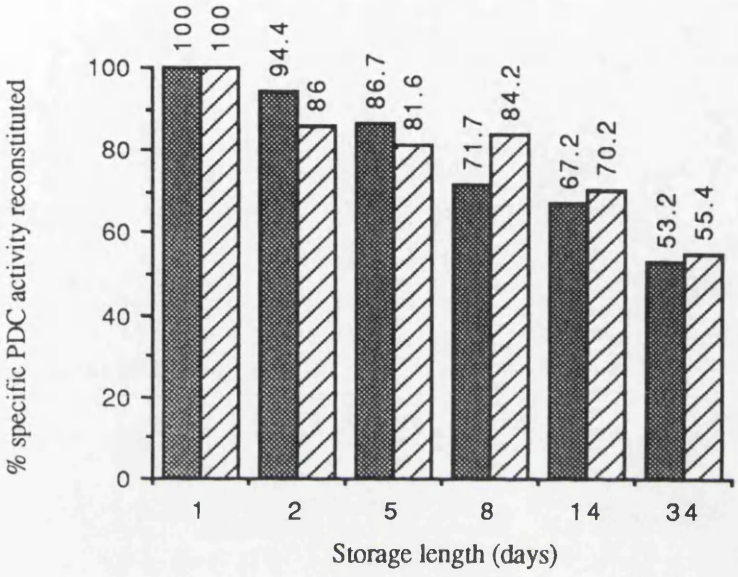


Figure 3.2.1.4.1 Stability profile of reconstitution after storage of the dissociated fractions at 4°C or -20°C

Duplicate 10-30µl aliquots of E2/X and E1/E3 (diluted as before), were preincubated at 33°C for 10min in an assay cuvette, in the presence of **Soln A** and **B**, and resultant activity assayed by the addition of substrate (see 2.2.11). % reconstitution was determined from duplicate values agreeing within 10%. Where (■) storage at 4°C; (▨) storage at -20°C in 50% (v/v) glycerol.

Clearly, the fractions exhibit a high degree of stability at either storage temperature with only a 50% reduction in activity reconstituted over 34 days. However, with subsequent preparations, glycerol was replaced by 1% (v/v) Triton X-100, as the former appeared to interfere with the specific proteolytic degradation of the E2/X core by Arg C. Benzamidine was also omitted as this is a potent inhibitor of Arg C. In the absence of both, the component fractions became considerably more unstable with large losses (i.e. 50%) in reconstitution after 4-5 days at 4°C (results not shown). This was presumably due to non-specific degradation by contaminating proteases in addition to minor conformational changes and possible aggregation, indicated by the presence of precipitation after a few days at 4°C. Instability of proteins increases with dilute solutions and evidently, the presence of 1% (v/v) Triton X-100 did not provide the same degree of viscosity and hence stability, as glycerol (see Kresze *et al.*, 1980). Furthermore, since glycerol was found to reduce the efficacy of Arg C, perhaps due to high viscosity, it is possible that some protection from proteolysis could be provided.

3.2.2 Proteolytic studies on the E3 binding function of protein X in reconstituted complex involving Arg C

Having established both a relatively non-denaturing method for the isolation of E2/X core and, the optimum conditions for maximum reconstitution with E1/E3 (i.e., 70-100%), it was then possible to examine the E3 binding function of protein X in reconstituted complex, following removal of the lipoyl domain by Arg C. This was carried out in the presence of cofactors, TPP and Mg^{2+}, to provide protection for residual E1α present in the purified core which has been shown previously to be susceptible to proteolysis by Arg C (Neagle & Lindsay, 1991).

3.2.2.1 Reconstitution in the presence or absence of excess E3 during the progressive cleavage of protein X by Arg C

Gopalakrishnan *et al.* (1989) and Powers-Greenwood *et al.* (1989), demonstrated that removal of the lipoyl domain of protein X, by Arg C, was accompanied by both an immediate loss in reconstituted complex activity and E3 binding potential. Thus, initial experiments investigated the validity of this result under the conditions described herein and in addition, in the presence of excess exogenous porcine E3.

This involved the proteolytic degradation of isolated E2/X core, with Arg C, in the presence of TPP and Mg^{2+}, and the stepwise removal of samples for reconstitution with E1/E3 at a ratio of 1:0.8 (w/w), in the presence or absence of porcine E3, at a 3-fold excess (w/w) with respect to the E1/E3 fraction. Samples

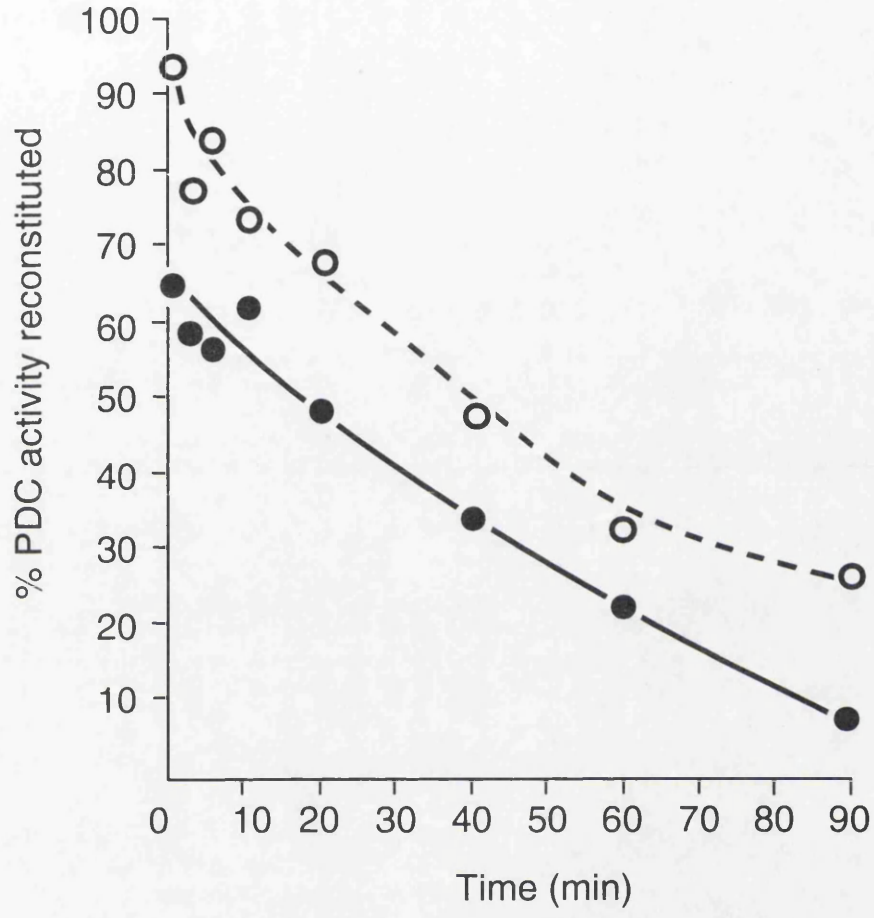
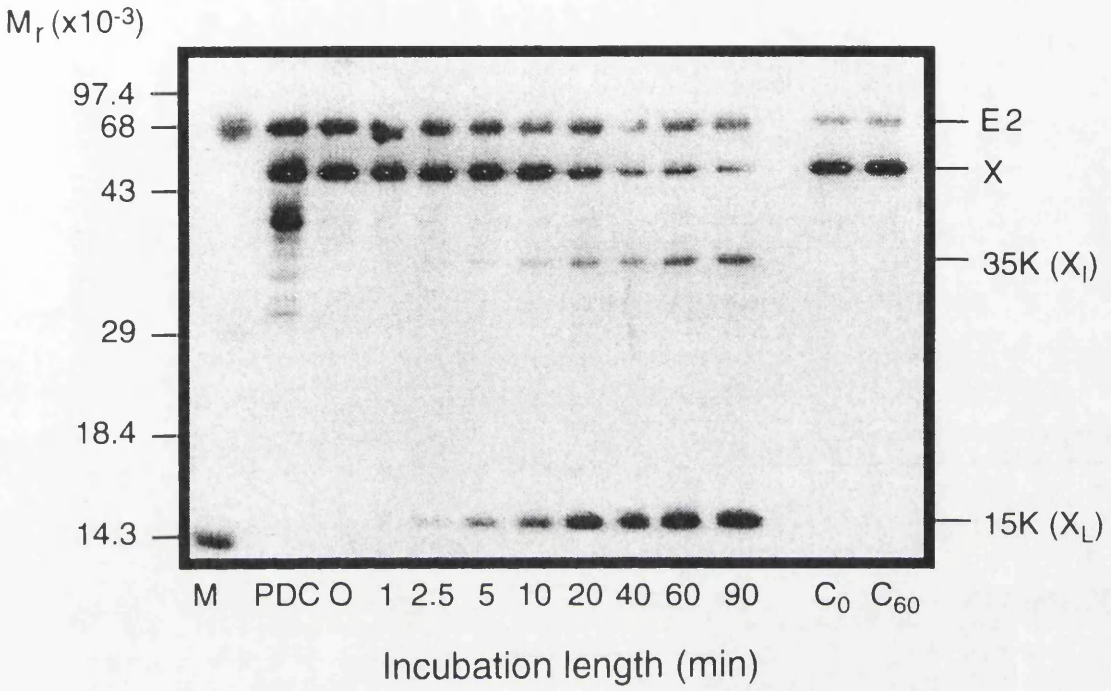


Figure 3.2.2.1.1a) Profile of reconstitution during the progressive cleavage of protein X by Arg C

E2/X core (1 : 5 in **Soln A**), was treated with 3% (w/v) Arg C over 90min at 33°C and at the intervals shown, samples removed and treated with 5mM benzamidine to arrest proteolysis. Duplicate aliquots were then preincubated for 10min at 33°C with 1 in 10 diluted E1/E3 at an E2/X:E1/E3 ratio of 1:0.8 (w/w), in an assay cuvette containing **Soln A** and **B**, and in the presence (o- -o) or absence (●—●) of a 3-fold excess (w/w) of porcine E3, with respect to E1/E3. PDC activity was then initiated by the addition of pyruvate to duplicate assay cuvettes and, the resultant specific activity expressed as % reconstitution calculated to within ± 5% error (see 2.2.10). (NB: the protein content of commercial E3 was not included in the calculation of specific activity).



b) Western analysis of isolated E2/X core treated with Arg C

The remainder of the benzamidine-arrested, Arg C-treated E2/X core was diluted in Laemmli buffer and analysed by immunological screening with antisera raised specifically to protein X, the immunoblot developed and overlaid with E2 antiserum. Lane M, 10μg low M_r marker proteins; PDC, 3μg purified, bovine heart PDC; C, control, undigested E2/X core; 4μg of E2/X / track .

were also removed for analysis by SDS-PAGE and immunological screening (see Fig. 3.2.2.1.1).

From the results it is clear that the immediate removal of the lipoyl domain by Arg C is accompanied by a parallel loss in the ability to reconstitute overall complex activity resulting, in the absence of excess E3, in a residual 9% of activity after 90min reflecting the significant reduction in intact protein X. Immunological analysis of the E2 component (Fig. 3.2.2.1.1b), and E1 component (result not shown), revealed minor and no detectable degradation by Arg C, respectively, in support of the findings of Rahmatullah *et al.* (1989a), where complete removal the lipoyl of protein X was accompanied by <6% E2 degradation. These results suggest that any loss in complex activity is due primarily, to the removal of the lipoyl domain of protein X, and therefore, not due to the degradation of residual core E1 proposed by Neagle & Lindsay (1991). However, in agreement with the criticisms of Neagle & Lindsay (1991), it is possible that the partial inactivation of the E2/X core noted during the preparation from purified complex (see section 3.2.1.2), has some effect on the binding ability of E3 and hence reconstitution, since in the presence of excess E3, the initial activity reconstituted is approximately 25% higher. Alternatively, this could simply reflect a return to 100% E3 occupancy following unavoidable E3 dissociation during the original purification of PDC from bovine heart mitochondria. (A 5-10% enhancement of intact PDC activity has been observed upon addition of excess porcine E3).

Notably, this constant 25% difference in real terms represented, at t_0, a 40% increase over and above that achieved in the absence of excess E3 and progressed to a 200% increase following extensive protein X degradation over 90min. This significant enhancement was also observed in the presence of excess E1/E3 (results not shown), and suggests that any reduction in the overall extent of E3 binding,

following the removal of the lipoyl domain of protein X, by Arg C, may be partially recovered in the presence of higher concentrations of E3.

Nevertheless, full recovery of reconstitution was not achieved and may reflect a requirement for yet higher concentrations of E3, where non-specific binding to the subunit binding domain of E2, and competition from the E1 component for the similar domain of protein X cannot be entirely ruled out. Alternatively, this could indicate a more profound effect on E3 binding than a simple lowering of affinity. The nature of the loss in E3 binding was therefore investigated further.

In a similar study, employing isolated E2/X core and E1/E3 fractions, Gopalakrishnan *et al.* (1989) noted that protein X degradation initially exceeded the corresponding loss in reconstituted PDC activity, suggesting an essential but non-rate limiting function for the lipoyl of protein X. Whilst a precise quantification of protein X degradation is not presented here, a similar pattern is possibly suggested by direct comparison of Figs. 3.2.2.1.1a & b, yet may reflect a lag in E3 dissociation due to the aforementioned <100% E3 occupancy of the reconstituted complex.

3.2.2.2 Analysis of the loss in E3 binding in intact, reconstituted and Arg C-treated reconstituted PDC following resolution by Superose 6 gel filtration

In section 3.2.2.1, it was observed that the activity of untreated reconstituted PDC could be increased by the presence of excess bovine heart E1/E3 or, porcine E3, suggestive of minor E3 dissociation. This could arise during the isolation from

Figure 3.2.2.2.1 Profile of E3 dissociation in intact, reconstituted and Arg C-treated reconstituted PDC, following resolution by Superose 6 gel filtration

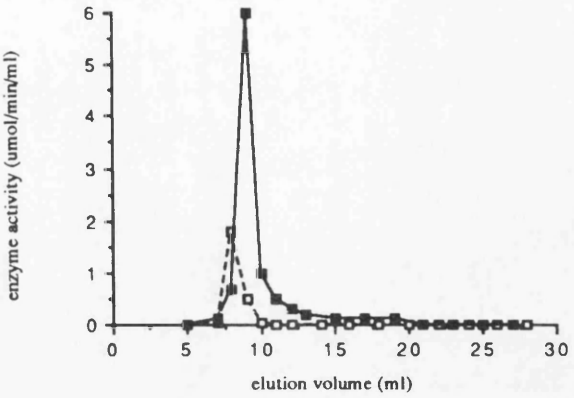
In each case, approx. 0.5mg of complex (in 1ml of **Soln A**), was resolved on a 25ml (1 x 25cm) Superose 6 column equilibrated in 50mM Tris/HCl buffer pH 7 (containing 0.01% (v/v) Triton X-100, 1mM DTT) at a flow rate of 0.5ml.min^{-1}, and the resultant 1ml fractions analysed for intact complex activity (□- -□), and E3 activity (■—■), as described in section 2.2.11.

a) Intact PDC: 0.5mg diluted to 1ml in **Soln A** prior to loading.

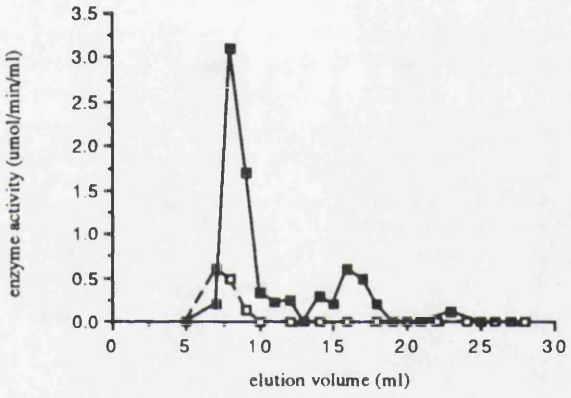
b) Reconstituted PDC: E2/X core was preincubated with E1/E3 at a ratio of 1:0.8 (w/w) for 10min at 33°C in the presence of **Soln A** and **B** (final volume 1ml), to give approx. 0.5mg of reconstituted complex, the % reconstitution determined by the removal of duplicate 5µl aliquots to assay cuvettes, and found to be 95%. The remaining sample was then loaded as described above.

c) Arg C treated reconstituted PDC: E2/X core was treated with 3% (w/v) Arg C over 75min at 33°C and the drop in activity monitored by the removal of timed aliquots for reconstitution with E1/E3 until only 10% remained. 5mM benzamidine was added and preincubation with E1/E3 then followed as described above. Total E3 activity in µmol.min^{-1}ml^{-1} was determined for each condition and from this the % dissociation of E3 was calculated and found to be as follows:-

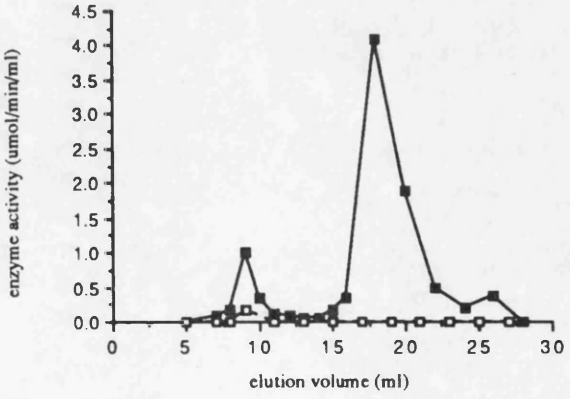
a) 3%; b) 22%; c) 88%.


enzyme activity (umol/min/ml)
6
5
4
3
2
1
0
0
5
10
15
20
25
30
elution volume (ml)

A)


enzyme activity (umol/min/ml)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
elution volume (ml)

B)


enzyme activity (umol/min/ml)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
elution volume (ml)

C)

bovine heart mitochondria and/or preparation of the dissociated fractions where slight denaturation and/or proteolytic degradation could result in both inactivation of the E1/E3 components and disruption of the E3 binding function of E2/X.

In addition, in agreement with the results of Powers-Greenwood *et al.* (1989), and Gopalakrishnan *et al.* (1989), following cleavage of the lipoyl domain of protein X, by Arg C, a rapid loss in reconstitution was noted presumably due to an accompanying loss in E3 binding. The extent of this loss in both untreated and Arg C treated complex was subsequently investigated by the following qualitative and semi-quantitative analysis of E3 dissociation employing gel filtration (Fig. 3.2.2.2.1).

From the results it is clear that whereas little E3 (i.e., 3%) becomes dissociated in intact complex following resolution by gel filtration, 20% was released or failed to rebind in the reconstituted sample. Since 95% reconstitution was achieved prior to column treatment, the release of E3 most likely results from the combination of a lowered E3 binding affinity and the subsequent effect of column treatment on the weakened core-E3 interactions, rather than the presence of unbound and/or inactive E3 in the reconstituted sample prior to resolution. Nevertheless, as the results of section 3.2.1.2 suggest the ability of PDC to function at substoichiometric levels of E1 and E3, and since the maximum % reconstitution achieved varied between preparations, from 70 to 100%, the presence of unbound and/or inactive E3 cannot be ruled out especially for cases of lower recovery. In addition, continued inactivation of the dissociated fractions was noted in section 3.2.1.4 where instability, upon storage, resulted in a rapid decline in reconstitution over the period of 4-5 days.

In contrast, following Arg C treatment a significantly larger % of E3 (i.e., 88%) eluted from the column as dissociated enzyme, suggesting that in the absence of intact protein X, the E3 binding affinity of the E2/X core is greatly reduced. Since only 10% of activity remained, it is likely that the majority of E3 in the reconstituted complex was unbound prior to column treatment.

The effect of column treatment, in conjunction with Arg C digestion, on E1 binding was also examined qualitatively by analysis of each fraction by SDS-PAGE and the resultant gels silver stained (results not shown). Unlike E3, E1 remained associated to the E2/X core in both untreated and treated reconstituted complex. Since E2 is thought to bind E1 and, since immunological analysis of the isolated core with anti-E2 serum revealed insignificant degradation by Arg C (results not shown), this result is in agreement with those of Powers-Greenwood *et al.* (1989), and Gopalakrishnan *et al.* (1989), which demonstrated no loss in E1 binding with the removal of intact protein X.

3.2.2.3 A study of reconstitution in the presence of varying concentrations of excess porcine E3 with untreated, partially and fully Arg C degraded E2/X core

The results described in section 3.2.2.1 indicated a lowering of E3 binding affinity with the removal of the lipoyl domain of protein X, which could be partially compensated for by the addition of excess E3. The following experiments were designed to investigate the effect of various concentrations of excess E3 on reconstitution with untreated, partially, and fully Arg C degraded E2/X core.

As before, isolated E2/X core was treated with 3% (w/v) Arg C at 33°C in the presence of TPP and Mg^{2+}, and reconstitution monitored until 40% (partial digestion), or 5% (full digestion), of the original activity remained. Samples were then used to study reconstitution in the presence of 0-10mg.ml^{-1} porcine E3 (i.e., a 0-50-fold (w/w) excess with respect to E1/E3). The results are presented in Fig. 3.2.2.3.1 where a) represents fully Arg C digested; b) partially Arg C digested; and c) undigested reconstituted complex.

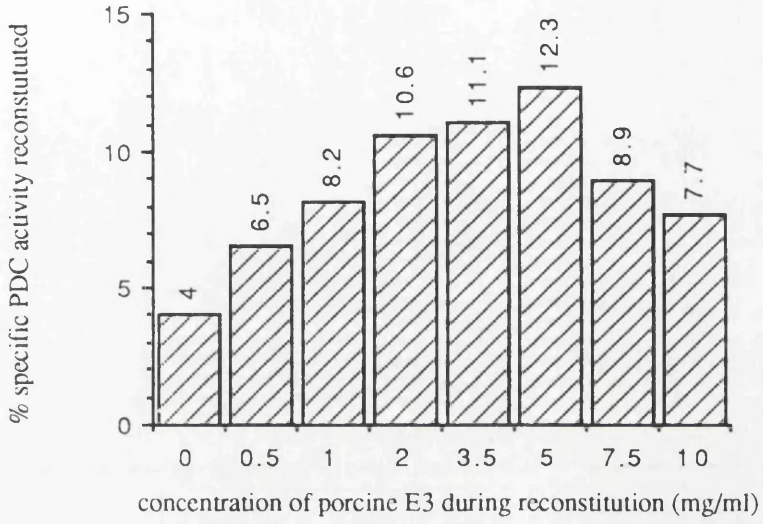
All three complexes exhibited increased reconstitution in the presence of excess porcine E3 suggestive of reduced E3 binding. However, with undegraded complex the approximate 30% increase in reconstitution (facilitated by a 1.6-fold excess in E3; 0.5mg.ml^{-1}) may simply represent recovery of E3, dissociated during isolation of PDC from bovine heart mitochondria and/or preparation of the dissociated fractions as originally proposed in section 3.2.2.1, and supported by the results of section 3.2.2.2. A similar 25% increase in reconstitution was observed with partially degraded complex following addition of a 1.6-fold excess of porcine E3, but rose to a 50% increase in the presence of 2mg.ml^{-1} porcine E3 and therefore, presumably reflects limited recovery from the combined effects of E3 disssociation as described above, and the additional lowering, or loss, of E3 binding affinity in those protein X molecules from which the lipoyl domains had been cleaved by Arg C. In contrast, with almost fully degraded complex the same range of excess porcine E3 (0.5-7.5mg.ml^{-1}; i.e., a 1.6-32 fold excess with respect to E1/E3), resulted in a 60-200% enhancement in reconstitution, suggesting an increasing dependence for recovery of complex activity upon higher concentrations of E3 which, could result from an increasing involvement in PDC activity of the low affinity binding of E3 to the subunit binding domain of E2 (see Discussion).

Figure 3.2.2.3.1 Profile of reconstitution, in the presence of various concentrations of porcine E3, of untreated, partially amd fully digested E2/X core

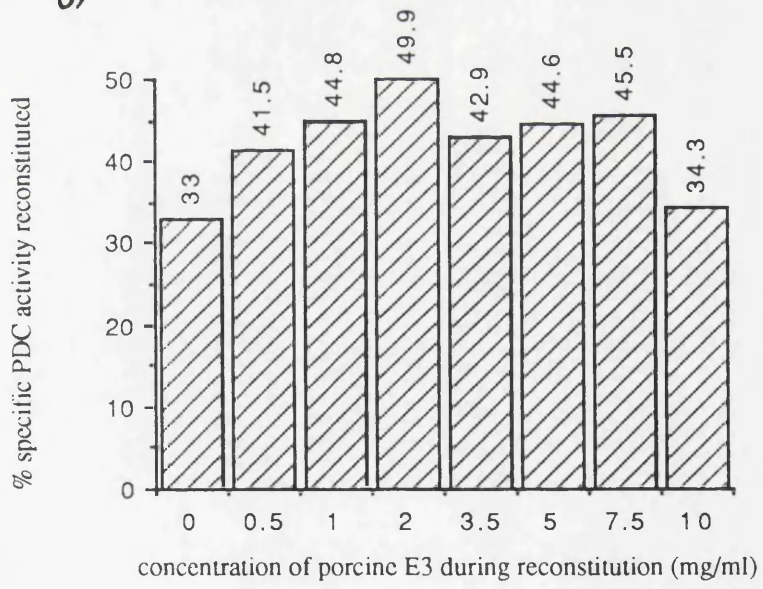
E2/X core was treated with 3% (w/v) Arg C in the presence of TPP and Mg^{2+}, and the resultant decrease in complex activity reconstituted monitored by the removal of timed aliquots for preincubation at 33°C for 10min with E1/E3 (ratio by weight of 1:0.8), in the presence of **Soln A** and **Soln B,** and activity assayed at 340nm. Digestion was arrested by the addition of 4mM benzamidine. For a) digestion proceeded until 95% of activity was lost, for b) until 60% was lost, and for c) undigested core used. Equivalent duplicate aliquots of each sample were then preincubated for 10min at 33°C with E1/E3 (ratio 1:0.8), in the presence of **Soln A** and **B** and a final concentration of 0-10mg.ml^{-1} (ie., 0-50 fold excess (w/w) with respect to E1/E3), porcine E3 (resuspended in **Soln A**), to a final volume of 50-80µl. Catalysis was initiated by the addition of 20µl to an assay cuvette and the resultant specific activity expressed as % reconstitution calculated from duplicate values of <5% difference (see section 2.2.10).

(NB: the protein content contributed by the presence of excess E3 was not included in the calculation of specific activity).

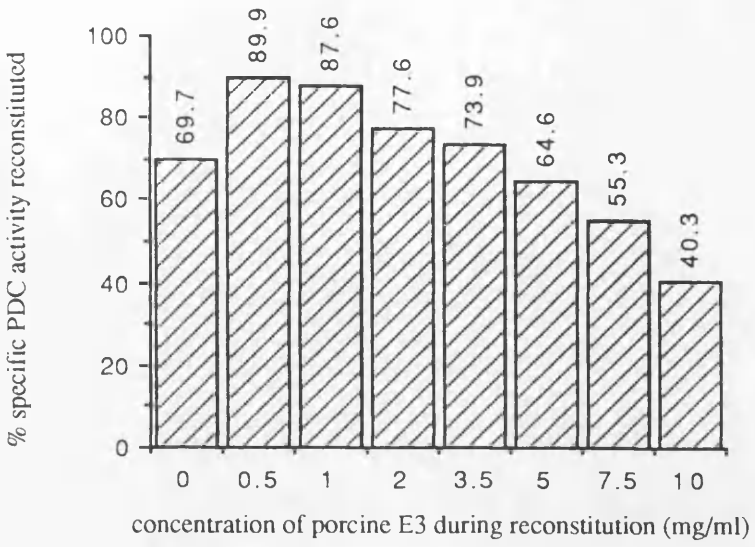
a)


% specific PDC activity reconstututed
15
10
5
0
4
6.5
8.2
10.6
11.1
12.3
8.9
7.7
0
0.5
1
2
3.5
5
7.5
10
concentration of porcine E3 during reconstitution (mg/ml)

b)


% specific PDC activity reconstituted
50
40
30
20
10
0
33
41.5
44.8
49.9
42.9
44.6
45.5
34.3
0
0.5
1
2
3.5
5
7.5
10
concentration of porcine E3 during reconstitution (mg/ml)

c)


% specific PDC activity reconstituted
100
80
60
40
20
0
69.7
89.9
87.6
77.6
73.9
64.6
55.3
40.3
0
0.5
1
2
3.5
5
7.5
10
concentration of porcine E3 during reconstitution (mg/ml)

It should be noted that a similar 200% increase in reconstitution accompanied only a 3-fold excess of porcine E3 in Fig. 3.2.2.1.1a. This discrepancy may have resulted from the incomplete removal here, of 3.2M NH_4SO_4 from the porcine E3 storage buffer. In support of this latter proposal, all three complexes exhibited decreased levels of reconstitution in the presence of highest E3 (and hence, trace salt) concentrations, where mild component dissociation could occur. However, such decreases could also reflect competition between E1 and E3 for binding sites on the E2/X core as suggested previously (see sections 3.2.1.2 & 3.2.2.1).

Importantly, these results imply that full recovery of reconstitution is not possible in the presence of up to a 50-fold excess of porcine E3, following the complete or partial removal of the lipoyl domains of protein X. In addition, no further increase in reconstitution was observed when preincubation was extended over 4h at either 33°C or 4°C, in the presence of a 19-fold excess of porcine E3 (i.e., 5mg.ml^{-1}; results not shown). Whilst these results indicate a dramatic decrease in E3 binding affinity, it is unclear whether the inability of porcine E3 to facilitate full recovery simply reflects: a) low specificity binding to the subunit binding domain of the truncated protein X and/or E2 component; or alternatively, b) limitations associated with small but significant differences in the catalytic and/or binding efficiencies of bovine heart and porcine E3 (Saiqa Khan, University of Glasgow, unpublished results). These results are therefore in apparent agreement with Rahmatullah *et al.* (1989b), and Powers-Greenwood *et al.* (1989), but in opposition to Neagle & Lindsay (1991), and Lawson *et al.* (1991), who ascribe the E3 binding function of protein X solely to the E3 binding domains. However, if the overall stability of this latter domain necessitates the presence of the lipoyl domain, its removal by Arg C could result in the loss of both stability and therefore, the E3 binding function.

Notably, large deletions in the proposed E1 binding site of E2 from *A. vinelandii* PDC, resulted in a complete and irreversible loss in E1 binding, yet full recovery of reconstitution was achieved with excess E1 following a small deletion or a single point mutation. This suggested a complete loss in binding function for the former, compared to an apparent lowering of binding affinity for the latter (Schulze *et al.*, 1991c). Nevertheless, the proposed indirect yet essential involvement of the lipoyl domain in E3 binding is not supported by the research of Neagle & Lindsay (1991), suggesting the possibility of an alternative explanation. To this end the following research was carried out.

3.2.2.4 Reconstitution, following Arg C treatment of isolated E2/X core, in the presence of mediating molecules and excess E3

As the loss in E3 binding could only be partially recovered by the addition of excess E3 (see section 3.2.2.3), this suggested either a dramatic reduction or complete loss in the E3 binding capacity of the E2/X core. In either case, the introduction of a mediating molecule which could interact catalytically with the E2/X/E1 core and dissociated (or weakly associated) E3, might provide a means for increased reconstitution. This possibility was briefly investigated employing a) free lipoamide or, b) lipoamide with redox dyes -- both in conjunction with excess porcine E3. Fig. 3.2.2.4.1 illustrates the possible mechanism for catalysis in the presence of such mediating molecules.

In agreement with the findings of Reed *et al.* (1958), whereby both free lipoic acid and lipoamide acted as substrates for E2 and E3 of *E. coli* PDC, this

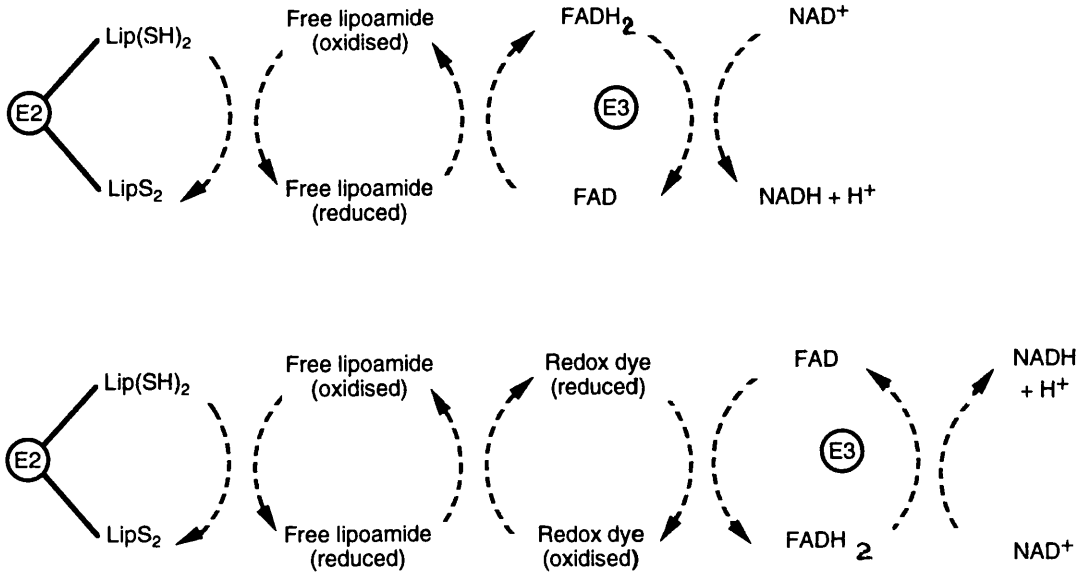


Figure 3.2.2.4.1 Proposed reaction mechanism of reconstituted complex catalysis in the presence of free lipoamide and redox dyes

proposes that free lipoamide might interact with reduced lipoyl domains on the E2 core, become converted to dihydrolipoamide and, due to accessibility as a free molecule in solution, interact with the weakly associated and/or dissociated E3 components which would then catalyse the reoxidation. Alternatively, the additional introduction of an artificial electron acceptor in the form of redox dyes might improve the rate of electron transfer between E2, lipoamide and E3, as shown. This required a dye which although stable to air oxidation, could be easily reduced with a redox value close to that of the FAD/$FADH_2$ pair (i.e. 0.26mV), and a minimum absorbance at 340nm. The following three dyes were chosen accordingly: 1) anthraquinone-sulphate; 2) hydroxynapthaquinone; 3) phenazine ethosulphate.

In both cases, samples of isolated E2/X core were treated with 3% (w/v) Arg C in the presence of TPP and Mg^{2+} and the decrease in reconstitution monitored until 5% remained at which point, digestion was arrested by the addition of benzamidine. Duplicate aliquots were then removed for reconstitution with E1/E3 in the presence or absence of lipoamide, excess E3 and redox dyes.

a) Reconstitution with lipoamide in the presence or absence of excess E3

From the results in Fig. 3.2.2.4.2 it is clear that the presence of lipoamide has little affect on reconstitution even at concentrations of $4mg.ml^{-1}$. Condition 8 and 9 show minor increases but this is more likely due to the addition of $1mg.ml^{-1}$ and $5mg.ml^{-1}$ E3, respectively, as subsequent experiments employing higher concentrations of porcine E3, revealed no change in % reconstitution with the addition of lipoamide (results not shown).

Figure 3.2.2.4.2 Profile of reconstitution in the presence of varying concentrations of lipoamide following Arg C digestion of the E2/X core

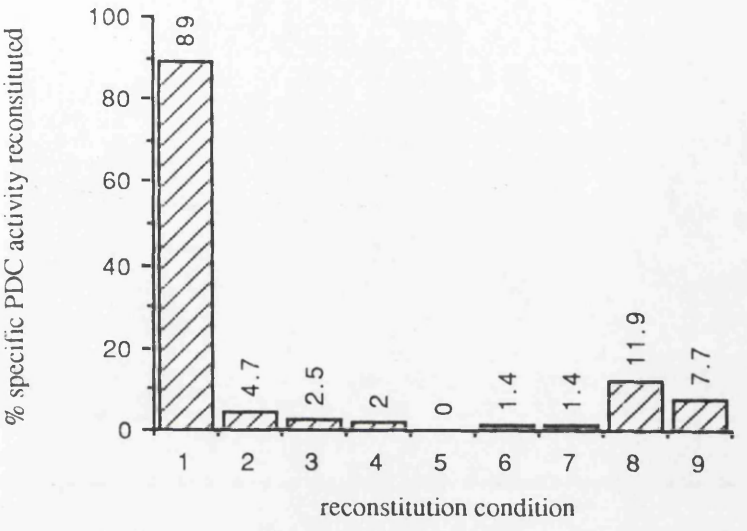
E2/X core was incubated with 3% (w/v) Arg C in the presence of TPP and Mg^{2+} and the drop in activity reconstituted monitored by the addition of E1/E3 (ratio of 1:0.8), as before (see 2.2.10). Once 95% of activity was lost, 4mM benzamidine was added and aliquots of E2/X core removed for a 10min preincubation at 33°C with E1/E3 and varying concentrations of lipoamide ± excess E3, in the presence of **Soln A** and **B** (final volume 50-60µl). 10µl duplicate samples were then removed to a cuvette for assaying (see 2.2.11). Lipoamide was made up in 50% (v/v) ethanol / **Soln A**.

