Structural and functional studies on component X of mammalian pyruvate dehydrogenase multienzyme complex

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To my family

(especially Rosemary, Gregg, Graeme and James)

CONTENTS

		Page
	LIST OF CONTENTS	i
	LIST OF FIGURES	viii
	LIST OF TABLES	xi
	ABBREVIATIONS	xii
	ACKNOWLEDGEMENTS	xiii
	SUMMARY	xiv
CHAPTER ONE		
1.	Introduction	1
1.1	Multienzyme complexes	1
1.1.1	Association of enzymes	2
1.2	2-oxo acid dehydrogenase multienzyme complexes	4
1.2.1	Role of 2-oxo acid dehydrogenase multienzyme complexes	6
1.2.2	Purification of 2-oxo acid dehydrogenase multienzyme complexes from various sources	6
1.2.3	Reaction mechanisms of the 2-oxo acid dehydrogenase multienzyme complexes	7
1.2.4	Pyruvate dehydrogenase (E1)	7
1.2.5	Lipoate acetyltransferase (E2)	9
1.2.6	Lipoamide dehydrogenase (E3)	10
1.2.7	Possible functions of lipoamide dehydrogenase (E3)	11
1.3	Pyruvate dehydrogenase complex	13
1.3.1	Subunit composition of pyruvate dehydrogenase from *E. coli*	14
1.3.2	Subunit composition of mammalian pyruvate dehydrogenase complex	15
1.3.3	Structure and function of pyruvate dehydrogenase complex	15
1.3.4	Lipoyl domains not associated with E2	18
1.3.5	Organisation of 2-oxo acid dehydrogenase complex	18
1.3.6	Regulation of mammalian pyruvate dehydrogenase complex	20
1.3.7	Sites of phosphorylation	22

		Page
1.4	Component X	23
1.4.1	Immunological studies on component X	24
1.4.2	Peptide mapping	25
1.4.3	Mitochondrial location of component X	26
1.4.4	Cross reactivity of anti-component X IgG with a 48000 M_r protein in the cytosolic fraction of BRL cells	27
1.4.5	Association of component X with E2 core	28
1.4.6	Cross linking of lipoyl groups on PDC	28
1.4.7	Stoichiometry of component X	29
1.4.8	Possible functions of component X	30
1.5	Eukaryotic gene expression	32
1.5.1	Recombinant DNA Technology	32
1.5.2	Molecular genetics of mammalian pyruvate PDC	33
1.5.3	Human pyruvate dehydrogenase (E1)	33
1.5.4	Genetics of eukaryotic component X	34
1.5.5	Genetics of prokaryotic and eukaryotic lipoamide dehydrogenase	35
1.6	Genetics of 2-oxo acid dehydrogenase multienzyme complex	35
1.6.1	Genetics of 2-oxo acid dehydrogenase complexes in *E. coli*	36
1.6.2	Genetic studies on E2 of PDC in *E. coli*	37
1.7	Aims of project	39

CHAPTER TWO

2.	Materials and Methods	41
2.1	Materials	41
2.1.1	Chemicals	41
2.1.2	Tissue culture materials	41
2.1.3	Radiochemicals	42
2.1.4	Enzymes and proteins	42
2.1.5	Animals	42
2.1.6	Miscellaneous	42
2.1.7	Photographic materials	43
2.1.8	Materials used in screening a λgt11 gene expression library	43
2.1.9	Reagents used in purification and sequencing of DNA	46

		Page
2.2	Methods	48
	2.2.1 Measurement of protein concentration	48
	2.2.2 Concentration of protein samples	48
	2.2.3 Measurement of incorporation of radioactivity into proteins	49
	(a) TCA precipitation	49
	(b) Quantitative determination of radioactive polypeptides excised from gel slices	49
	2.2.4 Polyacrylamide gel electrophoresis	50
	(a) Preparation of polypeptides prior to resolution by SDS PAGE	50
	(b) Conditions for resolution of proteins by SDS-PAGE	50
	(c) Preparative SDS-PAGE	51
	(d) Staining of gels with Coomassie blue	52
	(e) Destaining of gels	52
	(f) Processing of SDS gels for fluorography	52
	(g) Processing of SDS gels for autoradiography	52
	(h) Determination of M_r by SDS-PAGE	53
2.2.5	Purification of pyruvate dehydrogenase complex	53
	(a) Purification of pyruvate dehydrogenase from ox heart	53
	(b) Preparation of E2/X core assembly	55
2.2.6	Isolation of mitochondria and submitochondrial fractions	55
	(a) Isolation of rat liver mitochondria	55
	(b) Preparation of ox heart mitochondria	56
	(c) Preparation of cell extracts and subcellular fractions from cultured mammalian cells	56
2.2.7	Enzymatic assays	57
	(a) Pyruvate dehydrogenase complex activity	57
	(b) Pyruvate dehydrogenase kinase activity	57
	(c) Lipoamide dehydrogenase activity	58
2.2.8	Elution of PDC subunits from SDS polyacrylamide gels	58
2.2.9	Immunological procedures	59
	(a) Immune blotting	59
	(b) Preparation of IgG	60
	(c) Dot blots	61
2.2.10	Radiolabelling of proteins	61

Page

2.2.11 Modification of enzyme-bound lipoyl groups in pyruvate dehydrogenase complex 62

(a) Acetlytation assay 62

(b) Deacetylation assays 63

(c) N-ethylmaleimide modification of PDC 63

(d) N-ethyl [2,3-^{14}C] maleimide labelling of PDC subunits 63

2.2.12 Screening of a λgtll gene expression library with antibody probes 64

(a) Antibody screening procedure 64

(b) Growing λgtll 64

(c) Overlaying plaques with nitrocellulose filters 64

(d) Identification and rescreening of positive clones 65

(e) Estimation of the number of plaque forming units (P.F.U.) 66

(f) Drop titre (a rapid method for determining the number of plaque forming units) 67

(g) High titre lysate 67

(h) Confirmation of positive clones using high titre lysates 67

(i) Preparation of lysogens from recombinant phage 68

(j) Replica plating 68

(k) Preparation of crude lysate 68

(l) Permanent storage of lysogens 69

(m) Identification of fusion protein 69

2.2.13 Preparation of DNA 69

(a) Preparation of phage DNA 69

(b) Alternative preparation of bacteriophage DNA 71

(c) Separation of DNA fragments by agarose gel electrphoresis 72

(d) Restriction digests 72

(e) Restriction enzymes 73

Page

2.2.14 Subcloning into M13 73
(a) DNA sequencing 73
(b) Restriction digest of RF DNA 74
(c) Phenol extraction 74
(d) Ethanol precipitation 75
(e) Ligation of RF DNA to insert DNA 75
(f) Transformation of E. coli JM101 75
(g) Preparation of single stranded template DNA 76
(h) Annealing of primer and template 77
(i) Sequencing reactions 77
(j) Reaction mixes 78
(k) Polyacrylamide gel electrophoresis 78
(l) Autoradiography 79
2.2.15 Plasmid preparations 80
(a) Plasmid preparation of pTZ 18R 80
(b) Ligation of insert DNA into plasmid pTZ 18R 80

CHAPTER THREE

3. Role of component X in eukaryotic pyruvate dehydrogenase 82
3.1 Introduction 82
3.2 Results 85
3.2.1 Acetylation of E2 and component X during normal turnover of PDC 85
3.2.2 Low temperature labelling of E2 and component X with [2-^{14}C] pyruvate 85
3.2.3 Overall PDC activity with various substrate analogues 87
3.2.4 HPLC analysis of 2-oxobutyrate 87
3.2.5 Substrate analogue protection of E2 and component X from NEM incorporation 89
3.2.6 N-ethyl [2,3-^{14}C] maleimide incorporation into NADH reduced PDC incubated in various acyl CoA's 90
3.3 Discussion 91

CHAPTER FOUR

4. Selective proteolysis of mammalian puruvate dehydrogenase complex Part A : Effects of Trypsin Treatment 95

		Page
4.1	Introduction	95
4.1.1	Selective proteolysis of PDC with trypsin	98
4.1.2	The effect of tryptic digestion on the acetylation of PDC	99
4.1.3	Fluorographic decection of E2/X derived tryptic peptides after incubation with [^{14}C] pyruvate	99
4.1.4	Deacetylation of [2-^{14}C] pyruvate labelled intact and trypsin-treated PDC	100
4.1.5	Association of tryptic peptides with E2 core assembly	101
4.1.6	Incorporation of N-ethyl [2,3-^{14}C] maleimide into tryptic peptides of PDC	102
	Discussion	102
4.2	Part B	104
4.2.1	Limited proteolysis of [^{14}C] acetylated PDC by argC and its effect on overall complex activity	104
4.2.2	Immunological detection of proteolytic fragments from E2 and component X on incubation with protease argC	105
4.2.3	The effect of high salt concentration on PDC activity	106
4.2.4	The effect of high salt and argC on PDC activity	107
4.2.5	Relationship between decline in PDC activity and extent of degradation of component X in PDC treated with argC in the presence or absence of high salt	107
4.2.6	Release of E3 from E2/X core on incubation with argC	108
4.2.7	Study of the integrity of the E2/X core assembly after inactivation of the complex with argC and high salt	109
4.2.8	Labelling of PDC with [^{14}C] NEM after proteolysis with argC	110
4.2.9	Enhanced labelling of E3 may be at the active site	111
4.2.10	Effect of protease argC on PDC kinase	112
4.2.11	Proteolysis of PDC by argC in various buffer systems	112

Page

CHAPTER FIVE

5. Structural and Functional relationship of component X to the lipoate acetyltransferases (E2) component 120

5.1 Introduction 120

5.2 Results 121

5.3 Discussion - 124

CHAPTER SIX

6.1 Introduction 128

6.1.1 Nucleic acid hybridisation 130

6.1.2 Homologous probes 130

6.1.3 Partially homologous probes 130

6.1.4 Synthetic oligonucleotide probes 130

6.1.5 In vitro amplification of DNA by the polymerase chain reaction (PCR) 132

6.1.6 Antibody screening 133

6.2 Results 134

6.2.1 Reactivity of antisera with denatured PDC on a Western blot 134

6.2.2 Dot blot analysis (Levels of antigen detected by antisera) 135

6.2.3 Screening of λgtll gene expression library 136

6.2.4 Preparation of lysogens 137

6.2.5 Preparation of DNA that encodes for protein X 139

6.2.6 Restriction endonuclease digestion of DNA 139

6.2.7 DNA sequencing 140

6.2.8 Preparation of competent cells 141

6.2.9 Transformation of competent cells 141

6.2.10 Preparation of single stranded DNA 142

6.2.11 Nucleotide sequencing of insert DNA 142

6.2.12 Ligation of insert into vector PTZ 18 143

6.2.13 Preparation of recombinant plasmid PTZ 18 143

6.2.14 Restriction digest of recombinant plasmid DNA 143

6.3 Discussion 144

Page

CHAPTER 7

7. General Discussion 148

7.1 Functional role of component X 148

7.2 Limited proteolysis of component X 150

7.3 Structural and functional relationship of component X to the E2 core assembly 153

7.4 Cloning and sequence analysis of protein X 154

REFERENCES 155

FIGURE LEGENDS

Figure 1.1 Sequence of reactions catalysed by mammalian PDC

Figure 1.2 Mechanistic pathways for the reductive acetylation of lipoyl cofactors by 2-(1-hydroxyethylidene)-TPP at the active site of pyruvate dehydrogenase (E_1) (Frey et al., 1989)

Figure 1.3 Major routes of pyruvate metabolism in mammalian cells

Figure 1.4 Schematic diagram of domain structure of the E2 components

Figure 1.5 Schematic representation of the covalent modifications of pyruvate dehydrogenase and its regulation by various metabolites (Reed et al., 1980)

Figure 1.6 Phosphorylation sites on the E1 subunit pyruvate dehydrogenase

Figure 1.7 Possible functions of component X in mammalian PDC

Figure 2.1 Physical and genetic map of wild-type bacteriophage

Figure 2.2 Multiple cloning site on M13

Figure 2.3 Primer sequence and its annealing site in M13mp18

Figure 3.1 Substrate protection against NEM modification and inactivation of PDC

Figure 3.2 Acetylation of E2 and component X during normal turnover of the enzyme

Figure 3.3 Low temperature labelling of E2 and component X with [2-^{14}C] pyruvate

Figure 3.4 Structure of substrate analogues

Figure 3.5 HPLC analysis of 2-oxobutyrate and puruvate

Figure 3.6 Substrate analogue protection of E2 and component X from NEM incorporation

Figure 3.7 N-ethyl [2,3-^{14}C] maleimide incorporation into NADH reduced PDC

Figure 4.1A Selective release of [^{14}C] acetylated lipoyl domains from E2 and X polypeptides of native bovine heart PDC

Figure 4.1B Effect of trypsin on overall PDC activity

Figure 4.2 Time course of acetylation of native or trypsin treated PDC

Figure 4.3 Fluorographic detection of E2/X derived tryptic peptide

Figure 4.4 Deacetylation of [2-^{14}C] pyruvate labelled intact PDC and trypsin-treated PDC

Figure 4.4A B & C Deacetylation [2-^{14}C] pyruvate labelled intact PDC and trypsin-treatment PDC

Figure 4.5 Association of tryptic peptides with E2 core assembly

Figure 4.6 Incorporation of N-ethyl [2,3-^{14}C] maleimide into tryptic peptides of PDC

Figure 4.7A Limited proteolysis of [^{14}C] acetylated PDC with argC

Figure 4.7B Effect of protease argC on overall complex activity

Figure 4.7C Extent of degradation of E2 and component X after limited proteolysis with argC

Figure 4.8 Immunological detection of proteolytic fragments from E2 and component X on incubation with protease argC

Figure 4.9A SDS-PAGE analysis of constituent enzymes of PDC, after treatment in high salt followed by resolution on a gel filtration column

Figure 4.9B The effect of high salt concentration on PDC activity

Figure 4.10 The effect of high salt and argC on PDC activity

Figure 4.11 Relationship between enzymatic activity of PDC and degradation of component x in PDC treated with argC in the presence or absence of high salt

Figure 4.12 Release of E3 from E2/X core on incubation with argC

Figure 4.13 Study of the integrity of the E2/X core assembly after inactivation of the complex with argC and high salt

Figure 4.14 Enhanced susceptibility of E3 to [^{14}C] NEM modification after limited proteolysis of PDC with argC

Figure 4.15 NADH dependent inactivation of E3 by NEM

Figure 4.16A Effect of protease argC on PDC kinase

Figure 4.16B Incorporation of ^{32}P into E1 α subunit of PDC

Figure 4.17 Proteolysis of PDC by argC in various buffer systems

Figure 5.1 Immunoblotting analysis of lipoamide acetyltransferase (E2) and protein X from various cellular extracts

Figure 5.2 Selective tryptic release of ^{14}C acetylated lipoyl domains from E2 and X polypeptides of native bovine heart PDC

Figure 6.1 Immunological detection of PDC and PDC subunits with IgG prepared from antisera raised against PDC, E2 and component X

Figure 6.2 Dot blot analysis

Figure 6.3 Specificity of component X antisera for recombinant phage

Figure 6.4 Identification of fusion protein of component X by immunoblotting

Figure 6.5 Restriction endonuclease digestion of recombinant λ gt11

Figure 6.6 Example of a polyacrylamide sequencing gel

Figure 6.7 Nucleotide sequence and predicted amino acid sequence determined from cDNA encoding protein X

Figure 6.8 Comparison of nucleotide sequence of mammalian protein X cDNA and yeast protein X

Figure 4.15	NADH dependent inactivation of E3 by NEM
Figure 4.16A	Effect of protease argC on PDC kinase
Figure 4.16B	Incorporation of ^{32}P into E1α subunit of PDC
Figure 4.17	Proteolysis of PDC by argC in various buffer systems
Figure 5.1	Immunoblotting analysis of lipoamide acetyltransferase (E2) and protein X from various cellular extracts
Figure 5.2	Selective tryptic release of ^{14}C acetylated lipoyl domains from E2 and X polypeptides of native bovine heart PDC
Figure 6.1	Immunological detection of PDC and PDC subunits with IgG prepared from antisera raised against PDC, E2 and component X
Figure 6.2	Dot blot analysis
Figure 6.3	Specificity of component X antisera for recombinant phage
Figure 6.4	Identification of fusion protein of component X by immunoblotting
Figure 6.5	Restriction endonuclease digestion of recombinant λ gt11
Figure 6.6	Example of a polyacrylamide sequencing gel
Figure 6.7	Nucleotide sequence and predicted amino acid sequence determined from cDNA encoding protein X
Figure 6.8	Comparison of nucleotide sequence of mammalian protein X cDNA and yeast protein X
Figure 6.9	EcoRI digest of recombinant PTZ 18

TABLE LEGENDS

Table 1.1 Specific reactions catalysed by 2-oxo-acid multienzyme complexes Basic substrate - R CO-COOH

Table 1.2 Subunit composition of bovine heart pyruvate dehydrogenase complex

Table 1.3 The lipoate region on the acetyltransferase (E2) component

Table 1.4 Principal fractions of structure and assembly of the 2-oxo acid dehydrogenase complexes

Table 2.1 Caesium chloride solutions for step gradients prepared in SM (100ml)

Table 2.2 10 x Stock restriction enzyme buffers (µl)

Table 2.3 Final concentrations of each reagent in a sequencing reaction mix

Table 2.4 Quantities of reagents necessary to pour a single polyacrylamide gel for DNA sequence analysis

Table 3.1 Overall enzyme activity using substrate analogues

Table 5.1 N-terminal acid sequence of ox-heart E2 and component X

Table 5.2 Comparison of N-terminal amino acid sequences of protein X and various lipoate acetyltransferases (E2) from eukaryotic sources

Table 5.3 Internal amino acid sequences of component X

ABBREVIATIONS

ACSCoA	acetyl coenzyme A
BRL	buffalo rat liver cells
BSA	bovine serum albumin
bp	base pairs
cpm	counts per minute
DMSO	dimethylsulphoxide
DTT	dithiothreitol
EDTA	ethylenediamine tetra acetic acid
FPLC	fast protein liquid chromatography
HPLC	high pressure liquid chromatography
IPTG	isopropyl-1-thio-β-D-galactoside
Iodogen	1,3,4,6-tetrachloro-3α, 6α diphenyl glycoluril
LB	L broth
leupeptin	acetyl-L-leucyl-L-leucyl-L-argininal
2ME	2-mercaptoethanol
M_r	molecular mass (relative)
MM	minimal medium
Mops	4-morpholinopropanesulphonic acid
NBL-1	bovine kidney cells
NEM	N-ethylmaleimide
OGDC	2-oxoglutarate dehydrogenase complex
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDC	pyruvate dehydrogenase complex
PK15	pig kidney cells
PMSF	phenylmethyl sulphonylfluoride
RF	replicative form
RNase	ribonuclease
SBTI	soya bean trypsin inhibitor
SDS	sodium dodecyl sulphate
TBS	tris buffered saline
TCA	trichloracetic acid
TEMED	N,N,N'N'-tetramethylethylenediamine
Tween 20	polyoxyethylenesorbitan monolaureate
X-gal	5-brom-4-chloro-3 indoyl-β-D-galactoside

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SUMMARY

Pyruvate dehydrogenase is a multienzyme complex consisting of three enzymes, a decarboxylase (E1), an acetyltransferase (E2) and a dehydrogenase (E3). A fourth enzyme of unknown function component X or protein X has recently been identified. Several similarities between E2 and protein X have been demonstrated.

Further investigation into this unique polypeptide has shown monospecificity of antiserum raised against gel purified protein X and E2. In various cell lines, antiserum to protein X failed to recognise the E2 component, similarly with E2 antiserum no cross reactivity with protein X polypeptide was observed.

Although E2 and protein X are antigenetically distinct, comparison of the N-terminal amino acid sequence of the E2 and X polypeptides revealed significant homology between the two components. Limited amino acid sequence data on bovine heart protein X indicated that this protein contains a single lipoyl domain located at its N-terminus and belongs to a family of homologous enzymes, all of which possess acetyltransferase activity. Limited proteolysis of PDC with the specific protease trypsin selectively released the lipoyl domains from E2 and protein X. Release of these domains from the E2 core assembly resulted in the loss of overall complex activity. Although the peptides participate in the overall catalytic cycle of the enzyme, their release from the core effectively decreases the concentration of the peptides with the resultant loss of overall complex activity. Further studies on PDC with trypsin resulted in the loss of the enzymes ability on prolonged incubation with acetylating substrate to diacetylate the active site on E2 and thus protect this site from modification and inactivation with N-ethylmaleimide.

A possible role for protein X in eukaryotic PDC has been determined. Limited proteolysis of PDC by protease ARG C

selectively digests the lipoyl domain from protein X without significantly affecting the overall enzymes activity. Cleavage of the lipoyl domain from protein X results in the lowering of the affinity of the E3 component for the core assembly. Release of E3 from the core assembly exposed the active site on E3 rendering it susceptible to an NADH dependent labelling with [^{14}C] NEM. After limited proteolysis of PDC with ARG C, E3 activity studies revealed an NADH dependent inactivation of E3 by various active site inhibitors. By treatment of the complex with high salt (conditions which are known to release E3) enhanced labelling of E3 by [^{14}C] NEM was observed and NADH dependent inactivation of E3 confirmed, leading to the conclusion that protein X binds E3 to the core assembly and protects the active site on E3.

PDC is regulated by phosphorylation (inactivation) and dephosphorylation (activation) on the El α subunit. Limited proteolysis of PDC with ARG C resulted in a more rapid inactivation of the complex and incorporation ^{32}p into the El α subunit suggesting that the regulatory subunit of the associated kinase had been affected. An interesting observation was the presence of thiamine pyrophosphate and Mg^{2+} protected the El α subunit from proteolysis by ARG C.

A mammalian λ gtll gene expression library has been screened with antiserum to protein X. A partial cDNA clone of protein X has been isolated and nucleotide sequence obtained. Comparison of nucleotide sequence obtained from protein X and Saccharomyces cerevisiae and nucleotide sequence from the mammalian protein X clone revealed over 70% homology at the carboxyl-terminal of yeast protein X confirming the origin of the mammalian X clone.

Attempts to determine the route of acetyl group transfer between El and E2, El and X or between E2 and X have been made. Disruption of the interaction between these components by reductively acetylating E2 and X with [^{14}C] pyruvate at -20^{o}C or by employing

substrate analogues to observe this interaction has had limited success. At $-20^{o}C$ the turnover of the enzyme was successfully reduced although no disruption of the interaction between the components could be observed. The use of substrate analogues failed to show any preferential acylation between E2 and X or preferential interaction with El.

Finally, by monitoring the [^{14}C] reductive acetylation of PDC in the presence of Coenzyme A, participation of protein X in the normal catalytic cycle was established.

CHAPTER ONE

INTRODUCTION

1. Introduction

1.1 Multienzyme complexes

Enzymes are biological catalysts which usually function sequentially promoting consecutive chemical reactions linked by intermediate products. When a number of enzymes are associated to form a defined entity which can catalyse a series of linked biochemical conversions this is termed a multienzyme complex. Well characterised examples are: tryptophan synthase from Escherichia coli (Yanofsky and Crawford, 1972), fatty acid synthetase from both yeast (Lynen, 1972) and mammalian sources (Stoop et al., 1978) and the 2-oxo acid dehydrogenase complexes present in all organisms studied to date (Reed, 1974; Perham, 1975). A full description of several other multienzyme complexes may be found in reviews by Reed and Cox (1966) or Hardie and Coggins (1986).

Organisation into a multienzyme complex can provide a number of functional advantages including enhancement of catalytic or regulatory efficiency and substrate channelling (Perham, 1975). As a form of compartmentalisation, intermediates may be positioned on or within the complex in such a way that would exclude competition by another biochemical pathway, or prevent decomposition in aqueous solution of an unstable intermediate. Interaction of the component polypeptides, as in the case of tryptophan synthase, may produce aggregates that have catalytic properties not present in separate chains. Assembly of inter-related enzymes also makes it physically easier for the products of one enzyme to act as a substrate for the next enzyme in sequence. This may well increase the efficiency of the overall process, even if the intrinsic catalytic activity of each component is not altered by the association.

The increased efficiency of a complex as compared to that of separate enzymes dispersed at random is most obvious when all intermediate products are strongly bound to the complex. In such a case reactions of the intermediate products of one enzyme with the next one are more probable. Moreover, substrate can be channelled through a series of reactions to avoid competition with other enzymes for the intermediate products or to reduce sensitivity to inhibitors in the surrounding medium. Such a system would function as a mechanism for selecting the metabolic fates of the substrate.

1.1.1 Association of enzymes

The integration of metabolic function in cells and its regulation can largely be explained in terms of structural interaction and separations between elements (enzymes and substrates) of the diverse metabolic pathways (Welch, 1977). It is possible that no organisation exists between enzymes in some metabolic pathways and that metabolic interactions or regulatory behaviour are diffusion controlled rather than specifically modulated by the local structure or microenvironment of the cellular compartment in question.

The rates of all chemical reactions are limited by the time it takes for the two reactants to encounter each other. This time depends on the shape of the reactants, the viscosity of the medium, the distance between the two particles and other parameters of the system. Considering the small distances involved in living cells, the diffusion coefficients of both large and small biological molecules, and assuming that water is the medium, the calculated encounter times are very fast compared to the turnover of all but the most rapidly-catalysed bioreactions. It has been suggested, therefore, that many metabolic pathways do not require special conditions or structures to allow enzymes to encounter their substrates or effectors.

However, several authors have calculated that the difference between diffusion and enzyme turnover times may not be as large as first indicated if one imposes several corrections on the calculation of diffusion times (Welch, 1977). Another problem arises if randomly distributed enzymes depend solely on the diffusion of substrates. Atkinson (1969) has noted that unless special strategies are employed the volume occupied by total metabolites would be so large that the solvent capacity of the cell would be rate limiting.

Intracellular microenvironments, where local high concentrations of metabolites could be maintained, with a low total cellular metabolite concentration, can be created by arranging enzymes of a single biochemical pathway next to one another (Srere et al., 1973). Thus a high rate of enzyme activity could be maintained in a region of high metabolite concentration while the total average cellular concentration of the metabolite and therefore its volume of occupancy would be low. Increased rates of reaction have been reported when immobilised multi-step enzyme systems are compared to their soluble counterparts. Presumably a high local concentration exists as the substrate is produced in the immobilised system (Srere et al., 1973). However, the mechanism to explain these observations is not known precisely since diffusion times are thought to be much smaller than turnover times and some energy input seems necessary to maintain this situation of apparent non-equilibrium.