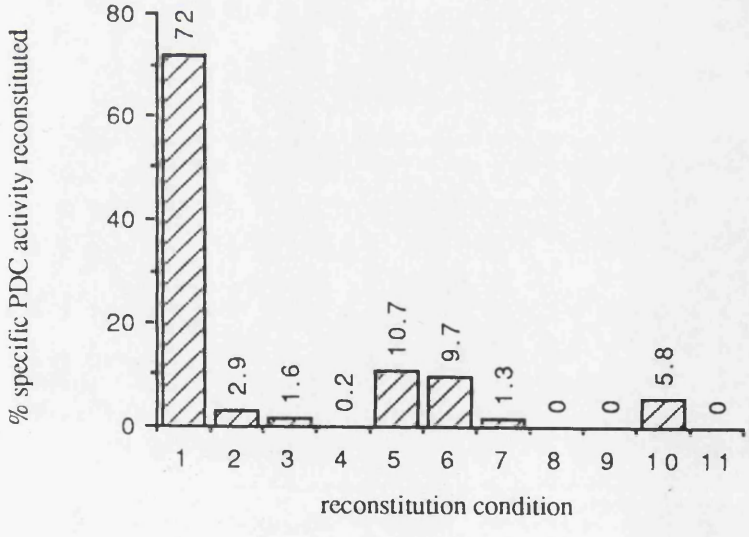
Condition 1: reconstitution before Arg C treatment.
2: " after " "
3: condition 2, in the presence of 0.3mg.ml^{-1} lipoamide.
4: " " 0.6mg.ml^{-1} "
5: " " 0.8mg.ml^{-1} "
6: " " 2.0mg.ml^{-1} "
7: " " 4.0mg.ml^{-1} "
8: as in 7, and 0.5mg.ml^{-1} porcine E3
9: " " 1.0mg.ml^{-1} "

Figure 3.2.2.4.3 Profile of reconstitution in the presence of various redox dyes, following Arg C digestion of the E2/X core

Arg C digestion was as described in 3.2.2.4.2, and conditions 1 and 2 illustrate activity reconstituted prior and following this treatment. Conditions 3 to 11 illustrate reconstitution following digestion in the presence of :-

3: **Soln C.**
4: " plus 0.1mg.ml^{-1} lipoamide.
5: as in 4, plus 5mg.ml^{-1} E3.
6: as in 5, plus 150µMolar anthraquinone - sulphate.
7: as in 5, " " hydroxynapthaquinone.
8: " " " phenazine ethosulphate.
9: " " " of all three dyes.
10: as in 9, but with **Soln B'** (minus L-cysteine).
11: as in 9, but with no lipoamide present.


% specific PDC activity reconstituted
100
80
60
40
20
0
89
4.7
2.5
2
0
1.4
1.4
11.9
7.7
1 2 3 4 5 6 7 8 9
reconstitution condition


% specific PDC activity reconstituted
80
60
40
20
0
72
2.9
1.6
0.2
10.7
9.7
1.3
0
0
5.8
0
1 2 3 4 5 6 7 8 9 10 11
reconstitution condition

The apparent failure of lipoamide to facilitate catalysis may reflect problems encountered with solubility which necessitated the presence of 50% (v/v) ethanol in the stock solution. High levels of solvent in the reconstitution volume were undesirable and kept between 2-8% (v/v) whereupon, lipoamide exhibited minor precipitation. Nevertheless, sufficient amounts could have remained in solution, but as lipoamide is a relatively poor substrate of E2, its conversion to dihydrolipoamide and subsequent reoxidation by E3, under such conditions could have occurred at a low rate requiring extended preincubation. Since previous experiments (not shown), highlighted problems with extended preincubation and, the insolubility of lipoamide presented further complications, this line of investigation was not pursued further.

b) Reconstitution with lipoamide and excess E3 in the presence or absence of redox dyes

From the results in Fig. 3.2.2.4.3 it is clear that, under the conditions used, the presence of redox dyes in the reconstitution volume has no apparent beneficial affect on activity recovered. The 200% increase expected (condition 5), upon the addition of $5mg.ml^{-1}$ porcine E3 (see section 3.2.2.3), actually decreased with subsequent additions of each or, all three dyes (conditions 6, 7, 8 and 9). However, it should be noted that preincubation was carried out (conditions 3-9), in the presence of **Soln C**, and not **B**, to eliminate any reduction of the dyes by L-cysteine (see section 2.2.11) -- present to maintain reduced CoA.

Since reconstitution in the presence of **Soln C** showed a slight reduction (compare conditions 2 and 3), reconstitution with all three dyes, $5mg.ml^{-1}$ E3 and

lipoamide, was carried out in the presence of modified **Soln B** lacking L-cysteine (condition 10). A 100% increase resulted but, with such low recovery in reconstitution -- below that achieved solely with excess E3, the significance of this result seemed in doubt. It is possible that higher concentrations of the dyes might have lead to increased reconstitution although, literature searches suggested lower concentrations to be more frequently employed.

In light of the results obtained in sections 3.2.2.4a & b, it seems that the initial recovery in reconstitution achieved with high concentrations of porcine E3 cannot be improved upon under the conditions described above.

3.2.2.5 N-terminal amino acid sequence analysis of the Arg C-truncated 35000-M_r fragment of protein X from bovine heart PDC

From the results presented so far in this chapter, the apparent consequence of the removal of the lipoyl domain of protein X, by Arg C, is a dramatic loss in reconstitution, thought to reflect an equivalent loss in E3 binding capacity. In the presence of high concentrations of porcine E3, only partial recovery was possible, suggestive of a severe decrease in E3 binding affinity and/or the total loss of the E3 binding function of protein X.

Both situations could arise if the lipoyl domain of protein X was involved in E3 binding either directly or indirectly, such as in the stabilisation of the E3 binding domain. This possibility is supported by similar studies in reconstituted bovine kidney PDC by Powers-Greenwood *et al.* (1989), and Gopalakrishnan *et al.* (1989)

but disputed by the findings of Neagle & Lindsay (1991) (working in intact bovine heart PDC), and Lawson *et al.* (1991) (*S. cerevisiae* PDC), which point to a minor but non-essential role. This discrepancy could be explained if Arg C was found to remove more than simply the lipoyl domain of protein X and hence, N-terminal amino acid sequencing of the truncated protein X was carried out to determine the precise site of cleavage.

This involved the proteolytic cleavage of isolated E2/X core, by Arg C, which resulted in the release of a highly immunogenic 15000-M_r fragment, known from previous N-terminal amino acid sequencing to contain the lipoyl domain (Rahmatullah *et al.* 1989b); and a truncated, core bound 35000-M_r fragment (see section 3.2.2.1). Samples were resolved by sequencing grade 15% (w/v) SDS-PAGE, and transferred to Immobilon PVDF membrane from which the 35000-M_r fragment was isolated and sent to the SERC Protein Sequencing Facility in Aberdeen (see Fig. 3.2.2.5.1 legend for details).

The sequence obtained was 23 residues in length and when matched with known acyltransferase sequences, a region of homology was identified within the E3 binding domain (see Fig. 3.2.2.5.1a). Previous N-terminal amino acid sequencing of the 35000-M_r fragment by Rahmatullah *et al.* (1989b), generated an almost identical sequence of 18 residues in length but failed to reveal any such sequence identity, probably owing to the misplaced assumption that Arg C cuts within the linker region joining the lipoyl and E3 binding domains.

Robien *et al.* (1992) published the 3-dimensional solution structure of a 51 residue synthetic peptide comprising the E3-binding domain of E2 from *E. coli* OGDC, determined via NMR spectroscopy, which revealed a disordered N-terminus (residues 0-12); followed by two parallel α-helices (residues 14-23 and 40-48),

separated by a short extended strand (residues 24-26), and an irregular loop (residues 31-39; see Fig. 3.2.2.5.1b). Although, this represented the E3-binding domain from an octahedral E2 core-containing 2-oxoacid dehydrogenase complex, alignment of 15 such sequences from all three complexes and of various sources, with strict identity at the N- and C-termini of both helices, revealed 4 regions of absolute or, highly conserved residues. This included: 1) a core of hydrophobic residues at positions corresponding to Ile-16, Leu-20, Leu-25, Ala-27, Ile-30, Leu-39, Val-44, with interhelical contacts providing stability; 2) glycine or asparagine at positions of tight turns (ie. 37 and 24); 3) a structurally restrictive proline at the N-terminal boundary of helix 1; and, 4) positively and negatively charged residues at specific positions corresponding to Arg-17, Arg-18, Arg-8, Arg-41; and Glu-22, Asp-43, respectively, thought to be involved in the E2/E3 interface. The 3-D NMR resolution of the subunit binding domain of E2 from *B. stearothermophilus* PDC (icosahedral core), by Kalia *et al.* (1993), revealed a similar pattern of two α-helices but here, separated by a structured 3_{10}-helix and overlapping β turns. In addition, the solvent exposed Thr-33 and Asp-43 of E2 from *E. coli* OGDC were buried, and therefore thought to be crucial for stability.

Alignment of the amino acid sequence obtained from the truncated 35000-M_r fragment of protein X (Fig. 3.2.2.5.1a), revealed the presence of conserved residues Pro-14, Arg-17, Arg-18, Leu-20, Glu-22, Gly-24, Thr-33; and, assuming that the subunit binding domain of protein X in bovine heart PDC is similarly positioned, these results place the site of Arg C cleavage at the extreme N-terminal edge of the E3 binding domain -- specifically, at the proline boundary (Fig. 3.2.2.5.1b). Hence, proteolysis would result in the release of the lipoyl domain with the attached linker region, exposing the truncated E3 binding domain (Fig. 3.2.2.5.1c). Subsequent inactivation, due to partial unfolding, would seem highly probable under such conditions and would therefore, explain more satisfactorily the

Figure 3.2.2.5.1 Isolation of the truncated 35000-M_r fragment of protein X for N-terminal amino acid sequencing

A preliminary digestion of isolated E2/X core by Arg C at 33°C followed by 12% (w/v) SDS-PAGE and immunological analysis, revealed the optimum incubation length for the production of the 35000-M_r fragment. This was then employed in a second digestion involving 300μg of isolated core, and the reaction arrested by the addition of an equal volume of sequencing gel sample buffer 1:1 (v/v) with Bromophenol blue. Resolution by 15% sequencing grade SDS-PAGE followed (as described in section 2.2.5), and duplicate 50μg samples subsequently transferred to either nitrocellulose membrane for Western analysis, or to sequencing Immobilon PVDF membrane for staining with Coomassie Blue. Comparison of either membrane identified the 35000-M_r band which was excised from the Immobilon and sent to Aberdeen for N-terminal protein sequence analysis.

Figure 3.2.2.5.1a) Alignment of 15 E3-binding domain amino acid sequences reproduced from Robien *et al.* (1992), and modified to show the 35000-M_r derived sequence from protein X

Areas of homology between the acyltransferases from OGDC (O), PDC (P), BCOADC (B) and protein X from PDC (X), isolated from *E. coli* (E), *Bacillus subtilis* (B), *A. vinelandii* (A), *B. stearothermophilus* (S), *S. cerevisiae* (Y), *Neurospora crassa* MRP3 (N), rat (R), human (H), *Pseudomonas putida* (P), and bovine (C), are shown underlined. Positions of identity with the equivalent region from the 35000-M_r fragment of protein X (35X), are represented by asterisks. Two gaps were inserted on either side of the EKG motif in allowance of possible evolutionary changes such as deletions or insertions.

```
        14            161718 1920  222324 25  27    30    33  35  373839  41
E-O:  SP      AIRRLLAEHNLDASAIKGTGVGGRLTRE
B-O:  SP      SARKLAREKGIDLSQVPTGDPLGRVRKQ
A-O:  SP      AARKIAEENAIAADSITGTGKGGRVTKE
E-P:  TP      LIRRLAREFGVNLAKVKGTGRKGRILRE
B-P:  MP      SVRKYAREKGVDIRKVTGSGNNGRVVKE
S-P:  MP      SVRKYAREKGVDIRLVQGTGKNGRVLKE
A-P:  GP      AVRQLAREFGVELAAINSTGPRGRILKE
Y-P:  SP      LAKTIALEKGISLKDVHGTGPRGRITKA
N-P:  LP      AAKRLAREKGIDLRNVKGSGPGGKITEE
R-P:  SP      LAKKLAAEKGIDLTQVKGTGPEGRIIKK
H-P:  SP      LAKKLAVEKGIDLTQVKGTGPDGRITKK
X-P:  LPSVSLLLAENNISKQKALKEIAPSGSNGRLLKE
P-B:  SP      AVRKRALDAGIELRYVHGSGPAGRILHE
B-B:  TP      AVRRLAMENNIKLSEVIGSGKDGRILKE
H-B:  TP      AVRRLAMENNIKLSEVVGSGKDGRILKE
       **     ***   *  ***   *     **
35X:LSP       AARNIL -EKG -ALDANQGTA?G
```

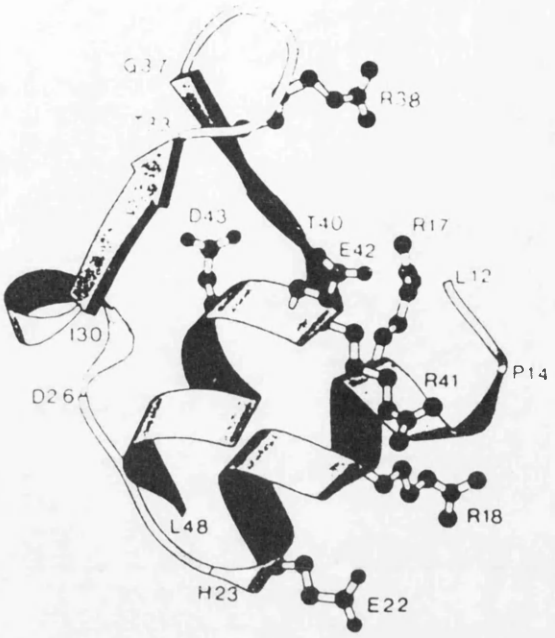


Figure 3.2.2.5.1b) Schematic ribbon drawing of the E3-binding domain of E2 from *E. coli* OGDC (reproduced from Robien *et al.*, 1992)

dramatic loss in E3 binding observed following Arg C treatment. Indeed, the close proximity of the cleavage site suggests that gross disruption, resulting in the complete loss of the E3 binding function may be more likely, then a partial unfolding leading to reduced affinity.

It should be noted however, that occasionally upon prelonged incubation (60min), further digestion of protein X to a 30000-M_r fragment occurred with the presumed release of the E3 binding domain (M_r 5000). Hence, a second site of Arg C cleavage is indicated in Fig. 3.2.2.5.1c. The involvement of this further digestion in the loss in reconstitution is unclear since significant loss in activity occurred before the appearance of the 30000-M_r fragment, which was often not detected by immunological analysis. Nevertheless, complete and irrecoverable loss in E3 binding must ensue upon the removal of the E3 binding domain, if protein X is solely responsible for the binding of this component as suggested by the gene deletion studies in *S. cerevisiae* PDC of Lawson *et al.* (1991).

Importantly, this result offers strong support for the proposed significant disruption of the E3 binding domain following Arg C treatment and therefore, also offers a possible explanation to the disparate findings of Powers-Greenwood *et al.* (1989), Gopalakrishnan *et al.* (1989), and Lawson *et al.* (1991). Hence, in agreement with the latter group, possible disruption of the E3 binding domain by Arg C, and not the direct result of the removal of the lipoyl domain, may be primarily responsible for the loss in E3 binding. However, at this point it was still unclear why the equivalent loss in E3 binding and, complex activity, following complete digestion of protein X, by Arg C, was absent in intact PDC (Neagle & Lindsay, 1991). The possibility that the pattern of cleavage differed between intact complex and isolated core resulting in altered cleavage sites was therefore investigated.

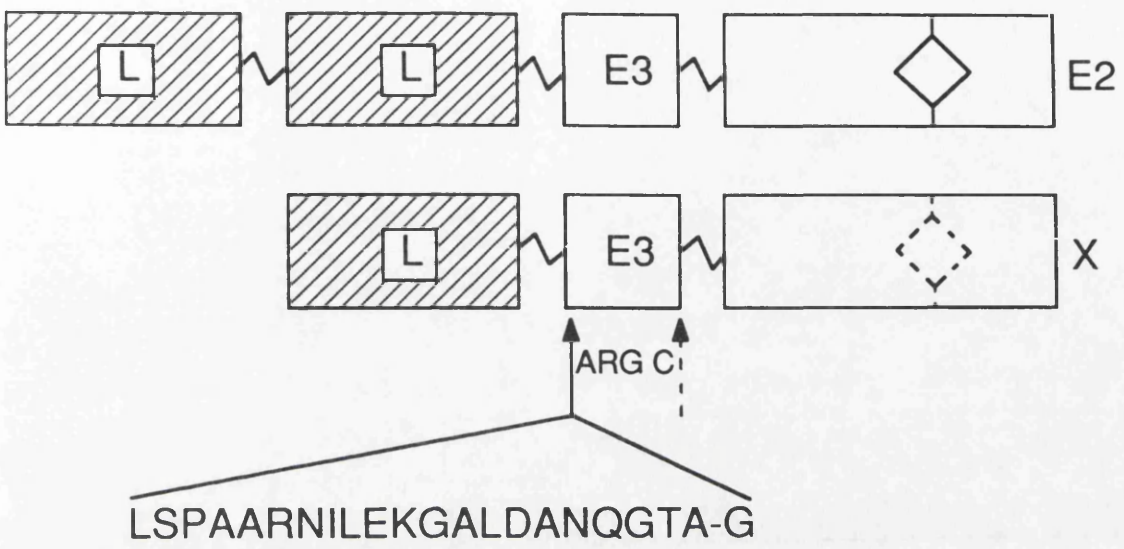


Figure 3.2.2.5.1C) A schematic representation of the E2 and protein X components of bovine heart PDC

The lipoyl domains are represented by hatched boxes; the lysine-linked lipoic acid moieties by L; the E3 binding domains by a boxed E3; the C terminal domains by open boxes; the active site of E2 by a diamond; and the flexible interdomain linkers by zigzag lines.

3.2.3 Comparative proteolytic studies on the E3 binding function of protein X in intact complex involving Arg C

From the evidence presented in section 3.2.2 it is clear that Arg C digestion of protein X in isolated E3/X core results in a dramatic loss in reconstitution upon the appearance of the truncated 35000-M_r core fragment and the accompanying release of the lipoyl domain. Results suggest the loss in activity reflects an equivalent loss in E3 binding due to the proposed disruption of the E3 binding domain, brought about by the close proximity of the Arg C cleavage site to the N-terminal boundary of this domain.

In contrast, Neagle & Lindsay (1991), accomplished the complete removal of the lipoyl domain of protein X, by Arg C, in intact bovine heart PDC, accompanied by only a minor loss in complex activity (i.e., 15-20%), although the affinity of E3 for the core was reduced, leading to dissociation upon gel filtration in low ionic buffer. Since Rahmatullah *et al.* (1989b), reported protection of protein X from Arg C degradation by E3 in intact bovine kidney PDC, a possible explanation for the above discrepancy could involve an alteration in the extent of Arg C digestion in isolated core compared to intact complex. This possibility was investigated as follows.

3.2.3.1 Analysis of the loss in intact complex activity following the selective degradation of protein X by Arg C

Intact PDC was treated with 3% or 6% (w/v) Arg C at 33°C over 2h in the presence of TPP and Mg^{2+}. At regular time intervals, samples were removed to measure complex activity, for analysis by SDS-PAGE of any non-specific E2/E1 degradation, and for specific protein X degradation by subsequent Western analysis. Decreases in the E2, E1 and X components were quantified by densitometric scanning of the resultant gels and immunoblot employing a Shimadzu FDU-3 gel scanner (see Figs. 3.2.3.1.1a-d).

In direct contrast to the findings of Neagle & Lindsay (1991), a gradual but extensive loss in intact complex activity was noted, with 50% loss over the first 60min (compared to 15% -- Neagle & Lindsay, 1991), and a further 20%-30% decrease (3%-6% (w/v) Arg C), over the following 60min. Quantification of protein X degradation illustrated a similar overall loss of 40% over 60min, and 65-90% over 2h although, the initial rate appears to exceed that of PDC activity loss (see Discussion). Whereas Neagle & Lindsay (1991) achieved complete protein X digestion with 3% (w/v) Arg C over 60min, repeated trials employing various buffer systems and a range of % (w/v) Arg C of both analytical (Boehringer Mannheim) and sequencing (Sigma) grade, failed to remove all traces of protein X (as shown in Figs. 3.2.3.1.1b & c), supporting the findings of Rahmatullah *et al.* (1989b), which indicated protection of protein X from Arg C by E3 (complete removal was achieved in the presence of high salt -- see Chapter 5). Notably, the proportion of protein X to Arg C (w/w), in isolated core, is two fold higher than in intact complex, yet more rapid and extensive degradation of protein X occurs with the former. In addition, this was unavoidably accompanied by significant non-specific Arg C degradation of

Figure 3.2.3.1.1 Analysis of the loss in intact complex activity following the degradation of intact protein X by Arg C

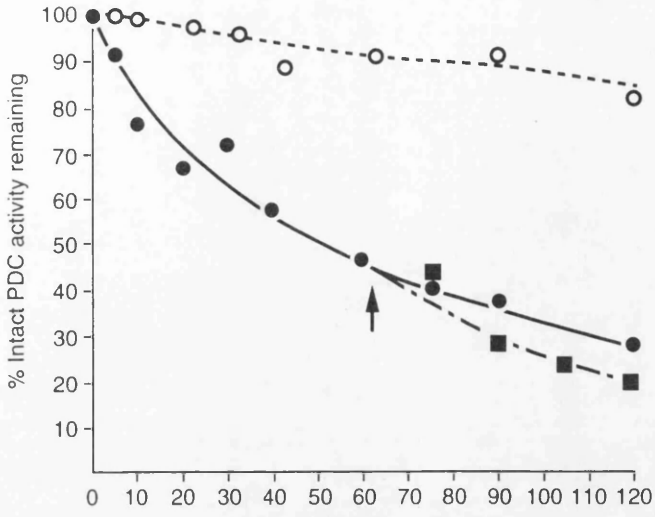
Freshly purified PDC was treated with 3% or 6% (w/v) Arg C at 33°C over 2h in the presence of TPP and Mg^{2+} (after 1h, 50% of the volume was removed and treated with a second addition of Arg C i.e., 6% (w/v)). At the times shown, samples were removed and the reaction stopped with 4mM benzamidine at 4°C. 5-10µl duplicate aliquots were used to assay complex activity at 33°C (see section 2.2.1) and the remaining volume diluted in Laemmli sample buffer for subsequent analysis by immunological screening (see section 2.2.4).

Figure 3.2.3.1.1a) Loss in intact complex activity with time following Arg C treatment

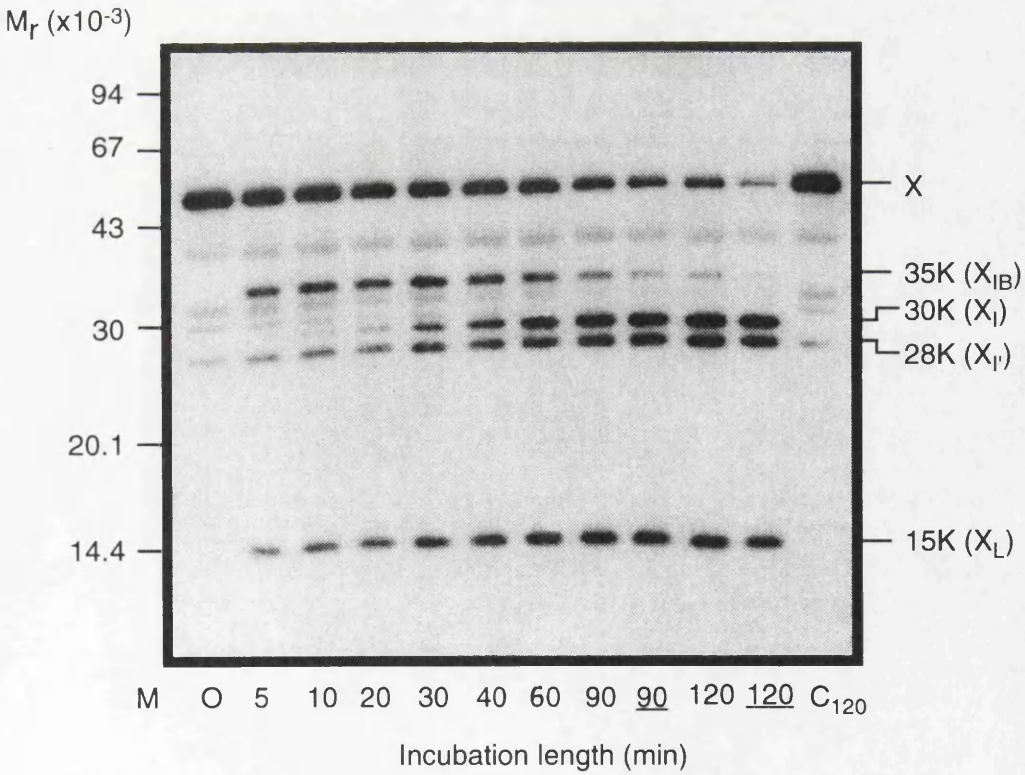
Loss in intact complex activity with time, expressed as a % of the original untreated activity. (•--•) represents addition of 3% (w/v) Arg C over 2h. (•-- -•) represents 3% (w/v) Arg C for 60min followed by an additional 3% (w/v) Arg C indicated by the arrow. (o- -o) indicates control untreated complex.

Figure 3.2.3.1.1b) Immunological analysis of the degradation of protein X by Arg C

Western analysis of Arg C degraded protein X resolved by 10% (w/v) SDS-PAGE, and screened with antiserum raised specifically to protein X. Lane M, 10µg low M_r marker proteins; samples were removed at the times shown (15µg/well), and 90', 120' represent samples removed after 6% (w/v) Arg C treatment. Intact protein X is represented by "X", and the various peptides generated are labelled "35, 30, 28, 15 K".


% Intact PDC activity remaining
100
90
80
70
60
50
40
30
20
10
0 10 20 30 40 50 60 70 80 90 100 110 120
Incubation length (min)

A)


M_r (x10^{-3})
94
67
43
30
20.1
14.4
X
35K (X_{IB})
30K (X_I)
28K ($X_{I'}$)
15K (X_L)
M O 5 10 20 30 40 60 90 90 120 120 C_{120}
Incubation length (min)

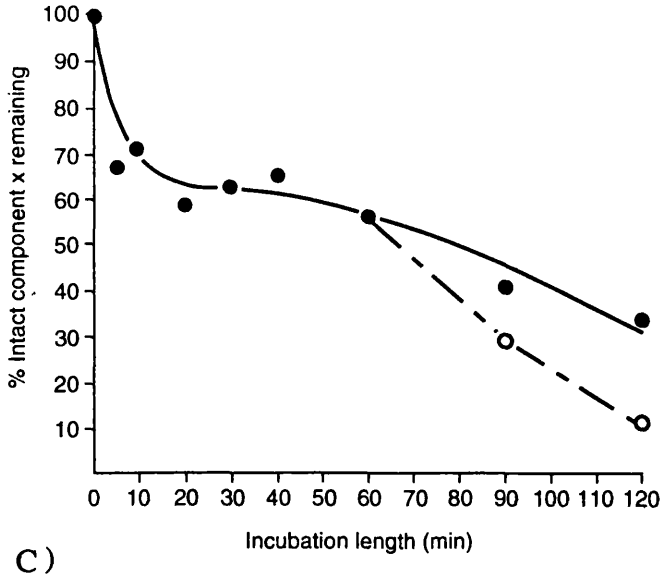
B)

Figure 3.2.3.1.1c) Decrease in intact protein X following Arg C treatment

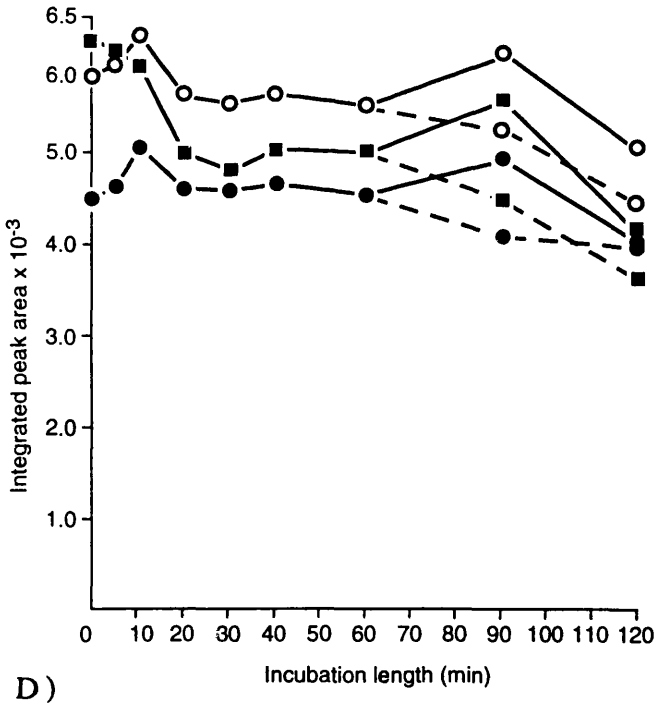
A scan of the above immunoblot was carried out employing preflashed hyperfilm and a Shimazu FDU-3 gel scanner at 500nm (see section 2.2.4). The % loss in intact protein X was thereby quantified, and presented with respect to the initial band absorbance. Where (•---•) represents 3% (w/v) Arg C; (o- - o) represents 6% (w/v) Arg C.

Figure 3.2.3.1.1d) Decrease in band intensity of components E2 and E1 from a 10% (w/v) SDS-PAGE Coomassie stained gel, following Arg C treatment

Samples removed at the time points shown above, were also resolved by 10% (w/v) SDS-PAGE and, following Coomassie blue staining, scanned with a Shimadzu FDU-3 gel scanner at 570nm (see section 2.2.4), to quantify the reduction in band intensity resulting from non-specific Arg C proteolysis. Where, (•---•) represents E1β; (•---•) E2; (o---o), E1α; and, hatched lines, a second addition of 3% (w/v) Arg C at 60min.


% Intact component x remaining
100
90
80
70
60
50
40
30
20
10
0 10 20 30 40 50 60 70 80 90 100 110 120
Incubation length (min)

C)


Integrated peak area x 10^{-3}
6.5
6.0
5.0
4.0
3.0
2.0
1.0
0 10 20 30 40 50 60 70 80 90 100 110 120
Incubation length (min)

D)

the E2 component, which at most exhibited a 30-40% decrease over 2h with 6% (w/v) Arg C illustrated by Fig. 3.2.3.1.1d -- with an initial 20% drop over the first hour.

Similar studies in isolated E2/X/K core employing 5% (w/v) Sigma Arg C (Rahmatullah *et al.*, 1989a), suggested an initial cleavage of E2 to the 28000-M_r inner catalytic domain ($E2_I$), and the combined 49000-M_r subunit binding domain, linker and lipoyl domain regions ($E2_{BL}$) -- identified through the ability to bind E1 and become acetylated; with further degradation of the latter species to a 39000-M_r lipoyl domain. An identical pattern of protein X cleavage to that illustrated in Fig. 3.2.3.1.1b, was also observed by this group whereby, release of the 15000-M_r lipoyl group resulted in a truncated 35000-M_r inner catalytic and subunit binding domain (X_{IB}), subsequently degraded to a 30000-M_r (X_I), then 28000-M_r ($X_{I'}$) inner catalytic domain fragment following the presumed cleavage of the subunit binding domain. Notably, the pattern of protein X cleavage here, differs from that observed for isolated E2/X core (see Fig. 3.2.2.1.1b) or, for intact complex cleaved in the presence of 1M NaCl (see Chapter 5; Fig., 5.2.2.2.2), and may therefore, reflect some degree of E3 mediated protection. However, as protein X degradation was accompanied by extensive complex activity loss, such protection cannot be offered as an explanation to the disparate results of Neagle & Lindsay (1991).

In addition, although relatively little degradation of the E1α and β subunits was noted in the presence of TPP and Mg^{2+} even over the extended 2h incubation where some loss resulted (i.e., a 10-12% and 20-30% decrease for β and α, respectively), the complete protection of E1α presented by Neagle & Lindsay (1991), was never fully reproduced. Therefore, it is highly likely that the additional Arg C degradation of E2, and possibly of E1α, contributed to the overall reduction

in PDC activity noted over 2h, and their apparent absence may explain the smaller loss in PDC activity recorded by Neagle & Lindsay (1991).

3.2.3.2 Release of E3 from intact complex following removal of the majority of intact protein X by Arg C

The results in section 3.2.3.1 revealed that removal of the majority of intact protein X, by Arg C, was accompanied by an equivalent loss in intact complex activity. However, due to the partial degradation of E2, this loss can only be partly attributed to the degradation of protein X. In reconstituted complex, this loss reflected a reduction in the E3 binding capacity of the isolated E2/X core, hence, a similar study was performed to monitor the dissociation of E3 from intact PDC following incubation with Arg C.

This involved the incubation of PDC with Arg C at 33°C in the presence of TPP and Mg^{2+} during which, complex activity was monitored by direct assay at 340nm (see section 2.2.11), and samples removed for the analysis of E3 dissociation involving centrifugation at 180000g. At this centrifugal field, dissociated E3 remains in solution whereas, bound E3 co-precipitates with the E2/X/E1 core fraction. Thus, by the removal of supernatants, and subsequent resuspension of pellets, the percentage bound and released E3 can be determined through E3 assays (see section 2.2.11).

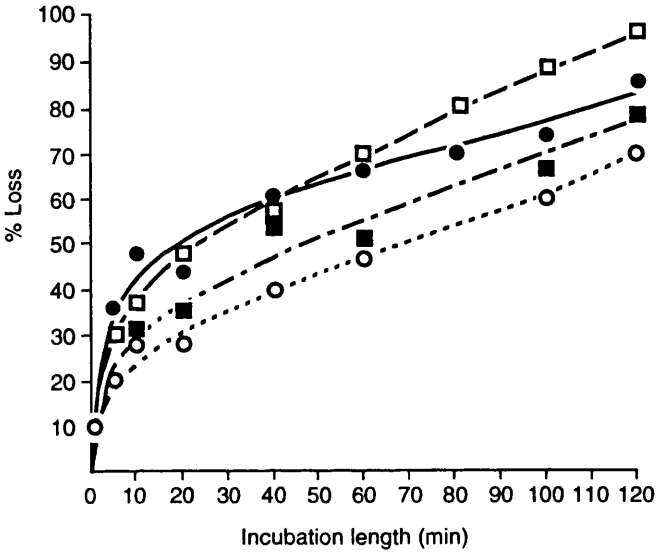
From the results in Fig. 3.2.3.2.1, the rate of loss of complex activity is presented in relation to the rates of loss of intact protein X and of bound E3. The loss in intact protein X was determined following Western analysis employing

Figure 3.2.3.2.1 Plot of the loss in intact complex activity, intact protein X and, bound E3 with time following Arg C digestion of intact PDC

Freshly prepared PDC was diluted to 3mg.ml^{-1} in 2ml **Soln A**, complex activity assayed at 340mm and aliquots (20µl) removed to Laemmli sample buffer. lml was then treated to 3+3+3% (w/v) Arg C at 0, 45 and 90min over a 2h incubation period at 33°C; and the remaining control sample treated likewise but with three additions of buffer. At the times shown, complex activity was assayed in duplicate by the removal of 5-10µl aliquots from a sample (20µl diluted to 100µl in **Soln A** and 4mM benzamidine), to assay cuvettes. Samples were also removed for immunological analysis; and to 1ml centrifuge tubes, kept at 4°C, for the quantification of E3 release (i.e. 83µl diluted to 500µl in 5mM benzamidine and **Soln A**). Centrifugation was at 180000g (r_{av} 2.5cm) for 2.5h at 4°C, the supernatants removed and the resultant pellets resuspended overnight in 1% (v/v) TX-100 buffer pH 6.8, before both were assayed in duplicate for total E3 activity at 340nm (see section 2.2.11). Protein X degradation was determined following resolution by 10% (w/v) SDS-PAGE of duplicate 5µg samples (for subsequent scanning); or, 10µg (for ^{125}I-incorporation), from each time point. Protein was then transferred to nitrocellulose and probed with protein X-specific sera, coupled to ^{125}I-protein A.

a) Quantification by light scanning: exposed preflashed hyperfilm was developed and the intact protein X band subsequently scanned at 600nm using a Shimadzu FDU-3 gel scanner (see section 2.2.4), to quantify the loss in intact protein X.

b) Quantification by ^{125}I-incorporation: exposed film was matched with the nitrocellulose filter to determine the precise location of the intact protein X bands which were then excised and the resultant γ radiation counted in duplicate in an iodine counter over 60 seconds. Re-exposure of the nitrocellulose blot revealed that the complete removal of each individual band was successful. Each point represented in Fig. 3.2.3.2.1, is an average of duplicate samples which differed by <15%.



PDC activity was determined in nkat.ml^{-1} and converted to a % of the initial activity (•—•). Released E3 activity, in nkat.ml^{-1}, was expressed as a % of the total E3 activity (released and bound), determined for each individual time point (o- -o). Loss of intact protein X-determined by scanning (■-- --■); or ^{125}I-incorporation (□--□), was also expressed as a % of the initial value.

protein X-specific antiserum by either : 1) quantification of ^{125}I- iodinated protein A incorporation of the nitrocellulose-bound intact protein X band; or, 2) by densitometric scanning of the intact protein X band from a preflashed hyperfilm exposure (see Fig. 3.2.3.2.1 for details). Both reveal a similar pattern of proteolytic degradation, but a slightly higher rate of loss is apparent for the latter method presumably reflecting differences in the degree of sensitivity in relation to experimental error. In agreement with the results of section 3.2.3.1, a close similarity between the extent of protein X degradation and loss in complex activity was apparent -- characterised by an initial higher rate of loss over the first 10-15min followed by a slow, extensive reduction. Yet, when compared to the degree of E3 dissociation it becomes clear that the reduction in complex activity and intact protein X over 2h, exceeds that of the former which exhibited only a 60% increase in E3 release over and above the 10% released in the absence of Arg C treatment, although Fig. 3.2.3.2.2 reveals an immediate increase in E3 release into the supernatant fractions.

As slight dissociation of the rate limiting E1 component was also observed in Fig. 3.2.3.2.2, the higher rate of complex activity loss may, therefore, result from minor E2 degradation, in support of the findings of section 3.2.3.1. Indeed, from Fig. 3.2.3.2.3, it is clear that whereas almost a direct correlation exists between loss in complex activity and intact protein X, the former loss initially exceeds that of E3 release, suggestive of an additional variable. Alternatively, the lag in E3 release may reflect the time taken for the subunit binding domain on X to unfold, following removal of the lipoyl domain by Arg C. In addition, if E3 binding were organised such that dissociation of each E3 dimer required the proteolysis of both or, one less accessible subunit, of an equivalent protein X dimer (see Chapter 5), significant degradation of the latter could precede a loss in E3 binding yet parallel PDC activity

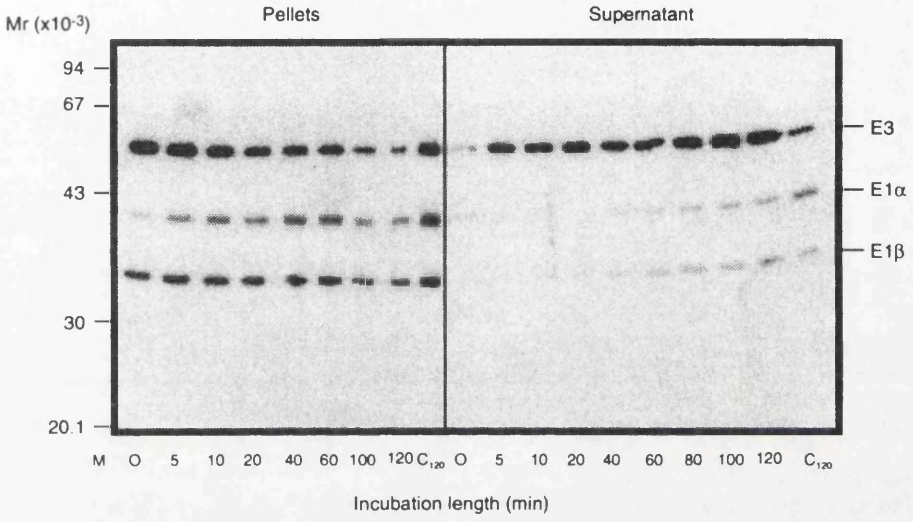


Figure 3.2.3.2.2 Immunoblot of the E3 and E1 components present in the resuspended pellets and isolated supernatants following Arg C treatment and centrifugation at 180 000g

5μg samples of each time point were resolved by 10% (w/v) SDS-PAGE for subsequent Western analysis employing E3 and E1-specific antisera. In either case, control samples were also loaded to illustrate the degree of dissociation, of either component, resulting from centrifugation treatment alone. Lane M, low M_r markers proteins; lane C, control undigested PDC.

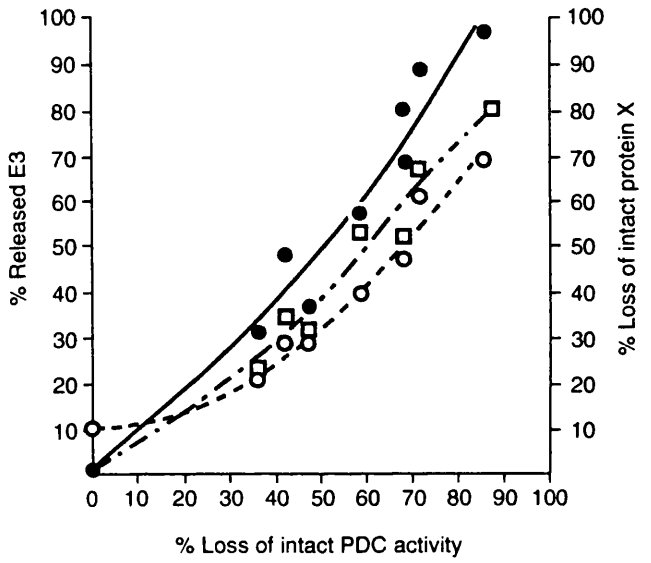


Figure 3.2.3.2.3 Comparison of the % loss in complex activity with both the % loss in intact protein X, and the resultant % release of E3

The % loss for each variable was determined as detailed in Fig. 3.2.3.2.1 (o- -o) represents the correlation between PDC activity and E3 release; (□– -□) represents the correlation between PDC activity and protein X degradation determined via ^{125}Iodine incorporation; and, (•—•) represents the correlation between PDC activity and protein X degradation determined via scanning.

loss, due to lowered E3 affinity and hence, catalytic efficiency. This possibility is discussed further in Chapter 4.

Thus, in conclusion it would appear that the proteolysis of protein X in intact complex results in an accompanying loss in PDC activity, which may be mainly attributed to the loss in E3 binding but may also partly reflect non-specific E2 degradation and the resulting minor E1 dissociation. This result is therefore in direct disagreement with those of Neagle & Lindsay (1991).

3.3 DISCUSSION

Central to the research presented in this chapter was the successful separation of bovine heart PDC into native, isolated E2/X core and dissociated E1/E3 fractions, which upon recombination, gave reproducibly high levels of reconstituted complex activity. Thus, in contrast to the harsh conditions employed by Rahmatullah *et al.* (1987; 1989b -- modified from Linn *et al.*, 1972; Stepp *et al.*, 1983), in the preparation of E2/X/kinase core, which involved high salt treatment at pH 9 for 1h at room temperature, followed by column separation and ammonium sulphate precipitation -- both in the presence of high salt and alkaline pH; with the additional use of 5M urea and monosodium hydroxymercuriphenyl sulphonate in further core purification (Powers-Greenwood *et al.*, 1989; Gopalakrishnan *et al.*, 1989); improved resolution was achieved here with the relatively mild conditions of 2M NaCl over 1h at neutral pH and 4°C. In addition, whereas 70-100% reconstitution of complex activity was routinely recovered here, the absence of a clear indication of the conditions and extent of control reconstitution in the above publications raised doubts over the degree of component denaturation and the interpretation of data (Neagle & Lindsay, 1991).

Excellent reconstitution of overall complex activity was observed with a range of E2/X:E1/E3 ratios of 1:0.7-1.3 (w/w), which compares well with that of intact complex (1:1.3), calculated from subunit stoichiometry and molecular mass values. A tendency to achieve maximal reconstitution was observed using sub-stoichiometric levels of E1 and E3. This may reflect partial loss of activity of E2/X during purification or, favoured by the high recoveries in reconstitution obtained, indicate that maximal occupancy of E1 and/or E3 binding is not required for maximal levels of reconstitution. Furthermore, this appeared to be independent of component

concentration, in contrast to the findings of Schulze *et al.* (1991c) which, in investigating E1 and E3 binding in *A. vinelandii* PDC found better recovery at higher protein concentrations, due to competition between the E1 and E3 components for E1 binding sites. Reassembly of the complex occurred rapidly, apparently within 1-2min, which is in agreement with the 10-15min preincubation employed by Schulze *et al.* (1991c), in similar reconstitution experiments involving *A. vinelandii* PDC, and of Kresze *et al.* (1980), and Rahmatullah *et al.* (1990), employing bovine kidney PDC.

Analysis of reconstitution, following the removal of the lipoyl domain of protein X by Arg C, revealed a dramatic loss in complex activity and subsequent resolution by gel filtration indicated a significant decrease in E3 binding affinity. Reconstitution of undegraded, partially and fully Arg C degraded E2/X core with E1/E3, in the presence of high concentrations of porcine E3 (0-10mg.ml^{-1}; 1.6-50 fold excess (w/w) with respect to E1/E3), resulted in successively higher recoveries yet failed to achieve full recovery of original activity. Notably, untreated reconstituted complex also exhibited an increase in activity with excess porcine E3 which, in agreement with earlier findings and an observed 5-10% enhancement of intact PDC activity following addition of excess E3 (results not shown), suggested the possibilty of both incomplete E3 occupancy and minor inactivation during complex purification and subsequent component isolation.

In contrast, the increasing dependency upon higher concentrations of porcine E3, during the progressive cleavage of the lipoyl domain of protein X (accompanied by enhanced recoveries), may reflect an increasing contribution to complex activity from the low affinity binding of E3 to the subunit binding domain of E2. This could arise following a dramatic reduction or, total loss, in E3 binding affinity for the equivalent domain on protein X. The low specificity of this binding would also

account for the inability of excess porcine E3 to completely overcome losses in reconstitution associated with the disruption of protein X/E3 interactions. Indeed, no further enchancement followed extension of the preincubation period from 10min to up to 4h, nor the addition of putative mediating molecules such as free lipoamide or redox dyes, to the reconstitution assay. The latter failure may, however, reflect problems of lipoamide insolubility and the incompatibility/non-feasibility of the proposed reaction mechanism, which required efficient rates of catalysis between the various components and mediating molecules, and therefore, that free lipoamide is a good substrate of both E2 and E3. Whilst this has been shown to be the case for *E. coli* PDC (Reed *et al.*, 1958), reductive acetylation by E1 and presumably, enhanced E2/E3 interactions, are dependent upon attachment to the lipoyl domain (see Perham, 1991).

Thus, the inability to achieve full recovery of reconstitution in the presence of excess porcine E3 led to the proposal that, following removal of the lipoyl domain of protein X by Arg C, the substantial reduction or complete loss in E3 binding affinity could result from disruption and hence, unfolding of the exposed subunit binding domain.

Support for this proposal came from the results of N-terminal amino acid sequencing of the truncated 35000-M_r fragment of protein X, which located the point of Arg C cleavage at the extreme N-terminal boundary of the subunit binding domain (Robien *et al.*, 1992; Kalia *et al.*, 1993). Although the exact location of this domain in mammalian protein X is as yet undetermined, such proximity of the cleavage site would most likely still cause disruption and hence instability if the margin of this domain was subsequently found to be several amino acids upstream. Further indication, came from previous reports of truncated E2/X core instability and losses in peripheral subunit binding, following selective proteolysis of the lipoyl

domain of E2 or, X, in the presence of detergent, CHAPS, (Rahmatullah *et al.*, 1990), or high salt (Neagle & Lindsay, 1991). Indeed, the proteolytic sensitivity of the E1/E3 binding region has also been observed in E2 from *A. vinelandii*, *B. stearothermophilus* and *E. coli* PDC (reviewed in Schulze *et al.*, 1991b).

At this point it remains unclear whether the action of Arg C promoted unfolding of the exposed subunit binding domain or, induced heat-lability of a specific E3 binding site within this domain. Initial attempts to address this question, involving Arg C treatment of intact PDC at 4°C over 24h, were unsuccessful owing to minimal protein X degradation at this temperature (results not shown), in agreement with previous observations (Rahmatullah *et al.*, 1989a,b).

Notably, complete removal of the lipoyl domain of protein X, by Arg C, in intact PDC, in the absence of accompanying E2 and E1 proteolysis, and with only minor losses in complex activity (i.e., 15% over 1h), was not accomplished, in contrast to the findings of Neagle & Lindsay (1991). Here, complex activity decreased to 50% over the first hour, in line with protein X cleavage, with a further 20-30% loss accompanying a parallel decrease in protein X although, possibly also in combination with minor E1 and E2 degradation. However in agreement, following Arg C treatment, E3 dissociation increased dramatically, yet from the results presented here, this release appeared to lag behind that of intact protein X and complex activity loss. Whilst some reduction in PDC activity may be attributed to <100% E3 occupancy and/or minor E2 degradation and the accompanying release of a small but significant population of E1 molecules, this disparity could also reflect the nature of E3 binding whereby dissociation of each E3 dimer requires the degradation of more than one protein X molecule. In addition, some variation in the accessibility of the protein X molecules could arise from its positioning within the E2/X core and, involvement in E3 binding. Indeed, although the proportion of

protein X to Arg C (w/w), in isolated core is two fold higher than with intact complex, the incomplete removal of protein X from intact PDC both here and elsewhere (Rahmatullah *et al.*, 1989b), indicates an enhanced inaccessibility in the presence of E3 (see Chapter 5). Furthermore, if loss of E3 binding affinity involves some conformational change in the subunit binding domain of protein X, E3 dissociation may not occur immediately, although any decrease in affinity could lead to reduced catalytic efficiency.

Thus in conclusion, the decrease in E3 binding affinity following Arg C treatment of both intact and reconstituted complex, appears to be a direct result of the proposed disruption of the subunit binding domain of protein X, following removal of a larger fragment comprising the lipoyl and linker regions; and is therefore in agreement with the findings in *S. cerevisiae* PDC of Lawson *et al.* (1991). A similar complete and irreversible loss in E1 binding followed the deletion of a large segment of the proposed E1 binding site from the E2 core of *A. vinelandii* PDC, yet E1/E3 binding and E2 core assembly were unaffected by the complete deletion of the lipoyl domain from E2 (Schulze *et al.*, 1991c). Nevertheless, the 30% loss in complex activity observed by Lawson *et al.* (1991), upon the precise and exclusive removal of the lipoyl domain of protein X, was noted upon closer examination to be accompanied by a small but significant decrease in E3 binding (not detailed by the authors). In addition, investigation of the dissociation constants (K_d) of E3 for the subunit binding domain and an overexpressed di-domain (comprising the lipoyl, linker and binding regions) of E2 from *B. stearothermophilus* PDC (Hipps *et al.*, 1994), revealed a tighter association with the latter domain, and as a previous study (Hipps & Perham, 1992) discounted any interaction of the lipoyl domain with E3, it was postulated that stability was provided by the linker region. Yet, removal of a lipoyl-like sequence from the N-terminus of component E1 of OGDC, by trypsin, resulted in both loss of E3 binding and complex activity (Rice *et al.*, 1992); where

sequence analysis of a full length cDNA clone for E2 from rat OGDC revealed the absence of a subunit binding domain motif (Nakano *et al.*, 1991). Hence, whilst the subunit binding domain of protein X is primarily responsible for E3 binding interactions within the E2/X core of bovine heart PDC, evidence suggests that the lipoyl and/or linker regions may also play an indirect role in the stabilisation of this domain.

Furthermore, evidence presented here suggests that, following loss of E3 binding potential from protein X, low affinity binding of E3 to the subunit binding domain of E2 results in partial recovery of complex activity. Whilst not observed in the protein X mutants of *S. cerevisiae* PDC (Lawson *et al.*, 1991a), this proposal is supported by the findings of two case studies in which residual PDC activity (i.e., 10-20%), was noted in the absence of immunologically detectable levels of protein X (levels of all other components including E3, were normal), in patients suffering from severe lactic acidemia resulting in early death (Marsac *et al.*, 1993; Robinson *et al.*, 1990). The significance of this low level binding to E2 may prove important especially in consideration of recent evidence (S. Khan, University of Glasgow, unpublished observations), which suggests that, in spite of the highly conserved nature of E3 and the successful employment of bovine heart E3 in the reconstitution of *E. coli* PDC, and, of porcine heart E3 with bovine kidney PDC (Kresze *et al.*, 1980), differences exist in the catalytic and/or binding requirements of E3 from porcine and bovine heart sources which led with the former, to reduced levels of reconstitution in bovine heart OGDC. This area is therefore the subject of current research.

RESULTS CHAPTER 4 :

Investigation into the catalytic role of protein X in bovine heart PDC.

4.0 INTRODUCTION

While involvement in E3 binding has unequivocally established a structural role for protein X (see Chapter 3), discussion still surrounds its precise contribution to overall complex catalysis. Initial functional characterisation of protein X by De Marcucci & Lindsay (1985), De Marcucci *et al.* (1985; 1986), Jilka *et al.* (1986) and Hodgson *et al.* (1986), revealed: pyruvate or NADH/acetyl CoA-linked acetylation -- including S^6,S^8-diacetylation (a phenomenon apparently unique to mammalian PDC); deacetylation; and, NEM incorporation identical to that of the E2 component. Furthermore, the discovery of significant N-terminal amino acid homology between the lipoyl domains of E2 and X in bovine heart PDC (Neagle *et al.*, 1989), raised the question of whether protein X might exist as an isoenzyme of E2. Yet production of component-specific antisera, and the subsequent isolation of a separate gene (PDX1), encoding *S. cerevisiae* protein X (Behal *et al.*, 1989), simultaneously ruled out exon splicing, pointing to gene duplication and independent evolution as a possible origin of this component.

Antibody screening of ten different rat tissues by De Marcucci *et al.* (1986), suggested a wide tissue distribution for both components. In addition, sequence analysis of the protein X gene (PDX1), from *S. cerevisiae* revealed the absence of a region equivalent to the extreme C-terminal portion of the inner catalytic domain of E2 in which resides the "His Xaa Xaa Xaa Asp Gly" active site consensus sequence proposed by Guest (1987), and therefore implied a structural/functional role distinct from that of E2 (Behal *et al.*, 1989).

Initially, the high affinity of protein X for the E2 core prevented elucidation of the precise nature of its catalytic properties. Clearly, (de)acetylation of protein X

could occur directly, although the requirement for acetyltransferase activity was not supported by the evidence of Behal *et al.* (1989), and of unpublished observations from the same group (Lawson *et al.*, 1991b). Alternatively, these processes could occur indirectly either, through the intramolecular transfer of acetyl groups between E2 and X lipoyl domains or, by the lipoyl domain of protein X visiting the active site of E2. However, by employing a procedure originally devised by Stepp *et al.* (1983), involving the use of high salt, alkaline pH and mercurial reagents over extended periods, Rahmatullah *et al.* (1987) purified a protein X/kinase fraction from bovine kidney PDC and thereby, produced the first evidence for direct E1-mediated acetylation of the lipoyl domain of protein X from pyruvate, in the absence of E2. In contrast, acetyl CoA-linked acetylation was dependent upon the inclusion of the inner catalytic and subunit binding domain of E2 ($E2_I$; prior treatment with papain facilitated removal of all lipoyl domains), suggestive of the absence of acetyltransferase activity, yet indicative of E2-active site servicing by the lipoyl domains of X. This was further supported by the discovery of a residual 12-15% complex activity in *S. cerevisiae* null mutants for the E2 gene (LAT1), following transformation with LAT1 single copy plasmids in which, the lipoyl domain plus or minus, linker-encoding regions were deleted; or, $lysine_{47}$ (essential for lipoic acid attachment), substituted for by an arginine. Whereas acetyltransferase activity and component binding remained unaffected, total loss of complex activity only occurred upon substitution of both $lysine_{47}$ and $lysine_{43}$ of $E2_L$ and X_L, respectively (Lawson *et al.*, 1991b).

In agreement, following the complete removal of the lipoyl domains of E2 from bovine kidney PDC, via collagenase treatment and centrifugal fractionation Rahmatullah *et al.* (1990), noted a residual, apparently X lipoyl domain-mediated, 10% complex activity. In addition, no loss in PDC activity was observed until 40% of the E2 lipoyls were removed, adding to the already extensive evidence for active

site coupling in which, a given E2 component may service more than one E1 or E3 active site and participate in transacetylation (see Graham *et al.*, 1986, for review; Stanley *et al.*, 1981). In contrast, in investigating the E3 binding function of protein X in bovine kidney PDC, Gopalakrishnan *et al.* (1989) noted that cleavage of the lipoyl domains, by Arg C, only slightly exceeded the initial rate of loss in complex activity, proposing an essential but non-rate limiting transfer of reducing equivalents from E2 to E3 via protein X, and citing the E3 binding potential of a protein X/kinase fraction ($X/K_c/K_b$), as strong evidence of direct interactions. Whilst Arg C was later found to remove both lipoyl and linker regions, overall loss in complex activity was therefore, correlated to the possible disruption of the E3 binding domain (see Chapter 3), although gene deletion of only the lipoyl domain of protein X from *S. cerevisiae* also resulted in a 30% reduction in PDC activity (Lawson *et al.*, 1991a). Further, through N-terminal amino acid sequencing, collagenase was also found to remove both lipoyl and linker, cleaving at the boundary of the E1 binding domain of E2 (Rahmatullah *et al.*, 1990), yet leaving E1 binding unaffected.

Hence, Rahmatullah *et al.* (1990) proposed preferential upstream utilisation of the lipoyls of E2 and, a more restricted downstream utilisation of protein X in the transfer of electron pairs from E2 to E3. Whether the inherent transacetylase potential of protein X is maintained in intact PDC at the residual (10-15%) level, therefore, only becoming apparent in the absence of E2 lipoyl domains or, suppressed through competition for acetyl groups and, only expressed in the absence of E2, is as yet unclear. Furthermore, the significance of this apparently superfluous activity in mammalian and yeast PDC, absent in all other 2-oxoacid dehydrogenase complexes from various species, is intriguing expecially in view of the unique diacetylation properties of mammalian PDC (see discussion). While the full extent of the involvement of protein X in complex activity remains to be seen, little evidence exists for acetyltransferase activity associated with X. In addition, as

debate continues over the organisation of protein X within the E2 core, growing evidence for a peripheral location fuels further doubt over the presence of a fully functional acetyltransferase active site in mammalian protein X (see Chapter 5 for references within).

Despite these considerations, the possibility of a protein X-mediated acetyltransferase activity should not be excluded at this stage. Indeed, in light of : an estimated 2000-M_r disparity between yeast (48000-M_r) and mammalian (50000-M_r) protein X -- compatible with the presence of an extra 20 C-terminal amino acid residues in the latter species; the presence of a single lipoyl domain on E2 from yeast PDC, in contrast to two in mammalian dihydrolipoamide acetyltransferase; and the absence of diacetylation in either E2 or X from *S. cerevisiae*, it seems quite plausible that differences in the structural/functional properties of protein X should exist between these species. Furthermore, the possibility of a partially degraded/ denatured C-terminal domain in protein X from bovine heart PDC cannot be completely ruled out, following employment of the exhaustive 5-day preparation of Rahmatullah *et al.* (1987), which involved high salt, alkaline pH and mercurial reagents together with repeated precipitation and overnight dialysis -- all in the absence of protease inhibitors (Rahmatullah *et al.*, 1987). In addition, initial sequence analysis of a 241 base pair cDNA clone from the extreme C-terminal segment of mammalian protein X revealed the presence of an active site-like "His Xaa Xaa Xaa Ser Gly" sequence (J.C. Neagle, University of Glasgow, unpublished observations). However, verification of this finding has been precluded by the absence of other published gene sequences for protein X from mammalian sources and has therefore, prompted more detailed analyses, at both nucleotide (J.E. Rice, University of Glasgow, unpublished observations), and amino acid level.

This latter work, involved N-terminal amino acid sequence analysis of the C-terminus of both E2 and X, following purification of E2/X core from bovine heart PDC, extensive tryptic degradation and, subsequent isolation of the extreme C-terminal peptides employing a specialised anhydrotrypsin resin. The preliminary results from this work are presented, together with additional investigations into the putative catalytic role of protein X in bovine heart PDC. These comprised studies of N-ethyl[2,3-^{14}C]maleimide incorporation in intact and reconstituted complex, following specific proteolysis of the lipoyl and linker regions of E2 or X, with collagenase or Arg C, respectively.

4.1 METHODS

4.1.1 Investigation into an improved method for E2/X core purification

Prior to the isolation and N-terminal amino acid sequencing of the tryptically cleaved C-terminal fragments of components E2 and X, following purification by resolution on an anhydrotrypsin column, endeavours to improve upon the standard E2/X isolation procedure described in section 2.2.9, were carried out. Whilst low yields and residual E1/E3 contamination were acceptable and unavoidable where retention of native activity was essential (see Chapter 3); here, the requirement for maximum purity and yield led to the investigation of various additional methods. These included: repeated high salt treatment with subsequent resolution by gel filtration or, centrifugal fractionation; treatment with the chaotrophic reagent, guanidinium chloride (GdnHCl) followed by gel filtration; or, prior proteolysis of the lipoyl domains of E2 and X in intact PDC, by trypsin, to facilitate the dissociation of E1 and E3 upon gel filtration.

A summary of the procedures used, together with a description of the component composition of each "purified" core fraction, as visualised on 10% (w/v) SDS-polyacrylamide gels stained with Coomassie blue, and the % recovery of total protein for each core fraction at each stage (with the overall % recovery in brackets), are presented in Table 4.1.1.1.

Whereas high salt treatment in conjunction with centrifugal fractionation gave poorer purification compared to that obtained for high salt treatment in combination

Treatment	Sample	Condition	% Recovery of original protein	Component composition
1a)	20mg PDC	2M NaCl, 1h, 4°C; core pelleted at 120000g, 16h.	54%	E2, X E1 (reduced) E3 (reduced)
1b)	9.6mg of "E2/X" from 1a)	as in 1a)	38% (20%)	E2, X E1 (reduced) E3 (trace)
2a)	20mg PDC	2M NaCl, 1h, 4°C; core resolved by 100ml Superose 6 column, pH 9 (see 2.2.9).	7-11%	E2, X E3 (trace) E1 (trace)
2b)	1.2mg of "E2/X" from 2a)	as in 1a)	85% (6-9%)	E2, X E1 (trace) E3 (no trace)
3a)	20mg PDC	4M NaCl (solid), 1h, 4°C; core pelleted at 120000g, 16h.	42%	E2, X, E3 E1 (reduced)
3b)	7mg of "E2/X" from 3a)	as in 3a).	41% (17%)	E2, X E3 (reduced) E1 (reduced)
4	30mg PDC	1M GdnHCl 1h, 4°C; core resolved by 100ml Superose 6 column, pH 9 (see 2.2.9 but GdnHCl used for NaCl). E2/X fractions spun at 120000g, 16h.	N.D.	E2, X E1 (reduced) E3 (trace)
5	30mg PDC	1%(w/v) trypsin, 5min, 30°C; inhibited with 10%(w/v) soyabean trypsin inhibitor then, as in 2a).	2%	27K fragment of E2 30K fragment of X E1 & E3 (no trace)

Table 4.1.1.1 Purification table of E2/X core preparations

The summarised results of five separate E2/X core purification procedures are presented detailing conditions used; descriptions of the purity of each E2/X core isolate, and % recoveries.

with column separation (Table 4.1.1.1, treatments 1 & 2), yields were considerably higher, although clearly reflecting greater contributions to total core protein from contaminating E1 and E3. Replacement of NaCl with the chaotroph, guanidinium chloride, resulted in a similar pattern of E1/E3 release to that of condition 1b (see treatment 4). Furthermore, tryptic degradation of intact PDC for the purpose of removing, or disrupting, the subunit binding domains of E2 and X, and thereby causing complete E1 and E3 dissociation, respectively, resulted in further complications involving yield, solubility and detection of the truncated core samples. Thus, the standard E2/X isolation procedure of section 2.2.9 (Table 4.1.1.1., treatment 2a), with additional salt treatment and centrifugal fractionation (treatment 2b), produced the purest samples of E2/X core where all traces of E3 and the majority of E1 were successfully removed. Yet the extremely poor yields following both procedures remained problematic, discouraging the additional purification step of 2b and hence, all E2/X purifications employed in this thesis followed the method outlined in section 2.2.9.

4.2 RESULTS

4.2.1 Preliminary research into the isolation of a C-terminal peptide of protein X containing an acetyltransferase active site motif

The surprising discovery of an acetyltransferase active site-like sequence motif within the C-terminal domain of protein X, as determined through nucleotide sequence analysis of a partial cDNA clone isolated from a human hepatoma λgt 11 expression library screened with protein X specific antiserum (J.C. Neagle, University of Glasgow, unpublished results), necessitated further attempts to verify the identity of the cDNA clone and the proposed active site motif within. Direct analysis, shown here, involved N-terminal amino acid sequencing of the extreme C-terminal portion of protein X in which, if present, would reside the acetyltransferase active site motif.

Isolation of the C-terminus relied upon extensive tryptic degradation of both E2 and X and the assumption, based on probability, of there being no trypsin-accessible lysine or arginine residues between the active site motif and the C-terminus, nor at the C-terminus itself. Fulfillment of these requirements would then enable purification, on immobilised anhydrotrypsin, of the C-terminal peptides of E2 and X which, would fail to bind to the modified matrix by virtue of the (presumed) absence of a C-terminal lysine or arginine residue. Anhydrotrypsin is a catalytically inert derivative of trypsin, in which the active site serine$_{195}$ has been converted to dehydroalanine, which selectively binds under weakly acidic conditions peptides with arginine, lysine or, S-amino-ethyl cysteine at the C-terminus. Hence, the

former would elute immediately leaving the latter tightly bound until addition of elution buffer and the alteration of the pH would facilitate dissociation. In addition, in the unlikely possibility of a C-terminal lysine/arginine residue on X, digestion of E2/X by chymotrypsin would result in peptides ending in tyrosine, tryptophan, phenylalanine or, methionine and, therefore, only the C-terminal peptides of X would adsorb to the matrix. Owing to constraints upon time, only preliminary data were obtained for direct sequencing and this is presented as follows.

4.2.1.1 Establishing optimal conditions for extensive tryptic degradation of isolated E2/X core

To enable efficient N-terminal amino acid sequencing, it was necessary to generate short (10-30 amino acid residues; M_r 1100-3100) C-terminal peptides, containing the proposed active site sequence. As rapid and extensive degradation requires initial unfolding of the proteins in conditions in which they are maintained in a soluble state, a range of denaturing conditions was employed including 3-6M urea, TCA precipitation (see Fig. 4.2.1.1.1), and brief heat treatment at 65-100°C (results not shown).

Following overnight treatment of native E2/X core with 1% (w/v) trypsin at 37°C (Fig. 4.2.1.1.1; lane 1), both E2 and X were substantially cleaved, with 34000, 22000 and 18000-M_r (E2 derived), and 29000-M_r (X derived) fragments remaining, and evidence of low M_r fragments (<15000), at the gel front (see Neagle *et al.*, 1989). Further degradation of the E2 component to the 22000 and 18000-M_r fragments (identified through immunological screening), was obtained with 3-6M urea (diluted to a compatible 2M prior to the addition of trypsin), indicating some

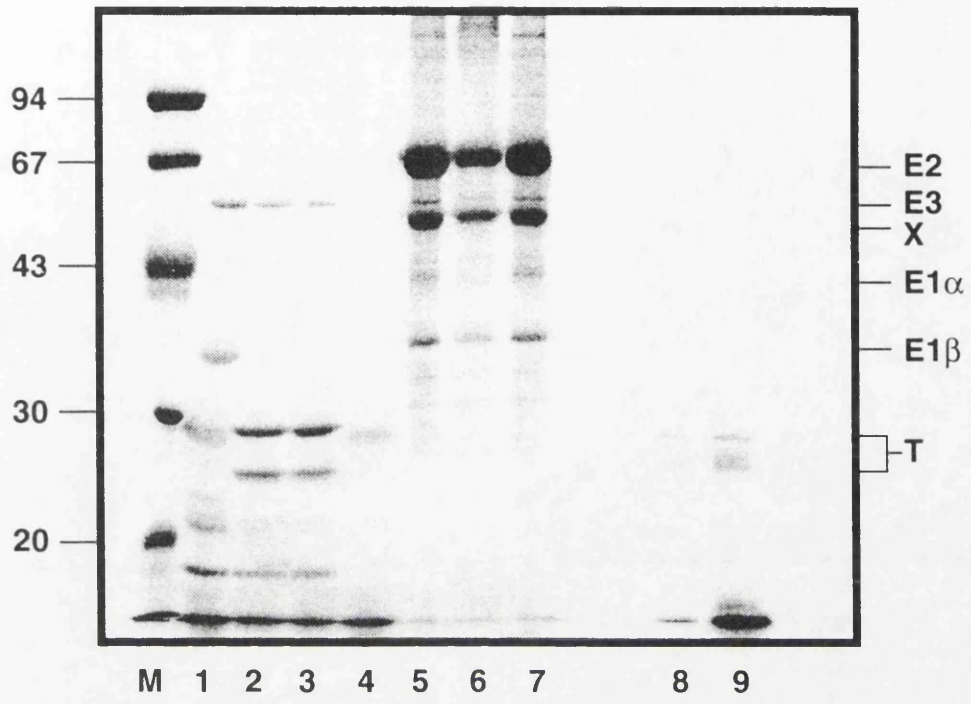


Figure 4.2.1.1.1 Analysis of SDS-PAGE resolved E2/X core samples following overnight treatment with 1% (w/v) trypsin at 37°C, under various conditions

Samples of E2/X core (diluted to 0.4mg.ml^{-1} in 0.1M NH_4HCO_3 buffer, pH 8 containing 0.1mM $CaCl_2$), were treated to denaturing conditions (sample 2: 3M urea, 30min; sample 3: 6M urea, 30min; sample 4: TCA precipitation). Buffer was then added to dilute urea to 2M, and pellets resuspended prior to overnight incubation with 1%(w/v) trypsin (diluted in buffer) at 37°C (conditions 1-4). Control, undigested samples were included to assess non-specific degradation (conditions 5 & 7), loss in yield from TCA precipitation (6), and autolysis of trypsin (8 & 9) where overnight incubations were omitted for conditions 7 and 9. E2/X core (15μg) and trypsin (10μg) were then resolved by 8% (w/v) SDS-PAGE as shown and stained with Coomassie Blue. Lane M, low M_r marker proteins (x10^{-3}).

increased accessibility due to unfolding (lanes 2 & 3). Notably, although the proteolytic resistance of the contaminating E3 traces was not affected by the presence of urea, TCA precipitation resulted in the successful cleavage of both E3 and the E2/X core to fragments <10000-M_r, with only traces of the 29000-M_r protein X-derived X_I fragment evident (lane 4).

From comparison of conditions 5, 6 and 7 it is clear that whilst no non-specific degradation of the core samples occurred with overnight incubation at 37°C (condition 5), approx. 50% loss in yield resulted from problems with solubilisation of the protein pellet obtained by TCA precipitation, despite overnight resuspension in 0.1M NH_4HCO_3 buffer. Equally efficient proteolysis coupled with smaller losses in yield, due to a more easily solubilised pellet, followed denaturation at 100°C for 5min (results not shown). Finally, although trypsin exhibited minor autolysis even in the presence of 0.1M $CaCl_2$, the undesirable presence of low levels of such proteolytic products, was outweighed by the improved degradation achieved with a second 1% (w/v) addition for a further 1h incubation, following overnight treatment. Hence, the standard method for maximum tryptic degradation listed in section 2.2.15 and employed in section 4.2.1.2, combined heat denaturation and treatment with 1+1% (w/v) trypsin at 37°C.

4.2.1.2 Preliminary isolation of the C-terminal peptides of E2 and X employing a specialised anhydrotrypsin column

Following elucidation of the optimal conditions for maximal tryptic degradation, trial separations of the resulting peptides on a pre-packed immobilised anhydrotrypsin column were carried out as described in the accompanying mannual, but with the modifications listed in section 2.2.15.