One reason for the acceptance that metabolic events are controlled by random diffusion stems from the fact that electron microscopy reveals areas of cell structure which have little or no obvious organisation. These areas include the cytosol and the

mitochondrial matrix. However, recent investigations of cytosolic structural elements have revealed a complex trabecular structure in both cytosol and the nucleus (Schliwa et al., 1981). In addition, experiments which have demonstrated interactions between soluble enzymes and subcellular structures have given rise to more intense considerations of metabolic control without the intervention of the diffusional process (Masters, 1981). Welch (1977) has presented forcefully the theoretical basis supporting the role of organised enzyme systems in metabolism and Srere (1982) has indicated that the inner membrane matrix compartment of mitochondria may have a degree of structural organisation in which the matrix proteins are adjacent and loosely-bound to the inner membrane. This proposal has important consequences relating to the control (diffusional or structural) of the main energy producing pathway of most aerobic cells and the biogenesis of mitochondria.

Association of enzymes with one another and with structural proteins in cells may be far more extensive than is generally imagined. Increased efficiency and new three dimensional arrangements with elaborate control mechanisms may arise from such organisation. Isolation and purification of multienzyme complexes cannot be assumed to preserve the integrity of these aggregates. Therefore, it is possible that their natural occurrence is more frequent than reported (Reed and Cox, 1966). It is also possible the enzymes exist as smaller aggregates which when diluted on cellular disruption form large complexes which are not normal cellular entities.

1.2 2-Oxo acid dehydrogenase multienzyme complexes

The 2-oxo acid dehydrogenase multienzyme complexes are associations of three enzymic activities, a substrate-specific dehydrogenase (E1), a distinct dihydrolipoyl acyltransferase (E2)

and dihydrolipoamide dehydrogenase (E3), a flavoprotein which is common to all three complexes.

Each complex catalyses a coordinated sequence of steps that can be represented by the overall reaction.

$$R\text{-}\overset{O}{\overset{\|}{C}}\text{-}CO_2H + CoA + NAD^+ \longrightarrow R\text{-}\overset{O}{\overset{\|}{C}}\text{-}S\text{-}CoA + NADH + H^+ + CO_2$$

Where R = CH_3 (pyruvic acid) or $HO_2C\text{-}(CH_2)_2$ (2-oxoglutarate) or the 2-oxo acids derived from the metabolism of valine, isoleucine and leucine (α-oxoisovaleric, α-oxoisocaproic and α-oxo-3 -methylvaleric acids). The acyltransferase constitutes the core of the complexes to which the E1 and E3 component enzymes are attached by non-covalent bonds. The total number of subunits is large and, depending on the type of complex and its source, is generally in the range of 60 to 100. These complexes may be self assembling and form ordered, symmetrical structures. There are no fewer than six coenzymes and prosthetic groups involved in the sequence of reactions catalysed by the complexes, viz; Mg^{2+}, thiamine pyrophosphate (TPP), lipoic acid, coenzyme A (CoASH), flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NAD^+).

In different organisms they are subject to regulation by ligand binding (Reed, 1974), covalent modification (Linn et al., 1969) control of gene expression (Langley and Guest, 1978), proteinase action (Linn et al., 1974), product inhibition (Garland and Randle, 1964), hormonal control (Reed and Yeaman, 1987; McCormack and Denton, 1984; Denton and Hughes, 1978) and glucose oxidation i.e. in starvation or diabetes (Wieland, 1983). The specific reactions catalysed by pyruvate dehydrogenase complex and the functionally analogous 2-oxoglutarate dehydrogenase (OGDC) and branched chain 2-oxo acid dehydrogenase complexes (BCOADC) are shown in Table 1.1.

Table 1.1 Specific reactions catalysed by 2-oxo-acid multienzyme complexes

Basic substrate - R-CO-COOH

Complex	R	Substrate	Product
Pyruvate dehydrogenase	CH_3-	Pyruvate	Acetyl-CoA
2-oxoglutarate dehydrogenase	$CO_2(CH_2)_2-$	2-oxoglutarate	succinyl-CoA
branch-chain 2-oxo acid dehydrogenase	$(CH_3)_2CH_2CH_2-$	2-oxoisocaproate	isovaleryl-CoA
	$(CH_3CH_2)CH_3CH-$	2-oxo-3-methyl valerate	2-methyl butyryl-CoA
	$(CH_3)_2CH-$	2-oxo-iso-valerate	isobutyryl-CoA
	$CH_3\ CH_2-$	2-oxo-butyrate	propionyl CoA
	$CH_3-S-(CH_2)_2-$	4-methylthio 2-oxobutyrate	3-methylthio propionyl CoA

1.2.1 Role of 2-oxo acid multienzyme complexes

Pyruvate dehydrogenase complex (PDC) provides acetyl CoA, an important biosynthetic metabolite used in the synthesis of fatty acids, leucine and steroids in eukaryotes. In most aerobic organisms, the oxidation of acetyl CoA via the tricarboxylic acid cycle provides a major source of reducing power in the form of NADH and metabolically utilizable energy in the form of ATP. The 2-oxoglutarate dehydrogenase complex (OGDC) is a major constituent of the tricarboxylic cycle regulating the flux of this pathway in its latter stages. OGDC catalyses the formation of succinyl CoA which is required in the biosynthesis of porphyrins, lysine and methionine.

The branched-chain 2-oxo acid dehydrogenase complex (BCOADC) catalyses the committed step in the degradation of branched-chain amino acids. In mammals this is nutrionally important in dealing with excess valine, leucine and isoleucine in the diet (Goldberg and Chang, 1978). These amino acids are ultimately converted to acetyl CoA (ketogenic), succinyl CoA (glucogenic) and propionyl CoA (glucogenic). BCOADC may also be involved in the catabolism of methionine and threonine since it is reported that 2-oxobutyrate (Pettit et al., 1978) and 4-methylthio-2-oxobutyrate can also be used as substrates by the complex (Jones and Yeaman, 1986). A deficiency in BCOADC activity in humans results in Maple Syrup Urine disease. The 2-oxo acid dehydrogenase complexes have been purified from a variety of prokaryotic and eukaryotic sources and a selected list follows.

1.2.2 Purification of 2-oxo acid multienzyme complexes from various sources

PDC has been isolated from E. coli (Reed and Mukherjee, 1969; Danson et al., 1979). Pseudomonas aeruginosa (Jeyasselan et al., 1980), Bacillus stearothermophilus (Henderson and Perham,

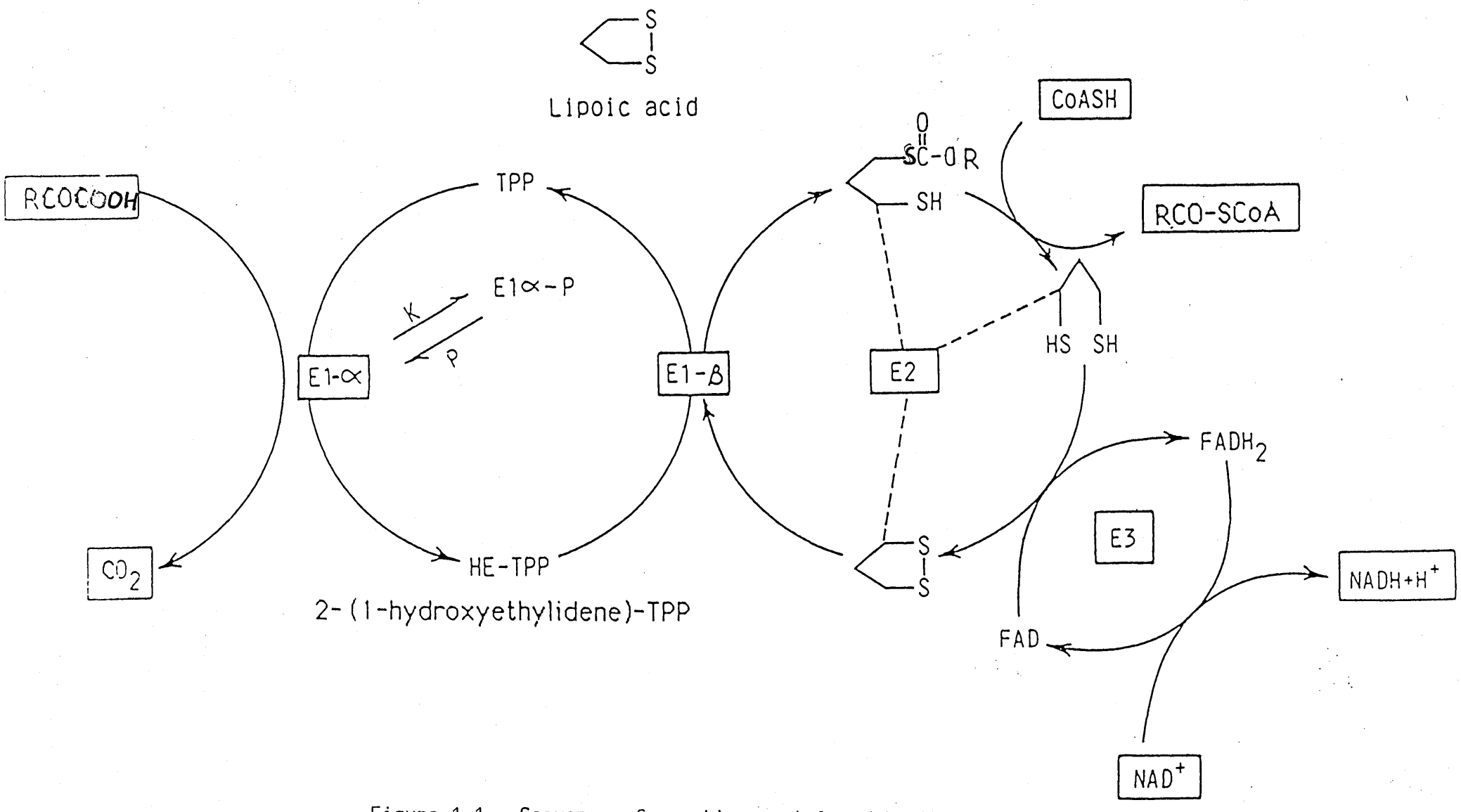


Figure 1.1 Sequence of reactions catalysed by the mammalian pyruvate dehydrogenase multienzyme complex

1980), Bacillus subtilis (Hodgson et al., 1983), Saccharomyces cerevisiae (Kresze and Ronft, 1981), ox kidney (Kresze and Steber, 1979; Cate and Roche, 1979), porcine liver (Roche and Cate, 1977) and ox heart (Stanley and Perham, 1980). OGDC has been purified from E. coli (Reed and Mukherjee, 1969), ox kidney (Reed and Oliver, 1968) and pig heart (Koike and Koike, 1976). BCOADC has been isolated from Pseudomonas putida (Sokatch et al., 1981b), bovine liver and heart mitochondria (Danner et al., 1979; Pettit et al., 1978) and Ascaris suum (Sokatch et al., 1981a).

1.2.3 Reaction mechanism of the 2-oxo acid dehydrogenase multienzyme complexes

As discussed previously (Section 1.2), the acyltransferase component of the multienzyme complexes forms a central core around which are arranged multiple copies of the E1 and E3 enzymes. The sequence of reactions catalysed by pyruvate dehydrogenase is shown schematically in Figure 1.1. Using pyruvate dehydrogenase complex as an example the reaction mechanism is discussed more fully.

1.2.4 Pyruvate dehydrogenase (E1)

The reaction catalysed by pyruvate dehydrogenase (E1) is the rate determining step governing overall PDC activity (Bates et al., 1977; Danson et al., 1978). Pyruvate is decarboxylated initially in a reaction involving the coenzyme thiamine pyrophosphate. Lipoic acid, covalently-bound to the ε-amino group of a lysine on the E2 component by an amide bond is then reductively acetylated with the concomitant opening of the dithiolane ring generating a free thiol. It has been shown recently by ^{1}H nmr that acetylation occurs initially at the S^8 position of the lipoic acid and not at the S^6 position although isomerisation can occur in aqueous solution (Yang and Frey, 1986).

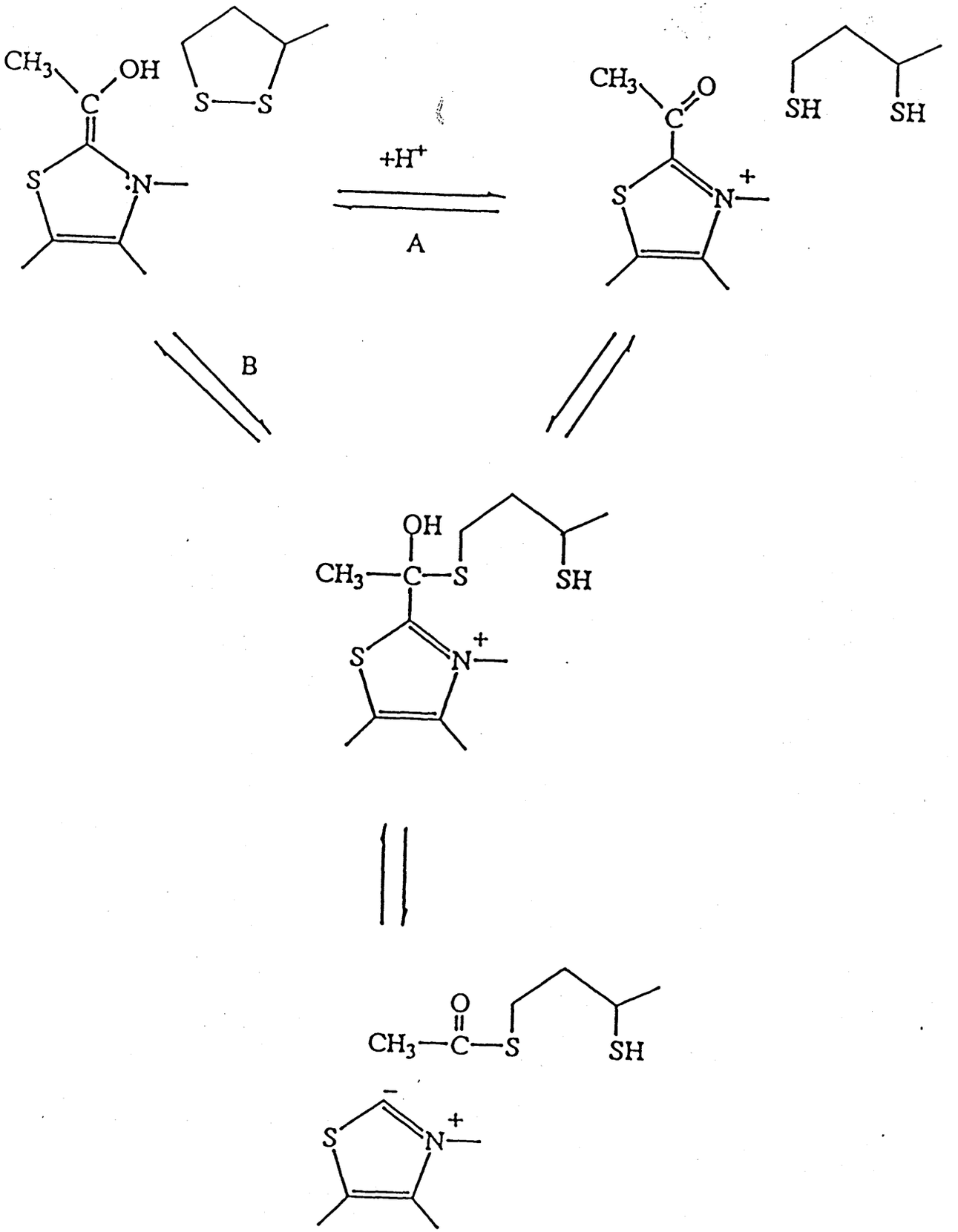


Figure 1.2 Mechanistic pathways for the reductive acetylation of lipoyl cofactors by 2-(1-hydroxyethylidene)-TPP at the active site of pyruvate dehydrogenase (E_1) (Frey et al., 1989)

Mammalian E1 of PDC and E1 of BCOADC are tetramers consisting of two subunits α and β while E1 of mammalian OGDC is a homodimer. The role of each subunit in the complexes has not yet been fully assigned but the β subunit is thought to bind the α subunit to the E2 core (Yeaman and Reed, 1987).

E1 catalyses the decarboxylation of pyruvate in a reaction that is dependent on thiamine pyrophosphate (TPP). The product of this reaction, 2-(1-hydroxyethylidene)-TPP [$CH_3C(OH)$=TPP] remains tightly bound to E1. The mechanism of reductive transacetylation between E1-$CH_3C(OH)$-TPP and a lipoyl group to give TPP and S-acetyldihydrolipoamide is the subject of much discussion (Fig. 1.2). The debate centres on the involvement of acetyl TPP as a compulsory intermediate in the decarboxylation and dehydrogenation of pyruvate (Pathway A Fig. 1.2) (Das et al., 1961; Daigo and Reed, 1962). An alternative intermediate has been proposed in which the enamine $CH_3C(OH)$=TPP reacts as a carbanion with lipoamide to form the tetrahedral adduct in a single step (Pathway B, Fig. 1.2) (Breslow and McNelis, 1962; White and Ingraham, 1962). Recent data have not clearly demonstrated the mechanism involving reductive acetylation intermediates (Frey et al., 1989) and further experimentation is required to address these questions.

It was observed that phosphorylation inhibited the ability of the enzyme to catalyse the formation of the intermediate 2-(1-hydroxyethylidene)-TPP with [1-^{14}C] pyruvate as substrate, but not with [2-^{14}C] hydroxyethyl TPP as substrate (Roche and Reed, 1972). Since phosphorylation occurs on the E1 α subunit and not on the β subunit, it was suggested that the α subunit catalysed the formation of 2-(1-hydroxyethylidene)-TPP. However this proposal has been questioned by Walsh et al. (1976) who reported that phosphorylation of the pig heart enzyme inhibits NADH-dependent

transfer of acetyl groups from enzyme-linked acetyldihydrolipoamide to TPP. These results await clarification, perhaps when the isolated enzymatically active α and β subunits are available.

1.2.5 Lipoate acetyltransferase (E2)

The lipoate acetyltransferase is involved in a series of reactions which includes reductive acetylation, deacetylation and finally reoxidation of the covalently-bound lipoamide moieties. This is facilitated by the lipoyl-lysyl "swinging arms" visiting the active sites of one or more E1, E2 and E3 components. The lipoate acetyltransferase specifically catalyses the reversible transacetylation of the enzyme bound 8-acetyldihydrolipoamide with formation of acetyl CoA (Yang and Frey, 1986). The E2 component also catalyses the intramolecular transfer of acetyl residues between lipoic acid prosthetic groups of different E2 components in the same complex (Bates et al., 1977; Collins and Reed, 1977). A detailed account of the structure of E2 can be found in Section 1.5.3.

The amino acid sequences of lipoate acetyltransferase and the corresponding succinyltransferase of the 2-oxoglutarate complex in E. coli have been deduced from the nucleotide sequences (Stephens et al., 1983b; Spencer et al., 1984) and have been used to identify a possible active site.

Little is known about the mechanism of acetyl group transfer by E2. There is no evidence for acetylation of the enzyme at sites other than lipoyl domains. However it has been suggested that conserved histidine residues in E2 of PDC and E2 of OGDC could have a catalytic function because they are located in the most highly conserved segments of the two acyltransferases (Spencer et al., 1984). Much more is known about chloramphenicol acetyltransferase (CAT) which catalyses the O-acetylation (and

inactivation) of chloramphenicol in antibiotic resistant bacteria (Shaw, 1983; Kleanthous et al., 1985). A specific histidine residue that is preferentially modified by non-specific and active site directed inhibitors has been identified and this residue is thought to function as a general base in catalysis.

Comparisons between the amino acid sequences of lipoamide acetyltransferase and lipoamide succinylltransferase of E. coli and several chloramphenicol acetyltransferases (CAT) have revealed some well-aligned homologies which may be indicative of an underlying structural similarity between the catalytic (acetyltransferase) domain of E2 and CAT. The region containing the active-site histidine residue in CAT is particularly well conserved in each E2 and has led to a proposed catalytic mechanism, involving a histidine residue in each E2 as a likely active-site residue (Guest, 1987).

1.2.6 Dihydrolipoamide dehydrogenase (E3)

The regeneration of oxidised lipoic acid is promoted by dihydrolipoamide dehydrogenase, (E3) an FAD-linked homodimer. During the catalytic cycle, the tightly-bound FAD moiety becomes transiently reduced with final production of NADH. This enzyme also employs, as part of its reaction mechanism, the alternative oxidation and reduction of a disulphide bridge at its active site (Massey et al., 1960; Massey and Veeger, 1961). This disulphide bridge has provided a focus for chemical studies of the active site (Brown and Perham, 1972, 1974; Pai and Schulz, 1983; Arscott et al., 1981). It consists of a reactive cysteine disulphide, which functions in catalysis; the two half cysteines are separated in the polypeptide chain by only four residues and evidence for involvement of this group in catalysis comes from studies in the presence of arsenite and NADH, in which flavin is oxidised completely (Williams, 1976; Danson et al., 1984).

A similar flavoprotein to lipoamide dehydrogenase, glutathione reductase also catalyses a specific pyridine nucleotide-disulphide oxidoreduction (Williams, 1976). Both structural and mechanistic similarities have been extensively documented. Thus each contains a redox active-disulphide bond (Searls and Sanadi, 1960; Massey and Veeger, 1961; Massey and Williams, 1965) which is located in a highly conserved section of polypeptide chain (Jones and Williams, 1975). Two electron reduction of either enzyme produces a charge transfer complex between a thiolate anion (as the donor) and oxidised flavin (as the acceptor). A base in the active-site accepts a second proton (Mathews and Williams, 1976; Mathews et al., 1977). In catalysis, these enzymes cycle between the oxidised E1 and the two electron reduced EH_2 state (Massey et al., 1960).

Catalysis by lipoamide dehydrogenase involves a sequential flow of electrons from dihydrolipoamide to the active centre disulphide and on reoxidation the electrons pass very rapidly via the FAD to NAD^+ (Thorpe and Williams, 1974). In glutathione reductase the sequence is reversed, beginning with NADPH and ending with thiol disulphide interchange between the active centre dithiol and glutathione.

As with lipoamide dehydrogenase the two thiols produced upon two electron reduction of glutathione reductase are not chemically equivalent and are readily distinguishable following chemical modification. The thiol closer to the amino terminus is involved in thiol-disulphide interchange while the other interacts with FAD (Thorpe and Williams, 1976; Williams et al., 1976).

Site-directed mutagenesis and molecular modelling have been used to identify a set of amino acid side chains in glutathione reductase that confer specificity for the coenzyme $NADP^+$ (Scrutton

et al., 1990). Systematic replacement of these amino acids, all of which occur in the $NADP^+$ binding domains, leaves the substrate specificity unchanged but converts the enzyme into one displaying a marked preference for the coenzyme NAD^+. It is probable that similar experiments can now be performed to redesign the coenzyme specificity of any nicotinamide nucleotide-specific enzyme that contains a conserved dinucleotide binding domain.

1.2.7 Possible function of lipoamide dehydrogenase (E3)

Lipoamide dehydrogenase has been isolated from many sources and has been found to be remarkably resistant to heat inactivation, proteolysis and urea treatment. E3 isolated from E. coli and Saccharomyces cerevisiae strongly resemble the pig heart enzyme in size and amino acid composition (Williams, 1976; Henrich et al., 1983). Stephens and co-workers have found considerable homology between the predicted nucleotide sequence of E3 from E. coli and peptides from pig heart E3 (Stephens et al., 1983c). In fact exchange of E. coli E3 component with pig heart lipoamide dehydrogenase supports overall activity and assembly into a hybrid multienzyme complex (Perham, 1975; Guest, 1978).

Lipoamide dehydrogenase has been isolated from organisms e.g. Trypanosoma brucei which do not possess pyruvate dehydrogenase or 2-oxoglutarate dehydrogenase complexes (Fairlamb, 1982; Danson et al., 1987). A similar situation exists in halophilic (Danson et al., 1984, 1986), thermoautophilic and methanogenic (Danson, 1984) archaebacteria. This enzyme has been purified also from Halobacterium halobium (Danson et al., 1986) and it was found to be similar to its counterpart in non-archaebacterial species in subunit arrangement and in its catalytic and kinetic properties.

Lipoamide dehydrogenase isolated from T. brucei was found to be associated with the plasma membrane and such an attachment may suggest a function specific to that location (Danson et al., 1987). There is growing evidence that dihydrolipoyl dehydrogenase, in addition to its established role in 2-oxo acid dehydrogenase complexes, is implicated in sugar transport in E. coli (Richarme and Heine, 1986). Its possible function in other membranes remains entirely speculative. However, the view that many processes at the plasma membrane may involve disulphide-thiol interchanges serves to heighten the interest in the biological function of this enzyme with its catalytic redox-active disulphide bond (Danson, 1987).

1.3 Pyruvate dehydrogenase complex

The conversion of pyruvate to acetyl CoA catalysed by PDC serves both bioenergetic and biosynthetic roles. It is essentially an irreversible reaction in vivo, occupying a key position in cellular metabolism controlling the supply of acetyl groups in mitochondria arising from the oxidation of carbohydrates and amino acids (Fig. 1.3). In many tissues including heart,muscle, brain and kidney, the acetyl CoA is almost exclusively metabolised through the citric acid cycle whilst in other tissues such as adipose tissue, mammary gland and liver a significant proportion is utilised in the synthesis of fatty acids and sterols. In animals, acetyl CoA cannot be employed in gluconeogenesis and thus conversion of pyruvate to acetyl CoA represents a net loss of carbohydrate reserves. Regulation of this step is of critical importance to the general energy balance and fuel economy of the cell. To control the activity of this important enzyme a range of regulatory mechanisms has evolved ensuring appropriate control as detailed in Section 1.3.4.