Fig. 4.2.1.2.1 illustrates one such elution profile of approx. 140μg of tryptically degraded E2/X core, from which fractions were collected and sent to the SERC-funded Protein Sequencing Facility at Aberdeen University for further purification by reverse phase micro-bore HPLC. Samples were also collected following proteolysis, both before and after precipitation of insoluble material and, following column resolution, for analysis on a silver stained 15% (w/v) SDS-PAGE gel (not shown). These results suggested that while extensive tryptic degradation had occurred as previously noted, significant levels of the partially digested 29000 and 15000-M_r (protein X), and 27000 and 18000-M_r (E2 derived), fragments remained -- although, subsequently substantially precipitated by a brief spin in a benchtop Eppendorf microfuge. Of more concern however, was the considerable accompanying removal of peptides <14000-M_r as evidenced, by a similar reduction in the level of silver stained protein migrating at the gel front. Nevertheless, it is possible that while some peptides of M_r 10000 were precipitated, smaller fragments remained in solution but, due to their relative size and quantity, were not easily visualised by silver stain.

Indeed, unlike the immediate elution of non-adsorbed peptides in peak 1 (fractions 2-4), including the 29000, 27000, 18000 and 15000-M_r fragments of E2

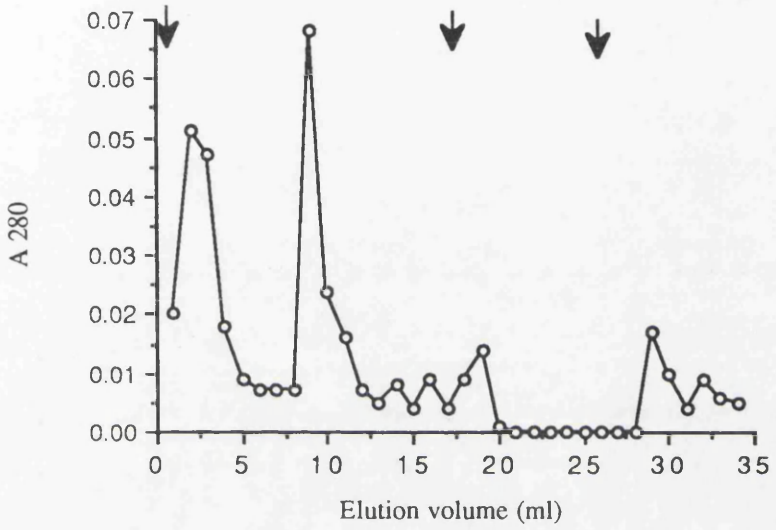


Figure 4.2.1.2.1 Elution profile of tryptically degraded E2/X core peptides from an anhydrotrypsin column

Purified E2/X core (100µg, 2.8mg.ml^{-1}) denatured, degraded and diluted as described in section 2.2.15, was briefly spun in an Eppendorf microfuge to pellet insoluble material, and 500µl (~37pmoles), analysed on anhydrotrypsin resin. 1ml fractions were analysed at 280nm. Arrows indicate changes in column buffer used (1st & 3rd arrow -- binding buffer; 2nd arrow -- elution buffer).

and X, and traces of undigested E3 (E2/X from the standard procedure of section 2.2.9 exhibited minor E3 contamination), no protein bands were apparent on the silver stained gel in samples taken from the second peak (fractions 9-11). In contrast, the elution of adsorbed peptides was signified by a small peak, and the predominance of the 18000-M_r peptide of E2, with traces of peptides <14000.

Peak fractions 2 and 9 (200µl each), were then subjected to reverse phase micro-bore HPLC analysis which resulted, in each case, in one major and 6-7 minor peak fractions (currently being sequenced in Aberdeen) -- clearly reflecting the incomplete proteolysis of the E2/X core and minor E3 contamination.

It is possible that improvements in the preparation of the trypically digested core could result in cleaner elution profiles and ultimately, less follow-up work. Of major concern were the relatively low yields of tryptic peptides generated. Whilst the manufacturer recommended a starting sample concentration of 10mg.ml^{-1} followed by a 1:20 (v/v) dilution in binding buffer to give 250µg of protein in 500µl (or, as exemplified, 20nmoles of ribonuclease), PEG concentration of the E2/X core isolate from volumes of 10-20ml to 0.5ml gave maximal concentrations of 2-4mg.ml^{-1} E2/X (see section 2.2.9). Furthermore, dilution in binding buffer at a reduced 1:10 (v/v) ratio, in order to maintain concentrations, may have proved counterproductive owing to the subsequent precipitation of insoluble material -- probably resulting from the hydrophobic nature of the C-terminal regions of E2 (and possibly X). This, combined with the large M_r of E2/X core of 3.6×10^6, resulted in a maximum loading of 26-37pmoles of digested core onto the anhydrotrypsin column. However, attempts to produce more E2/X and therefore increase the final concentration, were unsuccessful with poorer column resolution accompanying the dissociation of larger quantities of PDC (i.e., 50-100mg).

The solution may lie in the combination of several samples of E2/X core, precipitated through treatment at 65°C, rather than 100°C, with removal of the supernatant, prior to the addition of a small but sufficient volume of incubation buffer containing 1% (w/v) trypsin. This would achieve both the efficient removal of contaminating E3 (soluble by virtue of its heat stability), and further concentrate E2/X allowing the recommended 1:20 (v/v) dilution in binding buffer -- possibly also resulting in improved solubility. Adoption of the above improvements in a second attempt to isolate the C-terminal peptides of E2 and X will depend upon the outcome of the aforementioned amino acid sequencing, and the re-examination of the 241 nucleotide protein X clone (J.E. Rice, University of Glasgow). However, if problems with insolubility persist, due to the hydrophobic nature of the C-terminal region of E2 (and maybe of X), identification by this method may remain difficult. In this regard, employment of a molecular approach in the verification of the origin of the cDNA clone may prove more successful (see Chapter 6).

4.2.2 Investigation of a putative catalytic role for protein X employing selective proteolysis of E2 or X in conjunction with N-ethyl[2,3-^{14}C]maleimide labelling

4.2.2.1 Establishing optimal conditions for maximal E2 and minimal protein X cleavage in the presence of collagenase

To investigate the proposed involvement of protein X in overall complex catalysis, initial work centred on reproducing the findings of Rahmatullah *et al.* (1990), and thereby establishing optimal conditions for the controlled maximal removal of the lipoyl and adjoining linker region of E2, in isolated E2/X core, by collagenase, in the absence of similar protein X degradation. This is possible since collagenase only cleaves at a specific tetrapeptide sequence, "Pro Leu Gly Pro", which occurs only once in E2, and presumably, not at all in protein X (minor additional proteolysis by a contaminating protease was noted with some preparations of collagenase -- Rahmatullah *et al.*, 1990).

In bovine kidney E2/X core isolates, Rahmatullah *et al.* (1990) employed either, 20% (w/v) collagenase over 10min or, 2% (w/v) over 90min, at 30°C. However, the former condition was found to result in unfavourable protein X degradation and thus, Fig. 4.2.2.1.1 illustrates the results of a 60min incubation at 30°C with 10% (w/v) collagenase. The gradual loss in reconstituted PDC activity to approx. 20% (Fig. 4.2.2.1.1b) appeared to mirror the gradual cleavage of intact E2 until less than 5% remained (Fig. 4.2.2.1.1c; as determined through densitometer scanning of the Coomassie blue stained gel shown in Fig. 4.2.2.1.1a). A plot correlating one with the other, revealed a similar, although less marked, pattern to

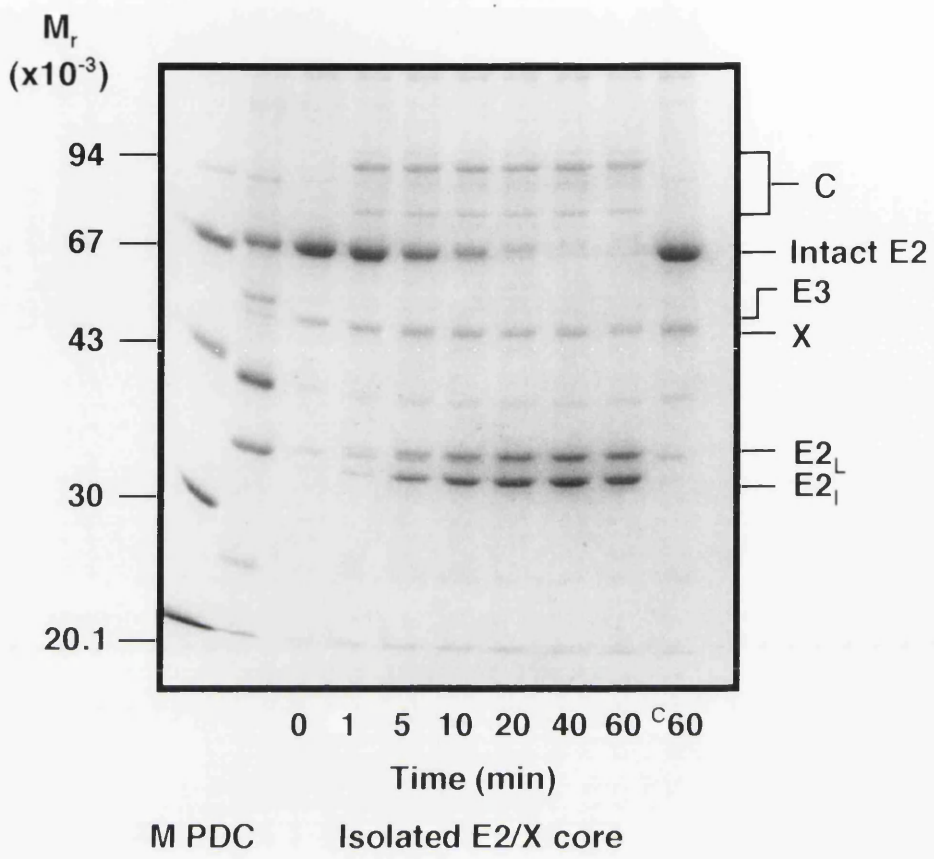
Figure 4.2.2.1.1 Effect of collagenase treatment of purified E2/X on its ability to reconstitute overall PDC activity

Freshly isolated E2/X core (lmg.ml^{-1}) diluted in 50mM Tris/HCl buffer, pH 7.5, containing 2mM $CaCl_2$, 3mM NAD^+, 0.5mM $MgCl_2$ and 0.2mM TPP was treated with 10% (w/v) collagenase (preincubated with 2mM $CaCl_2$ at 30°C for 10min), over 60min at 30°C, and at the intervals shown, aliquots (80µl) removed to 3mM EGTA at 4°C and 20µl portions preincubated at 30°C for 10min with E1/E3 in the presence of **Soln A** and **B** prior to measurement of reconstitution by the direct removal of aliquots (5µl) to pre-equilibrated assay mixtures. The remaining solution (60µl) was then removed to Laemmli sample buffer and used for subsequent SDS-PAGE analysis and antibody screening. A control, undigested sample was treated likewise.

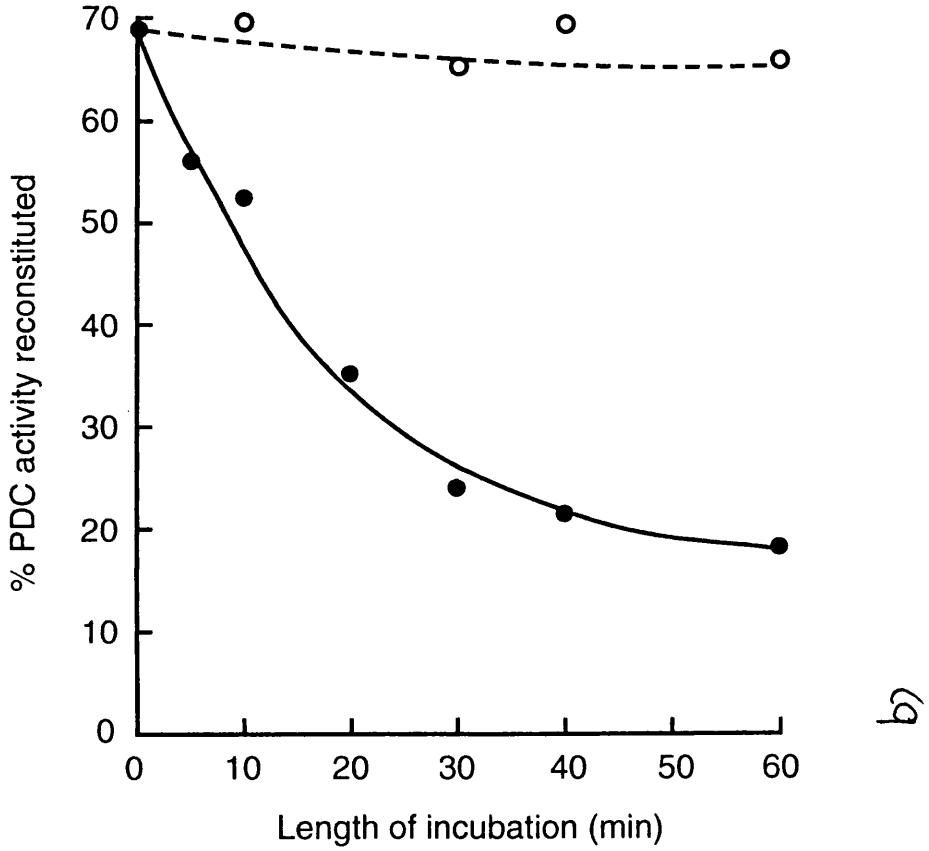
a) illustrates the successive cleavage with time of the lipoyl domain of E2 in isolated E2/X core following 10% (w/v) SDS-PAGE and Coomassie blue staining. (lane M, low M_r marker proteins; PDC, 10µg purified bovine heart PDC; E2/X, 5µg loadings where C_{60} represents control undigested samples; lane C, collagenase; $E2_L$ represents the released lipoyl and linker regions, and $E2_I$, the truncated subunit and catalytic domains).

b) illustrates the loss in reconstitution resulting from the successive cleavage of intact E2 over 60min. The % complex activity reconstituted was calculated as specific activity from duplicate results of <10% difference as described in section 2.2.10, where (•---•) represents 10% (w/v) collagenase treated reconstituted activity, and (o- -o) control, undigested reconstituted activity.

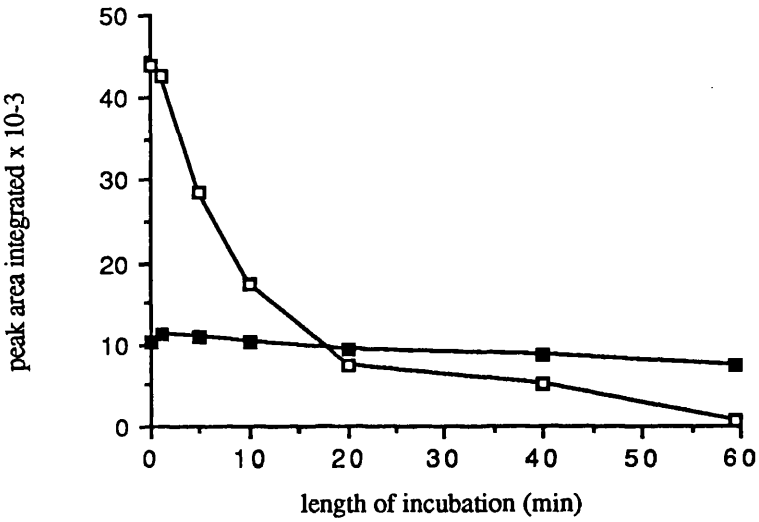
c) illustrates the results of densitometer scans at 550nm of intact E2 and X from Coomassie stained gels as described in section 2.2.4d, where (•- -•) represents intact E2, and (•---•) intact protein X peak areas (arbitrary units).


M_r
(x10^-3)
94
67
43
30
20.1
C
Intact E2
E3
X
E2_L
E2_I
0 1 5 10 20 40 60 C60
Time (min)
M PDC
Isolated E2/X core

a)


% PDC activity reconstituted
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
Length of incubation (min)

b)


peak area integrated x 10-3
50
40
30
20
10
0
0
10
20
30
40
50
60
length of incubation (min)

c)

that presented by Rahmatullah *et al.* (1990), whereby the release of E2 lipoyl domains initially exceeds that of complex activity loss (results not shown), suggestive of the active site coupling demonstrated in ox heart PDC by Stanley *et al.* (1981).

Through N-terminal amino acid sequencing and E1/E3 binding assays, Rahmatullah *et al.* (1990), identified the site of collagenase cleavage at the N-terminus of the E1 binding domain. Thus, the cleavage of intact E2 is revealed by the appearance of the released lipoyl and linker region and, the truncated inner catalytic and subunit binding domains of E2 on SDS-PAGE, represented by $E2_L$ (39000-M_r) and $E2_I$ (33000-M_r), respectively (see Fig. 4.2.2.1.1a). Extension of the incubation period by a further 30min ensured the complete removal of all E2 lipoyl domains, characterised by the presence of a basal level (i.e., 10-15%) of reconstituted PDC activity apparently, protein X lipoyl domain-mediated.

In close agreement, Lawson *et al.* (1991b) found gene deletion of the lipoyl, plus or minus, linker regions; or, substitution of the essential lipoic acid attachment site $lysine_{47}$, resulted in the retension of 12-15% complex activity -- deemed, in the presence of intact protein X and fully functional acetyltransferase activity, to represent servicing of the E2 active site by the lipoyl domains of protein X. In addition, while Rahmatullah *et al.* (1990) observed a residual 8% activity, this was clearly a reflection of the partial insolubility of the truncated E2/X, as addition of heparin resulted in both improved solubility and reconstitution (i.e., 12%). However, in the absence of relevant figures, this may also have resulted from minor protein X degradation. Of note, solubility problems were not encountered here under the different buffer conditions used.

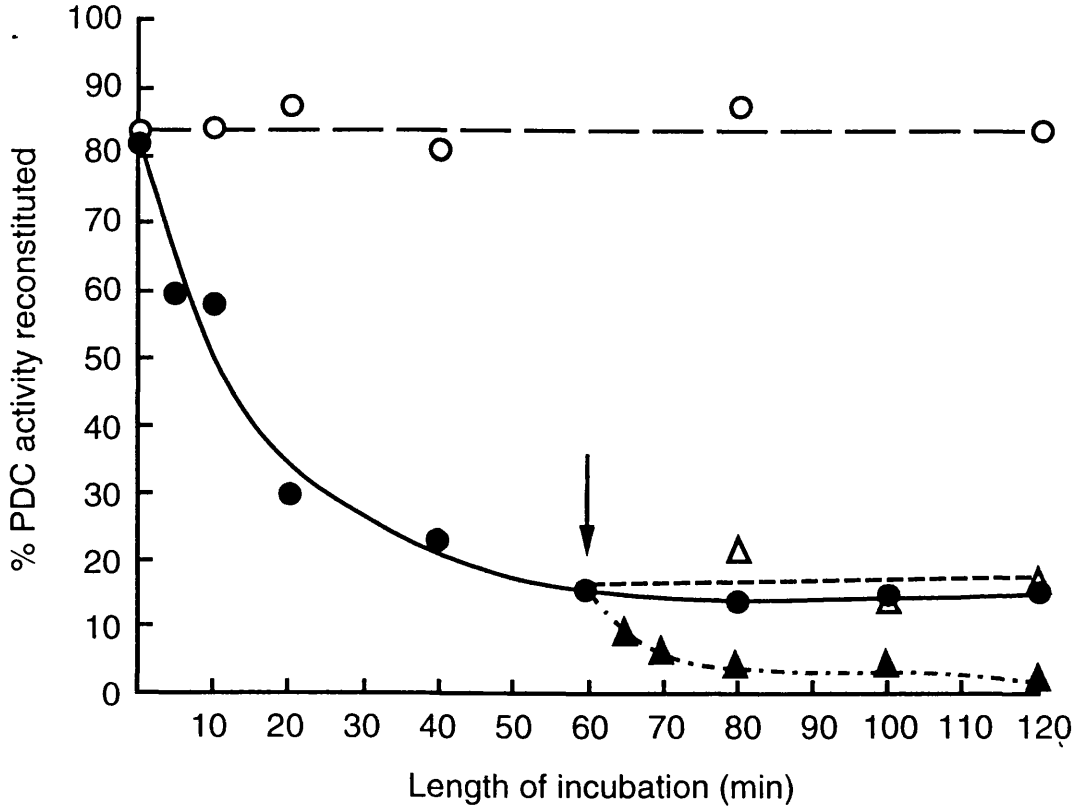
4.2.2.2 Analysis of reconstituted PDC activity during the sequential digestion of components E2 and X by collagenase and Arg C

In section 4.2.2.1, the optimal conditions for the complete removal of intact E2, by collagenase, were established whereby, following a 90min incubation with 10% (w/v) collagenase at 30°C, a residual apparently X-mediated 10-15% complex activity remained. This assumption was then verified by the analysis of component E2 and X degradation, and the resultant loss in reconstituted activity during the sequential digestion of isolated E2/X core employing 12% (w/v) collagenase, then 3% (w/v) Arg C.

As in Fig. 4.2.2.2.1, the characteristic decrease in reconstituted PDC activity (here to a residual 15%), was observed upon the digestion of more than 98% of intact E2 (as determined through densitometric scan analysis of Coomassie blue stained gels and immunoblots) over 60min. Equivalent analysis suggested only minor protein X degradation comparable to that of the control sample; however, following addition of 3% (w/v) Arg C, immunological screening and silver stain analysis of 10% (w/v) SDS-PAGE resolved samples (results also not included), revealed the rapid cleavage of the majority of protein X-derived lipoyl domains (i.e., within 20min), accompanied by an equally rapid decline in reconstituted activity to 3% -- apparently complete within 60min. Further cleavage of E2, by Arg C, was not observed. Notably, little difference was observed between the two control, non-Arg C treated samples, suggesting that extended treatment with collagenase results in insignificant protein X degradation (supported by immunological evidence). Taken together, these results provide further support for the presence of X-mediated PDC activity.

Figure 4.2.2.2.1 Analysis of reconstituted PDC activity during the successive treatment of isolated E2/X core with collagenase and Arg C

Isolated E2/X core (1mg.ml^{-1}) was treated with 12% (w/v) collagenase over 60min at 30°C, as described in Fig. 4.2.2.1.1, a control undigested sample included and subsequent analyses of components E2 and X carried out as listed therein. Where (•–•) represents % PDC activity reconstituted with 12% (w/v) collagenase treatment, and (o- -o) represents % PDC activity reconstituted with undigested core. After 60min, the collagenase treated sample was removed to ice, split into three equivalent samples and the following additions made: A: (•–•) no further addition; B: (Δ- -Δ) 3mM EGTA; C: (▲-- -▲) 3mM EGTA, 5mM $MgCl_2$, 3% (w/v) Arg C. All three were then placed at 30°C (indicated by arrow) and the % reconstitution (determined from duplicates of <10% difference and calculated as in section 2.2 10), and E2 and protein X degradation monitored as before.


% PDC activity reconstituted
100
90
80
70
60
50
40
30
20
10
0
10 20 30 40 50 60 70 80 90 100 110 120
Length of incubation (min)

4.2.2.3 Analysis of the involvement of components E2 and X, in intact or reconstituted complex activity, following treatment with proteases, collagenase and Arg C; determination of substrate/NADH induced incorporation of N-ethyl[2,3-^{14}C] maleimide

Following observations of an X lipoyl domain-mediated residual PDC activity from bovine sources both here and elsewhere (Rahmatullah *et al.*, 1990), and from *S. cerevisiae* (Lawson *et al.*, 1991b), analysis of the intimate component interactions in both protease treated and untreated, intact and reconstituted bovine heart PDC were carried out employing fluorographic visualisation of N-ethyl[2,3-^{14}C]maleimide ([2,3-^{14}C]NEM), incorporation in the presence of substrates -- pyruvate, acetyl CoA and NADH. Details of the five separate conditions used -- run simultaneously for both control and digested samples, are listed in section 2.2.14b.

Preliminary studies of [2,3-^{14}C]NEM incorporation, employing untreated intact complex at various concentrations, confirmed the findings of Hodgson *et al.* (1986), whereby component E2 and X incorporation was dependent upon the presence of pyruvate or NADH and, where prior incubation with acetyl CoA and NADH led to a time-dependent diminution in the extent of radiolabelling. As these results were not included this is best exemplified by the control sample of intact PDC in Fig. 4.2.2.3.3. Hence, whereas incubation with [2,3-^{14}C]NEM in the absence of all other substrates resulted in the prominent labelling of thiols of accessible cysteine moieties in E1 (E1β exhibited variable modification -- see Fig. 4.2.2.3.1; the reason for this is unclear); simultaneous incubation with pyruvate, led to the reductive acetylation of the S^{8}-thiols and [2,3-^{14}C]NEM modification of the adjacent, reduced S^{6}-thiols of the lipoyl domains of E2 and X and was therefore, indicative of normal

E1/E2 interactions. Upon NADH preincubation, greater modification by [2,3-^{14}C] NEM was observed, due to the availability of both reduced S^6,S^8-thiols. However, prior addition of acetyl CoA resulted in a dramatic diminution of radiolabel through competition, initially (i.e., 60s) for one thiol and, over longer preincubation (i.e., 60min) for both thiols, demonstrating both the S^6,S^8-diacetylation properties of E2 and protein X (believed to be unique to mammalian PDC -- Hodgson *et al.*, 1986), and normal E2/E3 interactions. Notably, minor modification of the active site thiols of E3 followed addition of [2,3-^{14}C]NEM in the presence of NADH, under which conditions, such thiols would be reduced.

Analysis and comparison of complex functioning and hence, component interactions, following the removal of the majority of the lipoyl domains of E2 in intact (Fig. 4.2.2.3.1), reconstituted (Fig. 4.2.2.3.2) and intact-column resolved complex (Fig. 4.2.2.3.3) were then carried out, as described in the next section.

a) Collagenase treated complex

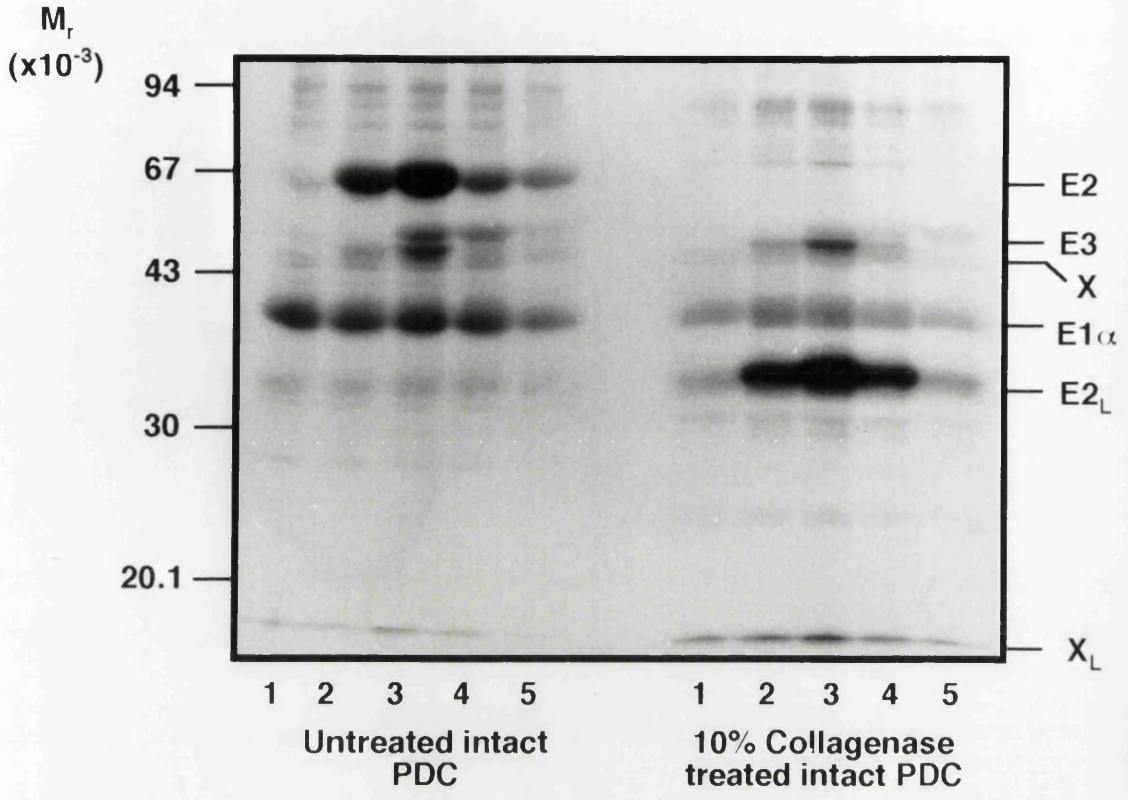
From Figs. 4.2.2.3.1-2 it is clear that the reduction in intact and reconstituted complex activity to approx. 20% of the t_0 activity was accompanied by the cleavage of the vast majority of intact E2 components (a trace of E2 can be seen in lanes 2 & 3). Notably, following the release of the lipoyl and linker regions (labelled in Fig. 4.2.2.3.1 as $E2_L$; and co-migrating with E1β in Fig. 4.2.2.3.2), intact protein X continued to exhibit pyruvate (lane 2) and, NADH (lane 3) dependent incorporation and, acetyl CoA-related diminution of radiolabel (lanes 4 & 5), in the collagenase treated complex, suggestive of functional interactions with E1 and E3. In addition, a similar pattern of [2,3-^{14}C]NEM modification was seen with

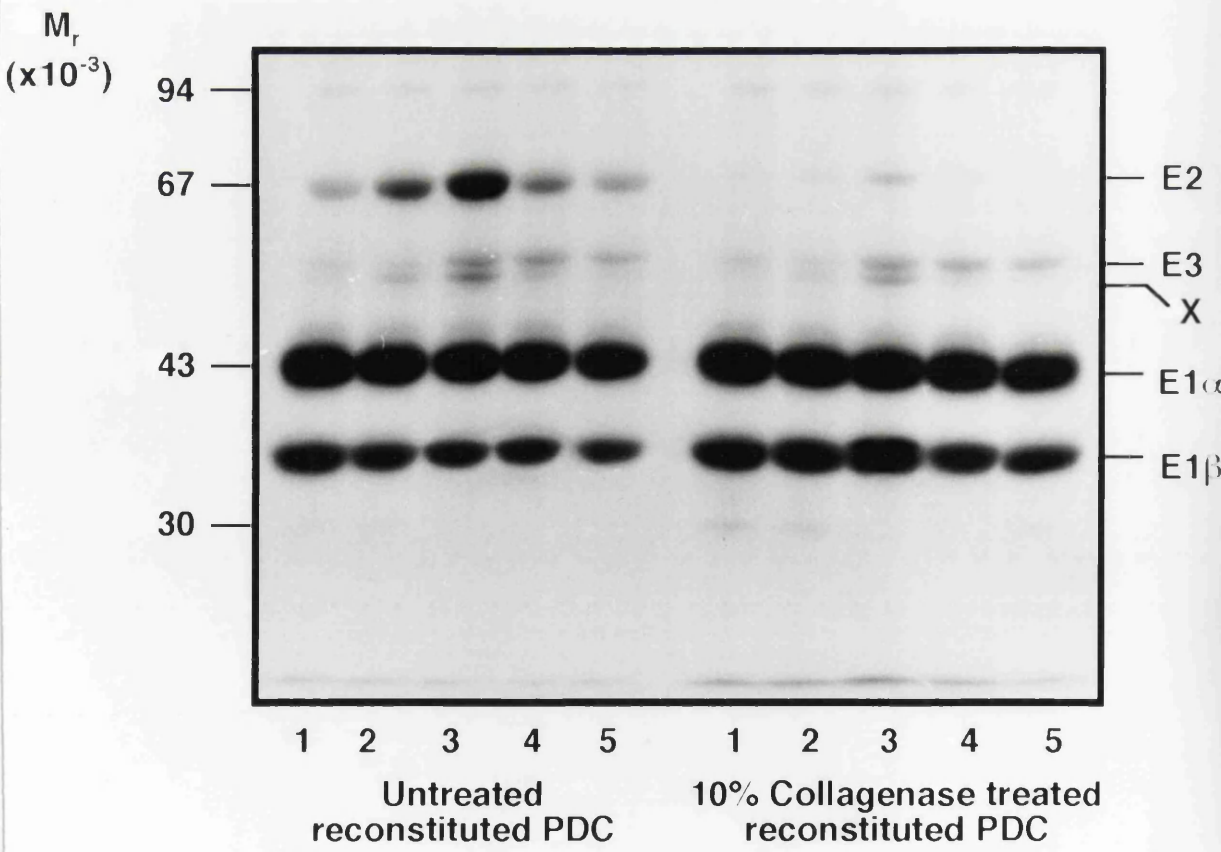
Figure 4.2.2.3.1 Fluorographic analysis of [2,3-^{14}C]NEM modification of collagenase treated and native bovine heart PDC

Sepharose CL-2B purified intact bovine heart PDC was diluted to 2mg.ml^{-1} and treated to a monitored 10% (w/v) collagenase digest at 30°C over 80min, as described in Fig. 4.2.2.1.1. Following addition of EGTA on ice, control and digested complex were found to be approx. 84% and 20% active, respectively, and both were then treated with [2,3-^{14}C]NEM as described in section 2.2.14b. Lane 1, 10min preincubation at room temperature with 0.85mM[2,3-^{14}C]NEM; lane 2, 0.85mM [2,3-^{14}C]NEM added prior to 4mM pyruvate and incubated for 10min; lane 3, 10min preincubation at room temperature with 0.2mM NADH, followed by incubation with 0.85mM [2,3-^{14}C]NEM for a further 10min; lane 4, 10min preincubation at room temperature with 0.2mM NADH, 1min with 0.4mM acetyl CoA, followed by incubation with 0.85mM [2,3-^{14}C] NEM for a further 10min; lane 5, as in lane 4, but with acetyl CoA incubation extended for 60min. Labelling was terminated by addition of 45mM 2-ME, the samples prepared in Laemmli sample buffer lacking DTT, and warmed to 70°C for 2min prior to resolution of 80μg samples by 10% (w/v) SDS-PAGE. The position of low M_r marker proteins is also shown.

Figure 4.2.2.3.2 Fluorographic analysis of [2,3-^{14}C]NEM modification of collagenase treated and control, reconstituted bovine heart PDC

Freshly isolated E2/X core and E1/E3 were analysed for optimal reconstitution prior to the monitored digestion of the former employing 10% (w/v) collagenase over 60min at 30°C, as described in Fig. 4.2.2.1.1. Control undigested sample was included, and both treated with 5mM EGTA on ice, prior to reconstitution with E1/E3 (to give approx. 1mg PDC), in the presence of **Soln A** and **B** (see section 2.2.10). Values for % reconstitution were 85% and 20% of the original t_0 value for control and digested PDC, respectively. [2,3-^{14}]NEM incorporation was then carried out as described in section 2.2.14b. A description of lane conditions is given in Fig. 4.2.2.3.1 but, 40μg PDC loaded per track.


M_r
(x10^-3)
94
67
43
30
20.1
E2
E3
X
E1α
E2_L
X_L
1 2 3 4 5
1 2 3 4 5
Untreated intact PDC
10% Collagenase treated intact PDC


M_r
(x10^-3)
94
67
43
30
E2
E3
X
E1α
E1β
1 2 3 4 5
1 2 3 4 5
Untreated reconstituted PDC
10% Collagenase treated reconstituted PDC

the released lipoyl and linker regions of E2 and X (resulting in the latter case, from low level collagenase degradation). The latter domain migrated with the gel front and also appeared to exhibit substrate-independent labelling (lane 1), suggestive of either additional modification of cysteine-derived thiols -- exposed through the action of collagenase or, of co-migrating low M_r species.

At this point it was unclear whether the characteristic labelling of the clipped lipoyl domains indicated a direct participation in PDC functioning or, indirect interactions with the active sites of the E1, E3 and truncated E2 components. Transformation of PDC-deficient mutant *E. coli* strains with two plasmids -- one encoding E2 lipoyl domain-depleted complexes, and the second, encoding discrete lipoyl domains, have demonstrated only low level complementation, suggestive of either the inability of untethered lipoyl domains to invade assembled complexes or, of only low affinity interactions between the two (Guest *et al.*, 1989). Indeed, following complete cleavage of intact E2 with 10% (w/v) collagenase (as seen by the disappearance of a Coomassie stained band and corresponding radiolabel at 72000-M_r), and removal of the clipped lipoyl domains by resolution on a Superose 6 column (see Fig. 4.2.2.3.3 legend for details), the residual complex activity and substrate-dependent [2,3-^{14}C]NEM incorporation observed clearly resulted from protein X-mediated complex activity. Furthermore, as the total yield and % recovery following column treatment, was identical for both control and treated complex, no additional decrease in complex activity appears to have occurred upon removal of the lipoyl domains of E2, in agreement with the findings of Rahmatullah *et al.* (1990) and Lawson *et al.* (1991b). Hence, whilst the released $E2_L$ fragments may interact and thereby participate in the partial reactions of the collagenase treated core and associated E1 and E3 components, this does not appear to contribute to the overall extent of complex activity. Although some loss in [2,3-^{14}C] NEM modification was apparent in the fluorograph shown here, equivalent labelling was repeatedly obtained

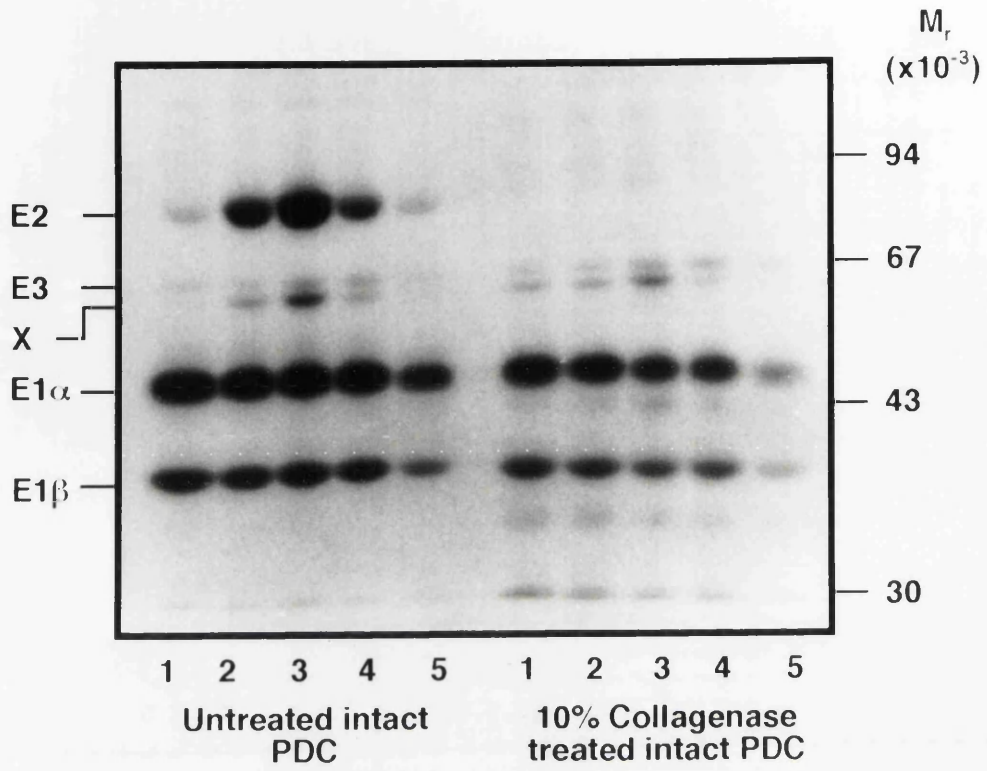


Figure 4.2.2.3.3 Fluorographic analysis of [2,3-^{14}C] NEM modification of collagenase treated and native bovine heart PDC subjected to gel filtration on Superose 6

Two 5mg samples of Sepharose CL-2B column purified bovine heart PDC were treated at 30°C over 90min -- one, in the presence of 10% (w/v) collagenase, as described in Fig. 4.2.2.1.1. Following addition of 5mM EGTA at 4°C, both samples were subjected to resolution on a 100x1cm Superose 6 column (flow rate 1ml.min^{-1}), by which,separation of the clipped lipoyl domains of E2 was achieved (as determined through A_{280}, E3 and PDC activity elution profiles). Column buffer contained 0.1M NaCl, and peak fractions corresponding to the resolved complexes were pooled for subsequent [2,3-^{14}] NEM incorporation as described in section 2.2.14b. A description of lane conditions is given in Fig. 4.2.2.3.1 but 50μg of PDC loaded per track.

for control and collagenase treated complex (results not shown). In addition, an increase in substrate-independent [2,3-^{14}C]NEM modification of all components, especially E3, was noted both here and with reconstituted complex (see Figs. 4.2.2.3.1 & 4.2.2.3.3), and was presumably the result of mild dissociation and exposure of active site thiols following high salt and column treatment.

Thus in conclusion, the results presented here provide strong support for the proposed servicing of the active site of truncated E2 by the lipoyl domains of protein X and therefore, the ability of this component to maintain complex activity at a residual level. In addition, normal protein X diacetylation is demonstrated in the absence of intact E2, indicating independence from the lipoyl domains of E2 in this latter process.

b) Arg C treated complex

In Chapter 3, it was observed that, following removal of the lipoyl and linker regions of protein X, loss in reconstituted PDC activity resulted from the proposed disruption to the exposed E3 binding domain of truncated protein X and, the accompanying reduction in E3 binding potential. Furthermore, as partial recovery of complex activity in the presence of excess porcine E3 was found to increase with increasing protein X degradation, this was thought to reflect enhancement of a low affinity binding of E3 to the subunit binding domain of E2 or of X.

Thus, in light of the possible involvement of E2 in E3 binding, and of the proposed role of protein X in the downstream channelling of reducing equivalents from E2 to E3 (Rahmatullah *et al.*, 1990), it was of interest to observe [2,3-^{14}C]

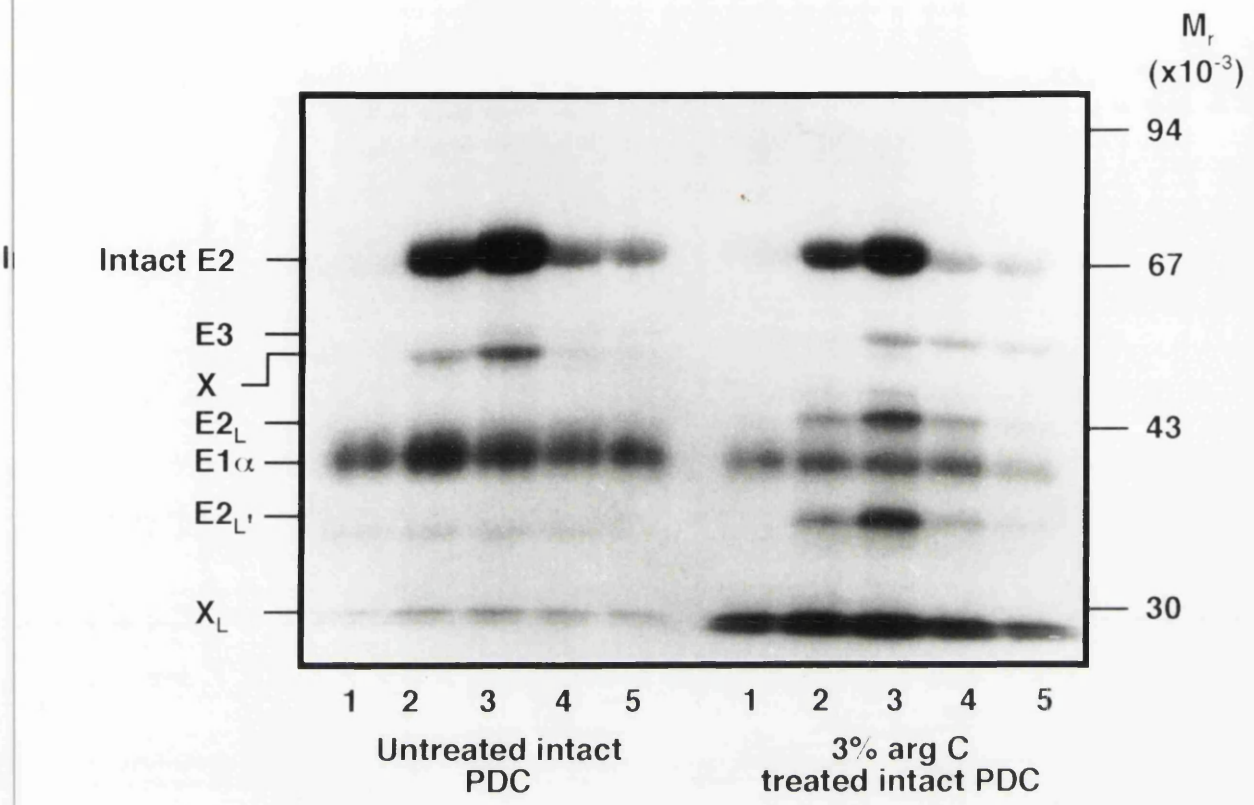


Figure 4.2.2.3.4 Fluorographic analysis of [2,3-^{14}C] NEM modification of Arg C treated and native bovine heart PDC

Freshly prepared bovine heart PDC was diluted in **Soln A** and treated to 6% (w/v) Arg C, as two 3% (w/v) additions at 0 and 60min, over 120min at 30°C. Activity was assayed, and protein X degradation monitored by immunological screening, until the majority of intact protein X had been cleaved and 25% of PDC activity remained. Control PDC, incubated for the equivalent time, exhibited 85% activity. To both, 5mM benzamidine was added, at 4°C, prior to [2,3-^{14}] NEM incorporation, as listed in 2.2.14b. A description of lane conditions is given in Fig. 4.2.2.3.1 and 80μg of PDC loaded per track.

NEM incorporation of E2, in both intact and reconstituted complex, following the extensive and complete removal, respectively, of the lipoyl and linker regions of protein X by Arg C.

In agreement with Chapter 3, the discrete degradation of protein X in intact complex by Arg C, was not possible in the absence of minor E2 proteolysis and Fig. 4.2.2.3.4, illustrates the results of an incubation over 120min at 33°C with 3+3% (w/v) Arg C. Here, cleavage of the majority (i.e., 85%) of protein X, as determined by immunological analysis and, from the low level of [2,3-^{14}C]NEM incorporation at the 50000-M_r position in Fig. 4.2.2.3.4, was accompanied by an approx. 75% loss in activity (in agreement with the findings of section 3.4.2) and, the apparent low level release of the 46000-M_r fragment comprising the lipoyl, linker and E1 binding domain of E2 (designated $E2_{BL}$ or, $E2_L$ in Fig. 4.2.2.3.4). These were subsequently degraded to an 39000-M_r lipoyl and linker fragment ($E2_L$ or, $E2_{L'}$ in Fig. 4.2.2.3.4), in agreement with Rahmatullah *et al.* (1989a, 1990). These latter lipoyl-containing species, together with the released lipoyl and linker regions of protein X, exhibited pyruvate (lane 2) and NADH (lane 3) induced [2,3-^{14}C]NEM incorporation and, an acetyl CoA-linked reduction in [2,3-^{14}C]NEM modification (lanes 4 & 5) identical to that observed in section 4.2.2.3a yet, presumably in the absence of the majority of bound E3. Once again, evidence of substrate-independent [2,3-^{14}C]NEM modification is apparent with the released lipoyl domains of protein X (compare lane 1 from Figs. 4.2.2.3.1-4).

In consideration of the results obtained in section 4.2.2.3a, it seems unlikely that the released lipoyl domains of either component E2 or X participate directly in overall PDC activity. However, whilst a small number of protein X molecules remained intact, it was unclear whether the residual complex activity also resulted from the low affinity binding of E3 to the subunit binding domain of E2 and hence,

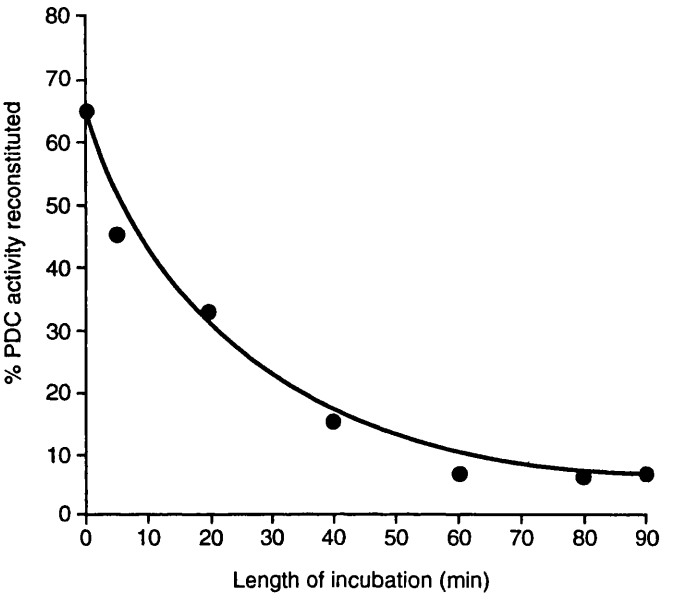
Figure 4.2.2.3.5 Effect of Arg C treatment of purified E2/X core on its ability to reconstitute overall PDC activity

E2/X core, diluted to 1ml in **Soln A** ($0.2mg.ml^{-1}$), was incubated at 33°C with 3% (w/v) Arg C over 90min, and at the times shown, aliquots (50µl) removed to 5mM benzamidine on ice prior to a 10min preincubation at 33°C with E1/E3 at a ratio by weight of 1:0.8 (E2/X:E1/E3). The % reconstitution was then determined by direct assay at 340nm (see sections 2.2.10-11). The remaining E2/X core was then diluted in Laemmli sample buffer for subsequent resolution on 10% (w/v) SDS-poylacrylamide gels, and immunological analysis with anti-X, E2 and E1α specific sera.

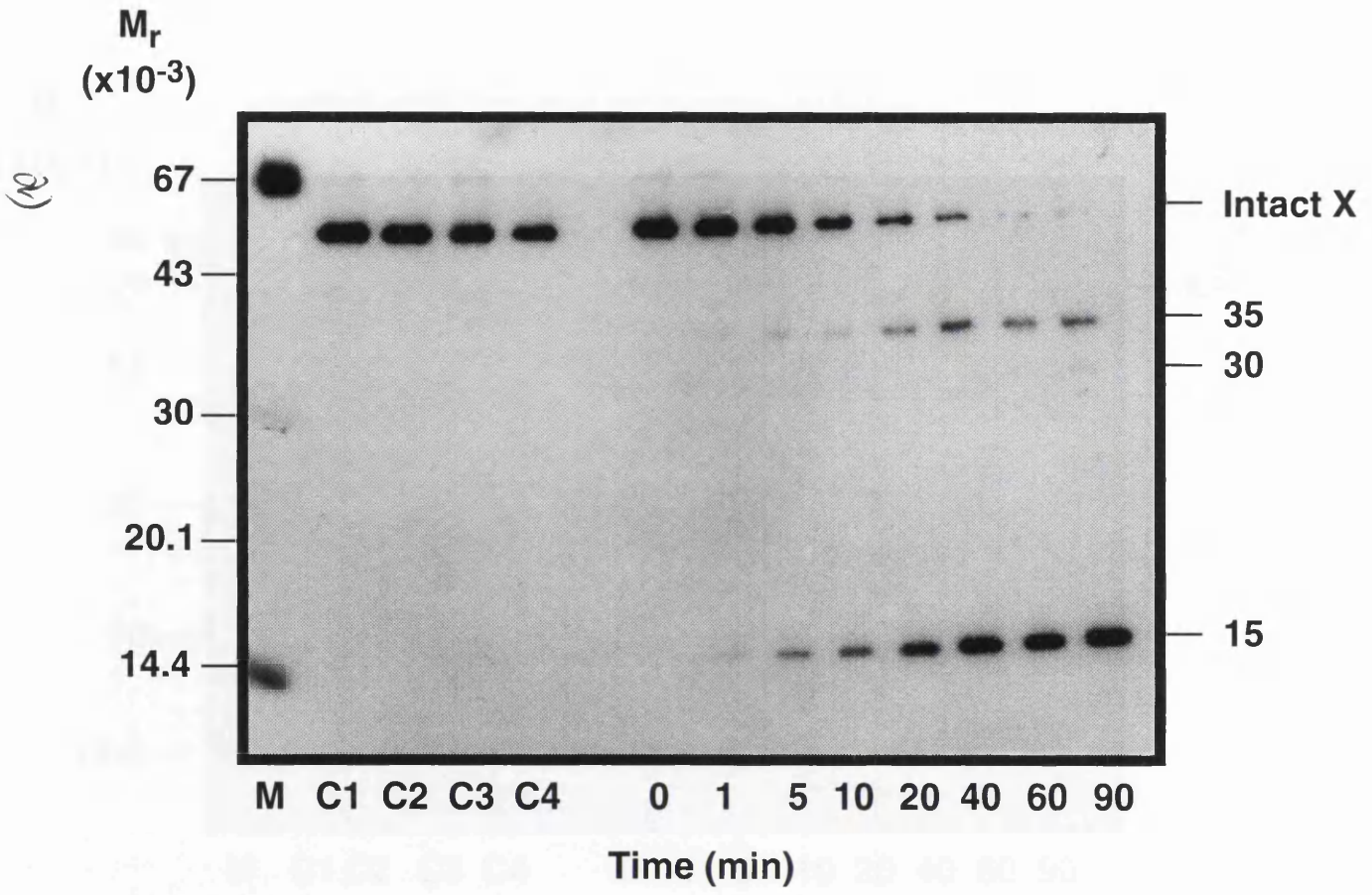
a) illustrates the loss in reconstituted PDC activity, with time, following the progressive cleavage of protein X by Arg C.

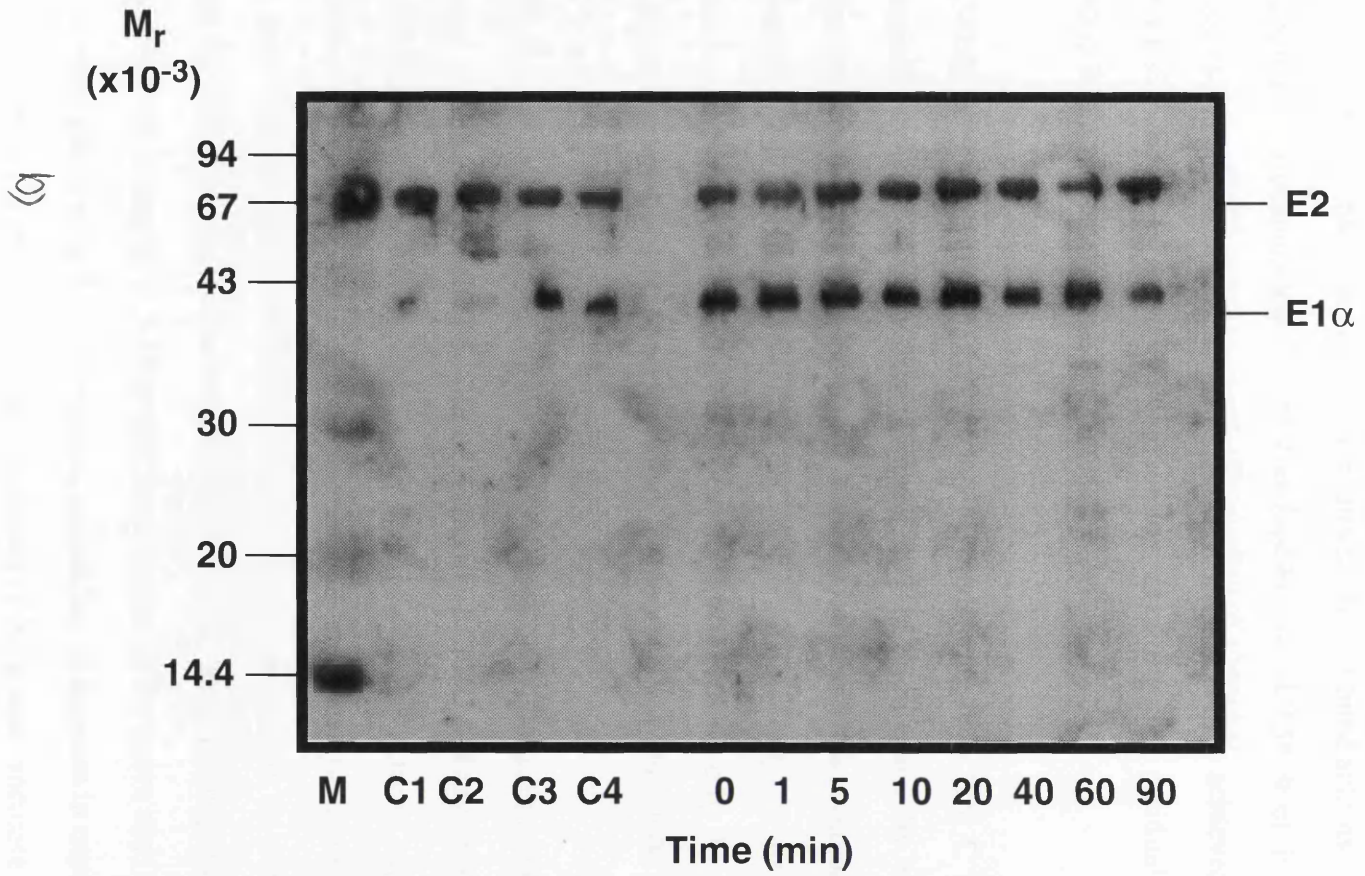
b) illustrates immunological analysis of the E2/X core with anti-serum specific to protein X, during incubation with Arg C. The cleavage of intact X (50000-M_r), to a 35000-M_r subunit binding and inner core domain (X_{IB}), the released 15000-M_r lipoyl and linker regions (X_L), and subsequently, to a 30000-M_r inner core domain (X_I), is shown. Lane M, low M_r marker proteins; lanes C1 & C2, duplicate control E2/X core samples incubated for 90min at 33°C in the absence of Arg C; lanes C3 & C4, duplicate control E2/X core samples incubated for 90min at 33°C in the absence of Arg C, and stored on ice for 60min.

c) illustrates an exposure from an immunoblot of the E2/X core with anti-serum specific to E2, overlaid with anti-serum specific to E1α. Lanes are defined as in 4.2.2.3.5b.


% PDC activity reconstituted
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
Length of incubation (min)

a)


M_r
(x10^{-3})
67
43
30
20.1
14.4
Intact X
35
30
15
M C1 C2 C3 C4 0 1 5 10 20 40 60 90
Time (min)


M_r
(x10^{-3})
94
67
43
30
20
14.4
E2
E1α
M C1 C2 C3 C4 0 1 5 10 20 40 60 90
Time (min)

the downstream channelling of reducing equivalents by E2 to E3. To answer this question, complete Arg C degradation of intact protein X was carried out in isolated E2/X core, employing incubation with 3% (w/v) Arg C at 33°C over 90min during which, loss in complex activity was monitored, following reconstitution with E1/E3 at a subunit stoichiometric ratio of 1:0.8 (E2/X:E1/E3) -- see section 2.2.10 and Chapter 3. Fig. 4.2.2.3.5a illustrates a typical plot of reconstituted activity loss, and from accompanying immunological analyses (see Fig. 4.2.2.3.5b & c), it is clear that the complete release of the lipoyl domains of protein X was achieved in the absence of significant E1α and E2 degradation, resulting in a residual 6% of (apparently) E2 lipoyl domain-mediated complex activity.

Subsequent analysis of [2,3-^{14}C]NEM modification in Arg C treated, reconstituted complex, following loss of approx. 90% of original activity, revealed an identical pattern of substrate-dependent E2 incorporation and diacetylation to that of untreated complex (see Fig. 4.2.2.3.6). Once again, complete protein X degradation, in the absence of similar E2 degradation, was evidenced by the absence of radiolabelled species at a M_r of 50000 (intact X), 46000 ($E2_{BL}$) or 39000 ($E2_L$).

Hence, assuming no involvement of the released lipoyl domains of protein X in complex activity, the residual 6-10% of reconstituted PDC activity observed in Figs. 4.2.2.3.5-6 would seem to reflect a low level of complex activity apparently independent of protein X lipoyl domain interactions and, presumably dependent upon a small number of E3 molecules, bound with low affinity to either, the truncated E3 binding domain of protein X or, the intact subunit binding domain of E2. Notably, little change in [2,3-^{14}C]NEM modification of the active site (di)thiols of E3 was visible following Arg C treatment, suggesting no increase in exposure of these residues upon removal of the lipoyl domains of X (a small increase in [2,3-^{14}C]NEM labelling was observed in Fig. 4.2.2.3.4 with Arg C treated intact

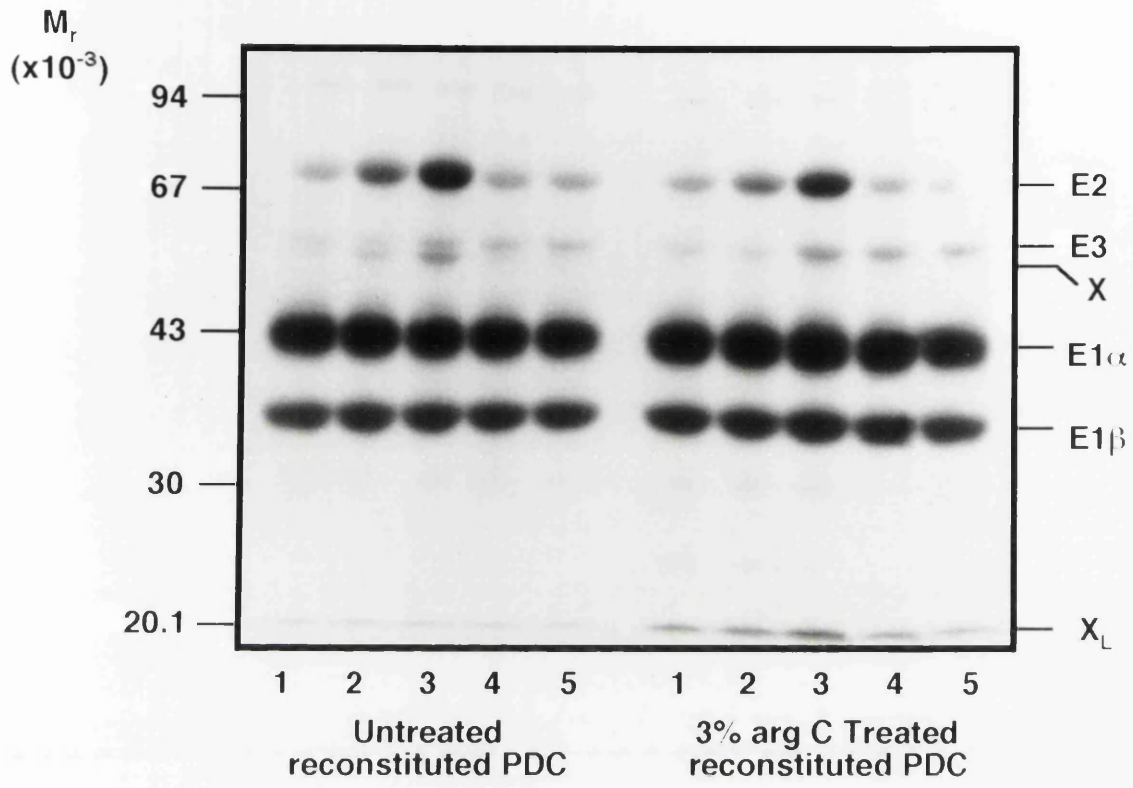


Figure 4.2.2.3.6 Fluorographic analysis of [2,3-^{14}C]NEM modification of Arg C treated and control, reconstituted bovine heart PDC

Identical samples of E2/X and E1/E3 prepared for Fig. 4.2.2.3.2 were used here whereby, complete removal of intact protein X was achieved with a 3% (w/v) Arg C digestion of E2/X core in **Soln A** over 90min at 33°C, until 10% reconstituted activity remained. Control and digested samples were then treated with 5mM benzamidine at 4°C, and 1mg of complex reconstituted from core and E1/E3 fractions in the presence of **Soln A** and **B** at an E2/X:E1/E3 ratio of 1:0.8 (w/w) (see section 2.2.10), prior to [2,3-^{14}C]NEM treatment as described in 2.2.14b. A description of lane conditions is given in Fig. 4.2.2.3.1, but 40μg of PDC loaded per track.

complex). As previously discussed in Chapter 3, support for the presence of a residual E2-mediated complex activity is provided by the findings of two independent clinical investigations of chronic lactic acidemia sufferers where, abnormally low levels of PDC activity were observed in the absence of immunologically detectable protein X (Robinson *et al.*, 1990; Marsac *et al.*, 1993).

4.3 DISCUSSION

Although the research presented in this chapter does not offer a definitive answer to the question of the existence of an acetyltransferase active site in mammalian protein X, support is provided for previous observations of a residual protein X-mediated complex activity in the absence of intact E2 from both mammalian (Rahmatullah *et al.*, 1990), and yeast (Lawson *et al.*, 1991b) PDC. Furthermore, confirmation and extension of the collagenase studies on reconstituted bovine kidney PDC of Rahmatullah *et al.* (1990), to include examination of component interactions, through [2,3-^{14}C]NEM labelling in both intact and reconstituted complex, revealed the ability of the lipoyl domains of protein X to undergo diacetylation in the absence of the equivalent domain on E2.

In addition, following complete cleavage of the lipoyl domains of protein X in isolated E2/X core, by Arg C, and subsequent reconstitution with E1/E3, similar [2,3-^{14}C]NEM modification studies indicated the presence of an exclusively E2 lipoyl domain-mediated residual (i.e., 6%) complex activity and, normal E2 diacetylation. Hence, the ability of the lipoyl domains of either component to promote residual PDC activity in the absence of the other, was suggestive of a non-essential but rate-limiting involvement for both in overall complex catalysis. This is supported by the discovery of residual PDC activity (i.e., 10-20%), in the absence of immunologically detectable levels of protein X, in chronic lactic acidemia sufferers by Robinson *et al.* (1990), and Marsac *et al.* (1993). Further evidence is also provided by the gene deletion studies of Lawson *et al.* (1991a,b) where a residual 15% and 66% complex activity were measured for $E2_L^-$ and X_L^- mutant *S. cerevisiae* cell lines, respectively. Loss of PDC activity in the latter case was thought, in Chapter 3, to partly result from a small yet significant reduction in E3

binding not mentioned by the authors, but nevertheless notable from the evidence presented. However, some contribution could result from reduced catalytic efficiency. Furthermore, the disparity in the extent of restriction upon the overall rate of catalysis was reflected in the relative levels of PDC activity remaining. Clearly, if a direct result of the reduced number and/or (possibly) efficiency of the protein X-derived lipoyl domains in the servicing of the acetyltransferase active site of E2, in contrast to the non-limiting active-site coupling exhibited by the E2 components, why has such activity remained intact in the face of evolutionary pressures? Hence, the question arises of whether the intrinsic potential of the lipoyl domains of protein X to undergo the various (de-, trans-) acetylation reactions of E2 could be a vestigial function, only expressed in the absence of competition from the equivalent domains of E2 or, a reflection of a low level participation in both up and downstream catalysis?

Examples of such apparently superfluous activity exist elsewhere. Indeed, at first glance, the very nature of the lipoyl domains -- varying in number across species and complex type and exhibiting active site coupling, suggests an intrinsic redundancy. For example, the apparently non-essential nature of two of the three lipoyl domains of E2 from *E. coli* PDC must confer some functional advantage in an organism so prone to rapid evolutionary change, where the potential for recombination/deletion in the tandemly repeated *lip* gene segments would be high (Graham *et al.*, 1986). Whether the "excess" lipoyl domains function to enhance the conformational flexibility of the one catalytically involved lipoyl as proposed, is as yet unclear, and still leaves unresolved the essential question. Similarly, recent research now points to a specific kinase binding function (leading to enhanced kinase function), for one of the two lipoic acid moieties attached to the lipoyl domains of component E2 in mammalian PDC, although whether this is in addition to normal lipoyl functioning remains to be seen (Radke *et al.*, 1993).

It is possible that for protein X, the answer lies in the unique diacetylation properties of mammalian PDC whereby, during conditions of excessive lipid oxidation such as with mild starvation or diabetes, control of the [NADH]:[NAD^+] ratio could be facilitated by the conversion of free acetyl CoA molecules to lipoic acid-bound acetyl groups. This would be accompanied by the reoxidation of NADH to NAD^+ and hence, inhibitory levels of both acetyl CoA and NADH would be removed from the cytosol. Thus, the ability of both protein X and E2 to undergo diacetylation increases the buffering potential of PDC from a theoretical 132 to 264 acetyl groups per PDC molecule. Following stabilisation of the [NADH]:[NAD^+] ratio to a level favouring PDC activity, these acetyl molecules would then be channelled to the TCA cycle and metabolised to generate energy. In the absence of any published evidence supporting the existence of an acetyltransferase active site sequence in protein X, it seems likely that such diacetylation and subsequent deacetylation would occur via transacetylation with E2.

Notably, as published evidence of acetylation studies in *S. cerevisiae* PDC are not available, proof for catalytically active lipoic acid moieties on protein X does not exist, which together, with the presence of a single lipoyl domain on E2 and, the apparent absence of diacetylation in PDC from this species, may point to the additional utilisation of acetyl CoA molecules in the glyoxylate pathway. Futhermore, whilst the lipoyl-like sequence at the N-terminus of E1 from bovine heart OGDC retains the ability to bind E3, in the absence of a catalytic requirement for this segment, no evidence for acetylation nor a lipoic acid attachment site exists (Rice *et al.*, 1992).

Owing to problems with insolubility, attempts to isolate and sequence the C-terminal portion of protein X, and thereby confirm the findings of J.C. Neagle of an active site-like "Glu His Xaa Xaa Xaa Ser Gly" sequence within a partial cDNA

clone (encoding the inner catalytic domain of human protein X and, also found to exhibit 70% homology to the equivalent region of the protein X gene from *S. cerevisiae*), remained in the initial stages. However, amino acid sequence analysis of the major tryptically-derived fragments of E2 and X, purified by anhydrotrypsin resin and subsequent HPLC analysis (B. Dunbar, SERC Protein Sequencing Facility, Aberdeen University); and, re-examination of the nucleotide sequence of the cDNA clone (J.E Rice, University of Glasgow), are currently underway and may provide further insight into the proposed catalytic role of protein X.

Following inference of the active site consensus sequence of chloramphenicol acetyltransferases by comparison to that of other acetyltransferases (Guest, 1987), and the proposed catalytic role of His_{602} and His_{375} of E2 from PDC and OGDC of *E. coli*, respectively (Spencer *et al.*, 1984); amino acid alignments have revealed the characteristic "His Xaa Xaa Xaa Asp Gly" motif in aminoglycoside acetyltransferases and, all dihydrolipoamide acetyltransferases sequenced to date. Furthermore, although substitution of the consensus site histidine residue in E2 from *E. coli* PDC (Russell & Guest, 1990), and in E2 from bovine BCOADC (Griffin & Chuang, 1990) resulted in loss of complex activity -- apparently supporting the proposed catalytic involvement in the nucleophilic attack of CoA on S^8-acyldihydrolipoamide; substitution of His_{427} within the truncated catalytic domain of E2 from *S. cerevisiae* PDC, whilst overexpressed in *E. coli*, had no affect on overall activity. In contrast, substitution of Asp_{431} (equivalent to Asp_{199} from chloramphenicol acetyltransferases and believed to be involved in structural stability) to asparagine, alanine or glutamate resulted in a 16, 24 or approx. 4-fold reduction in complex activity, respectively, which was proposed as evidence for involvement of Asp_{431} in catalysis (Niu *et al.*, 1990).

Whilst this apparent difference in the catalytic importance of the putative active site residues in yeast is disputed by J.R. Guest (University of Sheffield, personal communications), who points to incomplete substitution of the His_{427} and, interference from the imidazole buffers used, as possible solutions, it also questions the functional plausibility of an active site sequence for mammalian protein X in which the highly conserved aspartate is replaced by serine. Examination of the nucleotide composition of all the degenerate codons for either residue, reveals that this non-conservative substitution from an acidic to a neutral yet hydrophilic molecule, requires the alteration of the first two determining base pairs in any triplet code. When viewed in light of the dramatic 16-fold decrease in PDC activity following a similar substitution of Asp_{431} in E2 from *S. cerevisiae* PDC to the neutral amide derivative, asparagine, such findings do not offer much support for the presence of dihydrolipoamide acetyltransferase activity in protein X.

However, an explanation could lie in the mistaken inversion, during the deduction of the nucleotide sequence from sequencing gels, of the first two base pairs of the aspartate-encoding triplet (GAU/C), which resulted in one of the following serine codons (AGU/C). Alternatively, the presence of a serine residue may be indicative of a catalytic role distinct to that of E2 -- the nature of which remains unclear.

RESULTS CHAPTER 5:

Stoichiometry and organisation of the protein X subunit of bovine heart PDC

5.0 INTRODUCTION

Since the identification and characterisation of component X as a distinct polypeptide, related to the E2 component (De Marcucci *et al.*, 1985), knowledge of its multi-domain structure has progressed rapidly, derived from deacetylation/ acetylation studies in combination with various proteolytic and N-terminal amino acid sequencing investigations (De Marcucci & Lindsay, 1985; De Marcucci *et al.*, 1986; Hodgson *et al.*, 1986; Jilka *et al.*, 1986; Neagle *et al.*, 1989). In contrast, relatively little information has arisen concerning the subunit stoichiometry and detailed structural organisation of protein X within the E2 core, primarily owing to problems encountered in the isolation of intact native subunit, free of contaminating dihydrolipoamide acetyltransferase.