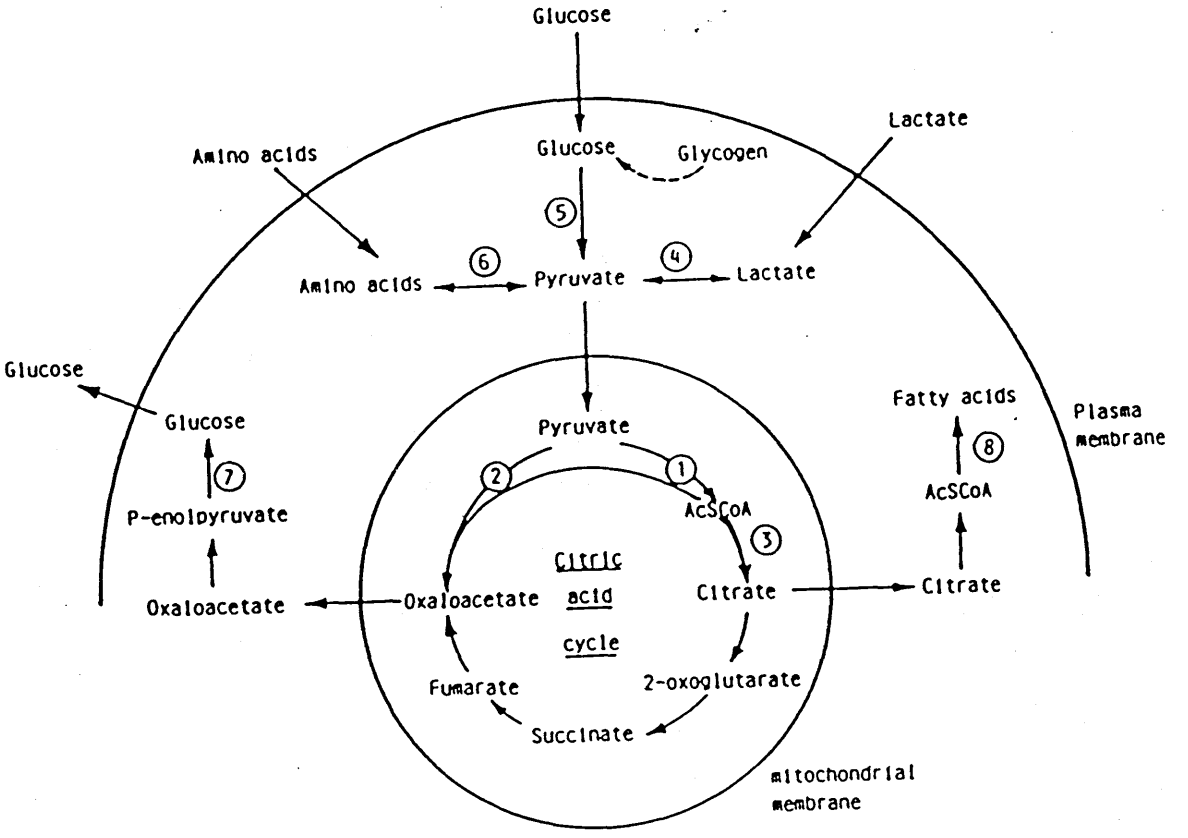


Figure 1.3 Major routes of pyruvate metabolism in mammalian cells

1, PDC; 2, pyruvate carboxylase; 3, citrate synthase;
4, lactate dehydrogenase; 5, glycolysis; 6, transamination;
7, gluconeogenesis; 8, lipogenesis.

1.3.1 Subunit composition of PDC from E. coli

Early research was carried out on the multienzyme complex purified from E. coli (Perham et al., 1987; Miles and Guest, 1987). The experience gained from studies on the PDC from prokaryotic sources has been of considerable value in stimulating parallel and subsequent research on mammalian PDC.

Analysis of purified PDC from E. coli by SDS gel electrophoresis (SDS-PAGE) reveals that it consists of three different types of polypeptide chain. Resolution of PDC from E. coli into its constituent enzymatic activities using a combination of gel filtration at high pH and chromatography on hydroxylapatite columns in 4M urea (Koike et al., 1963), permitted assignment of the specific functions of individual polypeptide chains: pyruvate dehydrogenase (E1) (a dimer, subunit M_r 100000), lipoate acetyltransferase (E2), (subunit M_r 80000) and lipoamide dehydrogenase (E3), a homodimer (subunit M_r 56000) (Perham and Thomas, 1971; Vogel and Henning, 1971; Eley et al., 1972). The M_r of the E2 chain estimated by sedimentation equilibrium in guanidinium-HCl is approximately 52000, suggesting that the value of M_r 80000 obtained by SDS gel electrophoresis is anomalous (Eley et al., 1972; Hayakawa et al., 1969; Linn et al., 1972).

More recently gene sequence analysis of the pyruvate and 2-oxoglutarate complexes of E. coli have been reported (Stephens et al., 1983a,b,c; Spencer et al., 1984; Packman et al., 1984). These studies have resolved many of the controversial features regarding size and number of lipoyl domain structures of the acetyltransferase and have confirmed that the apparent M_r for the E2 component as inferred from SDS-PAGE was significantly overestimated. The molecular genetics of the 2-oxo acid complexes will be discussed more fully in Section 1.6.

Table 1.2 Subunit composition of bovine heart pyruvate dehydrogenase complex

Enzyme	M_r	Subunits No	Subunits M_r	Subunits per molecu of complex
Native Complex	8,500,000			
E_1	154,000	4		
$E_1 \alpha$		2	42,000	60
$E_1 \beta$		2	37,000	60
E_2	3,100,000	60	52,000	60
E_3	110,000	2	55,000	12
Kinase	100,000	1	47,000	
		1	45,000	
Phosphatase	150,000	1	97,000	
		1	50,000	
Protein X	51,000	1	51,000	8-12

The above table is adapted from Reed and Pettit (1981) and Stepp et al. (1983)

1.3.2 Subunit composition of mammalian PDC

PDC isolated from bovine kidney and heart have M_r values approximately 7.0×10^6 and 8.5×10^6, respectively (Linn et al., 1972). The M_r values of the various polypeptide chains and the proposed stoichiometry of the bovine heart complex are presented in Table 1.2. The E1 component consists of non identical subunits α, M_r 42000, and β, M_r 37000, which form $\alpha_2\beta_2$ tetramers of M_r 154000. The core enzyme consists of 60 identical polypeptide chains of M_r 52000. E3 has an overall M_r 110000, comprising two identical subunits, M_r 55000, each containing a molecule of FAD. The stoichiometries of PDC kinase and phosphatase are not exactly known. The kinase and phosphatase have been purified to homogeneity and each of these enzymes consists of two subunits which have M_r values of 47000 and 45000 for the kinase (designated α and β respectively) and 97000-110000 and 50000 for the phosphatase (Pratt et al., 1982; Stepp et al., 1983).

1.3.3 Structure and function of PDC

The major contributor to organisation and catalytic efficiency of the respective 2-oxo acid complexes is their specific E2 components. This enzyme serves three major roles: (1) it forms a central symmetrical core; (2) it is an acyltransferase catalysing the formation of the acyl CoA product and (3) it provides attachment sites for lipoic acid prosthetic groups which are required to interact with the different active sites of all the constituent enzymes of the complex.

A number of researchers (Hale and Perham, 1979; Bliele et al., 1979; Kresze et al., 1980; Bliele et al., 1981) have demonstrated using limited proteolysis, that the E2 component consists of several functional domains (Fig. 1.4). These include a

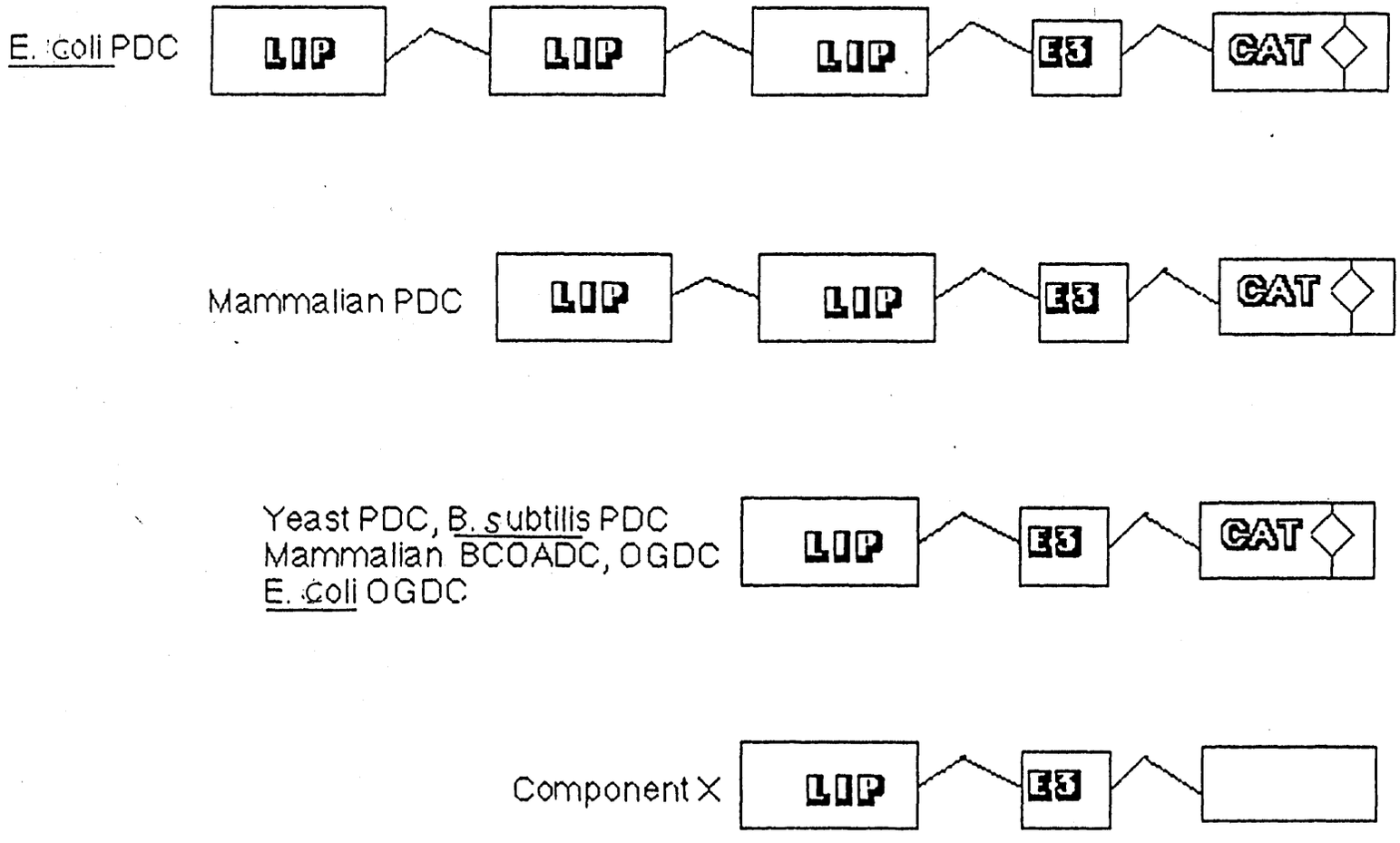


Figure 1.4 Schematic diagram of domain structure of the E2 components, LIP indicates the lipoate-containing domains, E3, the E3 binding region and CAT, the catalytic core domain. The linker regions are indicated by zigzags and the diamonds indicate the approximate positions of the putative active sites.

compact inner catalytic domain which, in addition to containing the active site, binds to other E2 catalytic domains to maintain the central core and is also responsible for binding the E1 and E3 components of the complexes.

The second type of domain is an extended, outer domain which contains the lysine group to which the lipoic acid moiety is attached. The lipoyl-bearing domains apparently interdigitate between the E1 and E3 components. Each E2 also contains a distinct region of polypeptide which is responsible for binding the E3 component (Packman and Perham, 1986). These different domains are joined by linker regions of polypeptide, which vary in length and are characterised by being rich in proline and alanine residues (Stephens et al., 1983b). ^{1}H nmr spectroscopy has shown these linker regions to be highly flexible, giving characteristic sharp resonances (Perham et al., 1981, Packman et al., 1984) which are diminished in genetically engineered complexes lacking one or more tandemly arranged lipoyl domains and their associated linker regions (Radford et al., 1987).

The complexes vary in the number of lipoyl domains which are contained within the E2 polypeptide. E2 from PDC of *E. coli* is remarkable in that it contains three highly conserved lipoyl domains, located in tandem repeat at the N-terminus of the polypeptide (Stephens et al., 1983b). Surprisingly deletion of two of the three lipoyl domains still allows assembly of a functional complex with full catalytic activity (Guest et al., 1985). Clearly additional domains are not required for the catalytic function of the complex, posing an interesting question as to the function of the two additional lipoate domains.

Table 1.3 Primary structure surrounding the lipoyl-lysine residues in proteins

Source	Enzyme					*										
Rat, Bovine	PDC E2	V I	E	T	D	K	A	T	V I	G	F	E	V	Q	E	E	G
Bovine	OGDC E2	I	E	T	D	K	T	S	V	Q	V	P	S	P	A	N	G
Bovine Human	BCOADC E2	V	Q	S	D	K	A	S	V	T	I	T	S	R	Y	D	G
E. coli	PDC E2	V	E	G	D	K	A	S	M	E	V	P	A S	P	F Q	A	G
E. coli	OGDC E2	I	E	T	D	K	V	V	L	E	V	P	A	S	A	D	G
Chicken	Glycine-cleavage H-protein	L	E	S	V	K	A	A	S	E	L	Y	S	P	L	T	G

The above table was adapted from Yeaman (1989).

Ox kidney PDC has been reported to possess two lipoic acid moieties per E2 chain (Cate and Roche, 1979) although earlier conflicting observations suggested the presence of only a single lipoic acid group (White et al., 1980; Hamada et al., 1975). Recent evidence suggests that the E2 polypeptide of mammalian PDC contains two functional lipoate residues (Hodgson et al., 1988). This has been demonstrated by cross-linking studies using phenylene bis-maleimide in the presence of 2-oxo acid substrate to form dimers, trimers and higher aggregates between lipoate residues, whereas E2 of OGDC is only capable of forming dimers consistent with the presence of a single lipoate residue. The observation of the formation of dimers, trimers and higher aggregates is best explained by the presence of two or more lipoate groups. Furthermore the predicted protein sequence of a partial cDNA clone of rat liver PDC E2 (Gershwin et al., 1987) is consistent with the possible presence of a second lipoate domain. More recently a full-length cDNA clone for E2 from human liver pyruvate dehydrogenase has been isolated and sequenced (Thekkumkara et al., 1988) which has confirmed the presence of two homologous lipoyl domains (Coppel et al., 1988).

The sequence surrounding the lipoate attachment site has now been determined from several bacterial and mammalian complexes (Stephens et al., 1983b; Spencer et al., 1984; Bradford et al., 1987; Hummel et al., 1988). The lipoate region is highly conserved as illustrated in Table 1.3.

Comparison of the lipoate acetyltransferase component of E. coli with mammalian acetyltransferase using electron microscopy reveals two polyhedral forms of the E2 component, the cube and the dodecahedron, both designs based on cubic point group symmetry.

The E2 component of pyruvate and 2-oxoglutarate dehydrogenases of E. coli and mammalian 2-oxoglutarate dehydrogenase have cubic design. The acetyltransferase and succinyltransferase components are similar in size and cube-like appearance as demonstrated by sedimentation equilibrium analysis and electron microscopy (Reed and Oliver, 1968).

1.3.4 Lipoyl domains of PDC not associated with E2

A recently discovered polypeptide of mammalian PDC has been the subject of intense investigation in recent years (De Marcucci and Lindsay, 1985; Jilka et al., 1986). This polypeptide, component X, has a close physical and functional union with the E2 core structure. In similar fashion to E2, component X can be reductively acetylated in the presence of E1 and is rapidly deacetylated in the presence of CoASH (De Marcucci et al., 1986). It can also be modified by N-ethylmaleimide (NEM) only in the presence of pyruvate or NADH. PDC was treated with N-ethyl [2,3-^{14}C] maleimide in the presence of pyruvate and gel purified component X was subjected to acid hydrolysis. The radiolabelled products were resolved on an amino acid analyser and found to co-elute with the product from similarly modified and hydrolysed lipoate acetyltransferase confirming the [^{14}C] acetylated group on component X as a lipoyl moiety (Hodgson et al., 1988).

1.3.5 Design of 2-oxo acid dehydrogenase complexes

The structural organisation of the 2-oxo acid dehydrogenase complexes can now be considered. Is there any correlation between the number of lipoyl domains per E2 chain and the symmetry of the E2 core? (Table 1.4). PDC and OGDC have three lipoyl and one lipoyl

Table 1.4

Some Principal Features of the Structure and Assembly of the 2-Oxo Acid Dehydrogenase Complexes

Organism	Gram Stain	Pyruvate Dehydrogenase Complex			2-Oxoglutarate Dehydrogenase Complex			Branched-Chain 2-Oxo Acid Dehydrogenase Complex		
		E2 Core	No. of Lipoyl Domains[a]	E1 Chains	E2 Core	No. of Lipoyl Domains	E1 Chains	E2 core	No. of Lipoyl Domains	E1 Chains
Escherichia coli	–	Octahedral	3	Single	Octahedral	1	Single			
Salmonella typhimurium	–	Octahedral	(3)	Single						
Azotobacter vinelandii	–	Octahedral	3	Single	Octahedral[b]	1	Single			
Pseudomonas spp.	–	n.k.[c]	n.k.[c]	Single	n.k.[c]	n.k.[c]	n.k.[c]	n.k.[c]	1	Split
Bacillus stearothermophilus	+	Icosahedral	1	Split				Icosahedral	1	Split
Bacillus subtilis	+	Icosahedral	1	Split	n.k.[c]	n.k.[c]	Single	Icosahedral	1	Split
Neurospora crassa		Icosahedral	(1)	Split						
Saccharomyces spp.		Icosahedral	1	Split	Octahedral[d]	n.k.[c]	n.k.[c]			
Mammals (rat, ox, pig, human)		Icosahedral	2	Split	Octahedral	1	Single	Octahedral	1	Split

[a]Numbers in parentheses denote number of lipoyl domains inferred from the apparent molecular mass of the E2 component on sodium dodecyl sulfate–polyacrylamide gels.

[b]Assumed symmetry based on the similarity of the complex to those of *E. coli* and pig, whose symmetries are known;

[c]n.k., not known.

[d]Tentative assignment;

The above table is adapted from Perham and Packman (1989)

domain per E2 chain respectively, although both have E2 cores of octahedral symmetry. The ability to create, by *in vitro* genetic manipulation of the *ace F* gene, active *E. coli* PDC with one, two or three lipoyl domains means that the symmetry is not limited by the number of lipoyl domains per E2 chain. Similarly, mammalian PDC, the Gram positive organism *B. stearothermophilus* and yeast enzymes contain one lipoyl domain, whereas human E2 contains two. However the E2 chain of *Azotobacter vinlandii*, which is Gram-negative like *E. coli*, also contains three lipoyl domains. As more E2 sequences are determined it will be interesting to see if correlation is at least sustained for Gram positive and Gram negative organisms. Significant homologies, however, indicate that the octahdral and icosahedral core forming E2 chains of prokaryotes and eukaryotes have arisen by divergent evolution from a common ancestor.

It is apparent from Table 1.4 that PDC from Gram positive organisms and eukaryotes are similar in symmetry and subunit structure and distinguishable from PDC complexes of Gram negative organisms in both regards. Thus the former have E2 cores of icosahedral symmetry and E1 components composed of E1 α and E1 β subunits whereas the latter have E2 cores of octahedral symmetry and an E1 component composed of a single polypeptide chain.

Normal association of E1 α and E1 β chains with an E2 core of icosahedral symmetry does not extend to BCOADC. In *B. subtilis*, the BCOADC possesses an icosahedral E2 core with associated E1 $\alpha_2\beta_2$ tetramers while mammalian BCOADC has an E2 of octahedral symmetry with E1 α and E1 β subunits. With increased detail of the principal features of the structure and assembly of 2-oxo acid complexes from other organisms a clear picture may emerge of the divergence from a common evolutionary ancestor (Perham and Packman, 1989).

1.3.6 Regulation of mammalian PDC

Mammalian PDC is subject to product inhibition by acetyl CoA and NADH with reversal of inhibition occurring in the presence of CoASH and NAD^+ respectively (Garland and Randle, 1964; Tsai et al., 1973). Mammalian PDC is also regulated by ATP-dependent phosphorylation (Reed, 1974; Denton et al., 1975) in which the E1α subunit (M_r 42000) is covalently modified by a specific PDC kinase, with concomitant loss of overall complex activity. In pig heart PDC (Sugden et al., 1978), ox heart and kidney PDC (Yeaman et al., 1978), inactivation is correlated with the phosphorylation of a specific serine residue in the E1 α chain. A specific phosphatase which is only loosely associated with the complex, exists to remove the phosphate group and restore activity (Reed, 1974). The activities of the PDC kinase and PDC phosphatase enzymes are regulated by various metabolites. The kinase is activated by Mg^{2+}, acetyl CoA and NADH and is inhibited by ADP, pyruvate and CoASH. The phosphatase is activated by Mg^{2+} and Ca^{2+} and inhibited by NADH (Reed et al., 1980) (Fig. 1.5). Ca^{2+} also inhibits kinase activity (Denton et al., 1975). Recently it has been postulated that PDC phosphatase, in the presence of E2, possesses an additional calcium binding site. It is possible that the second site is at the interface between the phosphatase and E2, with Ca^{2+} acting as a bridging ligand for specific attachment of the phosphatase to E2 or alternatively a conformational change may be produced in either enzyme, thus producing the second Ca^{2+} binding site. Favourable topographical positioning of the phosphatase and phosphorylated E1 on E2 apparently facilitates the Mg^{2+} dependent dephosphorylation (Reed and Yeaman, 1987).

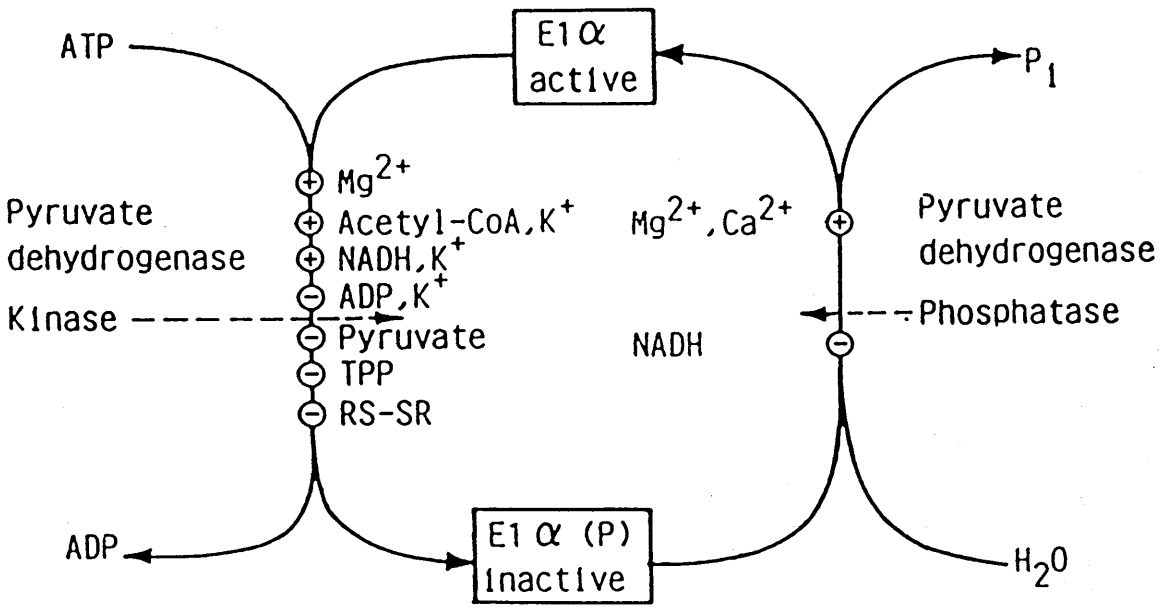


Figure 1.5 Schematic representation of the covalent modifications of pyruvate dehydrogenase and its regulation by various metabolites (Reed et al., 1980)

The steady state activity of the complex is sensitive to the ratios [ATP]/[ADP], [AcSCoA]/[CoA] and [NADH]/[NAD^+]. Pyruvate dehydrogenase and its two converter enzymes, kinase and phosphatase, comprise a monocyclic interconvertible enzyme cascade. The mechanism whereby acetyl CoA, CoASH, NAD^+ and NADH modify the activity of the kinase is controversial. It is possible that the kinase or its substrate has regulatory binding sites for these effectors but equally it is possible that these compounds act through binding to their substrate sites on E2 and E3. In view of the importance of the attachment of the kinase to the acetyltransferase core assembly for kinase activity, the mechanism of modification of the kinase by E2 may cause conformational changes on the kinase effecting the regulatory properties of this subunit.

A novel mechanism has been proposed whereby lipoate may act as a transmitter of regulatory interactions. This suggestion was originally made by Kerbey *et al.* (1976) and subsequently supported by Cate and Roche (1978). These latter authors have in a series of articles postulated a mechanism in which the regulatory effects on PDC kinase are mediated through changes in the acetylation and the oxidation reduction states of the E2 lipoyl moieties but, with the discovery of component X, they conclude that these changes may also be mediated by the lipoyl groups on component X (Jilka *et al.*, 1986). Reduction of lipoyl moieties bound to E2 appears to activate the kinase and acetyl CoA stimulation of the kinase requires acetylation of the lipoyl groups (Roche and Cate, 1977). The activating effects of low pyruvate concentrations in the presence of TPP could similarly be explained in terms of acetylation or reduction of lipoyl groups (Cate and Roche, 1978, 1979). However, this suggestion is in contradiction to the findings of Reed *et al.* (1980). With highly-purified pyruvate

dehydrogenase kinase and its dephosphotetradecapeptide substrate, it was demonstrated that the rate of phosphorylation was stimulated by acetyl CoA and NADH and inhibited by ADP and pyruvate. As E2 was absent in the preparation of the enzyme, these results indicate that the effectors can act directly on the kinase and not via lipoyl moieties bound to E2.

1.3.7 Sites of phosphorylation

During ATP-induced inactivation of pyruvate dehydrogenase complex phosphorylation occurs at three serine residues in the E1 subunit of the pyruvate dehydrogenase (E1) component (Yeaman et al., 1978; Sugden et al., 1979). Three sites of phosphorylation on the α subunit have been identified by tryptic digestion of ^{32}P-labelled PDC from bovine kidney and heart, a small monophosphorylated peptide containing site 1, a diphosphorylated tetradecapeptide (sites 1 and 2) and a monophosphorylated nonapeptide (site 3). Sites 1 and 2 are located in the same region of the peptide chain (Fig. 1.6).

Mammalian BCOADC is also subject to regulation by phosphorylation/dephosphorylation (Reed and Yeaman, 1987) but unlike PDC has only two serine residues on the E1 α subunit which undergo phosphorylation. As with PDC, phosphorylation of site 1 in BCOADC is responsible for inactivation of the complex. The role of the additional sites of phosphorylation on PDC and BCOADC has still to be firmly established.

Phosphorylation at site 1 in PDC occurs most rapidly and closely parallels the inactivation of the complex (Sugden et al., 1978; Yeaman et al., 1978). Phosphorylation at sites 2 and 3, in addition to site 1, markedly inhibit the rate of reactivation of

Figure 1.6 Phosphorylation sites on the E1α subunit of pyruvate dehydrogenase

Site 1 Site 2

TYR-HIS-GLY-HIS-SER(P)-MET-SER-ASP-PRO-GLY-VAL-SER(P)-TYR-ARG

Site 3

-TYR-GLY-MET-GLY-THU-SER(P)-VAL-GLU-ARG

From Yeaman and Reed,(1987)

complex by PDC phosphatase (Sugden et al., 1978; Kerbey and Randle, 1979). Partially phosphorylated complex is reactivated more rapidly than fully phosphorylated complex. On this basis these authors have proposed a role for multi-site phosphorylation as a mechanism for regulating the conversion of inactive complex into active complex (Randle et al., 1981).

This proposal has been criticised by Reed and his colleagues (Reed et al., 1980). From an observation by Teague et al. (1979), they concluded that the presence of phosphate groups on sites 2 and 3 in bovine kidney did not significantly affect its rate of reactivation by PDC phosphatase. These authors obtained evidence that dephosphorylation on sites 1, 2 and 3 occurred at different rates and relative rates of dephosphorylation were in the order site 2, site 3, site 1. They also found that phosphorylation of site 2 functions as well as phosphorylation of site 1 in inactivating the enzyme while phosphorylation at site 3 did not cause inactivation. The physiological significance of phosphorylation at site 2 is unknown. Phosphorylation of E1 results in essentially total loss of its enzymatic activity. No allosteric activators of the phosphorylated enzyme have been been reported (Hucho et al., 1972).

1.4 Component X

In mammalian pyruvate dehydrogenase complex, a distinct polypeptide of unknown function has been observed in recent years. Although this component had been noted previously (Stanley and Perham, 1980), it was considered originally to be a degradation product of E2 or possibly the associated PDC kinase activity.

The presence of this polypeptide may be unique to eukaryotic pyruvate dehydrogenase as no similar polypeptide has been detected in the analogous 2-oxo acid complexes, 2-oxoglutarate dehydrogenase and the branched-chain 2-oxo acid dehydrogenase. A similar polypeptide is present in yeast PDC (Kresze and Ronft, 1981). Although the relationship between component X of mammalian PDC and the polypeptide observed in yeast has not been confirmed conclusively, several similarities between the two polypeptides suggest that they may be functionally equivalent.

Incubation of pyruvate dehydrogenase complex with [2-^{14}C] pyruvate promotes reductive acetylation of covalently-bound lipoyl residues on the acetyl transferase (E2) core structure while apparently acetylating also an additional minor species of M_r 51000 (Bleile et al., 1979, 1981). Rapid deacetylation of acetyltransferase (E2) and the M_r 51000 polypeptide on the addition of CoASH gave rise to the belief initially that the polypeptide was a degradation product of E2.

1.4.1 Immunological studies on component X

The initial indicator that component X was a distinct polypeptide came from immunological studies. Production of subunit specific antisera in conjunction with sensitive immune replica and immune mapping studies (De Marcucci and Lindsay, 1985) indicated that component X was an immunologically distinct polypeptide.

Subunit-specific antisera to component X showed no cross reactivity with E2 as antigen and vice versa. In contrast antibodies prepared to component X by Roche's group (Jilka et al., 1986) did detect some cross-reactivity with E2 which was subsequently absorbed out to recognise only component X antigen. It is therefore, unlikely, that component X is a degradation product of E2.

Further confirmation of the distinct nature of component X came from immune mapping studies. Immune mapping permits the sensitive and specific detection of proteolytic fragments derived from a single subunit. For example only proteolytic fragments of E2 will be recognised even in the presence of a variety of cleavage products from the other components of the multienzyme complex. It was apparent that after digestion with elastase the E2 component degrades to two major peptides of M_r 45000 and M_r 42000 which are largely resistant to further digestion although after a longer time period a third, immunologically less reactive, M_r 29000 species is observed. Similar fragments have been observed previously (Kresze and Steber, 1979) by Coomassie blue staining after elastase digestion of purified E2 and PDC. These authors reported that the M_r 42000 fragment contained the lipoyl binding domain while the M_r 29000 fragment corresponds to the intersubunit binding region of E2. In contrast component X-derived fragments have M_r values of 29000 and 26000 which are cleaved rapidly to smaller peptides of M_r 15000 and below, the M_r 15000 peptide possibly containing the lipoyl binding domain of component X (Hodgson et al., 1986; Rahmatullah et al., 1989).

1.4.2 Peptide mapping

In separate studies De Marcucci et al. (1986) provided further independent evidence from one dimensional peptide 'maps' that E2 and X were structurally distinct polypeptides.

Two dimensional peptide mapping studies with ^{125}I-labelled and ^{14}C-labelled E2 and X polypeptides also demonstrate that the proteins are structurally distinct (Jilka et al., 1986). For example component X has been shown to produce two major fragments

M_r 30000-27000 and 28000-25000 which are not observed with limited proteolysis of E2. Limited proteolysis of E2 does not reveal an M_r 51000 fragment confirming that the M_r 51000 polypeptide is not a degradation product of the lipoate acetyltransferase M_r 70000 (De Marcucci et al., 1986).

1.4.3 Mitochondrial location of component X

Immune blotting studies of a variety of cellular extracts from Buffalo rat liver (BRL) bovine kidney (NBL-1) and pig kidney (PK-15) cells as well as purified mitochondrial, nuclear and cytosolic fractions of (BRL) cells have revealed that component X is a normal cellular component confined to the mitochondrial compartment. The mitochondrial localisation of protein X was further investigated. At high levels of digitonin, mitochondrial disruption of the inner membrane is induced with the concomitant release of inner membrane enzymes into the supernatant. It was observed that components of PDC were released into the soluble fraction only when the concentration of digitonin was high. From this it was concluded that component X is confined exclusively to the mitochondrial compartment, supporting the idea it is associated with PDC in intact cells and not an unrelated protein which is bound adventitiously to the complex during its isolation (De Marcucci, O. unpublished results).

One possibility considered initially was that component X is an isoenzyme of E2 as the site of acetylation on component X like on E2, is lipoic acid. In this respect, there could be two subpopulations of PDC arising from complexes containing either E2 or X core subunits.

However, at present, immunological inspection of PDC from several cell lines and rat tissues does not favour that idea, as component X shows no apparent preferential distribution between these cell types (Hodgson et al., 1988).

Although component X can be acetylated in the presence of pyruvate and deacetylated on the addition of CoASH, component X does not appear to have acetyltransferase activity (Jilka et al., 1986; Behal et al., 1989). Antisera prepared against the dihydrolipoyl acetyltransferase core was used to identify regions of acetyltransferase activity on E2 and component X. Failure of the acetyltransferase antiserum to detect component X was taken to be indicative that component X lacked acetyltransferase activity. This observation has been confirmed by nucleotide sequencing of component X from Saccharomyces cerevisiae (Behal et al., 1989).

1.4.4 Cross reactivity of anti-component X IgG with a 48000 M_r protein in the cytosolic fraction of BRL cells

Subcellular fractions obtained after fractionation by differential centrifugation (Attardi and Ching, 1979) and analysed by immunoblotting with antisera to component X revealed that the antiserum cross-reacted with a M_r 51000 polypeptide present in whole cells and mitochondrial fractions - protein X. An additional stronger cross reacting antigen of M_r 48000 was also observed in whole cell extracts and in post nuclear supernatant fractions but was absent from mitochondrial fractions. It has not yet been established if this antigen is a strongly cross reacting species or a major contaminant from the original X antigen preparation from the SDS gel (Neagle et al., 1989).

1.4.5 Association of component X with E2 core

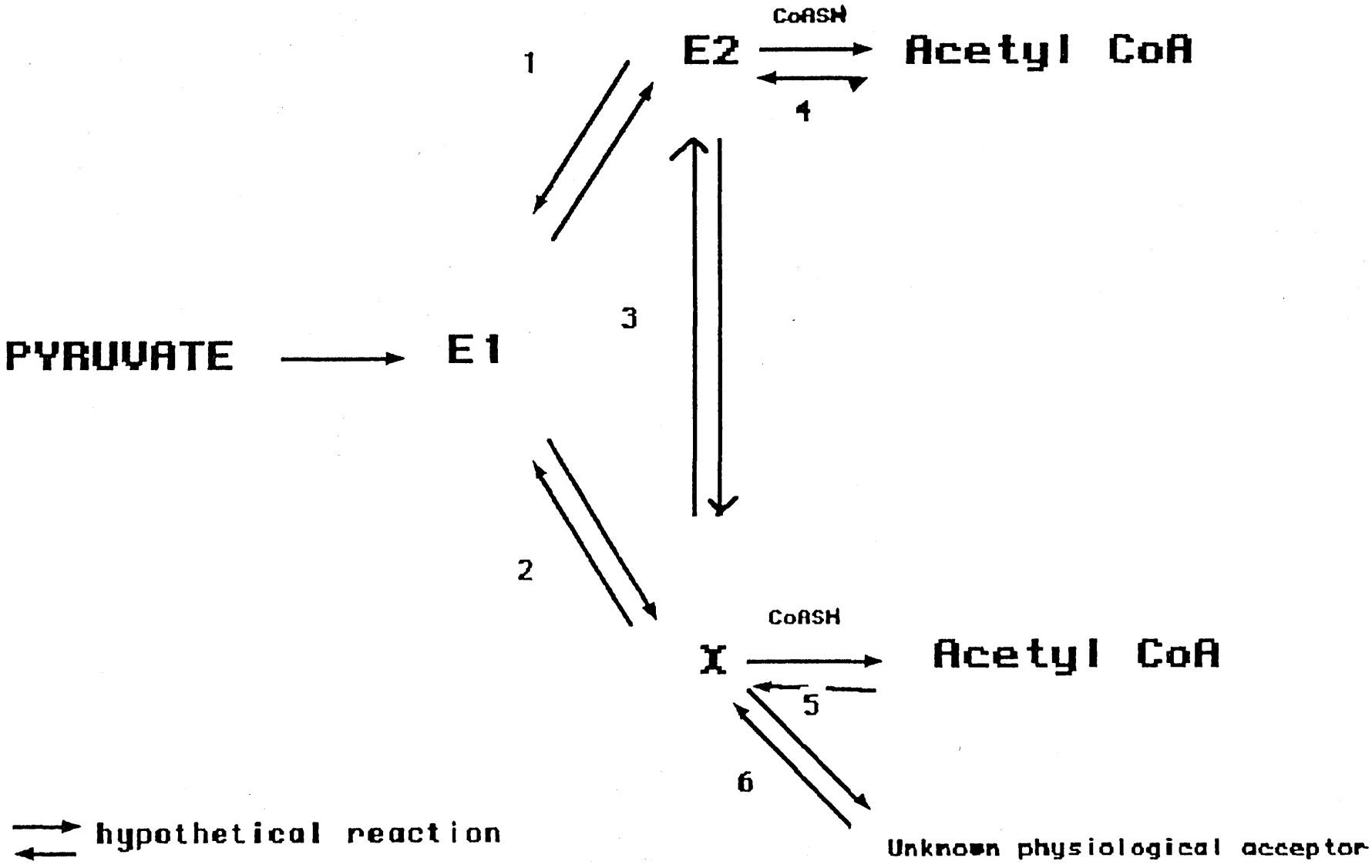
Further evidence for the nature of the association of component X with PDC is found when the intact complex is dissociated in 1M salt at alkaline pH (Linn et al., 1972), or 0.25M $MgCl_2$ (Kresze and Steber, 1979). Under these conditions the E2 core remains intact with an overall M_r value of 3.1×10^6 while homodimers of E3 (M_r 110000) and active tetramers of E1 (M_r 154000) are released from the complex. It is apparent the M_r 51000 polypeptide, component X, stays tightly associated with E2 on dissociation of the multienzyme aggregate.

Specific binding of component X IgG to native PDC has indicated also that there was at least some region of the component X subunit exposed at the periphery of the multienzyme complex. It was also observed that binding of antibody to component X caused little or no inhibition of overall complex activity (De Marcucci, O. and Lindsay, J.G. unpublished results) or on the rate of limited proteolysis by a specific protease (Neagle, J. unpublished results). Therefore the antibody has not proved useful for elucidating the function of X.

1.4.6 Cross linking of lipoyl groups on PDC

Studies employing electron microscopy have shown that the lipoyl domains on E2 subunits of PDC are located on peripherally extended regions of polypeptide (Bliele et al., 1979) and analysis by proton NMR has revealed these regions to be highly mobile relative to other parts of the complex (Wawrzynczak et al., 1981). This mobility would allow interaction of the lipoic acid at spatially distant active sites during the catalytic sequence. Pyruvate induced S-acetylation of lipoyl groups on PDC generates a thiol on the adjacent sulphur atoms of the dithiolane ring which is then susceptible to modification by maleimides (Brown and Perham, 1976).

Figure 1.7 Possible functions of component X in mammalian PDC



Reductive acetylation followed by reaction with phenylene-O bismaleimide has been used to achieve efficient cross-linking of the E2 subunits in PDC from E. coli via their covalently-attached lipoic acid residues (Collins and Reed, 1977).

Immunoblotting of cross-linked subunits of mammalian PDC with anti-X serum revealed that a number of aggregates containing X subunits were formed during cross-linking. A cross-linked species with an apparent M_r of approximately 105000, containing only component X was most probably a homodimer of X. Two higher M_r species were observed in which cross-linked X co-migrated with E2 cross linked products. Their estimated M_r values of 182000 and 127000 were consistent with the composition for these aggregates of E2/E2/X and E2/X respectively.

These results indicate that component X is exposed on the surface of the E2 core and the lipoyl domains of E2 and X interact in all possible combinations (Hodgson et al., 1988). An interesting observation was that no higher aggregates of component X, M_r 150000 and M_r 200000 were observed. This observation may indicate that component X has a single lipoyl domain and may exist as a dimer.

1.4.7 Stoichiometry of component X

The rate of incorporation of [2-^{14}C] pyruvate into E2 and component X is very rapid. (Estimates of incorporation of acetyl groups into E2 and X can be obtained by excising the bands of E2 and X after resolution by SDS-PAGE.) Measurement of incorporation of [2-^{14}C] pyruvate into E2 and X indicate that component X contains 16 $\pm$ 2% of the radiolabel bound to E2(Neagle, J. unpublished results). This value would allow for an estimated 8-12 molecules of component X per E2 'core' which approaches the levels of E3 in the complex and argues against its possible role as a regulatory component of the complex (De Marcucci and Lindsay, 1985).

However, recent evidence suggests that E2 contains two lipoyl domains per E2 chain (Hodgson et al., 1988; Cate and Roche, 1979). This value would suggest 16-24 molecules of component X per E2 'core'. Although two lipoyl domains per E2 chain may be present they may not be fully occupied with lipoyl groups which could explain the observations of one lipoyl group per E2 subunit (Barrera et al., 1972; White et al., 1980).

The data described above is only an estimate of the levels of incorporation of substrate into E2 and X. In E. coli, PDC, 0.5-0.7 moles of acetylating substrate is incorporated into each lipoyl domain on E2. This result led Perham and co-workers to the incorrect conclusion that there were two lipoyl domains per E2 chain (Danson and Perham, 1976). A similar result is observed with mammalian PDC. E2 loads a total of approximately 2 moles of acetylating substrate per E2 polypeptide. This result is much lower than anticipated, considering the complex's ability to diacetylate E2 and X, four moles of acetylating substrate would be expected to be incorporated into E2.

A more rigorous determination of incorporation of substrate into E2 and X needs to be performed before an accurate assessment can be made of the stoichiometry of component X in the complex.

1.4.8 Possible functions of component X in mammalian PDC

Inactivation of PDC by phosphorylation results in a parallel decrease in the rate of acetylation of E2 and X indicating that the latter protein can participate in the acetylation/ deacetylation reactions of the complex. This result suggests that the reductive acetylation of protein X must proceed through the formation of 2-hydroxyethylidene on the E1 component. It is not clear if X acts as an intermediate in this process i.e. if E1 can transfer acetyl

groups directly onto component X and then pass them onto E2, reaction 2, 3 and 4 (Fig. 1.7) where it is an essential intermediate of the catalytic process. Alternatively, X may be an associated enzyme in equilibrium with E2 reactions 1, 3 and 4 (Fig. 1.7). Component X may be directly involved in transferring acetyl groups onto CoASH, reaction 5 (Fig. 1.7) or onto other unknown physiological acceptors reaction 6 (Fig. 1.7).

Roche and coworkers (1978) originally advanced the idea that PDC may be regulated by the degree of acetylation of the complex. In support of this concept they have shown pyruvate dehydrogenase kinase activity to be stimulated at low levels of acetylation of the complex. They have suggested the involvement of a few lipoyl groups in this effect and suggested that it is the participation of the lipoyl group from protein X which functions as a regulatory subunit of the kinase (Cate and Roche, 1978, 1979; Rahmatullah and Roche, 1987).

More recently Roche and co-workers have abandoned this idea in favour of a role for component X in binding E3 to the E2/X core. They have demonstrated that protease argC preferentially degrades component X in the absence of the E3 component Rahmatullah et al., 1989) and it is argued that failure of the E3 component, but not E1, to bind to protease argC-digested core suggests that component X is involved in the association of the E3 component (Gopalakrishnan et al., 1989). Further evidence from this group argues that in intact complex the E3 component protects component X from proteolysis with argC and proteolysis of component X in intact complex parallels the loss of enzymatic activity.

A procedure for separating component X from the E2 core has now been described (Powers-Greenwood et al., 1989). In conditions of high urea and high salt concentrations an E2 oligomer, free of

component X, can be purified by elution from a Sephacryl S-400 column equilibrated in the same chaotropic reagents. The E2 oligomer retains acetyltransferase activity and is also able to bind the El component of pyruvate dehydrogenase complex. A reduced binding of the E3 component to the E2 oligomer is observed supporting the idea that protein X is involved in the binding of E3.

1.5 Eukaryotic gene expression

Until the last decade, classical and molecular genetic approaches were largely confined to the simpler prokaryotic systems. With the introduction of recombinant DNA technology, the development of appropriate plasmid and phage vectors and engineered bacterial hosts which are able to accept non-bacterial DNA, advances in mammalian genetics have made great strides in recent years.

1.5.1 Recombinant DNA technology

Recent progress in molecular biology has made it possible to alter the genetic information in bacterial cells by inserting non-bacterial DNA, thereby producing non-bacterial proteins. These bacteria contain recombinant DNA, i.e. they contain along with their own genes part or all of a gene from an animal cell. It is now possible to isolate a single gene from a mammalian cDNA or genomic library and insert into a suitable bacterial host. As these bacteria multiply they produce millions of copies of their own genes and of the inserted animal gene. If the animal gene is fused in such a way that a bacterium can treat the gene as its own the bacterium will produce the protein specified by the animal gene - a fusion protein. The protein produced by the recombinant DNA can be recognised by screening the plaques with antibodies raised against the animal protein. A more detailed study of screening, isolation and purification of plaques can be found in Section 6.6.6.

1.5.2 Molecular genetics of mammalian PDC

Recently several papers have been published reporting the molecular cloning and sequence analysis of cDNAs encoding all or part of the constituent polypeptides of the eukaryotic 2-oxo acid dehydrogenase complexes.

Nucleotide sequence of cDNA molecules encoding all or part of the E2 components of mammalian PDC and BCOADC has been reported recently (Gershwin et al., 1987; Hummel et al., 1988; and Lau et al., 1988). In rat liver PDC, the E2 polypeptide shows a high degree of similarity both with its counterparts from E. coli and with the E2 component of human and bovine BCOADC (Gershwin et al., 1987). The E2 clone from rat liver apparently encodes most of the polypeptide but is lacking the majority of the N-terminal lipoyl domain and a small C-terminal segment of the polypeptide. The complete nucleotide sequence for the dihydrolipoamide acetyltransferase component of human PDC has been obtained (Thekkumkara et al., 1988; Coppel et al., 1988). The presence of an amino terminal lipoyl domain and a carboxy terminal catalytic domain are confirmed. The lipoyl-bearing domain contains two repeating units, each harbouring a lipoic acid-binding lysine. Thus, mammalian PDC E2 component differs as to the number of lipoic acid binding sites from other E2 components from prokaryotic and eukaryotic organisms. A dihydrolipoamide dehydrogenase (E3) binding site has been identified on human PDC E2 which reveals 90% homology with the E3 site in rat liver (Gershwin et al., 1987).

1.5.3 Human pyruvate dehydrogenase (E1)

The nucleotide sequence of human pyruvate dehydrogenase E1 α and β subunits has been determined by Koike et al. (1988). The nucleotide sequence for the E1 α subunit has also been obtained by

Dhal et al. (1987) and Robinson et al. (1988). Although the reported E1α nucleotide sequences are significantly different, several important consistent features are found in each sequence. From the nucleotide sequence it was deduced that the proteins E1α and E1β were synthesised with typical mitochondrial import leader sequences (Schatz, 1983). The amino acid sequences of NH_2-terminal regions of the two subunits of human PDC E1 were highly homologous with these of mature porcine PDC (Koike et al., 1988). The amino acid sequences of phosphorylation sites determined in the E1α subunit were also conserved in bovine and porcine enzymes (Koike et al. 1988).

1.5.4 Genetics of eukaryotic protein X

The gene encoding the protein X component of pyruvate dehydrogenase complex from Saccharomyces cerevisiae has been cloned and sequenced recently (Behal et al., 1989). Comparison of the deduced amino acid sequences of yeast protein X and dihydrolipoamide acetyltransferase indicates that the two proteins evolved from a common ancestor. A high degree of homology exists between component X and the acetyltransferase at their N-terminal regions corresponding to the lipoyl domain; however, protein X lacks a highly conserved sequence near the carboxy terminus which is thought to be part of the active site of all dihydrolipoamide acetyltransferases which confirms the finding of Jilka et al. (1986) who observed that component X lacked acetyltransferase activity.

Also, comparison of the nucleotide sequence of the C-terminus region of protein X with the C-terminus of mammalian E2 reveals little homology which may suggest a possible role for component X in the complex which bears little relationship to the function of E2.

1.5.5 Genetics of prokaryotic and eukaryotic lipoamide dehydrogenase

The gene encoding E3 has been cloned and sequenced from a variety of sources - selected list follows:

E. coli (Stephens et al., 1983c), A. vinelandii (Westphal and De Kok, 1988), yeast (Ross et al., 1988), pig adrenal medulla (Otulakowski and Robinson, 1987), human liver (Pons et al., 1988) and human small cell carcinoma (Otalakowski and Robinson, 1987). These E3 proteins exhibit extensive similarity. In yeast the amino acid sequence surrounding the putative active site cystine (Reed et al., 1989) is identical with the sequence reported (a purified protein from pig heart) (Williams et al., 1982).

1.6 Genetics of 2-oxo acid dehydrogenase multienzyme complexes

An alliance of biochemistry and genetics has frequently proved the most powerful tool for investigating the control of gene expression and the mechanism and biological role of their products. Analysis of defective enzymes produced by mutants can allow conclusions as to which amino acids residues are essential to the mechanism of action e.g. the research performed on tryptophan synthetase (Yanofsky and Crawford, 1972) or on glutathione reductase (Scrutton et al., 1990).

The genetics of the 2-oxo acid dehydrogenase complexes is well advanced in E. coli but developed to lesser and varying degrees in other organisms. All of these studies have provided useful information concerning some aspects of the synthesis, assembly, operation and in vivo role of the 2-oxo acid dehydrogenase complexes.

1.6.1 Genetics of 2-oxo acid dehydrogenase complexes in E. coli

The structural genes for the pyruvate dehydrogenase (E1) and lipoate acetyltransferase (E2) components of PDC, aceE and aceF respectively, are closely linked in the leu region of the E. coli linkage map (Bachmann and Brooks, 1980). The corresponding genes (sucA and sucB) for the E1 and E2 components of the OGDC are closely linked to the gal region (Henning and Hertz, 1964; Herbert and Guest, 1969). There is a single gene lpd for lipoamide dehydrogenase (E3) and this maps next to the aceEF genes. A single structural gene for E3 is consistent with the finding that when the respective lipoamide dehydrogenases were purified from each of the complexes, they had identical peptide 'maps' and the same sequence for 42 amino acid residues (Perham et al., 1978).

Amber mutations in the aceE and sucA genes have polar effects on the aceF and sucB genes respectively and this defines the direction of transcription of aceEF and sucAB. The lpd gene maps immediately distal to the aceF gene, but can be transcribed independently of the aceEF and sucAB genes (Guest and Creaghan, 1974).

Mutations in aceE or aceF result in a deficiency in PDC activity and a requirement for acetate to sustain aerobic growth on glucose minimal medium. Mutations in sucA and sucB result in defective OGDC and a requirement for succinate to sustain aerobic growth on glucose minimal medium. A mutation in the lpd gene results in a deficiency in the activity of both complexes and a requirement for supplementation with acetate and succinate simultaneously for aerobic growth on glucose minimal medium. All these classes of mutant can grow anaerobically on unsupplemented glucose minimal medium because the complexes are not required for glucose fermentation, and other mechanisms are induced anaerobically for producing acetate e.g. the phosphoroclastic reaction :

Pyruvate ⟶ acetyl CoA + acetyl P + formate (Pascal et al., 1981) and succinate, viz: the reduction of oxaloacetate via fumarate (Haddock and Jones, 1977). Mutants requiring succinate for aerobic growth on glucose will also grow when supplemented with a mixture of lysine and methionine. This is probably because succinyl CoA is required for the biosynthesis of these amino acids and their presence diminishes the requirement of succinyl CoA derived from other endogenous sources for functions such as porphyin synthesis.

The structural genes of E. coli PDC, aceEF and lpd, have been cloned with phage λ vectors (Guest and Stephens, 1980). The expression of the PDC operon was investigated by infecting UV irradiated E. coli host with transducing phage λ housing the aceEF and lpd genes (Guest et al., 1989). These structures confirmed the existence of a single promoter for the aceE and aceF genes and an independent promoter for the lpd gene. The lpd gene was shown to be transcribed with the same polarity as the ace genes. The relative rates of expression of the three genes from the bacterial promoters were estimated to be 0.94: 1.0: 1.4-2.3, E1: E2: E3 on a molar basis.

1.6.2 Genetic studies on E2 of PDC from prokaryotes

The PDC of E. coli has a three-fold repeat of the lipoyl domain on its E2 chain which forms the N-terminal half of the polypeptide (Stephens et al., 1983). Strong homology in the amino acid sequences of these repeated domains, derived from the DNA sequence of the aceF gene (Stephens et al., 1983b) and the fact that all three domains can be isolated as functional entities after limited proteolysis of this PDC (Packman et al., 1984) strongly suggests that each domain is independently folded and can function in the reductive acetylation reaction of the entire complex.