De Marcucci & Lindsay (1985), noted that the dissociation of bovine heart PDC into the constituent components, E1, E2 and E3, following treatment with 0.25M $MgCl_2$ (or lM NaCl at pH 9.5), and separation by high pressure gel permeation on a TSK G3000 SW column (or employing Sepharose CL-6B), was not accompanied by dissociation of protein X from the E2 core. Furthermore, subsequent treatment with the chaotrophic reagent, p-hydroxymercuriphenyl sulphonate, failed to remove protein X whilst causing the dissociation of the tightly-bound intrinsic PDC kinase. Indeed, as a result of this tight association, and consequent copurification, initial reports of the presence of an additional species of 50000-M_r in preparations of porcine liver PDC, bovine heart PDC and core, and *S. cerevisiae* E2 core assemblies of PDC, misidentified protein X as a proteolytic fragment of E2 or, the core-bound kinase (Stanley & Perham, 1980; Bleile *et al.*, 1981; Rahmatullah *et al.*, 1984, 1985; Kresze *et al.*, 1980a, 1981).

To date, the isolation of protein X has only been possible under denaturing conditions such as: 6-8M urea; sodium dodecyl sulphate-PAGE (De Marcucci & Lindsay, 1985); or, by reverse phase HPLC employing a trifluoroacetic acid/acetonitrile gradient as detailed initially by Jilka *et al.* (1986).

In the quantification of protein X, Jilka *et al.* (1986) employed the above isolation techniques in the construction of a calibration curve relating various amounts of SDS-PAGE resolved bovine kidney PDC and protein X isolates, to the resultant absorbance of the silver stained bands, as determined through densitometric gel scanning. An estimate of 5mol protein X/mol bovine kidney PDC was calculated, and (apparently) substantiated by the determination, through E2 and X acylation in isolated core or complex samples employing [1-^{14}C]acetyl CoA, [1-^{14}C] propionyl CoA, [1-^{14}C]butyryl CoA and [2-^{14}C]pyruvate, of an average acylation ratio of 10:1 (E2:X).

As isotopic dilution analysis indicated the presence of a single lipoyl moiety in mammalian E2 (Cate & Roche, 1978; White *et al.*, 1980), a ratio of 10:1 (E2:X) pointed to 6 molecules of protein X per molecule of complex. In contrast, a similar yet less detailed study of ^{14}C-acetylation in bovine heart PDC, employing [2-^{14}C] pyruvate, noted that protein X incorporated 14-18% of that bound by E2 which, in line with the estimate of one lipoyl domain per E2 chain, suggested 8-12 molecules of protein X per PDC molecule (De Marcucci & Lindsay, 1985). However, subsequent sequencing of a cDNA clone of human PDC E2 by Thekkumkara *et al.* (1988), confirmed the existence of two lipoyl domains per E2 molecule, proposed by Hodgson *et al.* (1988), through covalent crosslinking studies of the lipoyl domains of E2 in bovine heart PDC. Hence, assuming equal rates and extent of ^{14}C incorporation, a ratio of 1:10 and a percentage incorporation of 14-18% would indicate 12 and, 16-24 molecules of X, respectively.

The question then arose, of how these protein X molecules would arrange within the E2 core whilst maintaining icosahedral (532) symmetry. In other words, is protein X an essential, integral core component, substituting for E2, and bound during assembly or, an external component bound after assembly is complete.

Initial investigations of Hodgson *et al.* (1988), involving careful molecular weight analysis and immunological screening of SDS-PAGE resolved samples of substrate-induced N,N' 1,2-phenylene-dimaleimide (PDM) crosslinking of the lipoyl domains of E2 and X in bovine heart PDC, revealed the presence of X/X and E2/X dimers and an E2/E2/X trimer, pointing to close interactions between the lipoyl domains of both adjacent protein X and E2 molecules. However, the binding of radiolabelled protein X-specific antiserum to native PDC discounted the possibility of an entirely internalised protein X and indicated a peripherally extended and therefore, accessible region, such as the lipoyl domain (Hodgson *et al.*, 1988).

In agreement, the isolation of active E2 core samples in the absence of protein X, suggests protein X is not an integral constituent of the dodecahedral core structure. For example, through the overexpression of the inner core domain of *S. cerevisiae* dihydrolipoamide acetyltransferase in *E. coli*, Niu *et al.* (1990), demonstrated self-assembly of a truncated yet active E2 core of apparent wild type core (532) symmetry, as determined by negative stain and cryoelectron microscopy (Stoops *et al.*, 1992). In addition, in bovine heart PDC, Powers-Greenwood *et al.* (1989), employed denaturing conditions to remove protein X from an active E2 core sample.

Further support for a partially accessible protein X, not involved in core assembly, came from the gene deletion experiments of Lawson *et al.* (1991a) where disruption of the protein X gene (PDX1), in *S. cerevisiae* resulted in a fully

functional E2 core. Furthermore, transformation of these mutant cells with a single or multi-copy plasmid containing the PDX1 gene, resulted in fully active complexes with equivalent protein X incorporation, suggestive of a limited number of binding sites. Indeed, in support of Jilka *et al.* (1986), differences in the sedimentation coefficients of the wild type and protein X-deficient PDH complexes were consistent with the absence of six E3 dimers and six protein X molecules. However, only partial reconstitution of complex activity (i.e., 20% of wild type), was achieved employing PDX1 null mutant E2/E1 core, recombinant yeast E3 and a crude isolate of protein X produced by the overexpression of the PDX1 gene in mutant *S. cerevisiae* cells. Clearly such poor recovery could be a reflection of either a core integrated protein X or, simply limitations in the concentration of this species within the crude extract.

In apparent opposition, Li *et al.* (1992), argued that the inability to facilitate the additional binding and/or exchange, of free protein X molecules for bound molecules, in various E2 core preparations of bovine kidney PDC, indicated a tight, irreversible integration during core assembly -- in direct contrast to kinase, which exhibited reversible binding. In addition, Li *et al.* (1992), proposed that the partial reconstitution of *S. cerevisiae* PDC upon incubation of the mutant E2/E1 core with E3 and X (Lawson *et al.*, 1991a), could result from partial core disassembly, followed by protein X binding and reassembly and therefore, did not exclude the possibility of an integrated protein X. Equally however, the results of Li *et al.* (1992), could simply reflect: partial inactivation of both the E2 and X components in the regions involved in binding; traces of mercurial reagents in the protein X preparation; or, inadequate reconstitution conditions such as, component concentration, and the temperature and length of preincubation.

Incidental information obtained from the recent sizing of frozen hydrated bovine heart and kidney PDC and E2 core (to 50-60nm and 20nm, respectively) by cryoelectron microscopy (Wagenknecht *et al.*, 1991), and quaisielastic light scattering (Roche *et al.*, 1993), indicated a uniform core distribution of protein X and, in conjunction with an equivalent study in *E. coli* (Wagenknecht *et al.*, 1992), revealed variable positioning of the E3 component. In light of the confirmed E3 binding function of protein X (see Chapter 3), and the postulated flexible tethering of E3 to the E2/X core (Wagenknecht *et al.*, 1991), these results apparently dispute the face-centred model of E3 binding proposed by Reed & Hackert (1990), and further complicates the elucidation of the organisation of protein X within the core assembly. In this regard, future cryoelectron microscopic images of E2/X cores containing bound antibody specific for the inner domain of protein X provide a promising approach to further advances in the understanding of E2/X core organisation (Wagenknecht *et al.*, 1991).

The work presented in this chapter represents a comprehensive investigation into the quantification and organisation of protein X from bovine heart PDC. Quantification involved the application of similar ^{14}C-acetylation experiments as those outlined previously by Jilka *et al.* (1986) and De Marcucci & Lindsay (1985). Furthermore, in the absence of published data concerning the quantification by densitometric scan analysis of silver stained SDS-PAGE resolved protein X and PDC samples by the former group, a more detailed analysis is described herein. Structural studies employed NADH-induced PDM crosslinking of protein X lipoyl domains in E2 lipoyl domain-depleted complex, following selective proteolysis by collagenase; and further proteolytic studies using ArgC.

5.1 METHODS

5.1.1 Isolation of a purified protein X polypeptide by electroelution following resolution of E2/X core by SDS-PAGE

The first step in the quantification of protein X from bovine heart PDC involved the isolation of a highly purified preparation from a sample of E2/X, previously isolated by the routine dissociation of Sepharose CL-2B column-purified PDC using 2M NaCl at pH 9, followed by resolution by gel filtration (see sections 2.2.8-9 for details). Analytical SDS-PAGE (7-8% (w/v) gels) were employed in the dissociation of the E2/X sample and gave maximum separation of protein X from traces of E3. In addition, the standard 20-well stacking gel was replaced to give one large (500µl) capacity well for loadings of 1mg, with an additional marker protein track.

The resolved protein bands were visualised by a non-fixative $CuCl_2$ stain, as transparent regions within an opaque blue background. The band corresponding to protein X was then carefully excised by scalpel, sacrificing yield for ensured purity, fragmented to 0.5cm^2 and destained in 0.25M Tris/HCl buffer pH 9, containing 0.25M EDTA. Following re-equilibration in 25mM Tris/192mM glycine/0.025% (w/v) SDS, the gel pieces were then transferred to a Biotrap apparatus, immersed in elution buffer, and the negatively charged proteins electroeluted into a minitrap compartment (positioned at the anode) over 16-18h at 100V, 4°C (see section 2.2.6 for details).

Sample purity was analysed by Coomassie blue stained 8% (w/v) SDS-polyacrylamide gels, and the protein concentration determined in quadruplicate by both the micro BCA and modified Lowry protein assays (see section 2.2.1), following dialysis into a compatible 50mM sodium phosphate buffer, pH 7, containing 0.5% (w/v) Triton X-100. As care was taken during excision of the protein bands to ensure purity, recoveries were typically low (i.e., 40-50%). Several unsuccessful attempts to establish a more accurate protein concentration through the precise determination of amino acid content (B. Dunbar, SERC Protein Sequencing Facility, Aberdeen University), were precluded by insolubility resulting from the necessary exclusion of various buffers and detergents. Samples were stored at -20°C in the presence of 0.5% (v/v) Triton X-100 to maintain solubility.

5.2 RESULTS

5.2.1 Quantification of the subunit stoichiometry of protein X in bovine heart PDC

5.2.1.1 Quantification of protein X by densitometric scan analysis of SDS-PAGE resolved, silver stained X standards and comparison with the equivalent band in highly purified PDC

To determine the exact number of protein X molecules per PDH complex, calibration curves were constructed in which the absorbances of various standard protein X samples were related to the relative amounts of protein loaded (typically, 0.05-1μg). From this, and the absorbances of the equivalent bands in various known duplicate loadings of purified PDC, the approximate quantities of protein X, in μg, were extrapolated. These were then expressed as a % of the total complex protein (in μg), and compared to those predicted, from the M_r values, for the presence of 6-12mol protein X/mol PDC.

Preliminary studies employing 10% (w/v) SDS-PAGE resolved Coomassie blue stained PDC (10-40μg) and (0.5-3μg) protein X revealed the need for increased resolution of the E3 and X components, in combination with reduced loadings, and an alternative more sensitive stain. Henceforth, 2.5-10μg of PDC and 0.05-1μg of protein X, were routinely resolved by 7-8% (w/v) SDS-PAGE and stained with the modified silver stain method of Wray *et al.* (1981).

Figs. 5.2.1.1.1-3 demonstrate one of five separate calibration curves (each comprising several scans), which were constructed from four independent PDC and two protein X preparations -- the results of which are summarised in Fig. 5.2.1.1.4.

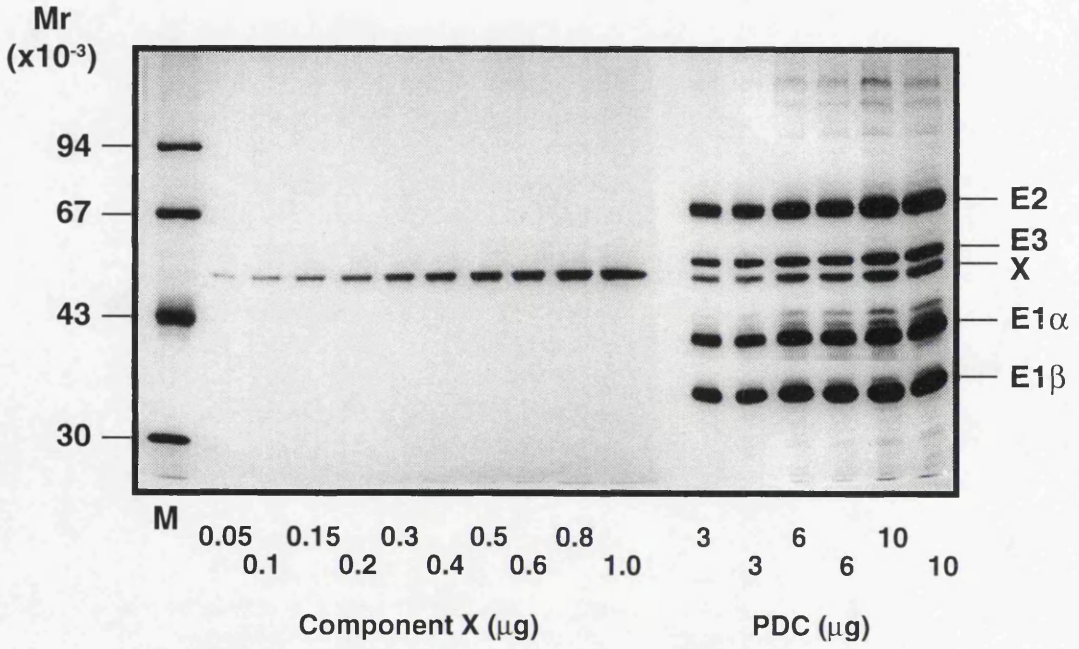
Fig. 5.2.1.1.1 clearly illustrates both the successful isolation of highly purified protein X and PDC (minor contamination by high M_r species was observed with the 10μg loading), and the improved component staining and resolution achieved with silver stained 7-8% (w/v) SDS-PAGE gels -- exemplified by the clear visualisation of the 46000-M_r and 43000-M_r subunits of PDC kinase (i.e., above E1α). High contrast between background and band staining resulted in two densitometer scans (for convenience only one is shown -- see Fig. 5.2.1.1.2), of high peak resolution and little base-line drift. From each, a plot was constructed relating integrated peak area to the amount of protein X loaded in μg (see Fig. 5.2.1.1.3). In agreement with Jilka *et al.* (1986), non-linear relationships were consistently obtained, indicating a degree of saturation in the band staining intensities. However, assuming equivalent rates of saturation for protein X in the isolated and complex samples, and by employing several samples of protein X and PDC, extrapolation of the results from the full length of each calibration curve would account for inaccuracies associated with decreased sensitivity.

Close duplicate protein X peak absorbances, differing by less than 5%, were obtained for the various loadings of purified complex. Direct extrapolation of five peak absorbances from both calibration curves resulted in an averaged estimate of 13.7±0.8mol protein X/mol PDH complex. Similar values were obtained from four separate calibration curves which are summarised in Fig. 5.2.1.1.4. In direct contrast to the findings of Jilka *et al.* (1986), of 4.8-5.3mol protein X/mol bovine kidney PDC, the evidence presented here strongly suggests the presence of approx. 12 molecules of protein X per molecule of bovine heart PDC. Since protein X is

Figure 5.2.1.1.1 Analysis of purified protein X and bovine heart PDC by SDS-PAGE

Standard protein X (0.2mg.ml^{-1}), and duplicate PDC (19.5mg.ml^{-1}), preparations were diluted in Laemmli sample buffer to 0.02mg.ml^{-1} and 0.5mg.ml^{-1}, respectively. Following brief treatment at 100°C in sealed Eppendorfs (to reduce evaporation), 2.4-47.6µl samples of protein X and, 6-18µl of the PDC duplicates were loaded on 8% (w/v) SDS-polyacrylaminde gels to give the protein amounts shown. Electrophoresis was carried out at 40mA to prevent curvature of peripheral bands, and once complete, the gel soaked in 50% (v/v) methanol for 24h prior to silver staining (see section 2.2.4).

8% (w/v) SDS analysis of purified component X and PDC



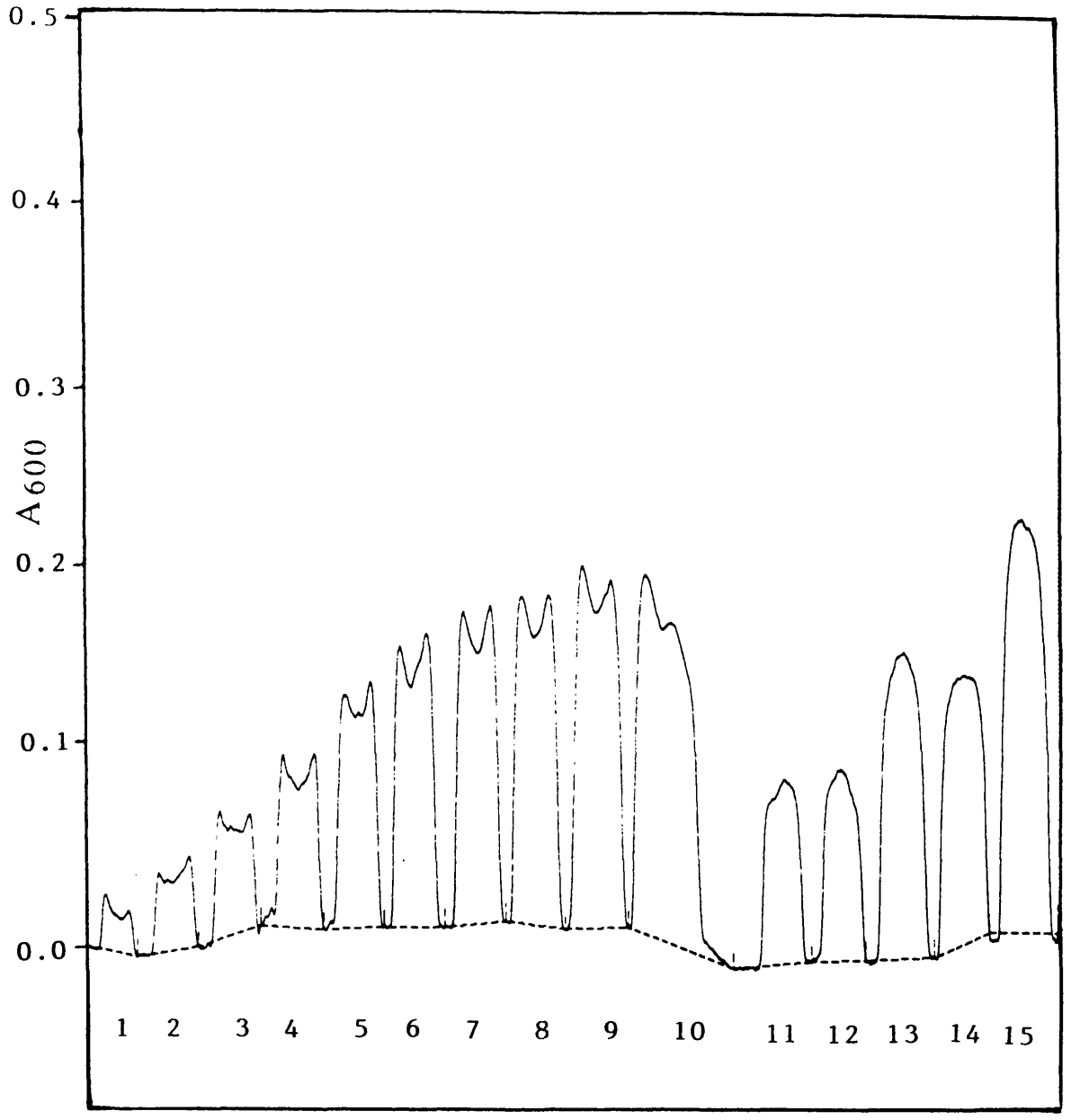


Figure 5.2.1.1.2 Densitometric scan analysis of SDS-PAGE resolved, silver stained protein X bands shown in Fig. 5.2.1.1.1

Densitometric scanning of the silver stained gel described in Fig. 5.2.1.1.1, employed a Shimadzu FDU-3 dual wavelength flying spot gel scanner in transmission mode at 600nm which was shown to give minimum background, and maximum band absorbance (variation in the wavelength used resulted from variable background staining). The standard parameters for silver stained gels listed in the user manual were adhered to except where: pK = 125; drift line = 0-0.5; smoothing = off. Gels were scanned several times with slight alteration in beam mode (linear or zigzag), and size to obtain both optimum resolution and band coverage. Accordingly, one of two scans, using a linear beam of 0.5 x 5mm, is shown here. Peaks 1-10, represent the protein X calibration curve of 0.05-1μg; peaks 11-15, represent duplicate loadings of 3, 6 & 10μg.

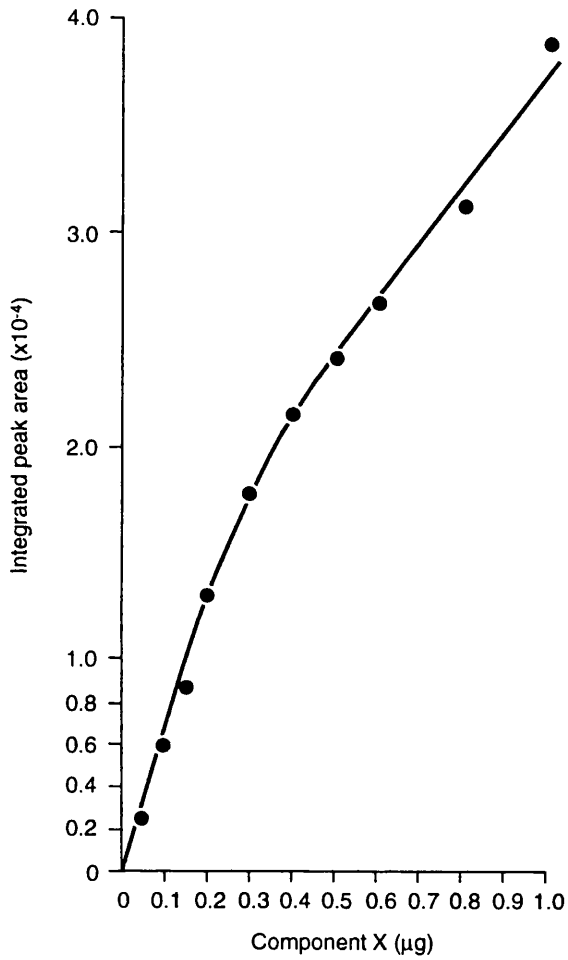


Figure 5.2.1.1.3 Correlation between band absorbance at 600nm and the amount of protein X

The integrated peak area (in arbitrary units), calculated for each band absorbance, was plotted against the relevant amount of protein X resolved, in μg, and used as a standard calibration curve in the determination of μg of protein per μg of complex and hence, calculation of the number of protein X subunits per complex.

PDC (μg)	Protein X (μg)	Nº of scans	Nº of protein X subunits / complex ± error.
5.0, 10.0	0.05-1.0	4	12.8 ± 0.5 : 1
20.0, 30.0	0.10-2.5	2	13.0 ± 2.4 : 1 (12.8 ± 2.3 : 1)
2.5, 5.0, 7.5	0.05-1.0	2	13.8 ± 2.0 : 1 (13.9 ± 1.3 : 1)
2.5, 5.0, 7.5	0.05-1.0	2	12.0 ± 0.9 : 1
3.0, 6.0, 10.0	0.05-1.0	2	13.7 ± 0.8 : 1

Figure 5.2.1.1.4 Estimation of protein X subunit stoichiometry from five independent determinations

Data represent the results of five independent evaluations employing four PDC and two protein X preparations. Duplicate quantities of PDC analysed (μg): the range of the calibration curves employed (μg): the number of scans taken for averaging and, the moles of protein X per mole of complex calculated, are shown. Bracketed values represent additional information determined from individual scans of component bands (see Fig. 5.2.1.1.5).

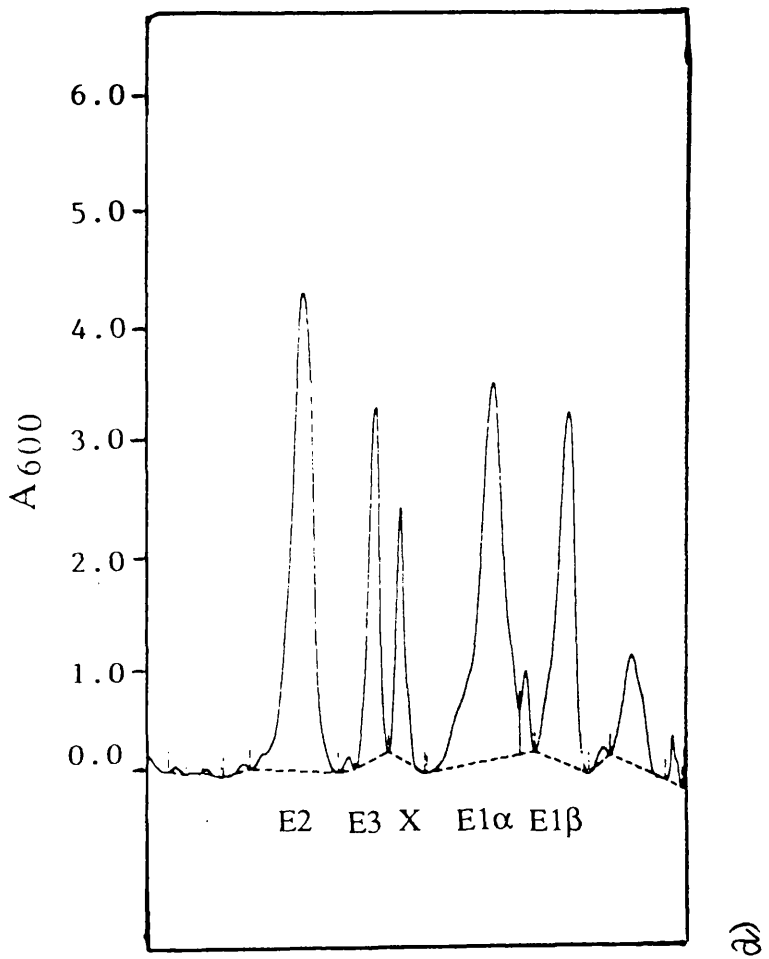
known to be directly involved in the binding of the six homodimers of E3 to the E2/X core in mammalian and yeast PDC (Rahmatullah *et al.*, 1989b; Lawson *et al.*, 1991a), it seems unlikely that such disparity would result from differences in bovine tissue. Equally, if correct, the different methods of protein concentration determination employed, are unlikely to result in a two fold disparity (the procedure of Fried *et al.*, 1985, was used by Jilka *et al.*, 1986). As with all three protein assays, significant deviation from the BSA calibration curve through variable lysine (Fried), or tyrosine and tryptophan (Lowry/BCA -- latter also, cysteine and cystine) content of the sample protein remain possibilities and therefore, potential sources of error. However, in light of the scant information provided by Jilka *et al.* (1986), beyond that several silver stained aliquots of protein X (i.e., a range of 7-28ng), and PDC (i.e., 30.6-612ng) were analysed by densitometer scans, and in the absence of any accompanying illustrative figures indicating amongst many important factors, sample purity, further discussion of the observed discrepancy is difficult.

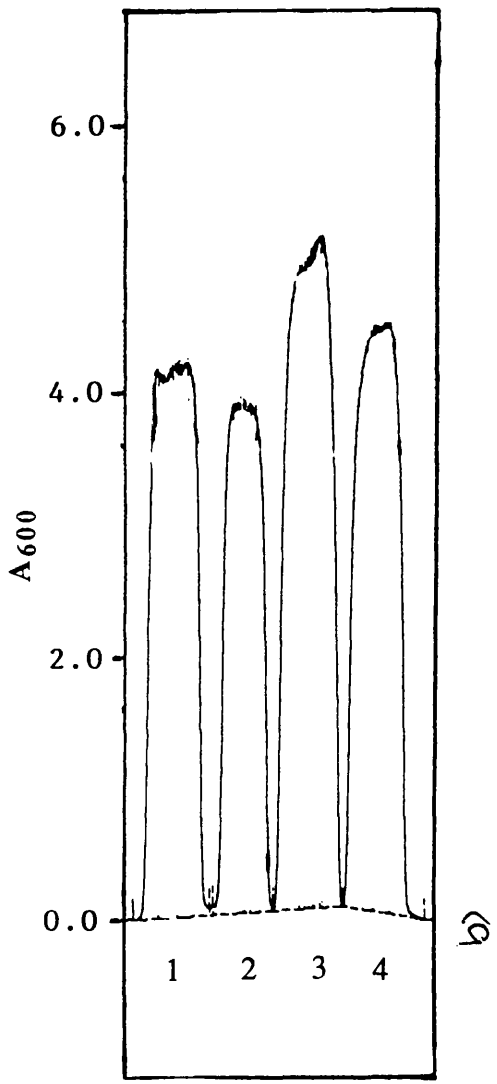
Fig. 5.2.1.1.5 illustrates additional scans, from a different calibration curve to that shown, carried out to assess variations in band staining intensity between individual components within and/or across duplicate PDC loadings. Independent scans of each component from duplicate 5 and 7.5μg loadings of PDC differed by approx. 10% indicating good reproducibility in loading and staining. Similarly, a constant peak ratio of 1 : 0.4 : 0.2 : 0.6 : 0.9 (E2 : E3 : X : Elα : E1β), was obtained in individual scans of duplicate 5 and 7.5μg PDC loadings which, assuming consistency in staining and, taking into consideration component molecular mass and stoichiometry, corresponds well with the staining profile of bovine heart PDC of 1 : 0.21 : 0.19 : 0.83 : 0.71 (E2 : E3 : X : E1α : E1β), with the exception of E3 (and E1β), which show disproportionately heavy staining. Although rough estimates of the number of protein X molecules per molecule of PDC (derived from the direct expression of integrated peak area for protein X, as a % of the total peak area of a

Figure 5.2.1.1.5 Assessment of reproducibility in loading and staining of protein bands

a) One of four control scans of duplicate loadings of 5 & 7.5μg of PDC showing all 5 component absorbances which yielded a peak ratio of 1:0.4:0.2:0.6:0.9 (E2:E3:X:E1α:E1β).

b) Control scan of the E2 band from duplicate loadings of 5μg (lanes 1&2), and 7.5μg (lanes 3&4), of PDC. Equivalent scans of E3, X, E1α, E1β were also carried out (results not shown).


6.0
5.0
4.0
3.0
2.0
1.0
0.0
A600
E2 E3 X E1α E1β
a)


6.0
4.0
2.0
0.0
A600
1 2 3 4
b)

given PDC sample), correspond closely to that determined from the calibration curves (Fig. 5.2.1.1.4 -- bracketed figures), the enhanced E3 and E1β silver staining exemplifies the potential for inaccuracy in such a direct determination.

The 10-20% error quoted in Fig. 5.2.1.1.4 suggests a range of 11-15 protein X molecules/PDH complex and apparently reflects cumulative error originating from inaccuracies in protein determination, sample dilution, pipetting, graph construction. However, subsequent statistical analysis employing a Minitab programme and the additive linear model, gave a mean value of 13.4±0.4, with a 95% probability of the true number of protein X molecules lying within two standard errors (ie., ± 0.8), of this mean (Tom Aitchison, Dept. of Statistics, University of Glasgow). The higher estimate of 13.4mol X/mol PDC may be due to variable occupancy of E1 and E3 binding sites in individual PDC preparations resulting from minor component dissociation, and leading to errors in M_r calculations. An internal control, involving quantification of the E2 component, was carried out to further assess the degree of inaccuracy involved.

5.2.1.2 Quantification of E2 by densitometric scan analysis of SDS-PAGE resolved, silver stained E2 and PDC samples -- an internal control

To verify the accuracy of the above method in the quantification of component X from bovine heart PDC, calibration curves of Biotrap-purified E2 were also constructed, and the stoichiometric ratio of E2 in PDC extrapolated, from the absorbances of the equivalent bands in silver-stained samples of highly purified complex. Knowledge of the stoichiometry, structure and organisation of the E2 core of mammalian PDC was initially gained through : the negative stain electron microscopic images of Oliver & Reed (1968, 1982), Junger & Reinauer (1972); molecular mass determination (Bleile *et al.*, 1981; reviewed by Perham, 1991); and, later verified by the cryoelectron microscopic and quasielastic light scattering studies of bovine heart and kidney PDC (Wagenknecht *et al.*, 1991; Roche *et al.*, 1993), together with additional information from equivalent electron microscopic analyses of *S. cerevisiae* PDC by Niu *et al.* (1990), and Stoops & Reed (1992). These studies indicated a core of 60 E2 components possibly arranged as cone-shaped trimers along the twenty vertices of a pentagonal dodecahedron of icosahedral (532) symmetry. Thus, comparison of the results obtained here with the predicted stoichiometry of 60 E2 molecules/PDH complex would reveal the degree of accuracy associated with this method of quantification.

Several E2 calibration curves were constructed employing identical component isolation, complex purification, silver staining and scanning procedures as detailed for protein X. Figs. 5.2.1.2.1-2 illustrate one of 5 separate scans at 450nm, and the resultant calibration curve from a silver stained gel of 0.2-2μg E2 and 1.5, 2.5 and 5μg of PDC (gel not shown). As before, good peak resolution,

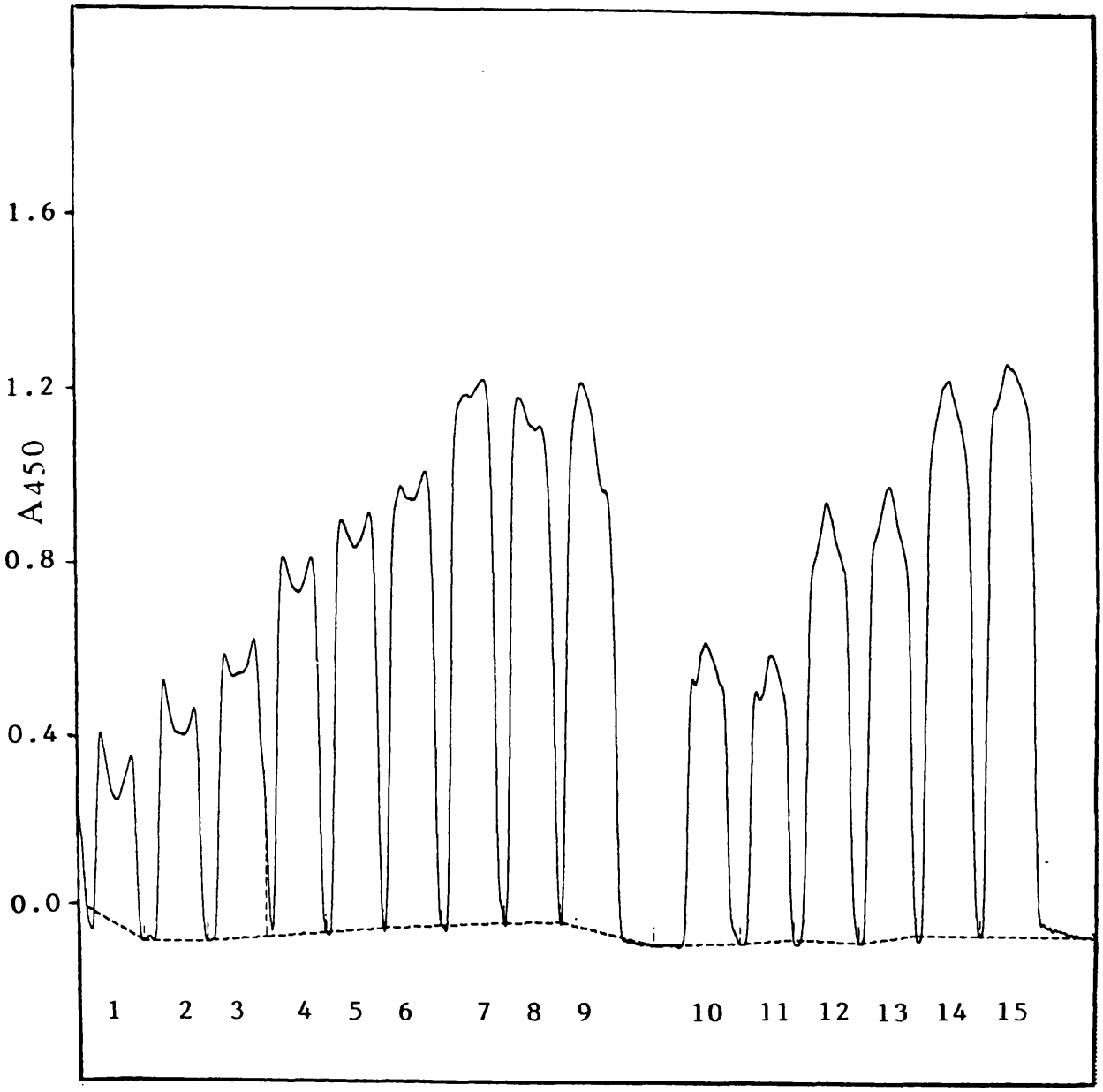


Figure 5.2.1.2.1 Densitometric scan of SDS-PAGE resolved, silver stained E2 and complex samples

Densitometric scanning of the E2 component from isolated and complex samples resolved by 10% (w/v) SDS-PAGE and silver stained as described in 2.2.4, employed a Shimadzu FDU-3 gel scanner at 450nm. Standard conditions, as outlined in the user manual were employed with the exceptions listed in Fig.5.2.1.1.2 and, with a linear 0.4 x 5 mm light beam.