Genetic reconstruction of the E2 chain (Graham et al., 1986) which permitted lowering of the number of lipoyl domains from 3 to 2 and from 2 to 1 per E2 polypeptide does not affect the assembly of a functional PDC. This is consistent with the view that the lipoyl domains are units of protein structure which can fold and function independently. These reconstructed lipoyl domains are catalytically active because they are hybrids created from homologous domains which are designed to resemble the original domains as closely as possible. The truncated E2 chains within the limits of experimental error are not impaired in catalytic efficiency. Thus, when an E2 construct containing only a single lipoyl domain is produced from plasmid PGS110 in E. coli, fully-active PDC is formed with normal octahedral symmetry. In this sense, therefore, the two extra lipoyl domains can be regarded as surplus to requirements.

At present separate functions have yet to be assigned to the E1α and E1β subunits in decarboxylation and acetyl transfer reactions in any PDC complex. The combination of site-directed mutagenesis of the enzyme together with binding and activity studies of TPP and its analogues is at present being used to understand the mechanism of the decarboxylation reaction.

In the E1 components of 2-oxo acid dehydrogenase complexes, as in all known TPP-binding enzymes, a common 30 residue motif, probably a $\beta\alpha\beta$ fold, akin to that previously identified for NAD(P)-binding enzymes interacts with the diphosphate moiety of the bound cofactor (Hawkins et al., 1989). The E1α subunit appears to bind the cofactor TPP (Stepp and Reed, 1985), in accord with the presence of the structural motif that has been identified in all known sequences of all TPP utilising enzymes. The resistance of the E1β subunit to proteolysis in the intact B. stearothermophilus complex (Lowe et al., 1985) and its role in protecting the mammalian E2 component from enzymatic digestion (Ramatullah et al., 1989) suggests E1β has some part to play in the binding of the E1α subunit to the E2 core.

1.7 Aims of project

Attempts to define the precise function of component X in mammalian PDC by protein chemical criteria have met with only limited success to date. A particular difficulty is that protein X is tightly associated with the E2 core assembly and it is not possible to separate these two polypeptides in the native state thus permitting standard reconstitution analysis in the presence or absence of component X.

An alternative strategy, which involves the isolation and characterisation of the gene encoding the protein X subunit offers long-term possibilities for a detailed investigation of protein X function. In *S. cerevisiae*, the molecular genetics are particularly advantageous in that isolation of a specific gene permits the creation of mutants which are specifically defective in that function. Gene replacement/disruption techniques is currently being exploited by Reed and co-workers.

In our laboratory it was decided to clone protein X gene from mammalian (human) sources for the reason detailed below:

(a) At the time the project was initiated it was not clear that yeast PDC had a functionally equivalent subunit for protein X.

(b) The availability of high quality antiserum to mammalian protein X and the availability of a human cDNA gene expression library allowed for an established protocol for the identification and isolation of specific clones to be followed.

(c) It was hoped that nucleotide and predicted amino acid sequence obtained from a protein X clone would establish a relationship with the E2 component and therefore shed light on a possible function for protein X.

In parallel with these molecular biological studies, it was also decided to adopt a number of biochemical strategies to elucidate possible functions of protein X.

In particular, low temperature and substrate analogues were employed in attempts to selectively disrupt the catalytic cycle at specific stages and thereby gain a clear understanding of the involvement of protein X in the catalytic process.

Finally, in view of the proteolytically-sensitive nature of the protein X subunit, extensive use of selective proteolysis with trypsin and argC were employed in an attempt to elucidate the function of the polypeptide in the complex. Initially, these studies involved reevaluation of the reports of Roche and co-workers that argC treated enzyme was defective in E3 binding but subsequently involved considerable progress on their original observations, leading to a possible role for component X in the complex.

CHAPTER TWO

MATERIALS and METHODS

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

The following reagents were obtained from Sigma Chemical Co., Poole, Dorset, U.K.: substrates and coenzymes for enzymatic assays, PMSF, TEMED, benzamidine-HCl, Coomassie brilliant blue type R, N-ethylmaleimide, Mops, leupeptin, Tween 20 and all proteases used in this study.

Poly(ethylene glycol) 6000 was obtained from Serva, Heidelberg, W. Germany. Iodogen™ was from Pierce Labs., Rockford, Illinois, U.S.A. DMSO, DTT were purchased from Koch-Light Laboratories, Colnbrooke, Berks, U.K. Sodium salicylate was obtained from Aldrich Chemical Co., Ltd., Gillingham, U.K.

All other chemicals including reagents for polyacrylamide gel electrphoresis were Analar grade from BDH Chemicals Ltd., Poole, U.K. or were of the highest grade available commercially.

2.1.2 Tissue Culture Materials

Eagle's medium (Glasgow modification), bovine kidney (NBL-1), pig kidney (PK-15) and Buffalo rat liver (BRL) cell lines were obtained from Flow Laboratories, Irvine, U.K.

New born calf serum and foetal calf serum were purchased from Gibco, Paisley, U.K.

"Versene" solution [0.6mM-EDTA, 0.17M-NaCl, 10mM-Na_2HPO_4, 2.4mM-KH_2PO_4, 0.002% (w/v) phenol red, pH7.8] and "Trypsin" solution [(0.25%) (w/v) trypsin, 10mM-NaCl, 1mM-sodium citrate, 0.002% (w/v) phenol red, pH7.8] were supplied as sterile solutions by the tissue culture unit of our department.

Disposable plasticware was obtained from Sterilin, Teddington, U.K.

2.1.3 Radiochemicals

N-ethyl [2,3-^{14}C] maleimide (6mCi/mmol), Na[^{125}I]I (carrier free)], [2-^{14}C] pyruvate (10.4Ci/mmol) were obtained from Amersham International, Bucks, U.K.

[γ-^{32}P] ATP (100-130Ci/mmol) was also purchased from Amersham International.

2.1.4 Enzymes and Proteins

The following commercial protein preparations were obtained from Sigma Chemical Co.: protein A (Staphylococcus aureus, Cowan I strain), bovine serum albumin, horse-radish peroxidase, TPCK-treated trypsin. Lipoamide dehydrogenase (diaphorase) from pig heart was from Boehringer Corpn. (London) Ltd., Sussex, U.K.

Marker proteins for M_r determinations by SDS-PAGE were obtained from Pharmacia Ltd., Milton Keynes.

2.1.5 Animals

Ox hearts were obtained from Paisley Abattoir, Sandyford Road, Paisley. The hearts were chilled on ice and brought to the laboratory within 2 h of slaughter.

2.1.6 Miscellaneous

Nitrocellulose paper (0.45um pore size) was obtained from Schleicher and Schüll, Dassel, W. Germany or obtained from Amersham International, Bucks., U.K. (Hybond C extra). Centricon^{TM} microconcentrates were from Amicon Ltd., Upper Mill, Gloucestershire, England.

Normal rabbit serum was from the Scottish Antibody Production Unit., (S.A.P.U.), Lanarkshire, U.K.

2.1.7 Photographic materials

"Lighting Plus" intensifying screens were purchased from Du Pont Cronex, Stirling, U.K.

Plast-X cassettes, for exposure of X-ray films were obtained from Anthony Monk (England) Ltd., Sutton in Ashfield, U.K.

X-Omat S and XAR-5 X-ray films, Kodak FX-40 liquid fixer and Kodak LX-24 X-ray developer were purchased from Kodak Ltd., Dallimore Road, Manchester, U.K.

2.1.8 Materials used in screening a λgtll gene expression library

L. Broth

per litre 10g Bacto-Tryptone (Difco 0123-01)
5g Bacto yeast extract (Difco 1880-17)
10g NaCl

LB Agar (plates)

per litre as above + 15g Bacto agar (Difco 0140-01)

LB Agar (top)

per litre as above + 7g Bacto agar

20% (w/v) Maltose

20g of maltose dissolved in 100ml of distilled water and filter sterilised. 1ml of 20% (w/v) maltose solution is added to every 100ml of medium.

SM Buffer

This medium is for phage storage and dilution.

per litre 5.8g NaCl
2g $MgSO_4$
50ml 1mM-Tris-HCl (pH7.5)
5ml 2% (w/v) gelatin
Sterilise by autoclaving

Tris buffered saline (TBS) pH7.5

per litre 9.86g NaCl

1.2g Tris

TBS + 3% (w/v) bovine serum albumin (BSA)

3g of BSA added to 100ml TBS

TBS + 0.05% (v/v) Tween 20

50ul of Tween 20 added to 100ml TBS

TBS + 3% (w/v) BSA + peroxidase conjugated anti rabbit IgG

For 100ml 100ul of peroxidase conjugated rabbit IgG added to 100ml TBS + 3% (w/v) BSA (1:1000 dilution)

Colour developing solution

60mg 4-chloro-1-naphthol

20ml ice cold methanol

100ml TBS containing 80ul of 30% (v/v) hydrogen peroxide

Prepared just before use.

Agarose

for 100ml The required weight of agarose (% w/v) was added to 100ml of water and melted under pressure for 7 min. When it had cooled to below 50^{o}C ethidium bromide was added to a final concentration of 0.5ug/ml.

Ethidium Bromide

per 10ml 10mg of ethidium bromide in 10ml of water

Lambda DNA-Hind III markers preparation per 100ul		Fragment size	No. of base pairs
		1	23130
		2	9416
Lambda DNA-Hind III markers	5ul	3	6557
		4	4361
TE Buffer	70ul	5	2322
		6	2027
Sample buffer	25ul	7	564
	100ul	8	125

Lysis solution

0.5M-Tris-HCl pH9.0

0.25M-EDTA

2.5% (w/v) SDS

8M Potassium acetate

per 100 ml 78.4g of potassium acetate in 100ml of water. Filter sterilised.

Proteinase K

1mg of proteinase K was dissolved in 1ml of distilled water and stored at -20°C until required.

E. coli strains

Y1088 [supE, supF, metB, trpR, hsdM$^+$, tonA21, StrA, Δ lacU169, proC :: Tn5 (pMC9)] pMC9 is a pBR322-lacI^Q derivative carrying laci^Q (lac repressor I_A. over-producer and is penicillin resistant).

Y1089 [Δlac (IOP2YA) U169, ProA$^+$, Δlon araD139, strA, thi$^-$, hflA150 Chr :: Tn10]. This strain is used to isolate the fusion protein produced by an isolated λgtll clone.

Y1090 [Δ lac U169 proA$^+$, Δ lon araD139 strA supF trpC22 :: Tn10 (pMC9)]. This is used to screen λgtll for the production of antigenic determinants.

BNN103 (λgtll) Source of λgtll phage. Induction of gtll at 42°C and subsequent treatment of the bacteria with chloroform will release the phage into the media. The structure of the expression vector λgtll is shown below

HB101 F$^-$, hsd S20 (r_B^- m_B^-), recA13, ara-14, proA2, lacY1, galK2, rpsL20 (Sm^r), xyl-5, mtl-1, supE44, λ$^-$

JM101 Δ lacpro, thi, supE, F' traD36, proAB, lacI^{q}Z Δ M15

TG1 SupE, hsdΔ 5', thi Δ(lac-proAB) F' (traD36 proAB$^+$ lacI^q lacZ Δ M15

2.1.9 Reagents used in purification and sequencing of recombinant DNA

10 x M9 salts

per litre 60g Na_2HPO_4

30g KH_2PO_4

10g NH_4Cl

5g NaCl Store at 4^oC

Glucose/minimal medium plates

15g minimal agar in 900ml H_2O

100ml 10 x M9 salt

1ml 1M $MgSO_4$

1ml 1M thiamine HCl

10ml 20% (w/v) glucose

2 x TY medium

per litre 16g bacto tryptone

10g yeast extract

5g NaCl

2 x TY agar

Add 15g/litre bacto agar to 2 x TY medium

This should give sufficient agar for approx. 40 plates.

H plates

per litre 10g bacto tryptone

8g NaCl

12g agar

H top agar

per litre 10g bacto tryptone

8g NaCl

8g agar

PEG/NaCl

20% (w/v) polyethylene glycol 8000

2.5M NaCl

TE buffer

10mM-Tris HCl pH8.0

1mM-EDTA

3M Na acetate

Prepare a concentrated solution of sodium acetate. Adjust to pH6.0 with glacial acetic acid and dilute to 3M with distilled H_2O.

IPTG (isopropyl-β-D-thio-galactopyranoside)

100mM (23.8mg/ml in H_2O). Prepare just before use.

X-gal (5-bromo-4-chloro-3 indolyl-β-galactoside)

2% (w/v) in dimethylformamide. Prepare just before use.

Phenol

Redistilled phenol is mixed with TE buffer 1:1. Use the lower phenol phase. (Wear gloves and handle with care).

Agarose gel running dye (sample buffer)

2.7ml glycerol

0.3ml 10 x TBE buffer

1ml 1% (w/v) SDS

1ml 0.5M EDTA

1mg bromophenol blue

Sterile $CaCl_2$

Sterile 50mM $CaCl_2$ was prepared and stored at $4^{o}C$.

TBE buffer (electrophoresis buffer 10 x stock)

108g Tris base

55g boric acid

9.3g $Na_2EDTA.2H_2O$

This gives a stock solution of pH8.3. Make to 1 litre with H_2O.

Klenow reaction buffer (10 x) (annealing/reaction buffer)

100mM Tris pH8.0

50mM $MgCl_2$

E. coli hosts

E. coli K12, JM101 Δ lacpro, thi, supE,F' trdD36, proAB, $lacI^qZ$ Δ M15

E. coli K12 (JM101) carries a chromosomal pro deletion which ensures selection on M.M. plates.

Ampicillin

per 100ml 500ug of ampicillin dissolved in distilled water and filter sterilised. Stored at -20°C.

Chloroamphenicol

34mg/ml in ethanol used at a final concentration of 170ug/ml

Chloroform/isoamyl alcohol

For 100ml 96ml of chloroform + 4ml isoamyl alcohol. Stored at 4°C.

2.2 Methods

2.2.1 Measurement of protein concentration

The concentration of protein was routinely determined by the method of Lowry et al. (1951), as modified by Markwell et al. (1976). Standard curves were constructed using bovine serum albumin as standard. The absorbance was measured at 660nm.

2.2.2 Concentration of protein samples

Protein samples were concentrated by freeze drying or by spinning in Centricon TM tubes until the required volume was reached (Amicon Ltd.).

Frequently, samples for SDS/polyacrylamide gel electrophoresis were concentrated by the addition of 4 vol acetone and stored overnight at -20°C.

Alternatively, to remove salts or other reagents which interfered with electrophoretic migration, samples were concentrated by adding 100% (w/v) TCA to a final concentration of 10% (w/v) and stored at 4°C for several hours. The protein pellets were washed with ether to remove traces of TCA, air dried and redissolved in an appropriate volume of Laemmli sample buffer.

2.2.3 Measurement of incorporation of radioactivity into proteins

(a) TCA precipitation

Aliquots (10-25µl) were spotted onto Whatman No. 1 filter paper discs (2.5cm diam.) and immersed in ice cold 10% (w/v) TCA. After stirring for 30 min at 4°C, the TCA solution was discarded and the filters were washed twice with similar volumes of 10% (w/v) TCA and once with absolute ethanol. Each wash was performed at 4°C for 30 min. Finally, the discs were washed in ether and air dried. Once dry they were transferred to scintillation vials and counted with 5ml of Ecoscint scintillation fluid. Background radioactivity was estimated using blank discs that were mixed with the sample discs during the final wash.

(b) Quantitative determination of radioactive polypeptides excised from gel slices

Samples of radioactive proteins were resolved by SDS/polyacrylamide gel electrophoresis. The gels were stained with Coomassie blue (see Section 2.2.4d). After destaining the gel was dried under vacuum on Whatman No. 3mm filter paper and the protein bands of interest were cut out with scissors. Slices were dropped into glass scintillation vials and reswollen in 0.5ml of distilled water. After 10 min, the pieces of filter paper were removed as

well as the remaining liquid and the slices were incubated overnight at 37°C with 1ml of 90% (v/v) Protosol in capped scintillation vials. Aliquots were removed and estimation of the radioactive incorporation determined on the addition of 5ml of Ecoscint scintillation fluid.

Pieces of gel of similar dimensions were cut from blank tracks and processed in an identical manner to determine background radioactivity.

2.2.4 Polyacrylamide gel electrophoresis

(a) Preparation of polypeptides prior to resolution by SDS/polyacrylamide gel electrophoresis

Protein solutions were mixed with an equal volume of Laemmli sample buffer (0.0625M Tris-HCl, pH6.8, 2% (w/v) SDS, 10% (w/v) sucrose and 0.001% (w/v) Pyronin Y) containing 5% (v/v) 2-mercaptoethanol or 10mM DTT and boiled for 5 min prior to electrophoresis. When protein samples were in pellet form they were solubilised in an appropriate volume of Laemmli sample buffer and boiled as above.

(b) Conditions for resolution of proteins by SDS-PAGE

Proteins were electrophoresed on SDS-containing polyacrylamide gels using the discontinuous Tris-glycine system according to Laemmli (1970). For analytical purposes, gels were cast using a home-made apparatus in slabs 19.0cm x 9.5cm x 0.15cm, or when greater resolution was required using Biorad Protean™ 16cm apparatus in slabs of 16.0cm x 14.0cm x 0.15cm.

SDS-PAGE was performed at a constant current of 40ma-60ma per gel until the Pyronin Y tracker dye was approx. 0.5cm from the bottom of the gel. The electrode buffer contained 0.192M glycine, 0.1% (w/v) SDS in 0.025M-Tris-HCl pH8.3.

(c) Preparative SDS-PAGE

Preparative SDS gels were polymerised as a large scale version of the analytical procedure described above by increasing the gel thickness to 3mm. This permitted application of up to 3mg of protein while maintaining most of the resolving power obtained on single track analytical gels.

Stock solutions

Main gel buffer

36.6g Tris/HCl pH8.8

250µl of TEMED per 100ml of solution

Acrylamide

28g of acrylamide

0.735g of bisacrylamide per 100ml of water

Stacking gel buffer

19.2ml, 0.1M Tris-HCl pH6.8

0.8ml of 20% (w/v) SDS

50ug of TEMED

Composition of SDS-PAGE gels

	10% Gel (ml)	12.5% Gel (ml)	5.2% stacking
Main gel Buffer	25	25	-
Acrylamide gel	71.5	89.3	17.5
20% (w/v) SDS	1.0	1.0	
Stacking gel buffer	-	-	10.0
Water	100	82.2	55
Total	197.5ml	197.5ml	82.5ml

After degassing, 150mg of ammonium persulphate was added to these solutions to initiate gel polymerisation. When the running gel was poured it was overlaid with propan-2-ol to maintain an even

surface during setting of the gel. Propan-2-ol was discarded and traces were removed by washing 3 or 4 times with distilled water. The top of the running gel was dried with filter paper and the stacking solution containing 150mg of ammonium persulphate poured onto the polymerised running gel.

Gels were wrapped in damp towel roll and could be stored for up to 3 weeks at $4^{o}C$.

(d) Staining of gels with Coomassie blue

Gels were stained for protein in 0.1% (v/v) Coomassie brilliant blue R250, 50% (v/v) methanol and 10% (v/v) acetic acid for 1 h.

(e) Destaining of gels

Unbound stain was removed by washing the gel several times in 10% (v/v) methanol and 10% (v/v) acetic acid solution.

(f) Processing of SDS gels for fluorography

Gels to be processed for fluorography were previously stained or directly fixed overnight by immersing in 25% (v/v) propan-2-ol, 10% (v/v) acetic acid.

Fluorography was performed according to the method of Chamberlain (1979) employing sodium salicylate as fluor. Slab gels were dried under vacuum and exposed for a suitable period of time using either X-OMAT S or XAR-5 Hyperfilm at $-80^{o}C$.

(g) Processing of SDS gels for autoradiography

Gels were stained or fixed as described above. Before drying, gels were soaked for 30 min in a solution containing 25% (v/v) propan-2-ol, 10% (v/v) acetic acid and 0.5% (v/v) glycerol to reduce the risk of cracking during drying.

(h) Determination of M_r value by SDS PAGE

The M_r values of polypeptides and peptides resolved by SDS-PAGE was determined by calibrating with a set of standard proteins on a 10% (w/v) slab gel. The standard proteins include phosphorylase b (M_r 94400); BSA (M_r 68000); ovalbumin (M_r 43000); carbonic anhydrase (M_r 31000); soyabean trypsin inhibitor (M_r 20100) and lysozyme (M_r 14000).

Relative mobility was calculated as the ratio:

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

A plot of Rf against log (M_r) of the standards yielded a curved line that was used for calibration. Standards and PDC samples were analysed on the same gel.

2.2.5 Purification of pyruvate dehydrogenase complex

(a) Purification of pyruvate dehydrogenase complex from ox heart

Pyruvate dehydrogenase complex (PDC) was purified from ox heart essentially as described by Stanley & Perham (1980) with the modifications stated below.

All operations were carried out at 4°C, starting with 250g of fresh ox heart or ox heart stored at -80°C (within 3 h of slaughter). Cubes of tissue were blended and extracted in 50mM Mops containing 2.7mM-EDTA, 3% (v/v) Triton X-100 and 0.1mM-DTT; adjusted to pH7.0 with NaOH. The residual material was discarded. The extracts contained assayable amounts of 2-oxoglutarate dehydrogenase activity; however the presence of lactate dehydrogenase and NADH oxidase interfered with the estimation of pyruvate dehydrogenase complex activity at the early stages of purification.

The complex was precipitated by the addition of 0.12 vol of a 35% (w/v) poly(ethylene glycol) solution at pH6.45. Pellets were resuspended in 50mM Mops containing 1% (v/v) Triton X-100, 2.7mM-EDTA, 0.1mM-DTT, 1.5uM-leupeptin, adjusted to pH6.8 with NaOH by homogenisation with a loose-fitting teflon-glass homogenizer. This extract was adjusted to 13mM-$MgCl_2$ and 50mM-sodium phosphate before the addition of 0.12 vol poly(ethylene glycol) for a second precipitation.

The 2-oxo acid dehydrogenase complexes were separated by differential precipitation with poly(ethylene glycol). Usually between 0.04-0.06 vol 35% (w/v) poly(ethylene glycol) were required to precipitate 90% of the 2-oxoglutarate dehydrogenase activity. OGDC was pelleted at 18000 x g for 10 min. The resulting supernatant fraction contained approximately 90-95% of the PDC activity. Pyruvate dehydrogenase complex was concentrated by centrifugation for 2.5 h at 176,000 x g in a Beckman Ti70 rotor or by the addition of 0.15 vol of 35% (w/v) poly(ethylene glycol) followed by centrifugation at 30,000 x g for 10 min. The pellet of pyruvate dehydrogenase was purified further by solubilising in column buffer (50mM-sodium phosphate buffer, pH7.0, 1% (v/v) Triton X-100, 2.7mM-EDTA, 0.2mM thiamine pyrophosphate and 1mM $MgCl_2$) and separating the residual 2-oxoglutarate dehydrogenase at $4^{o}C$ by gel filtration chromatography on a Sepharose CL-2B column (106cm x 3.5cm) equilibrated in column buffer at a flow rate of 24ml/h. Approximately 60 x 12ml fractions were collected and assayed for pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase activities. Fractions with maximal activity were pooled and concentrated by centrifugation at 170,000 x g for 2.5 h at $4^{o}C$.

The yellow pellets were resuspended by standing overnight in a small volume of column buffer containing 0.01% (w/v) NaN_3, 0.15μM leupeptin. The enzyme was stored at 4°C at a final concentration of 20-30mg/ml.

(b) Preparation of E2/X core assembly

E2/X core assembly was prepared as described by Kresze and Ronft (1981). Solid $MgCl_2$ was added to PDC (200μg) in phosphate buffer, pH7.0 to give a final concentration of 0.25M $MgCl_2$. After 30 min, the treated PDC was resolved on a Superose 12 column equilibrated in phosphate buffer by FPLC.

2.2.6 Isolation of mitochondria and submitochondrial fractions

(a) Isolation of rat liver mitochondria

Rat liver mitochondria were prepared by differential centrifugation (Chance and Hagihara, 1963).

Female rats (180-200g) were starved overnight and killed by cervical dislocation. The livers were removed, excess blood removed with blotting paper, and the livers were chopped finely with scissors before transfer into ice-cold isolation medium. Isolation medium contains 0.225M-mannitol, 0.075M-sucrose, 500μM-EGTA and 2mM MOPS. The solution was adjusted to pH7.2 with NaOH and stirred until the EGTA was completely dissolved; then the pH was adjusted to pH7.4.

Livers from 6 rats were homogenised in approximately 30ml isolation medium per liver with a tight-fitting Potter-Elvehjem homogeniser. After removal of the nuclei and cell debris at 800 x g for 7 min at 4°C, mitochondria were sedimented from the supernatant fluid by centrifugation at 6500 x g for 15 min. Pellets were washed by manual resuspension in the same buffer and centrifuged at 6500 x g for 15 min. To remove residual

contamination from nuclei, red blood cells and unbroken cells, mitochondrial pellets were resuspended in one-half the initial volume of isolation medium and re-centrifuged at 800 x g for 7 min. The pellets were discarded and the supernatant fractions carefully combined and centrifuged at 6500 x g for 15 min as previously. The final pellets contained highly-purified mitochondria which were kept frozen at -20°C until required.

(b) Preparation of ox heart mitochondria

Ox heart mitochondria were isolated as described by Smith (1967). Approx. 1.5-1.8g of mitochondrial protein were obtained from 300g tissue. Mitochondrial pellets were stored at -80°C until use.

Ox heart mitochondria utilised in this study were prepared by F. McCallum in our laboratory.

(c) Preparation of cellular extracts and subcellular fractions from Buffalo rat liver (BRL), bovine kidney (NBL-1) and porcine kidney (PK15) cells

Cellular extracts for immunoblotting analysis were prepared under conditions designed to minimise proteolytic degradation. Cells were grown in large roller bottles until they reached confluence. Monolayers were washed twice with ice-cold phosphate-buffered saline (20mM-sodium phosphate buffer, pH7.4, 0.15M-NaCl) prior to harvesting in a small volume of the same buffer by scraping with a rubber policeman. Small amounts of the suspension were immediately dissociated for SDS-PAGE by heating in Laemmli sample buffer at 100°C for 5 min.

The majority of the cells were fractionated to obtain nuclear and mitochondrial fractions as described by Attardi and Ching (1979).