Peaks 1-9, represent the E2 calibration curve of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.6, 1.8, and 2.0μg derived from loadings of 5-50μl of a 1 in 5 diluted E2 sample ($0.2mg.ml^{-1}$). Peaks 10-15 represent duplicate loadings of 1.5, 2.5 & 5μg purified PDC ($0.5mg.ml^{-1}$).

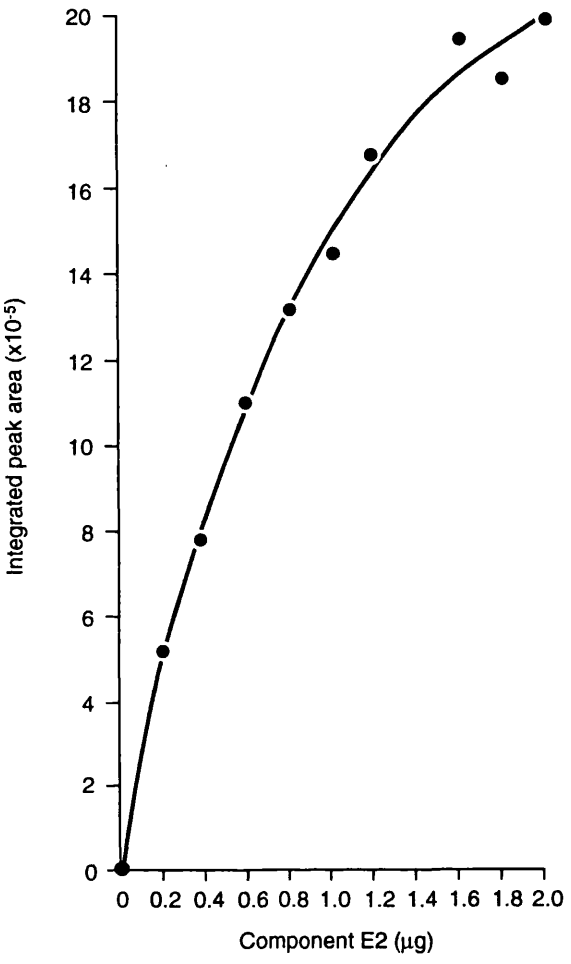


Figure 5.2.1.2.2 Correlation between E2 band absorbance at 450nm and the amount of E2

The integrated peak area (in arbitrary units) calculated for each band, was plotted against the relevant amount of protein resolved (in µg), and used to construct a standard curve for the quantification of the number of E2 subunits per PDC by the direct extrapolation of the amount of E2 present in each PDC sample (in µg), from the relevant E2 band absorbances.

with low base line drift and close duplicate values were obtained and an estimated 61.5±9.8mol E2/mol PDC determined. Two additional calibration curves, constructed from different E2 and PDC preparations, gave values of 53.6±5.4 and 69.8±4.2mol E2 /mol PDC (results not shown), which together, suggest a range of 48-74 E2 molecules (i.e., 60±20% error). Such scatter was originally thought to result from poor resolution of the heavier and therefore, more diffuse band staining which accompanied a 5-fold increase in resolved protein in samples of 2.5-10μg PDC (compared to protein X). However, calibration curves constructed with reduced 0.1-1μg and 0.5-2μg loadings of E2 and PDC, respectively, also exhibited the same degree of inaccuracy. Statistical analysis of this variance (Tom Aitchison, Dept. of Statistics, University of Glasgow), using the Minitab programme and the additive linear model employed in section 5.2.1.1, obtained a mean value of 61.8±2.5mol E2/mol PDC, with a 95% probability of the true number of E2 molecules lying within two standard errors of 61.8 (i.e., ± 5.0). This result is in close agreement with the proposed 60mer cores of icosahedral bovine heart PDC, although the larger standard error presumably reflects the inherent inaccuracies listed in section 5.2.1.1. Further analysis of protein X quantification was carried out employing ^{14}C-acetylation of both the E2 and X components.

5.2.1.3 Quantification of protein X via measurement of ^{14}C-acetylation of the lipoyl moieties of E2 and X

In contrast to the findings of Jilka *et al.* (1986), the results presented in section 5.2.1.1 point to the possible presence of 12mol protein X/mol bovine heart PDC. However, as this quantification procedure was subject to a 10-20% degree of error, an additional method for quantification was employed involving measurement

and comparison of the incorporation of ^{14}C-acetyl groups into both E2 and X components in the determination of an E2:X acetylation ratio. In this application, the elucidation of the precise level of component incorporation and hence, the exact substrate concentration is not necessary. In addition, and in contrast to the previous method, quantification of protein X here, is not subject to variable E1 and E3 occupancy within a given PDC preparation. For, assuming an equivalent rate and extent of incorporation for both components, the proportion of protein X to E2 and hence the estimated number of protein X molecules, can be determined.

This requires that all lipoyl domains of the assumed 60 E2 components and proposed 12 protein X molecules are fully and equally lipoylated, and therefore, all functional in the acetylation reaction. Supporting evidence includes : the observed formation of various PDM-crosslinked E2 species in bovine heart PDC, such as hairpins, trimers and tetramers, indicative of two functional lipoyl domains (Hodgson *et al.*, 1988); the presence of an essential lysine in both lipoic acid attachment sites of E2 from human PDC (Thekkumkara *et al.*, 1988); and, the maintenance of PDC activity following the successive removal, through gene deletion, of two of the three lipoyl domains of E2 from *E. coli* (Guest *et al.*, 1985), which were subsequently shown to exhibit indistinguishable acetylation (Graham *et al.*, 1986).

From the results presented in Fig. 5.2.1.3.1, it is clear that for 0.25-1mM [2-^{14}C]pyruvate, and for 0.5mM [1-^{14}C]acetyl CoA over 30min at room temperature, incorporation ratios of 9.2-8.2:1 (E2:X), were obtained which, assuming full acetylation of the E2 lipoyl domains, suggests the presence of 13-15 protein X molecules per PDC. In close agreement, Jilka *et al.* (1986), employing higher specific activity [2-^{14}C]pyruvate (65-82mCi.μmol^{-1}) at 20 or 100μM concentrations over 20-600s at 30°C, observed a range of protein X incorporation of

Figure 5.2.1.3.1a Summary of the ^{14}C-acetylation of the lipoyl domains of components E2 and X from bovine heart PDC employing [2-^{14}C] pyruvate and [1-^{14}C] acetyl CoA

Presented are the results of four independent ^{14}C-acetylation studies employing three PDC preparations. Control samples, lacking PDC were treated in an equivalent manner. In each case, purified PDC (1-4mg.ml^{-1}), was preincubated for several minutes in buffer prior to acetylation by [2-^{14}C] pyruvate or [1-^{14}C] acetyl CoA as described in section 2.2.13. Concentrations of the radioactive substrates, PDC and, additional reagents are shown, as are the amounts of PDC, resolved by SDS-PAGE, from which the % ^{14}C incorporations of the E2 and X components were calculated as listed.

Preparation of PDC	Radioactive substrate		Concentration of PDC used ($mg.ml^{-1}$)	Other additions	Triplicate loadings of PDC used for incorporation measurement (μg).	Ratio of incorporation (E2 : X) ± error
	Name	Conc^{n} (mM)				
1) PDC 30$mg.ml^{-1}$	[2-^{14}C] pyruvate	0.25	2.0	—	20, 30, 40	9.2 : 1 ± 0.92
2) PDC 35$mg.ml^{-1}$	[2-^{14}C] pyruvate	0.25	1.0	—	20, 40, 60	8.2 : 1 ± 0.66
3) PDC 20$mg.ml^{-1}$	[2-^{14}C] pyruvate	1.0	1.0	—	40, 80	8.2 : 1 ± 0.74
4) PDC 20$mg.ml^{-1}$	[1-^{14}C] acetyl CoA	0.5	4.0	0.3mM NADH	40, 80	8.4 : 1 ± 1.18

PDC (μg)	Average band incorporation ± error (cpm)		Ratio of incorporation (E2 : X)
	E2	X	
40μg	1897.8 ± 13	228 0 ± 20	8.3 : 1
40μg	1876.1 ± 130	206.8 ± 37	9.1 : 1
80μg	3078.9 ± 56	409.0 ± 20	7.6 : 1
80μg	3543.2 ± 81	468.6 ± 16	7.6 : 1

Figure 5.2.1.3.1b Detailed results of ^{14}C-incorporation of E2 and X of bovine heart PDC employing 1mM [2-^{14}C] pyruvate

An example of the levels of ^{14}C-acetylation of the E2 and X components following treatment with 1mM [2-^{14}C] pyruvate as outlined in Fig. 5.2.1.3.1a, is presented here as mean values of triplicate results from which averaged background counts were subtracted giving net values (± error) in cpm. Corresponding (E2:X) acetylation ratios are presented, for each loading of PDC. Mean background counts for loadings of 40 and 80μg PDC, were 108 and 119cpm for E2 and, 90 and 93cpm for protein X -- accounting for 3-6% and 15-30% of the incorporation into E2 and X, respectively.

9.8-13.7% of that observed for E2 in bovine kidney PDC. In addition, under equivalent conditions but employing 20μM [1-^{14}C]acetyl CoA (150μCi.nmol^{-1}), similar protein X incorporations of 9.7-9.9% were obtained. Furthermore, maximum acetylation with [2-^{14}C]pyruvate yielded 100-120 acetyl groups/mol of bovine kidney PDC of which, approx. 10 were accounted for by protein X incorporation.

Through densitometer scanning of silver stained gel samples of purified protein X and bovine kidney PDC, and in accordance with the above evidence, Jilka *et al.* (1986), argued for the presence of 5-6mol protein X/mol PDC, with two sites of acetylation per protein X molecule. However, following confirmation in mammalian PDC, of the presence of one lipoyl domain/protein X molecule and of two lipoyl domains/E2 molecule (De Marcucci *et al.*, 1986; Hodgson *et al.*, 1988; Neagle *et al.*, 1989; Thekkumkara *et al.*, 1988), such an explanation would only hold true if S^{6},S^{8}-diacetylation were limited to the single lipoyl moieties of protein X. Yet, in intact complex, diacetylation of protein X has only been demonstrated in the presence of equivalent E2 diacetylation (Hodgson *et al.*, 1986) and in this respect, the maximum acetylation recorded by Jilka *et al.* (1986), falls far short of a theoretical incorporation of 264 acetyl groups/PDC molecule. Indeed, as the slow, secondary acetylation of S^{6},S^{8}-diacetylation was only observed by Hodgson *et al.* (1986) over extended incubation (i.e., 5-60min), it is unlikely that diacetylation would occur with the relatively short incubations of Jilka *et al.* (1986), and hence, a protein X incorporation of approx. 10 acetyl groups would therefore support the estimate of 12mol protein X/mol PDC proposed herein.

The marginally higher protein X incorporation demonstrated here may reflect: differences in incubation length and temperature; concentrations and specific activities of the radioactive substrates; the corresponding concentration of TPP; or,

the method of isolation and quantification of the ^{14}C-acetylation of E2 and X. Notably, Jilka *et al.* (1986), demonstrated increasing inhibition of pyruvate-linked acetylation and increasing deacetylation of acetyl CoA-linked acetylation with both increasing incubation and TPP concentration, caused by the accumulation of side products and, the formation of 1-hydroxyethylidene-pyrophosphate on E1, respectively. Accordingly, levels of TPP were maintained at equivalent or reduced levels to that of substrate which here, were set above the optimum K_m values. Slight inhibition of acetyl CoA-linked acetylation may also have resulted from the accumulation of CoA which may be overcome by the inclusion of residual OGDC activity (Cate & Roche, 1979).

In contrast, De Marcucci & Lindsay (1985), employing [2-^{14}C]pyruvate (10.4mCi.mmol^{-1}), achieved a protein X incorporation of 16% that measured for E2 however, this may simply reflect incomplete acetylation owing to problems associated with the relative abundance of TPP (0.2mM), in relation to pyruvate (0-5μM) -- see Cate & Roche (1979). Nevertheless, it should be noted that (in the apparent absence of diacetylation), the maximum incorporation recorded by Jilka *et al.* (1986), still falls short of the potential acetylation of 132 lipoyl domains, and may therefore reflect a reduction in the number of functional lipoyl groups. Alternatively, this could reflect a problem of geometry where, as increasing numbers of lipoyl domains become irreversibly acetylated, accessibility of the remaining unlipoylated moieties to E1 decreases. In apparent agreement, incomplete acetylation of the three lipoyl domains of E2 from *E. coli* PDC, has been repeatedly reported as an incorporation of 1.7-2 acetyl groups/E2 chain (see Graham *et al.*, 1986, for overview), although this was later disputed in the recent review of Perham (1991), which cited the observation of three fully lipoylated lipoyl domains in E2 from *E. coli* PDC (Packman *et al.*, 1991) as an indication of previously incorrect determinations of reductive acetylation.

Thus in conclusion, the work presented here, in conjunction with the quantification of protein X detailed in sections 5.2.1.1-2, appears to support the proposed presence of 12mol protein X/mol bovine heart complex.

5.2.2 Organisational studies of protein X from bovine heart PDC

5.2.2.1 Organisational studies of protein X employing covalent N,N' 1,2-phenylene-dimaleimide (PDM) crosslinking of NADH-reduced lipoyl domains

Following the quantification of protein X from bovine heart PDC outlined in sections 5.2.1.1-3, an examination of the structural organisation of this component, within the E2 core of native complex, was carried out employing NADH-induced N,N'-1,2-phenylene-dimaleimide (PDM) covalent crosslinking of the lipoyl domains of protein X in the absence of the equivalent domain on E2. This required pre-treatment with non-radioactive N-ethylmaleimide (NEM) which, by the direct modification of reduced thiols present on E1 and E3, precluded substrate-independent crosslinking between these components and the lipoyl domains on protein X. The lipoyl domains of E2 were then removed by proteolysis with 10% (w/v) collagenase. NADH-induced PDM crosslinking of the remaining protein X-derived lipoyl domains was then carried out following the method of Hodgson *et al.* (1988), but with the modifications listed in section 2.2.12. In addition, control NADH and substrate-induced crosslinking of OGDC were included to reveal potential differences between single lipoyl domain crosslinking: where both S^6,S^8-thiols are reduced (NADH-induced); or, where the one thiol is acylated and the second remains reduced and therefore, available for modification by PDM (substrate-induced). Details of both PDC and OGDC crosslinking are given in section 2.2.12.

Figs. 5.2.2.1.1a-d illustrate the results of NADH-induced PDM crosslinking of the lipoyl domains of protein X in bovine heart PDC. In agreement with the findings of Rahmatullah *et al.* (1990), and of Chapter 4 (section 4.3.2.1), Fig. 5.3.1.1a illustrates the gradual loss in complex activity which reached a plateau at a residual 20% and accompanied cleavage of the E2 component into an inner catalytic and subunit binding domain fragment of M_r 33000 and a released lipoyl and linker region of M_r 39000 ($E2_L$ in Fig. 5.2.2.1.1b), following treatment with 10% (w/v) collagenase over 60min at 33°C. In the absence of intact E2 (see Fig. 5.2.2.1.2b), residual complex activity is presumably protein X lipoyl domain-mediated and this is discussed further in Chapter 4. Thus, further inhibition of the residual complex activity upon treatment at 30°C with a range of PDM concentrations (7.4-9.2μM), employed in the presence of 0.2mM NADH, for the determination of optimum NADH-induced crosslinking (Fig. 5.2.2.1.1c), resulted from modification and therefore, inactivation of the remaining protein X-derived lipoyl domains. Control crosslinking was also examined with 9.2μM PDM in the presence of NAD^+, the results of which are illustrated in Fig. 5.2.2.1.1c.

Following resolution of the above samples by 8% (w/v) SDS-PAGE and Western analysis, initial exposures of protein X crosslinking revealed a smear of immunoreactivity ranging from a M_r value of 100000 to 200000 (Fig. 5.2.2.1.1d; panel A, lane 1_5). Subsequent dilution of the specific activity of the 1.5μCi of ^{125}I-labelled protein A used, by the addition of 10μg non-radioactive protein A, improved the signal/noise ratio and revealed the pattern of discrete bands shown in panel B (lanes 1_5, 2_{10}, 3_{30}). A cluster of 4-5 bands of M_r 126000, 110000, 97-95000 and 92000 were repeatedly visible upon PDM treatment of various collagenase preparations of PDC. In addition, two further, occasionally less visible, protein X species of M_r 82000 and >200000 were also noted. Whilst anti-E3 screening suggested the absence of crosslinked aggregates involving E3 (panel C, lane 1_5), E1

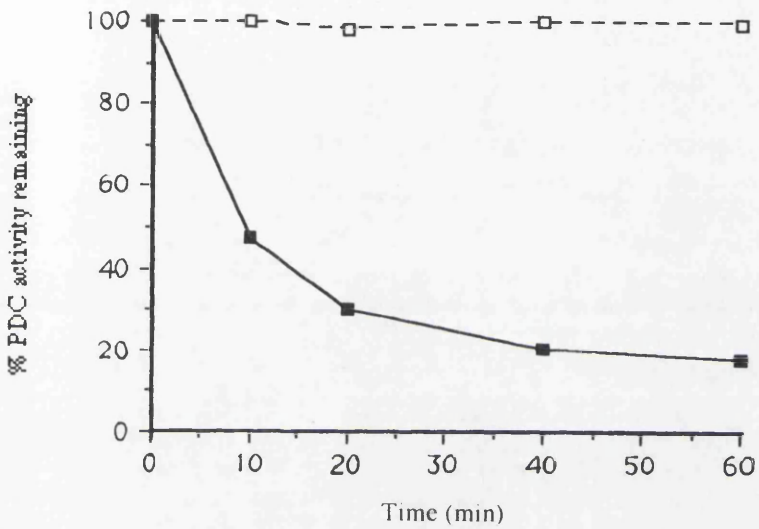


Figure 5.2.2.1.1a Loss in PDC activity following collagenase treatment

NEM pretreatment of a 2mg.ml^{-1} purified bovine heart PDC sample was carried out as described in 2.2.12 and all traces of unbound NEM removed. Following 10% (w/v) collagenase treatment at 30°C, during which time digestion was monitored by the removal of aliquots for assaying and immunological analysis, the sample was split into 4 x 100µg aliquots for subsequent NADH-induced crosslinking as described in section 2.2.12c.

(□- -□) control; (■—■) 10% (w/v) collagenase.

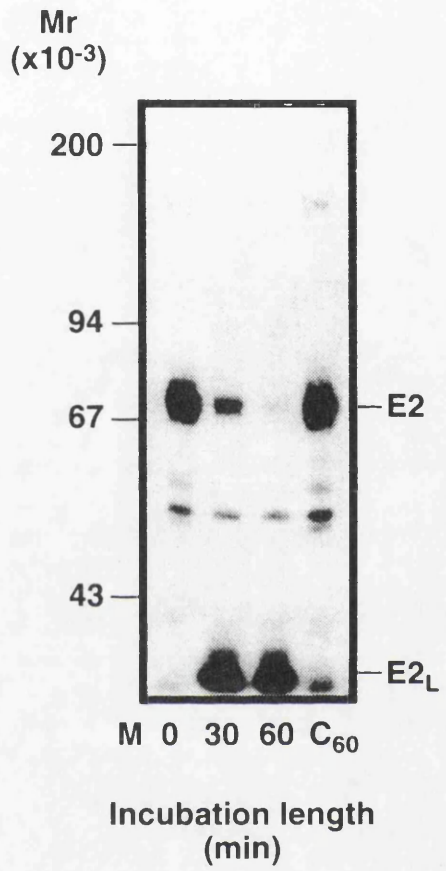


Figure 5.2.2.1.1b Western analysis of collagenase treated bovine heart PDC.

1μg samples of 10% (w/v) collagenase treated PDC were removed at the times shown and analysed by 8%(w/v) SDS-PAGE before immunological screening with anti-E2 specific serum. Where, M represents low M_r marker proteins; C_{60}, control undigested complex incubated for 60min; and $E2_L$, the released lipoyl and linker regions.

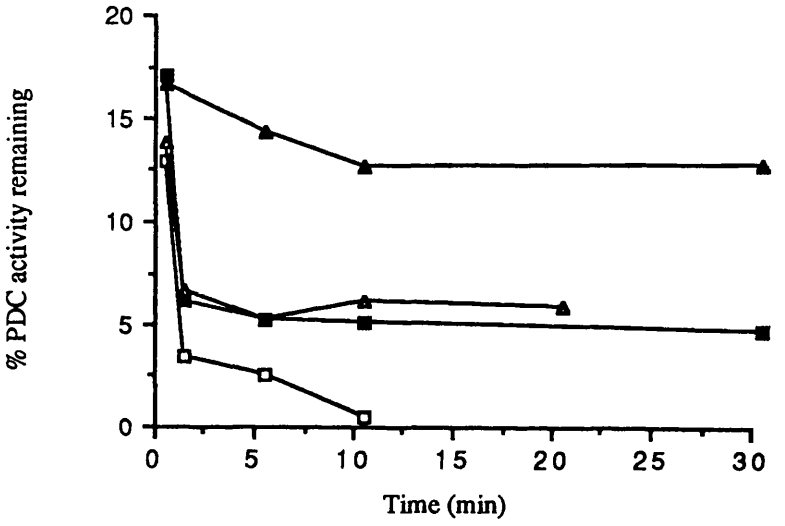
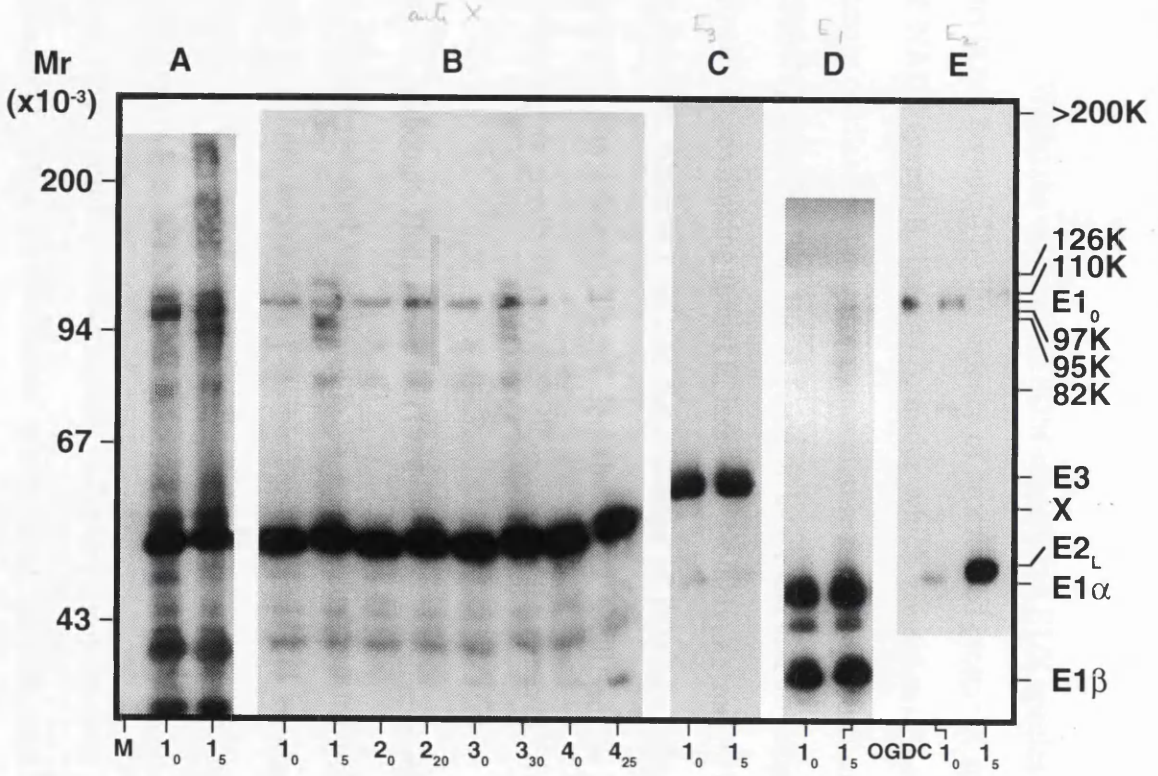


Figure 5.2.2.1.1c Loss in PDC activity following treatment with varying concentrations of PDM

Crosslinking of collagenase-treated PDC (see Fig.5.2.2.1.1a) was initiated by the addition of PDM at the following concentrations : 1, (□--□) 9.2μM PDM; 2, (Δ--Δ) 8.5μM PDM; 3, (■--■) 7.4μM PDM; 4, (▲--▲) 9.2μM PDM; in the presence of 0.2mM NADH (1-3) or, 0.2mM NAD^+ (4) as described in section 2.2.12. Crosslinking was monitored by direct assay at 340nm (see 2.2.10), and once <10% of the original complex activity remained, was arrested by addition of 20mM 2-ME on ice.

Figure 5.2.2.1.1d Immunological analysis of NADH-induced PDM crosslinking in collagenase treated PDC

PDM treated complex (4μg), previously modified with NEM and collagenase (see Fig. 5.2.2.1.1a-b), was diluted in 20mM 2-ME and Laemmli sample buffer, and resolved by 8% (w/v) SDS-PAGE. Formation of crosslinked products was analysed with anti-protein X serum (panels A & B), anti-E3 serum (panel C), anti-E1 serum (panel D) and anti-E2 serum (panel E). In panel A, immune complexes were detected with ^{125}Iodinated protein A (1.5μCi), whereas, in panels B-E its specific activity was reduced by addition of 10μg of non-radioactive protein A. Lanes denoted 1, 2, 3 represent crosslinking in the presence of 0.2mM NADH and 9.2μM, 8.5μM, 7.4μM PDM, respectively; and lane 4 represents control crosslinking with 0.2mM NAD^+ and 9.2μM PDM. Varying incubation lengths (indicated by subcripts) reflect the time taken for >90% activity loss, and X_0 denotes sample prior to addition of PDM. Lane M, low M_r marker proteins; OGDC, 05μg of purified complex.


Mr
(x10-3)
200
94
67
43
A
B
C
D
E
>200K
126K
110K
E1₀
97K
95K
82K
E3
X
E2L
E1α
E1β
M 1₀ 1₅ 1₀ 1₅ 2₀ 2₂₀ 3₀ 3₃₀ 4₀ 4₂₅ 1₀ 1₅ 1₀ 1₅ OGDC 1₀ 1₅

specific antiserum detected a faint but similar banding pattern to that of protein X (panel D, lane 1_5). However, in contrast to the NADH-dependent appearance of crosslinked species in panel B, equivalent E1 banding occurred in the presence of either NADH or NAD^+, suggestive of incomplete modification byNEM resulting in the low level NADH-independent formation of crosslinks between the individual subunits of E1 components.

Whilst the existence of PDM-crosslinked E1/X species remains a possibility, this is not favoured by the absence of crosslinked protein X species in the presence of NAD^+ (panel B, lane 4_{25}), under which conditions, the S^6,S^8-thiols would remain oxidised and therefore, unavailable for PDM modification. Finally, anti-E2 screening revealed both the absence of intact E2, and any apparent involvement in protein X crosslinking (panel E, lane 1_5). Notably, a strongly immunogenic 42000-M_r species, observed only in the presence of PDM and NADH, was thought to represent the released lipoyl domain of E2 modified by PDM and therefore exhibiting marginally slower mobility to that of the unmodified domain.

Although, Hodgson *et al.* (1988), cite the singular appearance upon substrate or NADH-induced crosslinking, of a 105000-M_r protein X dimer, the results presented here suggest the possible formation of 3-4 conformationally different protein X dimers of correspondingly altered mobilities (M_r 110000-92000), postulated in Fig. 5.2.2.1.2, to result from 2-3 different lipoyl-lipoyl, and/or one or more lipoyl-thiol cross reactions. Further support was gained through the appearance of a single crosslinked species of M_r 100000, upon resolution by the low resistance continuous phosphate-buffered gel electrophoresis system of Weber & Osborne (results not shown). However, the appearance of conformationally distinct X/X dimers may simply reflect increased interaction between the individual lipoyl domains of protein X in the absence of the equivalent E2 lipoyl domains, and thus,

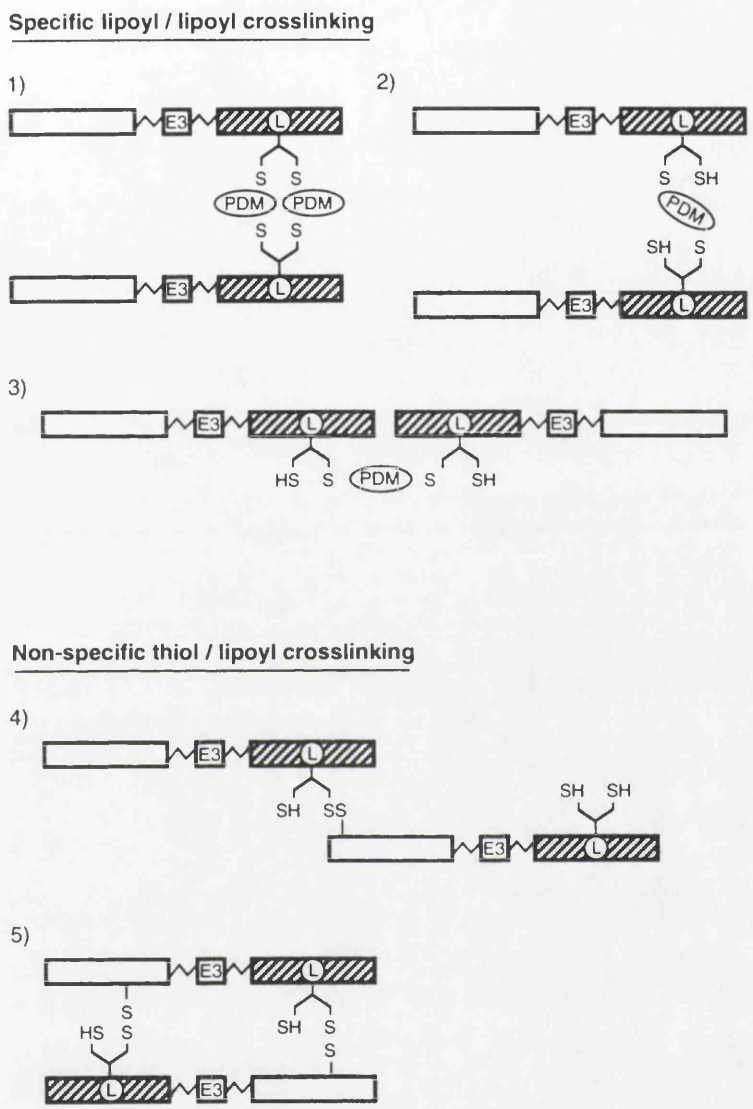


Figure 5.2.2.1.2 Schematic representation of proposed protein X dimer conformations

Shown are schematic representations of various permutations of the proposed protein X dimer conformation crosslinked by specific lipoyl/lipoyl interactions (numbered 1-3), or by non-specific lipoyl/thiol interactions (numbered 4-5). Protein X is represented as in Fig. 3.2.2.5.1b, where the lipoyl domain is represented by hatched box; the essential lysine by L; the dithiolane ring of the attached lipoic acid moiety by Y; the E3 binding domain by a boxed E3; the C-terminal domain by an open box; and the flexible interdomain linkers by zig-zag lines.

may provide an explanation for minor discrepancies with respect to Hodgson *et al.* (1988), in intact PDC.

In addition, it should be noted that the 110000-M_r species present in both anti-X and E2 blots in the absence of PDM was identified through direct comparison with purified OGDC (panel E), to be the E1 component of this latter complex, known to exhibit minor crossreactivity with both aforementioned sera following the discovery of N-terminal lipoyl-like sequences (Rice *et al.*, 1992). Upon addition of PDM, this band appeared more immunoreactive with protein X, but not E2 specific anti-sera suggestive of an X/X species of identical mobility.

Furthermore, whilst the evidence of panel E suggests the absence of E2/X crosslinking as expected, the minor 126000 and >200000-M_r species detected with anti-X serum, closely resemble a dimer and unspecified aggregate of E2 and X identified through immunological and M_r analysis by Hodgson *et al.* (1988). Alternatively, the high M_r species of 200000 could represent an aggregate of protein X (possibly of two protein X dimers), although this would require the involvement of both S^6 and S^8 thiols in the crosslinking of adjacent molecules (see later, Fig. 5.2.2.1.3).

Lastly, the identity of the minor 82000-M_r species remains unclear, although this could represent a protein X dimer of particularly compact conformation and therefore, enhanced mobility or, protein X crosslinked to the released E2 lipoyl domains. However, in light of the strong immunoreactivity of this latter domain, the corresponding absence of a 82000-M_r species in panel E is not supportive.

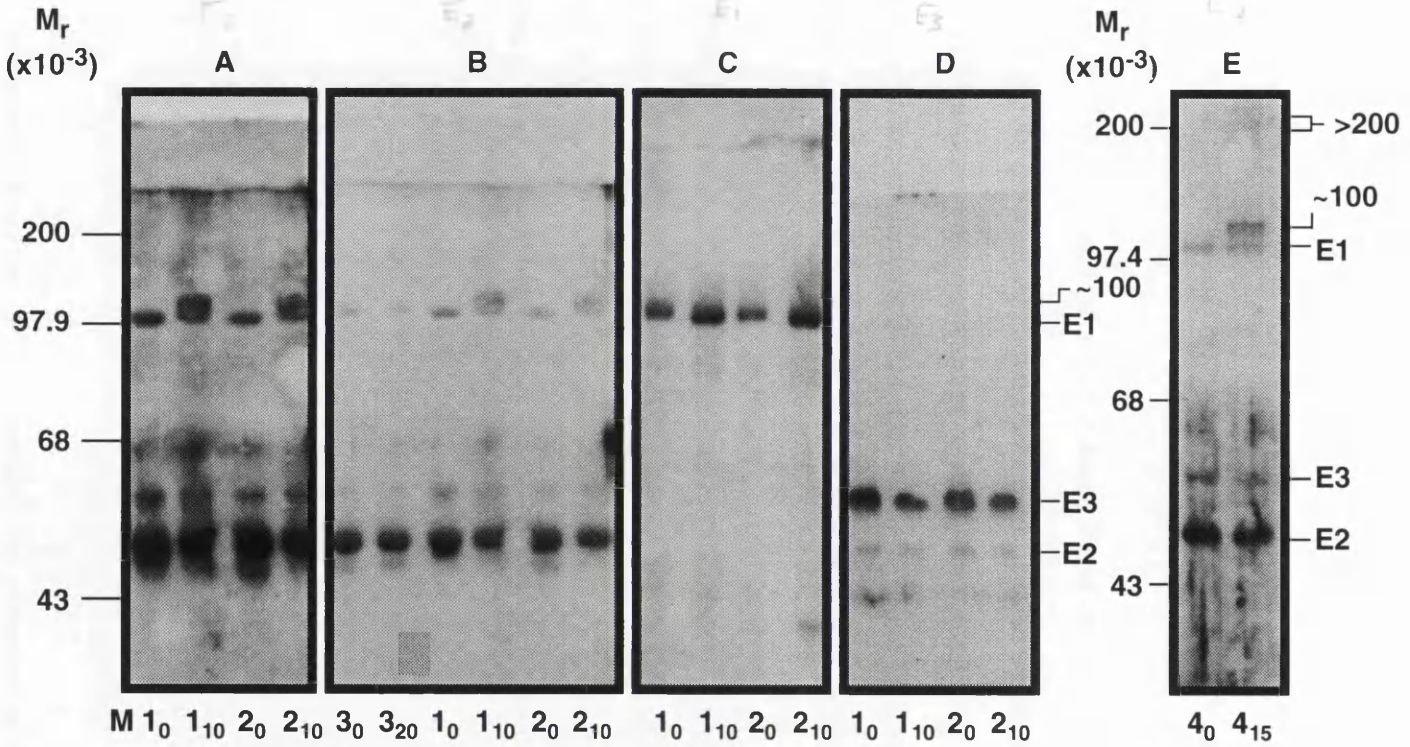
Initial studies of both substrate and NADH induced PDM crosslinking of the single-lipoyl domain containing E2 components of OGDC were then performed to

verify the observation of Hodgson *et al.* (1988) of a single E2 dimer of M_r 100000. As before, pretreatment with NEM was followed by incubation with PDM (5-15μM) at 30°C in the presence of 2mM 2-oxoglutarate or, 0.2mM NADH, until less than 10% of the original activity remained (results not shown). At this point, crosslinking was terminated by the addition of 20mM 2-ME, and samples resolved by 8% (w/v) SDS-PAGE for subsequent screening with component specific antisera. Whilst the conditions used here differ from those of Hodgson *et al.* (1988), where a constant 7.5μM PDM concentration was employed at 21°C, under N_2, and crosslinking initiated by addition of substrate, and not PDM; it is clear from the results presented (Fig. 5.2.2.1.3; panel A), that an increase in immunoreactivity at M_r 100000 followed either substrate or, NADH-induced crosslinking (compare lanes 1_0 & 1_{10} and, 2_0 & 2_{10}, respectively). Dilution of the 1.5μCi used, into 10μg of non-radiolabelled protein A improved the signal/noise ratio and crossreactivity of the E2 serum to the E1 and E3 components (due to the presence of lipoyl-like sequences on the 96000-M_r E1 component, minor crossreactivity remains), and revealed the presence of a discrete band at 100000-M_r (compare lanes 1_{10} & 2_{10} of panels A & B), not apparent in the presence of NAD^+ (lane 3_{20}). Immunological analysis with sera specific for E1 (panel C), and E3 (panel D) indicated no involvement in crosslinking although, PDM modification of E1 appeared to result in increased immunoreactivity.