To obtain particulate and cytosolic fractions, cells were fractionated by the method of Zurrendonk and Tager (1974) as modified by Mori et al. (1981).

Aliquots (1ml) of the cell suspensions were mixed at 0°C with an isotonic digitonin solution at a final concentration of 0.25 to 5mg/ml. After standing at 0°C for 2 min, the samples were centrifuged for 1 min at 14,000 x g in an MSE Micro-Centaur centrifuge. Samples were kept at -20°C until required.

2.2.7 Enzymatic assays

(a) Pyruvate dehydrogenase complex activity

The overall activity of pyruvate dehydrogenase complex was determined spectrophotometrically by measuring NADH formation at 340nm at 30°C (Brown and Perham, 1976). Assays were performed in a final volume of 0.67ml in 50mM-potassium phosphate buffer, pH7.4 containing 2mM-TPP, 1mM-$MgCl_2$, 2.5mM-NAD^+, 0.13mM-CoA, 2.6mM cysteine-HCl and 2mM-sodium pyruvate. A unit of activity (Kat) was defined as the amount of enzyme which produced 1mol of NADH/sec at 30°C under the conditions of assay.

(b) Pyruvate dehydrogenase kinase

Pyruvate dehydrogenase kinase (EC 2.7.1.99) was assayed by measuring the rate of inactivation of the complex in the presence of 0.2mM-ATP at 30°C (Hucho et al., 1972) or by estimation of the rate of incorporation of ^{32}P-labelled phosphoryl groups from [γ-^{32}P] ATP into serine residues on the E1α subunit of PDC (Stepp et al., 1983). Incubation mixtures contained 20mM potassium phosphate buffer, pH7.5, 1mM-$MgCl_2$, 0.1mM-EDTA, 2mM-DTT and 20-50ug of PDC in 200ul of assay mix.

After equilibration at 30°C for 1 min, the reaction was initiated by the addition of 0.02ml of 2.5mM [γ-^{32}P] ATP (approx. 13,000 d.p.m./nmol). At the indicated times, aliquots (20ul) were applied to filter discs (Whatman No. 1). The discs were immediately dropped into 10% (w/v) TCA, washed, dried and utilised for radioactivity counting as described in Section 2.2.3a.

(c) Lipoamide dehydrogenase (E3) activity

Reduced lipoic acid was prepared as described by Kochi and Kikuchi (1976). Reduced lipoic acid (20ug) was added to 0.67ml of 50mM potassium phosphate buffer pH7.4 containing 2mM TPP, 1mM $MgCl_2$, 2.5mM-NAD^+. The reaction was initiated by the addition of 10ug PDC and the activity determined spectrophotometrically by measuring NADH formation at 340nm at 30°C.

2.2.8 Elution of PDC subunits from SDS/polyacrylamide gels

Purified subunits of PDC were recovered from SDS/polyacrylamide gels by a modified version of the procedure of Tolan et al. (1980). 3mg PDC was resolved by 10% (w/v) SDS-PAGE (Section 2.2.4b). Gels were stained with Coomassie blue and destained with several changes of 10% (v/v) acetic acid, 20% (v/v) methanol. Stained protein bands were cut from the gel and rinsed with distilled water for about 30 min. After extruding gel strips through the barrels of 2ml disposable syringes to fragment the gel, the gel was transferred into scintillation vials and dried in a vacuum dessicator over NaOH pellets to remove acid from the destaining solution and to facilitate extraction. Dried gel pieces were swollen in a minimal volume (2-3ml) of 50mM-triethanolamine, 1% (w/v) SDS, 1mM-DTT, adjusted to pH8.0 with HCl (extraction buffer). The suspensions were heated for 5 min at 65-70°C and the gel

slurry extracted by continuous stirring at room temperature for 16-20 h. Fragments of gel were removed by centrifugation at 1,000 x g for 5 min to yield a clear blue-coloured extract. The gel slurry was extracted once more in a similar manner before pooling the extracts and concentrating them by freeze-drying.

Samples were redissolved in a minimal volume of water and clarified by a brief centrifugation as before to remove any residual fragments of gel. Proteins were precipitated by the addition of 4 vol of acetone and stored at -20°C, a procedure which also partially removed the dye. Pellets were redissolved by boiling in a small volume of 1% (w/v) SDS and stored at -20°C until required.

It was convenient to repeat the acetone precipitation step when large white precipitates of SDS were obtained after the first acetone precipitation.

The presence of SDS and traces of dye interfered with the estimation of protein concentration (Bradford, 1976; Lowry et al., 1951). The amount of recovered proteins was determined approximately by quantitative scanning of Coomassie blue-stained bands of protein subjected to SDS-PAGE by comparison with BSA as a standard.

2.2.9 Immunological procedures

(a) Immune blotting (Immune Replica Analysis, Western Blotting)

The immune blotting procedure was employed to permit immunological detection of polypeptides after their electrophoretic transfer from SDS/polyacrylamide gels to nitrocellulose paper (Towbin et al., 1979; Batteiger et al., 1982).

Polypeptides were resolved by SDS-PAGE (Section 2.2.4) and transferred electrophoretically onto nitrocellulose paper at 400ma for 3 h or 40ma for 16-20 h using a Bio-Rad Trans-Blot™-cell.

Transfer buffer comprised 25mM-Tris, pH8.3, 192mM glycine, 0.02% (w/v) SDS, 20% (v/v) methanol and was stored at 4°C before use.

After transfer the nitrocellulose paper was removed and incubated, face up, in 20mM Tris-HCl, pH7.2, 0.15M NaCl 0.5% (v/v) Tween 20, 0.005% (w/v) NaN_3 (blocking buffer) for 1 h at room temperature or overnight at 4°C.

Nitrocellulose paper was then incubated in fresh blocking buffer supplemented with antiserum at a dilution of 1:50-1:100 and 5% (v/v) heat-inactivated horse serum for 90 min at room temperature. Excess antibody was removed by washing with 5 changes of blocking buffer over a period of 60 min before incubation for 90 min at room temperature with blocking buffer containing ^{125}I-labelled protein A (approx. 3 x 10^6 c.p.m. per blot). Excess ^{125}I-labelled protein was removed by several washes with blocking buffer as above. The processed nitrocellulose was allowed to dry at room temperature before analysis by autoradiography.

(b) Preparation of IgG

Three separate antisera, anti PDC, anti-E2 and anti-component X (7ml each), were dialysed against 2 x 500ml of 20mM-Tris HCl, pH8.0, 28mM NaCl for 20 h. A DEAE Affigel Blue column (40ml) was washed with 2 vol 8M urea followed by 3 vol 2M NaCl and, at 4°C, 7 vol of dialysis buffer. Antiserum was loaded onto the column and eluted at a flow rate of 12ml/h. 6ml fractions were collected and the A_{280} monitored to detect eluted proteins. Peak fractions containing IgG, which is the only major class of serum protein not retained, were pooled, freeze dried and reconstituted to 80% of their original volume. The serum was then dialysed against 2 x 1 l of dialysis buffer at 4°C.

After each run, the DEAE Affigel Blue column was regenerated as described in the table below.

Temp	Reagent	Column Vol.	Reason
RI	8M urea	2.3	Remove CN^-
RI	2M NaCl	3-4	Elute proteins
4^oC	Dialysis buffer	6-7	equilibrate column

(c) Dot blots

Dot blots were employed to test the sensitivity of the serum IgG prepared from the DEAE affigel blue column and to compare the titre of purified IgG with the original antiserum. PDC was diluted in BSA (100µg/ml); further dilutions were made with water until 1ul of mixture, containing 50, 10, 5, 1 and 0.5ng of complex respectively could be spotted onto strips of nitrocellulose. The complex was challenged with the appropriate IgG or antisera and processed as described in the antibody screening method (Section 2.2.12a).

2.2.10 Radiolabelling of proteins by Iodogen method

Iodogen™ (1,3,4,6-tetrachloro-3∝, 6∝-diphenylglycoluril), is a mild solid phase reagent that efficiently reacts with aqueous mixtures of I^- and tyrosine groups on proteins to produce iodinated proteins (Fraher and Speck, 1978).

Iodogen™ (1mg) was dissolved in 1ml of chloroform. The solvent was removed with a gentle air stream by rotating the reaction container until a thin film of Iodogen was formed on the base.

Proteins for iodination (1mg of protein A or 600µg low M_r value standards) were dissolved in 0.5ml 0.1M-Tris-HCl buffer pH7.2, 0.15M NaCl and transferred into the container with the Iodogen.

After the addition of 200-400uCi [^{125}I]NaI, incubations were carried out for 15 min at room temperature with occasional mixing. Reactions were terminated by removing the mixture from the container and passing through a 10ml Sephadex G-25 column equilibrated in 0.1M-Tris-HCl pH7.2 and 0.15M NaCl. Fractions (1ml) were collected in disposable Eppendorf tubes. The iodinated protein was generally found to be between fractions 4 and 7. This was confirmed by counting 5ul aliquots. The peak fractions were pooled and stored at -20^{o}C. Protein A for immunoblotting analyses was dispensed in 0.05-0.1ml aliquots containing approximately 3 x 10^{6} c.p.m. each.

2.2.11 Modification of enzyme-bound lipoyl groups in pyruvate dehydrogenase complex

(a) Acetylation Assay

The acetylation of covalently-associated lipoyl groups in PDC was assayed by measuring incorporation of radioactivity from [2-^{14}C] pyruvate into TCA-precipitable material (Cate and Roche, 1979).

Standard mixtures (200ul) contained 50mM potassium phosphate buffer, pH7.2, 0.2mM-TPP, 0.5mM-$MgCl_2$, 0.25mM-EDTA and 200ug PDC. Catalysis was initiated after 1 min preincubation at 30^{o}C by addition of 0.1mM [2-^{14}C] pyruvate (10.4mCi/mmol).

Samples (10ul) were withdrawn at the indicated times, spotted onto Whatman No. 1 filter paper discs and processed for measurement of TCA-insoluble radioactivity (see Section 2.2.3).

In some experiments NEM was added to a final concentration of 0.5mM before incubation with [2-^{14}C] pyruvate. Stock 10mM NEM solution in 50mM KP_i (pH7.0) was prepared freshly each time.

(b) Deacetylation assays

PDC was incubated, as described in Section 2.2.11b, for 30-40 min to allow the complete acetylation of the complex. CoASH was added to a final concentration of 1mM and the deacetylation reaction was followed by removing 10µl aliquots of reaction mixtures, spotting onto filter paper discs and measuring TCA-insoluble radioactivity.

To study the effects of NEM on the deacetylation reaction, NEM (0.5mM) was added to PDC before preincubation with CoASH.

(c) NEM modification of PDC

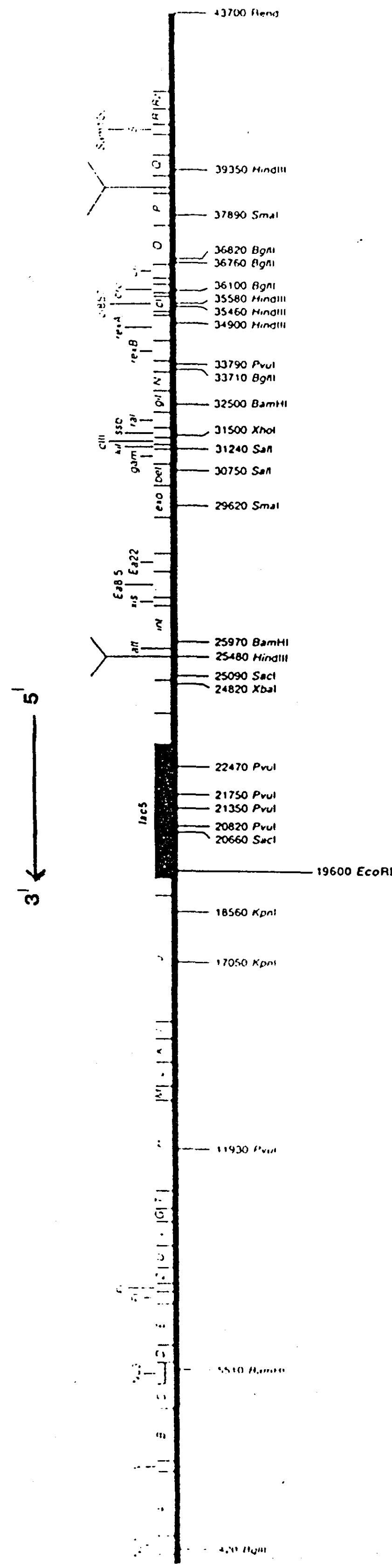
PDC (6mg), dissolved in 600ul 50mM-potassium phosphate buffer, pH7.5, 0.25% (v/v) Triton X-100, 1mM-NAD^+, 5mM-$MgCl_2$, 2mM-TPP, and 0.5mM-NEM, was incubated for 24 h at 4°C. Unreacted reagents were removed by gel filtration in 1ml columns of Sephadex G-25 equilibrated in 50mM-potassium phosphate buffer, pH7.5, 1% (v/v) Triton X-100, 1mM-NAD^+, 5mM-$MgCl_2$, 2mM-TPP (Penefsky, 1977). This procedure blocked all NEM-sensitive sites available to the complex in the absence of pyruvate in a time-dependent manner (Hodgson, 1986).

(d) [^{14}C] NEM modification of PDC subunits

PDC (200µg) was added to 200µl Laemmli sample buffer (minus DTT or 2ME). The mixture was heated briefly to 100°C, prior to incubation with 5µCi N-ethyl [2,3-^{14}C] maleimide. After 30 min at room temperature, precipitation was induced by the addition of 4 vol of acetone and the mixture incubated overnight at -20°C. After spinning at full speed for 5 min in a Microfuge, the pellet was resuspended in Laemmli sample buffer containing 10mM-DTT.

Figure 2.1 Structure of expression vector λGT11

The structure of the expression vector λ gt11 (lacZ, c1857, nin 5, S100) is outlined. The position of the unique EcoRI site in the lac Z gene into which foreign cDNA sequences are inserted, and of the adjacent KpnI and SstI (SacI) sites are shown. The transcriptional orientation of lac Z is indicated by the horizontal arrow and the size of the vector is shown in kilobase pairs. [adapted from Sambrook et al., 1989].



43700 Rend
39350 HindIII
37890 SmaI
36820 BglII
36760 BglII
36100 BglII
35580 HindIII
35460 HindIII
34900 HindIII
33790 PvuI
33710 BglII
32500 BamHI
31500 XhoI
31240 SalI
30750 SalI
29620 SmaI
25970 BamHI
25480 HindIII
25090 SacI
24820 XbaI
22470 PvuI
21750 PvuI
21350 PvuI
20820 PvuI
20660 SacI
19600 EcoRI
18560 KpnI
17050 KpnI
11930 PvuI
lac5
exo
bet
gam
cIII
ssc
ral
gal
N
rexB
rexA
cI
cII
O
P
Q
att
int
xis
Ea8.5
Ea22
5'
3'

2.2.12 Screening of a λ gtll gene expression library with antibody probes

A human heptoma λ gtll cDNA gene expression library was a gift from J. De Wet (Fig. 2.1). The library was constructed from cDNA copied from poly A^+ RNA isolated from the human hepatoma line li 7 which was grown on Balb/c athymic mice. The library contains approximately 8 x 10^6 independent clones, and 75% of the phage carry inserts. Although the cDNAs were inserted into λ gtll with EcoRI linkers it has been reported that approximately 20% of the clones are missing one of the EcoRI sites which should bound the insert. The screening method is essentially that of Young and Davis (1983) with the major modification of the use of a chromogenic agent to identify recombinant phage which give a positive immunological response owing to the expression of fusion protein which is recognised by the subunit-specific antisera.

(a) Antibody screening procedure

(b) Growing λ gtll

Host E. coli strain Y1090 was grown overnight at 37°C in LB medium containing 10mM $MgSO_4$ and 0.2% (w/v) maltose. Phage were diluted in SM buffer to give approximately 1 x 10^6 PFU per ml and 10-100ul added to 200ul of the overnight culture of Y1090. After 15 min at 37°C 7.5ml soft agar was added and mixture poured onto LB plates (150mm diam.) containing 10mM $MgSO_4$. The plates were incubated for 2.5 h at 42°C with the lids removed for the final 15 min.

(c) Overlaying plaques with nitrocellulose filters

Nitrocellulose filters, cut to the dimensions of the plates (diam. 150mm) were soaked in 10mM IPTG. When dry, 0.5ng of antigen was spotted on to the edge of the filter adjacent to a pencil mark used to establish the orientation of the filter (the pencil mark

face down onto the plate). The plates were carefully covered with the prepared nitrocellulose filters and the filters marked at the edges with a syringe needle for identification. Inverted plates with filters were incubated for a further 2 h at 37°C to lyse the cells. After 2 h the filters were removed and washed in TBS for 5 min. If required, fresh IPTG impregnated nitrocellulose filters could be used to overlay the plates. Otherwise the plates were stored at 4°C. A further 5 min wash in 3% (w/v) BSA in TBS, pH7.5 was followed by an overnight wash in TBS, 3% (w/v) BSA + 0.02%, sodium azide and 1:100 dilution of either of the antibodies to the components of PDC. The BSA blocks non-specific sites on the nitrocellulose, reducing the background signal.

The first antibody mixture was decanted and stored at -20°C as it can be used on several subsequent occasions. Filters were again washed in TBS for 10 min before the addition of TBS + 0.05% (v/v) Tween 20 for 5 sec. Prolonged exposure of the filter to the TBS/Tween 20 solution resulted in a loss of signal. On completion of the washing stage the TBS was discarded and TBS, 3% (w/v) BSA in TBS the second antibody, peroxidase conjugated goat-anti rabbit IgG was added to each filter and incubated for 3 h. The second antibody was discarded and the filters incubated in colour development reagent containing 40ml of ice cold methanol, 200ml of TBS, 120ul of 60 vol hydrogen peroxide and 4-chloronaphthol to a final concentration of 5mM. When the purple background had started to develop the filters were removed and washed in water to stop the colour development reaction.

(d) Identification and rescreening of positive clones

A positive clone, usually identified by its doughnut appearance and darker blue colour, was pinpointed and picked by using the fine end of a sterile Pasteur pipette. The agar plug was

added to 500ul of sterile SM buffer followed by one drop of chloroform. The solution was vortexed for 30 sec before storage overnight at 4°C.

After a brief vortex the samples were microfuged for 8 min and 200µl removed to a sterile polytube, one drop of chloroform was added and the phage stored at 4°C. The concentration of phage was determined (Section 2.2.12e) and approximately 40,000 (75µl) used to infect 50µl of a fresh overnight culture of Y1090. After 15 min at 37°C, 3ml of LB soft agar was added and the mixture poured onto 90mm LB plates (pH7.5) and the screening process repeated (Section 2.2.12b).

After each screening the number of positive phage used to infect an overnight culture was reduced by a factor of 10 until 100% positives were obtained.

(e) Estimation of the number of plaque forming units (Titre)

The lon protease deficient host Y1090 used to plate out λ gt11 libraries for screening with antibody probes is not defective for host-controlled restriction and modification enzyme activities. Therefore, the initial packaging mix of libraries cloned in λgt11 should not be plated directly on Y1090 for screening, but should first be amplified through Y1088.

It is important to use Y1088 when determining the titre of the recombinant phage as it is important to repress the production of toxic polypeptides (in the form of β -galactosidase fusion proteins) encoded by the recombinant phage. Therefore, E. coli Y1088, which produces lac repressor, is used as the host to amplify the library.

(f) Drop titre

A rapid method for determining the number of plaque forming units (p.f.u.)

A single colony of Y1088 was used to inoculate 10ml of LB media and grown overnight at 37°C. 3ml of molten top LB agar was added to 200ul of the overnight culture and the mixture poured over a LB plate and allowed to dry. The plate was divided into four sections and 20ul of different dilutions of recombinant phage added to each of 3 sections. 20ul SM buffer was added to the fourth section as a control. After the phage had dried onto the plate, the plates were incubated overnight at 34°C.

(g) High titre lysate

200ul of a Y1088 overnight culture was added to approximately 10^5 p.f.u. of the isolated recombinant phage. After 15 min at 39°C, 3ml of soft agar was added to the mixture and poured onto a prewarmed (37°C) LB + Mg^{2+} plate. After incubation at 42°C for 6 h, lysis occurred and the plates were overlaid with 3ml SM buffer and stored overnight at 4°C. The SM buffer was decanted and centrifuged for 15 min at 8000 x g. The supernatant fluid was removed and stored at 4°C. A drop of chloroform was added to prevent bacterial degradation of the lysate.

(h) Confirmation of positive clones using the high titre lysate

An accurate titre was performed on the high titre lysate (Section 2.2.12e). At a ratio of 50:50, p.f.u. from recombinant and non recombinant λgt11, 100µl of the mixture was then used to infect 50ul of host Y1090 which were grown overnight at 37°C in LB medium. After 15 min at 37°C 3ml of soft agar was added and the mixture poured onto LB plates (100mm) containing 10mM $MgSO_4$. The plates were incubated for 2.5 h with the lid removed for the final 15 min. Plates were then overlaid with nitrocellulose filters presoaked in IPTG and the filters were processed as described in Section 2.2.12b). Approximately 10% recombinant phage were observed.

(i) Preparation of lysogens from recombinant phage

The cells from a 2ml overnight culture at 37°C of host Y1089 grown in L Broth, pH7.5, 0.02% (w/v) maltose and 10mM $MgSO_4$, were pelleted before resuspension in 2ml of SM buffer. After a repeat washing of the cells, the cells were finally resuspended in 2ml of SM buffer and incubated for 1 h at 37°C.

100ul of these cells were infected with a five fold excess of viable phage (a multiplicity of infection of 1:5). The samples were left for 1 h at room temperature before the number of p.f.u. determined (titred). The lysates were streaked out on LB plates and incubated overnight at 30°C.

(j) Replica plating

An overnight L-Broth agar plate impregnated with lysogens is rolled onto filter paper. A fresh L-Broth agar plate is placed over the filter paper and the plate stored overnight at 42°C. The procedure is repeated with a second L-Broth agar plate and this plate is stored at 30°C overnight.

The two plates are compared and temperature sensitive lysogens containing the component X phage will be present at 30°C but not 42°C. At 42°C the temperature sensitive phage repressor is non functional. Clones which grow at 30°C but not at 42°C are assumed to be lysogens. Lysogens arise at a frequency of between 10% and 70% under these conditions.

(k) Preparation of crude lysate

After inoculation with a single colony of the Y1089 recombinant lysogen 10ml of L Broth was incubated overnight at 30°C. After incubation for 20 min at 45°C, IPTG was added to a final concentration of 10mM and the cells incubated at 37°C for a further 80 min. The lac-Z directed fusion protein production is induced by the addition of IPTG to the medium.

The clear supernatant fraction obtained after centrifugation at 5000 x g for 10 min was discarded and the pellet resuspended in lysate buffer. The cells were disrupted by passing them through a 21 gauge needle and heating them for 3 min at 70°C. After a 10 min high speed spin on a microcentrifuge a very viscous solution was obtained. DNase was added to a final concentration of 0.5µg/ml and the suspension incubated for 1 h at 37°C.

(l) Permanent storage of lysogens

A single colony from a Y1089 recombinant lysogen grown overnight at 30°C was used to inoculate 10ml LB medium, pH7.5. After overnight incubation at 30°C, 200µl was removed and added to 200ul of glycerol. The mixture was rapidly frozen in alcohol/dry ice and stored at -80°C. Cells frozen in the absence of glycerol are damaged by the formation of ice crystals.

(m) Identification of fusion protein

An aliquot of crude lysate was removed, added to an equal volume of Laemmli sample buffer and resolved by a 10% SDS-PAGE before blotting onto nitrocellulose (Section 2.2.9a). The nitrocellulose was challenged with appropriate antisera and the presence of the appropriate protein identified using [^{125}I] protein A (Section 2.2.9a).

2.2.13 Preparation of DNA

(a) Preparation of phage DNA (Maniatis et al., 1982)

This procedure is based on the method of Yamamoto et al. 1970. A Y1089 recombinant lysogen was grown overnight in LB medium at 32°C. 4ml of the overnight culture was diluted into 400ml LB medium and aerated at 32°C until the cell density was A_{600} = 0.4.

The phage were induced by shaking the flasks at 42°C for 20 min and then aerated for a further 3 h at 37°C. Cells were harvested by centrifugation at 8000 x g for 10 min and resuspended in 40ml SM buffer. Following centrifugation at 10000 x g for 10 min, the resultant pellet was resuspended in phage buffer. Cells were lysed by freeze thawing in dry ice/alcohol and a 37°C water bath respectively. This was repeated three times. DNase I and RNase I (heat treated) were added to a final concentration of 0.5µg/ml to digest chromosomal DNA and the mixture allowed to stand overnight on the bench.

Solid sodium chloride 0.5M followed by PEG 8000 [to a final concentration of 10% (w/v)] were added and the solution left for 1 h at 4°C. Following a spin at 6000 x g for 30 min, the resultant pellet was resuspended in 5ml SM buffer, the mixture vortexed for 30 sec and then centrifuged at 1600 x g for 15 min at 4°C. The aqueous layer (top) was removed and added to 0.5g ml^{-1} of solid caesium chloride.

Caesium Chloride Gradient

Table 2.1 Caesium Chloride solutions for step gradients prepared in SM (100ml)

Density (ρ)	CsCl (g)	SM (ml)	Refractive Index (ρ)
1.45	60	85	1.3768
1.50	67	82	1.3815
1.70	95	75	1.3990

The step gradients were made by carefully and sequentially layering solutions of decreasing density on top of one another.

The aqueous layer was added to the $CsCl_2$ gradient and samples were centrifuged at 50000 x g for 80 min. The DNA was visualised in the middle of the ρ 1.5 band. A needle was inserted

below the DNA and the DNA was extracted into a sterile Eppendorf tube. Caesium chloride was removed by overnight dialysis against SM buffer. The DNA was incubated at 70°C for 30 min at 0.2 vol lysis buffer followed by 30 min incubation on ice with 0.25 vol 8M potassium acetate. The precipitate was spun at 10,000 x g for 20 min and the DNA precipitated from the supernatant by the addition of 3M Na acetate, pH6.0, followed by 2.5 vol of 80% (v/v) ethanol. DNA was dissolved finally in TE buffer.

(b) Alternative preparation of bacteriophage DNA

It was found that preparation of DNA through a caesium chloride gradient resulted in pure DNA but with reduced yields. An alternative hybrid preparation based on the method of Yamamoto (1970) resulted in improved yields.

The original method was followed as in the preparation of phage DNA up to and including the extraction of phage with chloroform.

Bacteriophage particles were then collected by centrifugation at 50,000 x g for 2 h in a Beckmann SW27 rotor. 2ml of SM buffer was added to the glassy pellet which was left to solubilise overnight. The pellet may be pipetted gently to ensure the bacteriophage particles have resuspended.

Proteinase K was added to a final concentration of 50ug/ml followed by SDS to 0.5% (w/v) and the bacteriophage particles incubated for 1 h at 65°C. An equal volume of TE-equilibrated phenol was added and the tube inverted several times to ensure thorough mixing. After a brief spin, the aqueous layer was removed and extracted with 1:1 phenol/chloroform. The aqueous layer was dialysed against TE buffer overnight.

(c) Separation of DNA fragments by agarose gel electrophoresis

Agarose gel electrophoresis was the standard procedure used to separate, identify and purify DNA fragments.

Agarose gels were prepared by heating at 100°C, under pressure for 7 min, the required amount of agarose in electrophoresis buffer (Section 2.1.9). This was usually enough time to completely melt the agarose. When the agarose had cooled to around 50°C, ethidium bromide was added (to 0.5µg/ul) before quickly casting the gel, concentrations of between 0.75% (w/v) agarose and 2% (w/v) agarose were routinely used, depending on the size of the DNA fragments. Electrophoresis was performed in a horizontal mini gel apparatus.

The gel was immersed in electrophoresis buffer containing ethidium bromide (0.5µg/ml) and electrophoresis was performed at 50 or 100 V until the dye front had almost reached the end of the gel. DNA was visualised by ethidium bromide fluorescence on a U.V. trans-illuminator. The gels were photographed with a polaroid CU-5 camera. The size of fragments were estimated by comparison with DNA Hind III marker fragments derived from λcI857 DNA digested to completion by Hind III.

(d) Restriction Digests

Restriction enzymes were used as recommended by the supliers.

Buffers

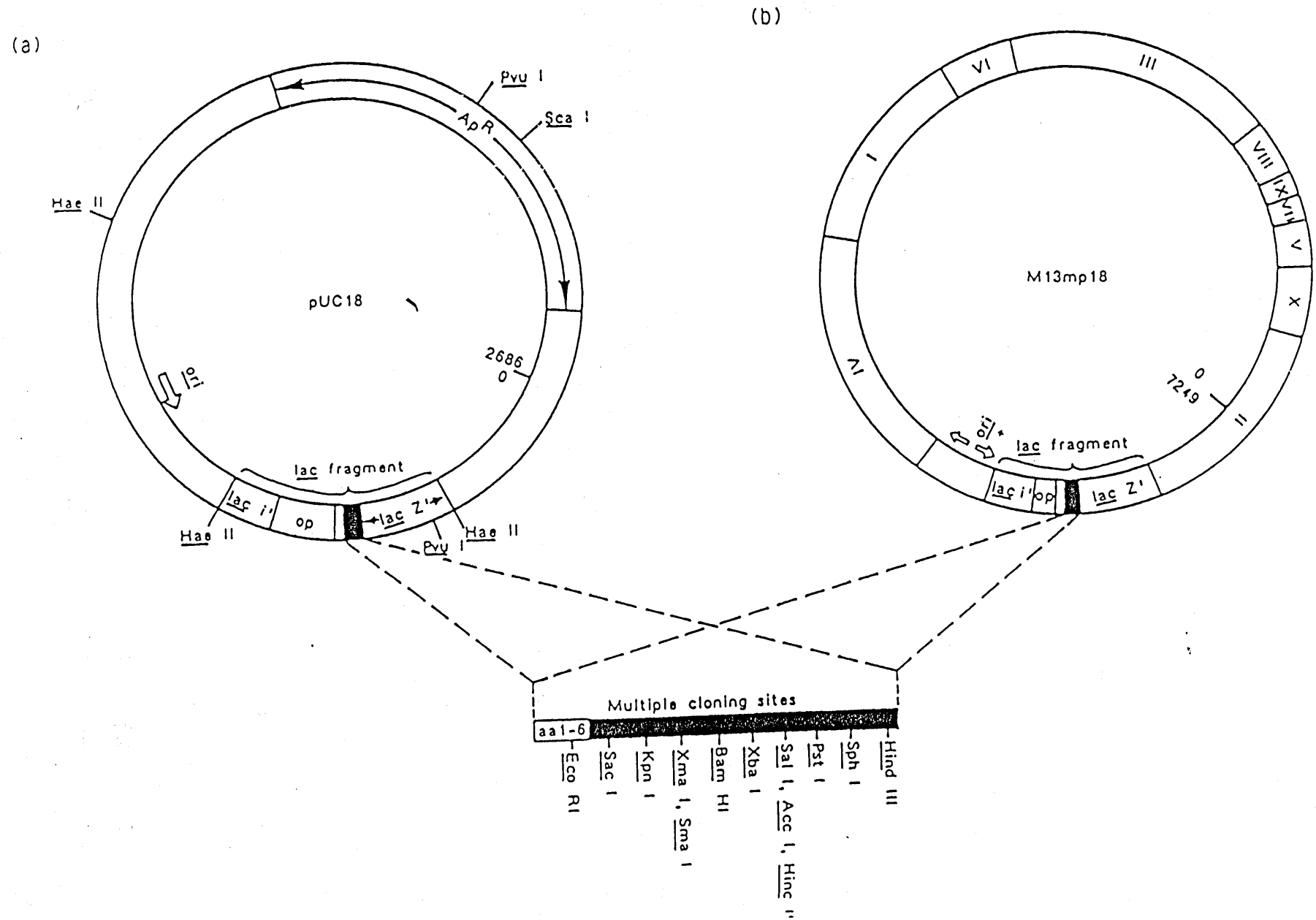
Table 2.2 10 x Stock restriction enzyme buffers (µl)

Salt conc.	H_2O	4MNaCl	1M Tris	$1mMgCl_2$	1M DTT
Low	790	0	100	100	10
Medium	640	50	200	100	10
High	300	100	500	100	0

Figure 2.2 The multiple cloning sites of (a) the plasmid vector pUC18, and (b) the double-stranded replicative form of bacteriophage M13mp18

Maps of plasmid vector pUC18 (a) and the replicative form of bacteriophage M13 (b) are shown. The plasmid vector pUC18 is derived from a fragment of pBR322 by insertion of a fragment of the lac gene at HaeII sites; the multiple cloning sites interrupt the lacZ' (truncated β-galactosidase) gene. Ap^R: ampicillin resistance gene. The same lac fragment including the multiple cloning sites has also been inserted into phage M13. I-X: the genes of bacteriophage M13.

Figure 2.2


(a)
(b)
pUC18
Pvu I
Sca I
ApR
Hae II
ori
2686
0
lac fragment
lacI'
op
lac Z'
Hae II
Pvu I
Hae II
M13mp18
VI
III
VIII
IX
VII
V
X
II
IV
I
7249
0
ori
lac fragment
lacI'
op
lac Z'
Multiple cloning sites
aa1-6
Eco RI
Sac I
Kpn I
Xma I, Sma I
Bam HI
Xba I
Sal I, Acc I, Hinc II
Pst I
Sph I
Hind III

(e) Restriction enzymes

One unit of enzyme activity is defined as the amount of enzyme required to digest lug of DNA to completion in 60 min. Typically, 1µg of DNA was digested with a slight excess of restriction enzyme. The DNA was mixed with 0.1 vol of the appropriate ten fold stock buffer (see Table 2.2) and twice the recommended quantity of restriction enzyme. Sterile water was added to the required volume. The reaction mixture was incubated for 60 min at the required temperature.

2.2.14 Subcloning into M13 (M13mp18)

M13 is a specific filamentous bacteriophage of E. coli. The virus particles contain single stranded, circular DNA which following infection, serves as a template for the synthesis of the complementary strand. The double-stranded form of the viral DNA present in infected cells is called the replicative form (RF form). Several modifications were introduced into the phage and host cells to engineer an efficient vector/host system on the basis of these bacteriophage (Messing, 1983), making it an ideal source for producing single stranded DNA for sequencing by the dideoxy method (Sanger, 1981). The double stranded RF-form was modified to serve as an efficient cloning vector, by introducing the same multiple cloning region and colour selection system for the identification of recombinants (on the basis of β-galactosidase activity) (Fig. 2.2).

All procedures used for cloning were according to the M13 cloning and sequencing handbook (Amersham International).

(a) DNA sequencing

All DNA sequencing was carried out using the M13/dideoxy method (Sanger, 1981; Messing, 1983). Initially the components

were purchased in kit form; latterly, individual components were purchased and substituted. All the protocols for cloning and sequencing were supplied with the kits in the form of the "M13 cloning and sequencing handbook" (Amersham International plc). In the following sections, page numbers in the format (Handbook p1) refer to the Amersham Handbook.

(b) Restriction Digest of RF DNA

RF-form of M13mp18 (1µg) was digested with 4 units of EcoR1 at 37°C for 40 min and then placed on ice. After checking that all the vector had been cut (by running a small aliquot on an agarose gel), the vector was purified by phenol extraction and ethanol precipitation.

(c) Phenol extraction

TE buffer (50µl) was added to the digested vector and extracted with an equal volume of phenol. After a brief vortex (20 s) the sample was microfuged and the upper aqueous layer removed and saved. Another 50ul of phenol was added to the aqueous phase and the process repeated. Care was taken to avoid material at the interface. After the phenol extractions, 500µl of diethyl ether was added to the aqueous layer and the sample vortexed briefly. The aqueous phase (lower) was saved and the process repeated twice more.

(d) Ethanol precipitation

To the phenol/ether extracted DNA 0.1 vol 3M Na acetate pH6.0 was added followed by 2.5 vol of (-20°C) ethanol. After an overnight incubation at -20°C the sample was microfuged for 5 min and the supernatant removed and discarded. The pellet was washed in 750µl of (-20°C) ethanol, care being taken not to disturb the pellet, and, after a brief spin (30 s), the ethanol was discarded.

The pellet was vacuum-dried and the DNA dissolved in TE buffer to give a final concentration of approx. 10ng/ml. The samples were stored in small aliquots at -20°C.

(e) Ligation of RF DNA to insert DNA (Handbook p24)

Ligation mixes for ligation of RF DNA to insert DNA were as follows:

RF DNA (20ng)	2µl
Insert DNA (100ng)	5µl
10 x ligase reaction buffer	1µl
ATP 10mM	1µl
Dithiothreitol	1µl
T4 DNA ligase	1µl

Incubation was for 4-6 h at 14°C.

The final volume was varied from 10-20µl, depending on the volume of buffer in which the insert DNA was resuspended.

(f) Transformation of E. coli JM101 (Handbook p25)

E. coli JM101 was streaked out on minimal medium plates and, after overnight growth, the plate was stored at 4°C (for approx.
1 month before re-streaking). A single colony was innoculated into 2 x TY medium (10ml) and shaken overnight at 37°C. 40ml 2 x TY was inoculated with 2ml fresh overnight culture and shaken (37°C) for 3 h. 10ml 2 x TY was also inoculated with a drop of overnight culture to provide fresh cells. Cells from the 40ml 2 x TY culture were harvested (5000 x g, 5 min) resuspended in 20ml 50mM $CaCl_2$ and left on ice for 20 min before re-harvesting (5000 x g, 5min) and resuspending in 4ml 50mM $CaCl_2$. 0.3ml of competent cells were added to 5ul of DNA ligation mix and kept on ice for 40 min. Cells were then heat shocked at 42° for 3 min and returned to ice.

The following were added to each tube:

100mM IPTG	40µl
2%(w/v)-X-gal	40µl
Fresh cells	200µl
Molten top agar	3ml

and the mix poured onto H plates prewarmed at 37°C.

(g) Preparation of single stranded template DNA (Handbook p27)

A single plaque was mixed (using a sterile Eppendorf pipette tip) into fresh 2 x TY (1.5ml) containing 1ml/100ml medium of an overnight culture of *E. coli* JM101. This culture was shaken for 5 h at 37°C and the cells harvested by spinning in a microfuge for 5 min. The supernatant fluid was carefully transferred to a sterile Eppendorf and microfuged for 5 min to ensure all the cells were removed. The supernatant fraction was added to 200µl PEG/NaCl [20% (w/v) polyethyleneglycol 8000/2.5M-NaCl], shaken and left for 15 min; then centrifuged for 5 min and the supernatant discarded. Following a further 2 min spin, all remaining traces of PEG were removed using a drawn out Pasteur pipette. 100µl TE buffer and 50ul of phenol saturated with TE buffer were added to the viral pellet and vortexed for 15 sec. After 15 min at room temperature the contents were again vortexed for 15 sec and microfuged for 3 min. The upper layer was transferrred to a fresh Eppendorf tube and 1ml of chloroform added. The chloroform was mixed by vortexing for 15 sec and separated after a 1 min microfuge spin. The aqueous (upper) layer was transferred to a sterile Eppendorf tube. 3M-Na acetate, pH6.0 (10µl) and ethanol (250µl) were added and DNA precipitated by standing overnight at -20°C or by placing the tube at -70°C (dry ice/methanol) for 15 min. The DNA was recovered by centrifugation for 10 min and washed with 1ml of -20°C, ethanol.

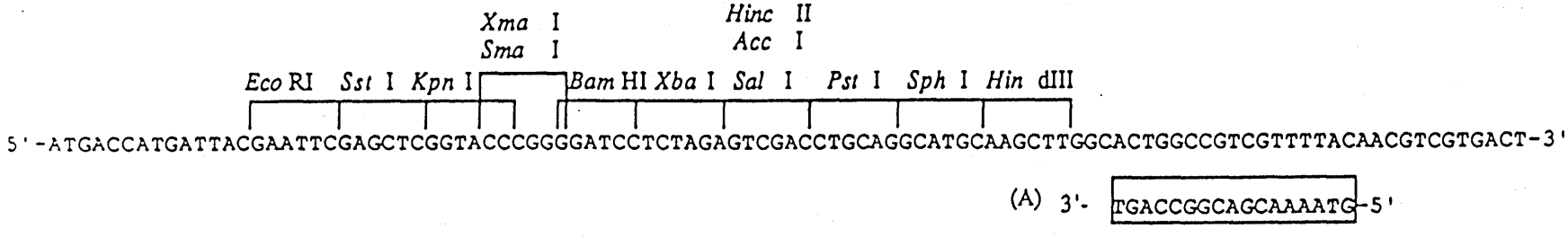
Figure 2.3 Sequence and annealing site of 17-mer primer

The sequences of the multiple cloning sites in the single-stranded templates of M13mp18 and M13mp19 and the restriction sites are indicated. The sequence and the position of annealing of the universal sequencing primer, 17 bases (A) long, is shown.

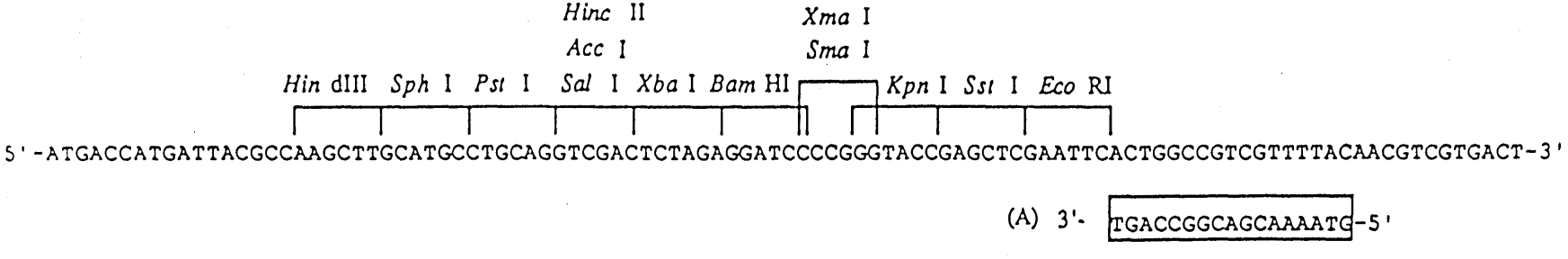
Figure 2.3

(c)

M13mp18



M13mp19



The ethanol was discarded and the tube left to dry. The DNA pellet was redissolved in TE buffer (50ul) and stored at -20°C.

(h) Annealing of primer and template (Handbook p33)

For each clone, the following were placed in a 1.5ml microfuge tube:

single stranded template DNA	5µl
primer	1µl
10 x Klenow reaction buffer (10mM-Tris pH8.5, 100mM $MgCl_2$)	1.5µl
Distilled water	2.5µl

The primer used is a 17-mer, its sequence and its annealing site in M13mp18 can be observed in Figure 2.3.

(i) The sequencing reactions (Handbook p34)

To the annealed primer/template mix 1.5µl (15µci) of (α-^{35}S) dATPαS at > 600 Ci/mmol (Amersham SJ304) and 1µl (1u/ul) of Klenow fragment of E. coli DNA polymerase I. 2.5µl of the mixture was placed on the rim of each of four tubes, marked A, C, G or T in a microfuge rotor. 2µl of the relevant dNTP/ddNTP mix (see below) was placed inside the rim of each tube and a brief spin mixed the contents. After 20 min, 2µl of chase mixture (0.5mM uniform mixture of dATP, dCTP, dGTP and dTTP) was placed on the rim of the tube and mixed in with a brief spin. After a further 15 min chase reaction, 4µl of formamide dye mix was added. Formamide dye mix was prepared as follows: 100ml of formamide was deionised by stirring with 5g Amberlite (BDH) for 20 min, then filtered to remove the resin, to this was added 0.03g xylene cyanol FF, 0.03g bromophenol blue and 4ml 0.5mM-EDTA.

(j) Reaction mixes (Handbook p32)

The final concentration of each reagent in a sequencing reaction mix is listed in Table 2.3.

	A reaction mix	C reaction mix	G reaction mix	T reaction mix
dATP	16.6	16.6	16.6	16.6
dCTP	28	0.3	28	28
dGTP	28	28	0.3	28
dTTP	28	28	28	0.3
ddATP	22.2			
ddCTP		22.6		
ddGTP			66.6	
ddTTP				111

Final concentration of reagents in sequencing reaction mixes. All concentrations are μM.

(k) Polyacrylamide gel electrophoresis (Handbook pp36 and 45)

The DNA fragments in the sequencing reaction mixes were resolved by electrophoresis on polyacrylamide gels (20 x 40 x 0.4cm). The notched plate was siliconised before use with Repelcote.

Two types of gel were employed, linear and buffer gradient. The quantities of reagents necessary to pour a 6% (w/v) acrylamide, 8M-urea tris borate gel are shown in Table 2.4.

Before loading, the wells were flushed out with running buffer (1 x tris-borate) to remove unpolymerised acrylamide and urea, which leaches into the gel slots, preventing the samples from forming a precise layer.

The samples were heated to 95^{o}C for 3 min, then loaded immediately onto the gel. Gels were run at 28mA for 2 to 4 h.

Gels were fixed after removing the notched plate by soaking in a 2:1 bath of 10% (v/v)acetic acid, 10% (v/v) methanol for 30 sec to remove the urea. The gel was removed from the bath and a sheet of Whatman 3MM paper laid on top. The gel was peeled off with the paper backing, placed on a Bio-rad Model 1125 gel dryer and covered in saran wrap. Sixty minutes under vacuum was usually sufficient to dry the gel.

Table 2.4 Quantities of reagents necessary to pour a single polyacrylamide gel for DNA sequence analysis

		Linear	buffer gradient	
			Top	Bottom
Urea	(g)	21	19.2	3.8
acrylamide stock	(ml)	7.5	6	1.125
10 x tris-borate	(ml)	5.0	2	1.875
sucrose	(g)			0.75
bromophenol blue	(ml)			0.075
water	(ml)	50	40	7.5
TEMED	(µl)	50	80	15
AMPS (conc)	(µl)	300	80	15

Acrylamide stock 40% (w/v) 38:2 acrylamide : NN'methylene bisacrylamide deionised by stirring with 5g Amberlite MB1 and filtered through a scintered glass funnel.

(1) Autoradiography

The dried gel was placed in a light proof cassette and covered by a sheet of Kodak X-Omat X-ray film. The film was exposed overnight at room temperature before developing.

2.2.15 Plasmid preparations

(a) Plasmid preparation of PTZ18

Plasmid PTZ in host HB101 was used to inoculate a 10ml culture of L broth containing 50µg ml^{-1} of ampicillin. After overnight growth at 37°C, 500µl was removed and used to inoculate a second 10ml LB + ampicillin medium and incubated at 37°C until the A_{620} was 0.6. At this time chloroamphenicol was added to a final concentration of 170µl/ml and the culture incubated for a further 20 h at 37°C.

The culture was pelleted by centrifugation at 5000 x g for 10 min, the supernatant was discarded and 2.5mg ml^{-1} lysosyme added. After 5 min at room temperature, the cells were lysed by the addition of 200µl 0.1% (w/v) SDS, 0.2M NaOH. 0.5M potassium acetate pH4.8 followed and after 15 min in ice the cells were transferred to sterile Eppendorf tubes and pelleted at 16000 g for 15 min. The supernatant fluid was phenol extracted followed by extraction with equal volumes of chloroform isoamyl alcohol (24:1 v/v). Chloroform denatures the proteins while the isoamyl alcohol reduces foaming during the extraction phase and facilitates the separation of the aqueous and organic phases. DNA was precipitated by the addition of 3M Na acetate followed by 4 vol of ethanol and storage overnight at -20°C. The DNA was pelleted in a microfuge and washed 3 times in 80% (v/v) ice cold ethanol before being dried under vacuum. The DNA was solubilised in an appropriate volume of TE buffer and RNA was removed by the addition of heat-treated RNase I (0.5µg/ml final).

(b) Ligation of insert DNA into plasmid PTZ 18R

PTZ 18R and PTZ 19R are specially designed plasmids which permit DNA cloning, dideoxy DNA sequencing, *in vitro* mutagenesis and

in vitro transcription in one system. The size of PTZ 18R is approximately 2880 base pairs and a 300bp insert ligated into the vector would constitute 10% of the plasmid. Therefore greater amounts of insert can be produced when ligated with PTZ 18R and amplified by a plasmid preparation.

Ligation of insert was carried out essentially as described in Section 2.2.14e. A single colony from *E. coli* host TG1 was inoculated into 2 x TY medium (10ml) and grown overnight at 37°C and processed as described in Section 2.2.14f. Selection of recombinant was slightly different. IPTG and X-gal were spread onto H plates and allowed to dry before 100µl of ligation mixture was spread on to the plate. Clear colonies were assumed to be successfully ligated plasmid and insert. Purified plasmid and insert was prepared as described above.

CHAPTER THREE

ACETYLATION REACTIONS OF COMPONENT X OF MAMMALIAN PYRUVATE DEHYDROGENASE COMPLEX

3. Acetylation reactions of component X of mammalian pyruvate dehydrogenase complex

3.1 Introduction

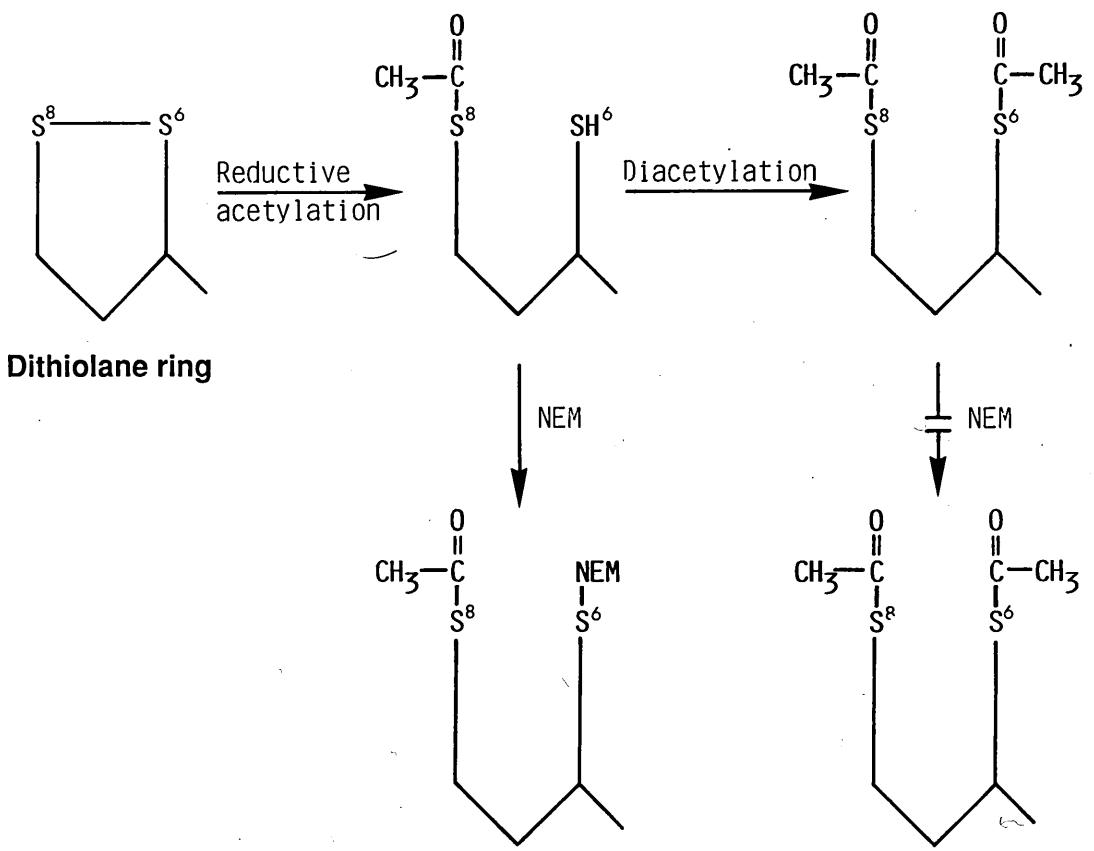
A recently recognised subunit of mammalian PDC, M_r 51000 forms a close physical and functional union with the E2 core assembly (De Marcucci et al., 1986; Jilka et al., 1986). In addition, the E2 assembly, isolated from PDC of Saccharomyces cerevisiae, contains a tightly-bound polypeptide M_r 48000 which exhibits similar properties to mammalian protein or component X (Kresze and Ronft, 1981). While the distinctive nature of this subunit in eukaryotic PDC has been confirmed recently (De Marcucci and Lindsay, 1985), it was originally thought to be a degradation product of E2 or possibly the intrinsic kinase (Bliele et al., 1981). However, by employing refined immunological techniques and protein chemical criteria it has been possible to establish that component X is a specific polypeptide distinct from the other major components of the complex (De Marcucci and Lindsay, 1985; Jilka et al., 1986).