In addition to the findings of Hodgson *et al.* (1988), a faint doublet of approx. M_r 200000 was also detected upon anti-E2 screening of NADH-induced PDM crosslinked OGDC (panel E, lane 4_{15}). Whilst the presence of an E2 dimer necessitates the crosslinking of either the S^6 or S^8-thiol on adjacent E2 components following substrate and NADH-induced crosslinking, the possibility of E2 trimers, tetramers etc., requires the involvement of both S^6 and S^8-thiols within individual lipoyl moieties. Indeed, in view of the growing evidence for trimeric E2

Figure 5.2.2.1.3 Immunological evidence for the production of 2-oxoglutarate and NADH-induced PDM crosslinking in E2 of bovine heart OGDC

Purified OGDC (0.5mg.ml^{-1}) previously modified with NEM, was treated to NADH or substrate-induced PDM crosslinking at 30°C, pH 7.5, as decribed in sections 2.2.12a-c, and 4μg samples removed to 20mM 2-ME in Laemmli sample buffer for resolution by 8% (w/v) SDS-PAGE and subsequent immunological analysis with subunit-specific antisera. Formation of crosslinked products was analysed with anti-E2 serum (panels A, B & E), anti-E1 serum (panel C) and anti-E3 serum (panel D). In panel A, immune complexes were detected with ^{125}Iodinated protein A (1.5μCi), whereas, in panels B-E its specific activity was reduced by addition of 10μg of non-radioactive protein A. Lanes denoted 1 represent crosslinking in the presence of 2mM 2-oxoglutarate and 0.2mM NAD^+, with 15μM PDM. Lanes labelled 2 and 4 represent crosslinking in the presence of 0.2mM NADH and 15μM or 7μM PDM, respectively. Lane 3 represents a control crosslinking with 0.2mM NAD^+ and 15μM PDM. Varying incubation lengths (indicated by subcripts) reflect the time taken for >90% activity loss, and X_0 denotes sample prior to addition of PDM. Lane M, low M_r marker proteins.


Mr (x10-3)
A
B
C
D
Mr (x10-3)
E
200
97.9
68
43
200
97.4
68
43
>200
~100
E1
~100
E1
E3
E2
E3
E2
M 1₀ 1₁₀ 2₀ 2₁₀ 3₀ 3₂₀ 1₀ 1₁₀ 2₀ 2₁₀
1₀ 1₁₀ 2₀ 2₁₀ 1₀ 1₁₀ 2₀ 2₁₀
4₀ 4₁₅

organisation within the octahedral and icosahedral cores of *A. vinelandii* and *S. cerevisiae* PDC, respectively (Mattevi *et al.*, 1992; Stoops *et al.*, 1992), the formation of trimeric species would not be unexpected for NADH-induced crosslinking in OGDC. However, the low levels of these higher M_r species of E2 in OGDC, and of X in PDC, may simply result from the inflexibility of the crosslinked dimers and/or the steric arrangement of the adjacent sulphydryl groups which could preclude modification by two molecules of PDM. Nevertheless, in OGDC, the formation of E2 dimers with substrate-induced PDM-crosslinking clearly indicates sufficient space for both succinyl and PDM modification within a single lipoic acid moiety. Furthermore, previous studies have demonstrated both diacetylation, and acetylation with accompanying NEM-modification, in the E2 and X components of bovine heart PDC (De Marcucci *et al.*, 1986; Hodgson *et al.*, 1986).

Thus in conclusion, in PDC the predominance of X/X dimers may indeed indicate pairwise symmetry for this component within the E2/X core. However, at this stage, it is unclear whether the minor formation of a larger aggregate points to limitations in inter-dimeric interactions involving both S^6,S^8-thiols within individual lipoyl moieties on X.

5.2.2.2 Structural analysis of protein X employing Arg C treatment

The results of section 5.2.2.1 implied the presence of dimeric protein X organisation within collagenase-treated E2 core, in agreement with the findings of Hodgson *et al.* (1988) in intact complex, for both NADH and substrate-induced PDM crosslinking. Furthermore, previous observations of the incomplete cleavage

of protein X, by Arg C, in intact complex (see Chapter 3; Gopalakrishnan *et al.*, 1989 and Rahmatullah *et al.*, 1989b), hinted at a population of less accessible, presumably E3-protected protein X molecules. Hence, in addition to the proteolytic studies listed in Chapter 3, further investigations employing Arg C in the presence of mildly dissociating conditions such as, 1M NaCl or 0.25M $MgCl_2$, pH 7.6, were carried out here.

From the plot of intact complex activity over 135min at 33°C presented in Fig. 5.2.2.2.1, it is clear that activity loss in the presence of 1M NaCl proceeded at a faster rate than in its absence (i.e., almost a 2-fold difference), following addition of 3+3+3% (w/v) Arg C at 0, 45 and 90min. By 135min only 10% of the original complex activity remained in the presence of 1M NaCl, and was associated with the complete removal of intact protein X (see Fig. 5.2.2.2.2a). A similar result was obtained with 0.25M $MgCl_2$, pH 7.6, indicating a general effect of ionic strength, rather than a specific metal ion activation of Arg C. In regard to the recognised E3 binding role of protein X, and the observed protection offered by E3 (Rahmatullah *et al.*, 1989b), high ionic strength will presumably give rise to mild E3 dissociation and therefore, increased accessibility to protein X. As the pattern of degradation remained unchanged, new cleavage sites did not appear to be rendered, and in agreement with the observations of Chapter 3, immunological evidence suggested only minor E1 (Fig 5.2.2.2.2b) and E2 (not shown) degradation. In contrast, in the absence of salt, a more gradual decline to a residual 30% complex activity was accompanied by incomplete protein X removal (Fig. 5.2.2.2.2a). Similar results were also obtained in sections 3.2.3.1-2.

As recent research points to flexible, and therefore, apparently variable tethering of this latter component to the E2/X core (Wagenknecht *et al.*, 1991), one or both monomers of an X/X dimer could derive protection via E3 binding

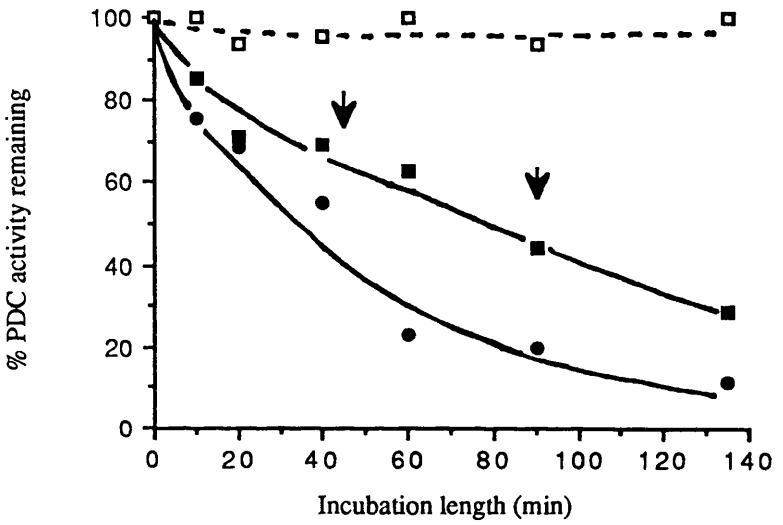


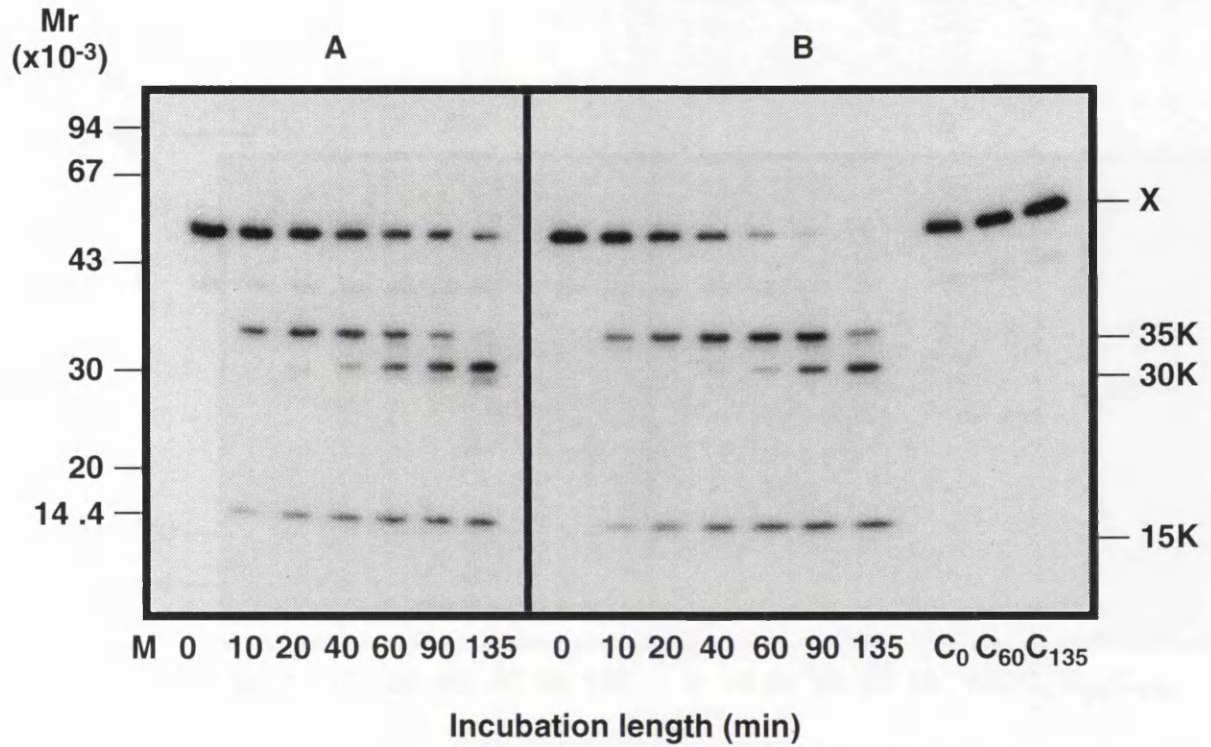
Figure 5.2.2.2.1 Affect of Arg C treatment on overall PDC activity in the presence and absence of high salt

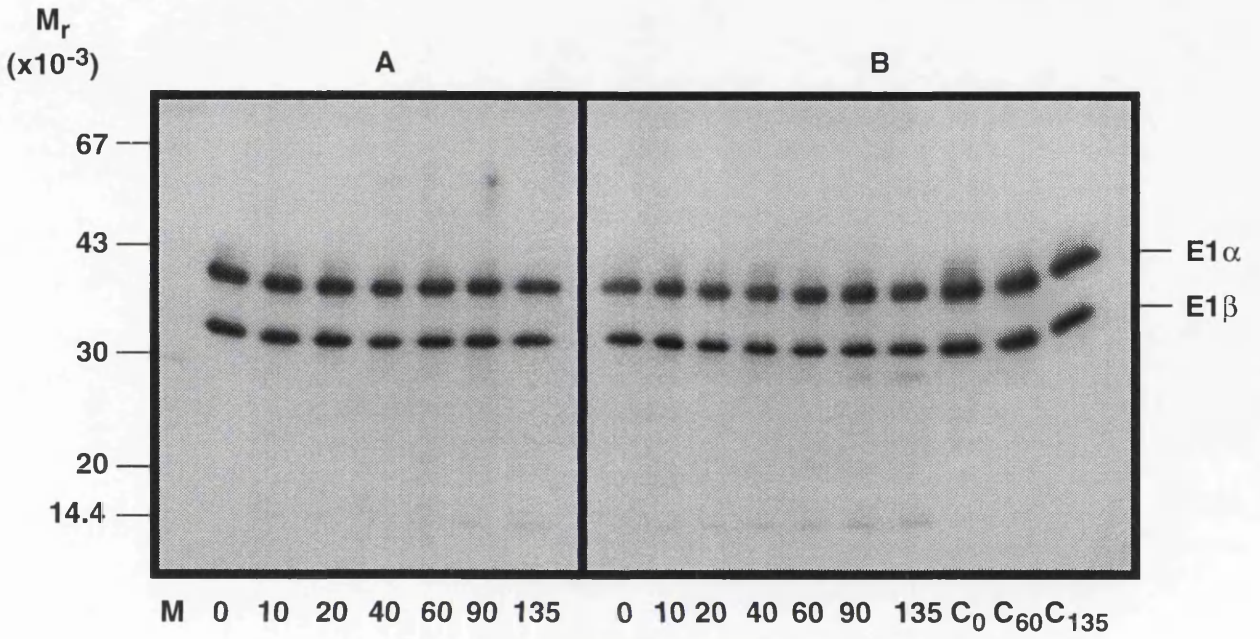
A final concentration of 1M NaCl was added to 2 of 3 identical samples of freshly purified PDC, diluted in **Soln A** to 1mg.ml^{-1}, one labelled as a control and all three preincubated at 33°C prior to the addition of 3% (w/v) Arg C to the remaining two samples. Complex activity was monitored over the 135min incubation (by the removal of 20μl aliquots to 4mM benzamidine at 4°C prior to assay), during which two further additions of 3% (w/v) ArgC were made at 45 and 90min (indicated by arrows). Activity was then expressed as a % of the original at t_0.

(□- - □) control + 1M NaCl; (■—■) Arg C; (●—●) Arg C + 1M NaCl.

Figure 5.2.2.2.2 Immunological analysis of protein X degradation during Arg C treatment of PDC in the presence of 1M NaCl

Following removal to 4mM benzamidine on ice and dilution in Laemmli sample buffer, the samples described in Fig. 5.2.2.2.1 were resolved by 10% (w/v) SDS-PAGE, transferred to nitrocellulose and screened with protein X or E1-specific anti-sera Fig. 5.2.2.2.2 a & b, respectively. Lane M, denotes the position of low M_r marker proteins; lanes 0-135, samples removed for analysis (3.5μg/track); lanes C_0-C_{135}, controls; PANEL A, in the absence of 1M NaCl; PANEL B, in the presence of 1M NaCl.


Mr
(x10-3)
A
B
94
67
43
30
20
14 .4
X
35K
30K
15K
M 0 10 20 40 60 90 135 0 10 20 40 60 90 135 C0 C60 C135
Incubation length (min)


M_r
(x10^{-3})
A
B
67
43
30
20
14.4
E1α
E1β
M 0 10 20 40 60 90 135 0 10 20 40 60 90 135 C_0 C_{60} C_{135}
Incubation length (min)

b)

interactions. Thus, any heterogeneity in the proteolytic sensitivity of a population of protein X molecules could reflect both transitory and variable E3 positioning. Alternatively, more protection may be offered to one subunit of each pair, which would necessarily indicate a differential E3 binding involvement (overcome in conditions of mild dissociation), within each dimer.

5.3 DISCUSSION

In contrast to the estimated 5mol protein X/mol PDC proposed by Jilka *et al.* (1986), similar densitometric scanning experiments presented here indicated 13.4±0.4mol X/mol PDC, with a 95% probability of the true value lying within two standard errors (i.e., ± 0.8). This value is slightly higher than the 12moles expected, and may reflect incomplete occupancy of E1 and E3 binding sites in the PDH complex leading to overestimations of protein X stoichiometry. Notably, equivalent determinations of the degree of protein X acetylation obtained by Jilka *et al.* (1986), by the precise measurement of the incorporation of radiolabelled substrates, were achieved here. Hence, both the estimated acylation of protein X of approx. 10% that of E2 (Jilka *et al.*, 1986), and the estimated incorporation ratio of 8-9:1 (E2:X) presented here, apparently support the proposed molar ratio of 12:1 (X:PDC), deduced herein from the quantification of purified protein X and PDC samples.

As the latter method of quantification is based on the pivotal assumption that all bovine heart PDC preparations contain a constant component ratio of E2:X and furthermore, that all lipoyl domains of E2 and X exhibit equivalent acetylation potentials, the resultant 10-20% error noted may therefore reflect deviations from the above. Indeed, whilst E2 of *E. coli* possesses three apparently functionally equivalent lipoyl domains, numerous reports of maximum acetylation point to an incorporation of 1.7-2 acetyl groups/E2 chain (see Graham *et al.*, 1986 for review). In addition, previous publications cite the existence of heterogenous populations of purified pig heart PDC ranging from a M_r of 1-8 x 10^6, and raise questions over the true size and composition *in situ* (Sumegi *et al.*, 1987). Though not an isolated report, the significance of this latter finding is unclear, and although variable

acetylation may exist within populations of E2 from both mammalian and bacterial PDC, the maximum acetylation of bovine heart PDC reported by Jilka *et al.* (1986), is 75-90% that expected for the total number of lipoyl domains, in comparison to 57-67% for *E. coli* PDC. Furthermore, in a recent review Perham (1991) disputed the accuracy of previous maximum acetylation determinations, following the discovery of the full lipoylation of the three lipoyl domains of E2p of *E. coli* by Packman *et al.* (1991).

The estimate of 12 molecules of protein X per molecule of complex becomes more intriguing when viewed in light of the suggested dimeric organisation of protein X within the E2 core, presented here. The formation of X/X dimers employing NADH-induced PDM crosslinking mirrored the results of similar substrate and NADH-linked crosslinking in intact PDC (Hodgson *et al.*, 1988), and suggested that even in the absence of E2 lipoyl domains, protein X interactions are limited and indicative of pairwise symmetry. In addition, and in contrast to the easy removal of protein X from isolated core (see Chapter 3), complete degradation of protein X, by Arg C, in intact PDC appeared dependent upon the presence of mild dissociating conditions (i.e., 1M NaCl or 0.25M $MgCl_2$), which hinted at a population of less accessible molecules. Indeed, initial reports of an E3-mediated protection of protein X in bovine heart PDC (Rahmatullah *et al.*, 1989b) were substantiated by further reports by Powers-Greenwood *et al.* (1989); and Gopalakrishan *et al.* (1989), and later consolidated by the confirmation of an E3 binding role for protein X in *S. cerevisiae* (Lawson *et al.*, 1991a).

In line with the 1:1 (E3:E1) binding relationship in bovine heart OGDC, where recent evidence indicated a role in E3 binding for the N-terminal lipoyl-like sequences of E1 (Rice *et al.*, 1992), and where both components exhibit dimeric organisation about an octahedral E2 core (Reed & Yeaman, 1987), a model of six E3

dimers bound to the E2 core by six protein X dimers is suggested. Whilst it is difficult to reconcile the integration of these molecules of protein X into a core of icosahedral (532) symmetry without an accompanying disarray, post-assembly binding appears a more plausible explanation. Support comes from the demonstration of apparently native E2 core self-assembly in *S. cerevisiae* (Niu *et al.*, 1990; Lawson *et al.*, 1991a), and from preparations of active E2 core bovine heart PDC (Powers-Greenwood *et al.*, 1989) -- all in the absence of protein X. Yet, it is clear from the tight association of protein X for the E2 core, and the necessary employment of denaturing conditions in its removal, that such external binding must involve closer interactions to those of the peripherally bound E1 and E3 components, and of the kinase molecules which also exhibit high core affinity (Li *et al.*, 1992).

Although, the integration of protein X during core assembly is not yet discounted, the recent elucidation through electron microscopy of the *S. cerevisiae* E2 core as 60 E2 molecules arranged along 20 vertices as cone-shaped trimers (Stoops *et al.*, 1992), in agreement with the similar trimeric symmetries of *A. vinelandii* PDC, and of chloramphenicol acetyltransferases (Mattevi *et al.*, 1992; DeRosier, 1992), would necessitate either quasi-icosahdral symmetry or the substitution of E2 by protein X molecules. Importantly, the latter suggestion is not supported by the determination of a 95% probability of the true number of E2 molecules falling within two standard errors (i.e., ± 5.0) of a mean value of 61.8 (Tom Aitchson, Dept. of Statistics, University of Glasgow). In addition, a study of protein X distribution within bovine kidney PDC, through the relative positioning of E3, revealed a wide yet variable distribution, thought to reflect the postulated flexible tethering of E3 to the E2/X core (Wagenknecht *et al.*, 1991). Whilst it remains unclear which component contributes to such tethering (Schulze *et al.*, 1993), this new information raises questions over the face-centred model of E3 binding

originally proposed by Reed & Hackert (1990), and demonstrates the need for further structural and component interaction analysis. Furthermore, in view of the proposed flexible E3 tethering, variable and transitory E3 positioning relative to the E2/X core could explain variations in the proteolytic sensitivity of some protein X molecules which was not apparent in the absence of E3 in isolated core.

CHAPTER 6 :

DISCUSSION AND FUTURE DIRECTIONS

6.0 DISCUSSION AND FUTURE DIRECTIONS

6.1 An E3 binding function for protein X in bovine heart PDC

The work presented in Chapter 3 confirmed and extended previous observations in *S. cerevisiae* PDC of an E3 binding function for the subunit binding domain of protein X (Lawson *et al.*, 1991a).

Selective proteolysis of the lipoyl-bearing region of X from reconstituted or intact bovine heart PDC, employing Arg C, resulted in the loss of both E3 binding potential and complex activity but had little affect on E1 binding, in agreement with the findings of similar work carried out in bovine kidney PDC (Rahmatullah *et al.*, 1989a; Powers-Greenwood *et al.*, 1989; Gopalakrishnan *et al.*, 1989). These results pointed to a role in E3 binding for the lipoyl domain of X. In contrast, removal of this domain from X in intact bovine heart PDC, via selective proteolysis with Arg C (Neagle & Lindsay, 1991); or, via gene deletion experiments in *S. cerevisiae* PDC (Lawson *et al.*, 1991a), had been shown previously to have little overall effect on complex catalysis or E3 binding.

N-terminal amino acid sequencing of the truncated protein X here, revealed significant homology to the highly conserved E3 binding domain motif of E2 components from various species and of X from *S. cerevisiae* PDC. Thus, the close proximity of the Arg C cleavage site to the boundary of the E3 domain suggests that both lipoyl and linker regions are released by selective proteolysis. From these observations, it was proposed that loss of E3 binding follows disruption or

destabilisation of the adjacent subunit binding domain and/or a specific E3 binding site within. However, attempts to investigate the possibility of a temperature-sensitive destabilisation were unsuccessful (results not shown) and hence, were in agreement with the previous findings of Rahmatullah *et al.* (1989a).

Direct or indirect participation, possibly through the stabilisation of the subunit binding domain, should not be ruled out for the lipoyl and linker regions of protein X. Indeed, minor losses in both E3 binding and PDC activity accompanied the removal of the lipoyl domain of X in *S. cerevisiae* (Lawson *et al.*, 1991a). Furthermore, enhanced peripheral subunit binding in E2 from *B. stearothermophilus* and *A. vinelandii* PDC have been linked to additional binding sites in the linker regions separating the subunit binding and lipoyl domains (Hipps *et al.*, 1994; Schulze *et al.*, 1991b). This possibility is given more weight following the discovery of E3 binding properties ascribed to a lipoyl-like sequence at the N-terminus of E1 from bovine heart OGDC (Rice *et al.*, 1992). Although smaller than a lipoyl domain (i.e., 10000-M_r), and lacking a functional lipoamide group, this region of E1 exhibits many characteristics associated with lipoyl domains. In addition, while OGDC lacks a separate protein X species, the presence of the lipoyl-like sequence on E1 appears to confer X-like properties such as, E3 binding and tight core affinity -- both of which are lost, together with overall complex (but not E1) activity, upon its removal from E1. The authors cite this as a clear case of "domain shuffling" and, in the absence of a subunit binding domain motif in the related E2 from rat OGDC (Nakano *et al.*, 1991), it is not altogether surprising that both E1 and E3 binding affinities are affected by this removal.

As protein X has only been identified as an independent species in yeast and mammalian PDC, in all other 2-oxoacid dehydrogenase complexes characterised to date, peripheral subunit binding is the sole responsibility of the E2 component --

necessitating in some cases, both subunit binding and inner catalytic domains. Clearly then, the evolutionary emergence of an additional subunit binding component in *S. cerevisiae* and mammalian PDC has led to differentiation, where E3 binds to X, and E1 to E2 (Lawson *et al.*, 1991a,b; Gopalakrishnan *et al.*, 1989). Although participation in E3 binding may not have provided the selective pressure necessary for the maintenance of X (see section 6.2), it is of interest to note that in the absence of X or X-like sequences in bovine kidney BCOADC, E3 affinity for the E2 core appears less than that observed in PDC and OGDC from the same source, and leads to significant dissociation during purification from mitochondria (Clarkson & Lindsay, 1991).

Physiological evidence, together with work presented in Chapter 3, suggest however, that E2 may maintain a low binding affinity for E3. In the former case, reports of lactic acidemia sufferers exhibiting residual (i.e., 10-20%) PDC activity in the apparent absence of protein X, have provided direct support (Robinson *et al.*, 1990; Marsac *et al.*, 1993). In the latter, following increasing protein X degradation in isolated E2/X core, employing Arg C, successively higher levels of recovery were achieved upon reconstitution with E1/E3 in the presence of excess porcine E3, suggestive of a second independent binding site -- presumably on E2. Full recovery of reconstitution was not achieved and may indicate the low specificity of this second E3 binding site. Alternatively, this may be a reflection of the incompatibility of porcine E3 in the reconstitution of bovine complexes.

This possibility has recently come to light following preliminary findings from current research in this laboratory. Competition assays involving reconstitution of bovine heart OGDC from constituent E1, E2 and E3 components in the presence of increasing concentrations of E3 from various sources, indicates significant differences in the catalytic and/or binding requirements of the otherwise

highly conserved E3 components, across species (S. Khan, University of Glasgow, personal communication). One area of future research will, therefore, involve the extension of such competition binding assays to reconstituted bovine heart PDC. Since it is now clear that Arg C cleaves not only the lipoyl but also the linker regions of X, with the resultant disruption of the subunit binding domain, it would be of great advantage to identify a protease specific for the removal of only the lipoyl domain of X. In the absence of subunit binding domain instability, elucidation of the precise nature of the involvement of the lipoyl domain in E3 binding would be made posssible. An alternative approach, employing transfection studies in protein X-deficient cell lines (C. Marsac, INSERM U75 Institute, Paris, personal communication) has also been proposed and could provide fresh insight into the E3 binding functions of both E2 and X.

6.2 The role of protein X in complex catalysis

In agreement with previous observations of a residual X lipoyl domain-mediated complex activity following removal of the lipoyl domains of E2, in *S. cerevisiae* PDC via genetic manipulation (Lawson *et al.*, 1991b) or, in bovine kidney PDC after treatment with protease, collagenase (Rahmatullah *et al.*, 1990); proteolytic cleavage of the lipoyl domains of either X or E2 in reconstituted or intact PDC resulted here, in a residual 10-15% complex activity.

Subsequent [2,3-^{14}C]NEM labelling of the treated complexes, in the presence of various substrates and products, confirmed the ability of either component to substitute for the lipoyl domains of the other and furthermore, to undergo normal diacetylation. The results suggested a non-essential but clearly,

rate-limiting involvement of either component in complex catalysis. This supported previous observations of low level PDC activity and, presumably E2/E3 binding, in the absence of intact X presented in Chapter 3, and the discovery of residual 10-20% PDC activity in X-deficient lactic acidemia sufferers (Robinson *et al.*, 1990; Marsac *et al.*, 1993). Whilst X_L- mutants in *S. cerevisiae* exhibited approximately 70% wild type activity (Lawson *et al.*, 1991a), the considerably lower E2-lipoyl domain mediated activity noted here, in bovine heart PDC following Arg C treatment, is accounted for by the loss in high affinity E3 binding to X. In contrast, a residual 10-15% protein X lipoyl domain-mediated activity was observed both in *S. cerevisiae* and mammalian PDC, reflecting limitations in the number and (possibly) efficiency of X.

As the majority of publications dispute the existence of an acetyltransferase active site on X (Behal *et al.*, 1989; Lawson *et al.*, 1991b; Rahmatullah *et al.*, 1987), and attempts to isolate and sequence the C-terminal portion of X and thereby, confirm previous observations of an active site-like "His Xaa Xaa Xaa Ser Gly" sequence on X from human PDC (J.C. Neagle, University of Glasgow, unpublished results), remain in the initial stages; it is still unclear whether, and perhaps somewhat unlikely that, X functions as an isoenzyme of E2. Although, the close structural identity between E2 and X, and maintenance of a multi-domain conformation suggest the potential for more than just an E3 binding role, overwhelming evidence for an external core positioning (discussed in Chapter 5), is not supportive of an intimate participation in catalysis. In contrast, in servicing the active site on E2, protein X may function in the downstream channelling of reducing equivalents from E2 to E3 as initially proposed by Gopalakrishnan *et al.* (1989), in view of the E3 binding role.

The ability of X to undergo the various acetylation reactions of E2 could therefore, exist as a vestigial function -- secondary to that of E3 binding. A similar situation arises in *E. coli* PDC, where all three lipoyl domains on E2 remain fully functional yet, only one (presumably the outermost) is necessary for catalysis, with the inner domains thought to provide increased length and flexibility facilitating enhanced active site coupling by the extended "superarm" (Turner *et al.*, 1993). Indeed, it is of interest to note that while the lipoyl domain-like sequence at the N-terminus of E1 from bovine heart OGDC exhibits characteristics common to protein X (such as, E3 binding and tight core associations), and of its lipoyl domain, suggestive of domain shuffling, no evidence exists for an involvement in catalysis (Rice *et al.*, 1992).

The answer to the maintenance of the acetylation function on X may lie in the unique diacetylation properties of E2 and X from mammalian PDC. During conditions of excessive lipid metabolism such as, mild starvation or diabetes, high levels of products, NADH and acetyl CoA, are removed from the cytosol via E3-directed acetylation of the lipoyls of E2 and X. Following stabilisation of the [NADH]:[NAD^+] ratio, acetyls are channelled to the TCA cycle to produce energy. The ability of both S^6,S^8-thiols of the lipoic acid moiety dithiolane ring to undergo acetylation, and the additional presence of X, increases the storage potential more than 2-fold to 264mol of acetyl groups/mol PDC. The apparent absence of X and diacetylation in OGDC and BCOADC presumably reflects differing positions in metabolism, and of diacetylation in PDH complexes from species such as plants and yeast, possibly the additional presence of acetyl CoA-utilising pathways like the glyoxylate cycle. One discrepancy still persists however, in that whilst E1-directed acetylation does not require acetyltransferase activity, acetylation from precursors, acetyl CoA and NADH, does and, in the absence of such activity on X, the lipoyl domains of this latter component must therefore, service the active site on E2. If

diacetylation is one of the selective pressures for the emergence and maintenance of a functional lipoyl domain on X, it seems odd that acetyltransferase activity is not similarily maintained. However, it should be noted that as the precise mechanism for diacetylation remains to be elucidated, an additional acetylation activity may be involved, which could quite plausibly reside on X.

In consideration of this fact, re-assessment of the active site-like motif observed in a partial cDNA clone of the human protein X gene (J.C. Neagle, University of Glasgow, unpublished results), is currently underway. Involving re-sequencing of the nucleotide code (J.E. Rice, University of Glasgow), together with amino acid sequence analysis of the C-terminal tryptic peptides of X, obtained through anhydrotrypsin column purification (see Chapter 4), these results may provide new insight into the precise role of protein X in complex catalysis. In addition, if good correlation exists between both sequences, the partial cDNA clone, fluorescently or radioactively labelled, could then provide a powerful tool for the isolation of the entire protein X gene from a λgt10 library, and localisation on a specific chromosome.

6.3 Quantification and organisation of protein X within bovine heart PDC

In Chapter 5, densitometric scan analysis and ^{14}C-acetylation of the lipoic acid moieties on E2 and X yielded an estimate of 12-13mol X/mol PDC. This result was in contrast to previous determinations of 5mol X/mol PDC in bovine kidney PDC, employing similar methods but, based on the incorrect assumption of there being only one lipoyl domain on E2 (Jilka *et al.*, 1986). Statistical analysis of the

data obtained here from densitometer scans of E2 and X, indicated a 95% probability of the true number of either component lying within two standard errors of the calculated mean values of 61.8 ± 2.5mol E2/mol PDC and 13.4 ± 0.4mol X/mol PDC, respectively (Tom Aitchson, Dept. of Statistics, University of Glasgow). This gave a slightly higher range for X of 12.6-14.2 molecules per complex than expected, and may arise from variable E1 and E3 occupancy leading to overestimations of protein X stoichiometry. Nevertheless, the small standard error suggested close agreement between individual determinations -- supported further, by a good internal control value of 61.8 molecules of E2 per complex.

Organisational studies, employing covalent NADH-induced PDM crosslinking of the lipoyl domains of X in E2-lipoyl domain-depleted complex, revealed protein X interactions indicative of dimeric organisation and similar to that observed previously, in intact PDC, with substrate or NADH-induced crosslinking (Hodgson *et al.*, 1988). In regard to the E3 binding function of X, and the dimeric nature of E3, a model of 6 homodimers of E3 bound via 6 dimers of X was proposed.

In support, additional findings in Chapter 3, pointed to an initial lag in E3 release, compared to PDC activity loss, following cleavage of the lipoyl and linker regions of protein X in intact PDC, by Arg C. Clearly, this situation could arise if each E3 dimer were bound to more than one X molecule, necessitating disruption of the subunit binding domain on both monomers of each X dimer. Alternatively, if one monomer of each pair were engaged in core associations and the other in E3 binding, accessibility of the latter subunit to Arg C could be partially obstructed by the presence of E3. Indeed, reports of E3-mediated protection in intact complex exist both here, where complete protein X degradation required use of mild dissociating conditions (see Chapter 5) and, elsewhere (Rahmatullah *et al.*, 1989;

Powers-Greenwood *et al.*, 1989; Gopalakrishnan *et al.*, 1989). However, it should be noted that the lag in E3 release may simply reflect a time-dependent conformational change and gradual lowering or loss of E3 affinity, following destabilisation of the subunit binding domain. Nevertheless, a 1:1 (E1:E3) binding relationship also exists in bovine heart OGDC where, both components arrange with dimeric symmetry and, where similarities have been drawn between the N-terminal portion of E1 (responsible for E3 binding) and the lipoyls of X (Rice *et al.*, 1992). Furthermore, preliminary results suggest that both subunits of the E1 dimer must be cleaved by trypsin, prior to E3 release (J.E. Rice, University of Glasgow, unpublished observations).

The unusually close core interactions and structural identity between E2 and X prompted proposals of an internal, integral positioning for X -- again recently suggested by Li & coworkers (1992). However, it is difficult to reconcile the integration of 12 molecules of X into a core of icosahedral symmetry unless X substitutes for E2. This possibility is disputed by the successful isolation of native E2 core, devoid of protein X, from *S. cerevisiae* (Niu *et al.*, 1990) and, bovine kidney PDC (Powers-Greenwood *et al.*, 1989). In addition, the statistical analysis of E2 quantification shown here, in bovine heart PDC, gave a 95% probability of the true number of E2 lying in the range of 57-67mol E2/mol PDC, discounting the possibility of an icosahedral core comprising 12X and 48E2 molecules (Tom Aitchson, Statistical Dept., University of Glasgow). Indeed, in light of the proposed dimeric protein X organisation illustrated here, and the growing evidence for trimeric symmetry in both cubic (*A. vinelandii* -- Mattevi *et al.*, 1992) and icosahedral E2 cores (*S. cerevisiae* -- Stoops *et al.*, 1992), neither organisation appears compatible with the other, adding to the mounting evidence in favour of an external positioning for X.

Following establishment of the number and organisation of X in bovine heart PDC, future objectives in this area will probably involve completion of E3 binding studies in OGDC and, extension of previous research into component interactions (concentrating specifically on E2/X and E3/X binding), during import of the nuclear encoded precursors into the mitochondrion, and subsequent complex assembly (De Marcucci *et al.*, 1988).

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