In similar manner to E2, component X is reductively acetylated by E1 in the absence of CoASH (De Marcucci et al., 1986). Acetylation of E2 and component X requires an active E1 subunit and rapid deacetylation occurs on the addition of CoASH. The site of acetyl group incorporation on E2 polypeptides is the lipoic acid prosthetic group which is covalently attached via an amide bond to the N^6-amino group of specific lysine residues in the polypeptide chain. Recently the site of acetylation on component X has also been established to be lipoic acid (Hodgson et al., 1986). Comparison of the deduced amino acid sequences of yeast protein X and E2 reveals that the amino terminal part of protein X resembles E2, but the carboxyl-terminal is completely different (Behal et al., 1989). Protein X lacks the

Figure 3.1 Substrate protection against NEM modification and inactivation of PDC

The diagram indicates only the site of chemical attack by NEM not the exact product formed after modification of the reduced thiol on the dithiolane ring.

Substrate protection against NEM modification and inactivation of PDC



Dithiolane ring

carboxy-terminal segment of E2 that contains a highly conserved sequence which is thought to be part of the putative catalytic site of all dihydrolipoamide acyltransferases (Miles and Guest, 1987). This finding suggests that yeast protein X, in contrast to E2, is unable to catalyse an acetyl transfer between the protein-bound S-acetyldihydrolipoyl moiety and CoASH.

A unique feature of eukaryotic PDC is its ability to diacetylate the lipoic acid moieties on E2 (Fig. 3.1). Treatment of PDC with N-ethylmaleimide (NEM) results in a substrate or product-dependent inhibition of complex activity owing to the generation of a free thiol on the S-acetyldihydrolipoamide intermediate. However, prolonged incubation of PDC with pyruvate or NADH plus acetyl CoA prior to the addition of N-ethylmaleimide diminishes the inhibition of the complex activity by NEM and also reduces incorporation of N-ethyl [2,3-^{14}C] maleimide into both E2 and component X. The increase in protection against inhibition by NEM occurs in parallel with a second, slow phase of incorporation of acetyl groups into both E2 and protein X which is in agreement with a previous report (Cate *et al.*, 1980). It has been suggested that prolonged incubation with acetylating substrate under these conditions produces an $\underline{S}^{6}\ \underline{S}^{8}$ (diacetyl) lipoyl group (Fig. 3.1). The formation of $\underline{S}^{6}\ \underline{S}^{8}$ (diacetyl) lipoamide by mammalian PDC has been detected previously by ^{13}C-nmr spectroscopy using high concentrations of exogenous dihydrolipoamide as substrate (O'Connor *et al.*, 1982). The physiological significance of diacetylation is discussed more fully in Chapter 7.

Preparation of PDC free from component X employing standard biochemical separation techniques would greatly facilitate studies on the precise function of component X in the complex. It is possible to dissociate E1, E2 and E3 in active form with relative ease (Linn *et al.*, 1972). Unfortunately under these conditions

component X remains tightly bound to E2. However, in a recent report it has been claimed that an active E2 oligomer can be prepared which is devoid of component X (Powers-Greenwood et al., 1989). By employing severe dissociating conditions i.e. 5.2M urea, 0.35M NaCl and 0.2M EDTA, it has proved possible to separate an oligomeric form of E2 by gel filtration. The isolated E2 oligomer apparently retains its acetyltransferase activity and capacity to bind E1. The E2 oligomers have a greatly diminished ability to bind the E3 component leading the authors to suggest that protein X is responsible for binding E3 to the core structure. Such data must be viewed with caution as the integrity of the E2 core and damage to the outerdomains under such severe dissociating conditions has not been fully assessed.

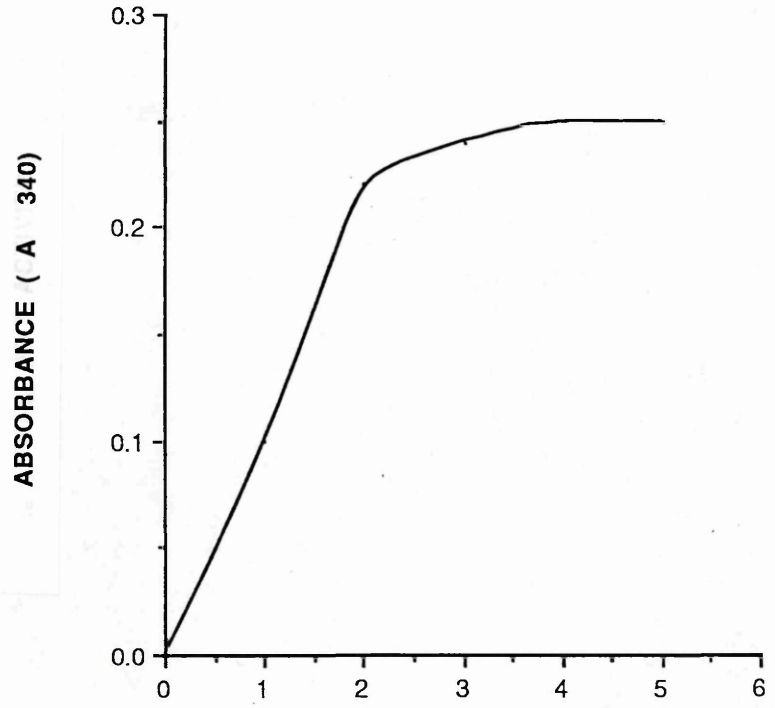
In view of the close physical and functional relationship between E2 and protein X within the E2 core structure, it has been difficult to determine the precise function of this polypeptide in the multienzyme complex. Does component X mediate directly in the catalytic mechanism for the transfer of two carbon units from E1 to the final acceptor CoASH or is component X in equilibrium with E2 with which it interacts strongly, perhaps promoting acetyl group transfer to an unknown physiological acceptor? It is also important to establish if E2 and X polypeptides can interact independently of each other with pyruvate dehydrogenase (E1) or is there a defined sequence of acetyl group transfer between these polypeptides (Fig. 1.7). Similarly while both polypeptides can be deacetylated by the addition of CoASH, it is not known if both can interact independently with this cofactor with X providing an alternative pathway for the transfer of acetyl groups and electrons into CoASH and NAD^+ respectively.

A number of approaches were employed in this chapter in an attempt to delineate the route of acetyl group transfer within the PDC complex and the involvement of protein X.

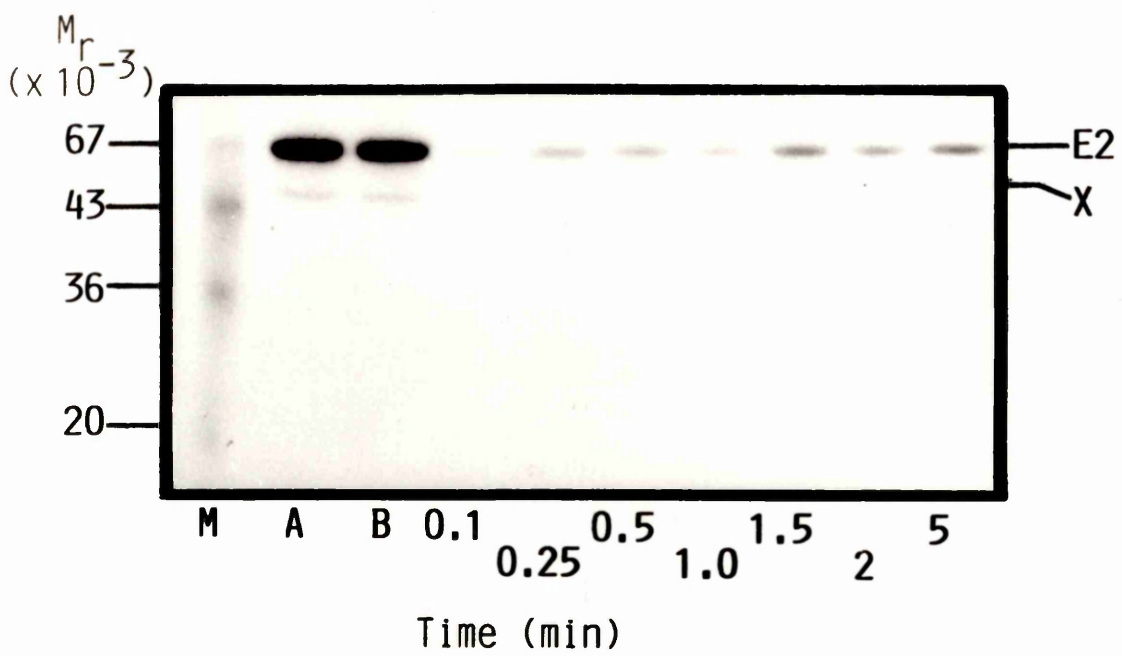
Figure 3.2 Acetylation of E2 and component X during normal turnover of the complex

250μg of PDC was made to 100μl with 20mM potassium phosphate buffer pH7.4 containing 2.5mM NAD^+, 1mM $MgCl_2$ and 0.1mM TPP. CoASH was added to a final concentration of 0.2mM and the mixture incubated for 10 min. 5μCi of [2-^{14}C] pyruvate was added and 5μg of enzyme withdrawn and assayed (A). After 6s, 12s, 30s, 1 min, 1.5 min, 2min and 5 min, 25μg aliquots were withdrawn and added directly to Laemmli sample buffer. The samples were resolved by SDS-PAGE on a 10% (w/v) gel and processed for fluorography (B). Lane A, 25μg of PDC labelled with [2-^{14}C] pyruvate in the absence of CoASH; lane B, PDC labelled with [2-^{14}C] pyruvate in the presence of NEM, final concentration 0.5mM Lane M ^{125}I M_r markers.

A


ABSORBANCE (A 340)
0.3
0.2
0.1
0.0
0
1
2
3
4
5
6
TIME (MIN)

B


Mr
(x 10-3)
67
43
36
20
E2
X
M
A
B
0.1
0.25
0.5
1.0
1.5
2
5
Time (min)

3.2 Results

3.2.1 Acetylation of E2 and component X during normal turnover of PDC

As indicated previously to test the possibility that [^{14}C] acetylation of protein X was a non physiological side reaction promoted by conditions required to trap the [^{14}C] acetyl dihydrolipoamide intermediate, samples were removed for analysis of the extent of acetylation of E2 and component X at various stages during the time course of the standard enzyme reaction, resolved by SDS-PAGE and processed for fluorography as described in Section 2.2.4f.

Clearly component X is not observed in Figure 3.2 although it is the easily detectable original in the fluorograph. Therefore the conclusions drawn are based on the data obtained from the fluorograph. In the presence of CoASH low levels of acetylating substrate are incorporated into both E2 and protein X during the initial stages of the enzyme reaction (Fig. 3.2). As the reaction rate declines and the reaction reaches equilibrium, increased labelling of both E2 and component X is observed until at equilibrium the degree of incorporation of radio-label remains constant. Thus it appears that E2 and X equally participate in the kinetics of acetylation during normal turnover of the enzyme, and acetylation of protein X does not appear to represent a non-physiological side reaction of the acetyltransferase and is compatible with it acting as an normal catalytic intermediate.

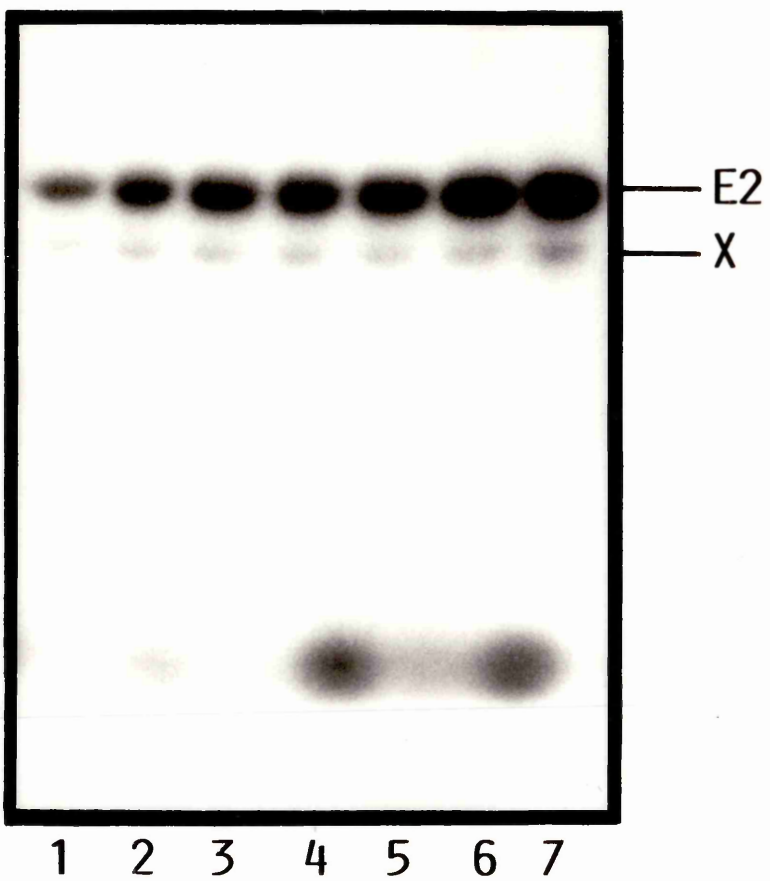
3.2.2 Low temperature labelling of E2 and component X with [2-^{14}C] pyruvate

In operational terms both E2 and X can be acetylated at similar rates and to similar extents at room temperature by E1 in a

Figure 3.3 Low temperature labelling of E2 and component X with [2-^{14}C] pyruvate

A sample of PDC (250µg) was incubated at -20°C overnight in 50mM potassium phosphate buffer, pH7.2 containing 1mM $MgCl_2$, TPP, NAD^+ and 50% (w/v) glycerol. On the addition of [2-^{14}C] pyruvate (0.1mM) samples (30µg) were withdrawn and added immediately to boiling Laemmli sample buffer before resolution on a 10% (w/v) SDS/polyacrylamide gel and processing for fluorography. Lanes 1-6 PDC incubated with [2-^{14}C] pyruvate at -20°C for 0s, 5s, 30s, 45s, 60s and 120s.

As a control lane (7), PDC (30ug) in 50% (w/v) glycerol was incubated in [2-^{14}C] pyruvate at room temperature for 5s before treatment as above.


E2
X
1 2 3 4 5 6 7

TPP-dependent reaction which is also affected by the phosphorylation state of E1. The E1-mediated transfer of reducing equivalents and acetyl group in E2 and X is reported to be the rate-limiting step in overall complex activity (Cate and Roche, 1979). In an attempt to determine a defined sequence of acetyl group migration between these components loading of [2-^{14}C] pyruvate was performed at -20^{o}C. It was hoped that the temperature-dependence of the individual steps was sufficiently altered such that the sequence of acetyl group transfer between E1 and E2/X would no longer be rate limiting and the route of acetyl group transfer between E2 and X could be elucidated.

At -20^{o}C in 50% (w/v) glycerol PDC was incubated with [2-^{14}C] pyruvate and samples were withdrawn at different time intervals from the incubation mixture. These were added immediately to boiling Laemmli sample buffer before analysis by SDS polyacrylamide gel electrophoresis and processing for fluorography (Section 2.2.4).

The extent of [2-^{14}C] pyruvate incorporation into E2 and component X at -20^{o}C can be visualised in Figure 3.3. It is apparent that the rate of loading of the enzyme has been significantly reduced, as throughout the length of the time course, at -20^{o}C, compared to incubation at room temperature for 5 s (lane 7), the rate of acetyl group incorporation into E2 and component X was increased slowly. E2 and component X undergo reductive acetylation almost immediately and throughout the time course with increased labelling of the subunits occurring in a time-dependent manner. However, at all times labelling of E2 and X occurred in parallel and therefore the sequence of acetyl group transfer under these conditions could not be elucidated.

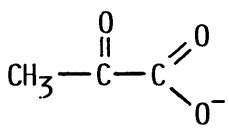
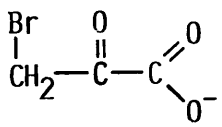
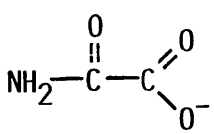
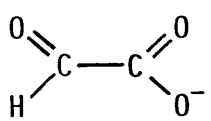
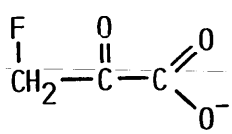
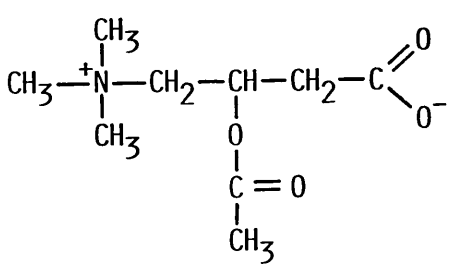
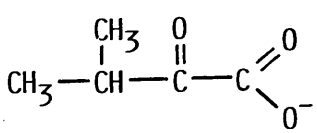
Table 3.1 Overall enzyme activity using substrate analogues

	Substrate analogue (20mM)	PDC activity using substrate analogues	PDC activity with pyruvate 30 min preincubation in substrate analogue
1	Pyruvic acid	100	100%
2	β-bromopyruvate	-	0
3	α-oxamate	-	96
4	glyoxalate	-	91
5	β-fluoropyruvate	-	0
6	acetylcarnitine	-	91
7	α-ketoisovalerate	-	92
8	oxaloacetic acid	-*	0
9	α-keto-β-methyl-N-valerate	-	94
10	α-keto-isocaproic acid	-	99
11	α- ketobutyrate	40	50
12	α-ketoglutarate	-	99
13	α-ketovalerate	-	90
14	ρ-hydroxyphenylpyruvic acid	-	0
15	β-hydroxypyruvic acid	-	0

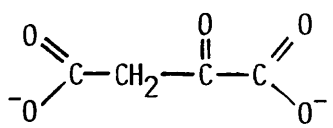
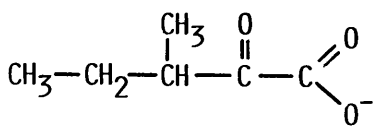
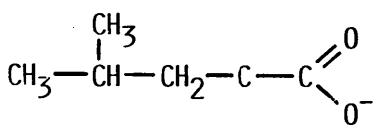
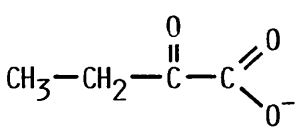
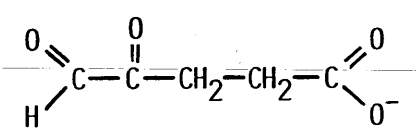
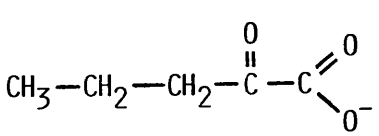
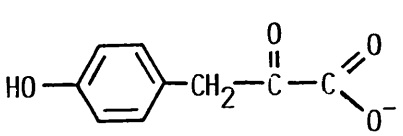
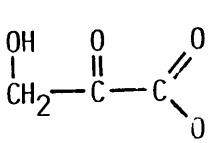
* See text for explanation of recorded oxaloacetic activity

Each determination is the result of two assays which differed by less than 10%..

FIG. 3.4 **Structure of substrates**

Name	Structure
Pyruvate	
β bromopyruvate	
α oxamate	
Glyoxylate	
β fluoropyruvate	
Acetyl carnitine	
α ketoisovalerate	

Structure of substrates

Name	Structure
Oxaloacetate	
α keto-β-methyl-N-valerate	
α ketoisocaproate	
α ketobutyrate	
α ketoglutarate	
α ketovalerate	
P-hydroxyphenylpyruvate	
β hydroxypyruvate	

3.2.3 Overall PDC activity with various substrate analogues

In a separate approach, a series of substrate analogues were employed with a view to assessing their potential as inhibitors of acetyl group transfer between E1 and E2 or X. This idea stemmed from an earlier observation that [2-^{14}C] pyruvate can be transferred onto the lipoic acid on E2 of the 2-oxoglutarate dehydrogenase complex, although it cannot be released subsequently by CoASH addition. The 2-oxoglutarate dehydrogenase is therefore able to utilise a substrate analogue of 2-oxoglutarate, pyruvate (J.A. Hodgson, unpublished results). By adopting a similar approach with substrate analogues of pyruvate, an attempt was made to disrupt the interaction between E1 and E2/X and therefore inhibit the transfer of substrate analogue between E2 and X or X and E2. In preliminary experiments a variety of possible analogues were tested for their efficiency as substrates for pyruvate dehydrogenase complex (Table 3.1). The structures of the substrate analogues can be seen in Figure 3.4.

With the notable exceptions of oxaloacetate and α-ketobutyrate the remaining analogues were inactive as substrates for the pyruvate dehydrogenase complex. It was found that with freshly prepared oxaloacetate no PDC activity could be detected but that an increase of activity was observed with time resulting from the rapid decomposition of oxaloacetate to pyruvate and CO_2.

3.2.4 HPLC analysis of α ketobutyrate

The possibility that the activity recorded using α-ketobutyrate as substrate was a result of contaminating pyruvate was investigated. Samples assayed from freshly prepared α-ketobutyrate over a time period revealed no increase in activity suggesting that unlike oxaloacetate, α-ketobutyrate was not decomposing to pyruvate.

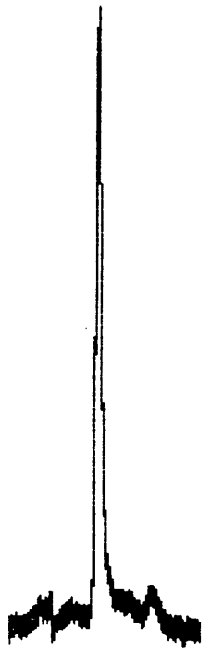
Figure 3.5 HPLC analysis of 2-oxobutyrate and pyruvate

(a) Elution profile from 20µl of 1mM ∝-ketobutyrate passed through a fast organic acid HPLC column at a flow rate of 1ml/min and detected by UV absorbance at 210nm.

(b) Elution profile from 20µl of equilmolar mixture of ∝-ketobutyrate and pyruvate passed through a fast organic acid HPLC column at a flow rate of 1ml/min and detected by UV absorbance at 210nm

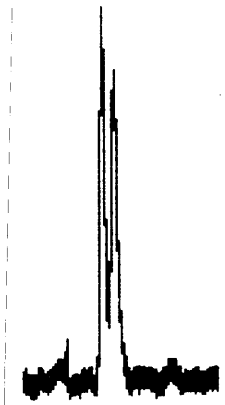
(c) Elution profile from 20µl of 1mM pyruvate passed through a fast organic acid HPLC column at a flow rate of 1ml/min and detected by UV absorbance at 210nm.

α-ketobutyrate



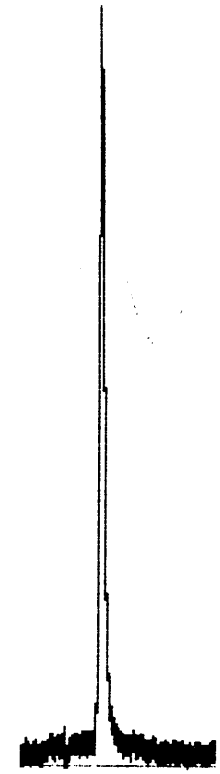
RT	Area%
1.28	0.834
2.62	86.484

α-ketobutyrate/pyruvate



RT	Area%
2.30	44.794
2.63	52.141

pyruvate



RT	Area%
1.30	0.942
2.31	99.058

To eliminate the possibility that the overall complex activity observed with ∝-ketobutyrate was caused by contamination with pyruvate, ∝-ketobutyrate and pyruvate were subjected to HPLC analysis. HPLC analysis of ∝-ketobutyrate detected no pyruvate (Fig. 3.5). ∝-ketobutyrate is therefore a genuine substrate producing a rate of reaction approximately 50% of that obtained with pyruvate. During the course of this work ∝-ketobutyrate was confirmed as a substrate for pyruvate dehydrogenase (Paxton et al., 1986).

Although with the exception of ∝-ketobutyrate, the substrate analogues failed to promote PDC activity, the possibility exists that several of these compounds are trapped on the lipoic acid sites on E2 and component X and are unable to be transferred to a suitable physiological acceptor. Alternatively the substrate analogues may inhibit E1 activity by binding to the active site or reacting with TPP in such a way that the substrate cannot be transferred onto the E2 core assembly. To investigate the possibility that the substrate analogues could inhibit pyruvate-dependent enzyme activity, PDC was preincubated in substrate analogue (final concentration 0.4mM) for 1 min before pyruvate dependent enzyme activity was measured (Table 3.1). The assay was repeated at time intervals of 15 min and 30 min. Prolonged incubation of PDC in bromopyruvate, fluoropyruvate, hydroxypyruvate and hydroxyphenylpyruvate resulted in a time-dependent inactivation of pyruvate-dependent complex activity. No such effect was recorded with the remaining substrates. Incubation in ∝ ketobutyrate resulted in a 50% decrease in overall enzyme activity.

3.2.5 Substrate analogue protection of E2 and component X from NEM incorporation

Prolonged incubation of PDC in substrate or NADH plus acetyl CoA prior to the addition of NEM protects the complex from NEM inhibition and also reduces the incorporation of [^{14}C] NEM into E2 and X. As radiolabelled substrate analogues were unavailable this offered a means of visualising, by [^{14}C] NEM incorporation, the degree of substrate analogue incorporation into E2 and X and the possibility of observing any lack of catalytic exchange of substrate between E2 and X by means of preferential labelling with [^{14}C] NEM of E2 or X.

Radiolabelled NEM and substrate analogue were added simultaneously to PDC to test if there was immediate activation of the dithiolane rings. Alternatively PDC, was preincubated for 120 min with substrate analogue to examine the level of protection on E2 and X. The reaction was terminated by addition of 2-mercaptoethanol and the products resolved on SDS-PAGE before processing for fluorography. The results of the experiment are visualised in Figure 3.6. The substrate analogues listed in Table 3.1 were employed as possible substrates for pyruvate dehydrogenase (E1) in the absence of CoASH. Several of the substrate analogues were ineffective in activating the dithiolane ring, (lanes 4,5; 8,9; 10,11; 12,13; 25,26; 29, 30) i.e. β-bromopyruvate, glyoxalate β-fluoropyruvate, acetyl carnitine, α-ketoglutarate and p-hydroxphenylpyruvate. All revealed on simultaneous addition with [^{14}C] NEM, little incorporation of radio label into E2 and X, results which are in agreement with the measurement of overall enzyme activity using these analogues as substrates. It is observed in (lanes 14,15; 16,17; 27,28), α-ketoisovalerate, oxaloacetate, α-ketovalerate, reveal little increase in NEM

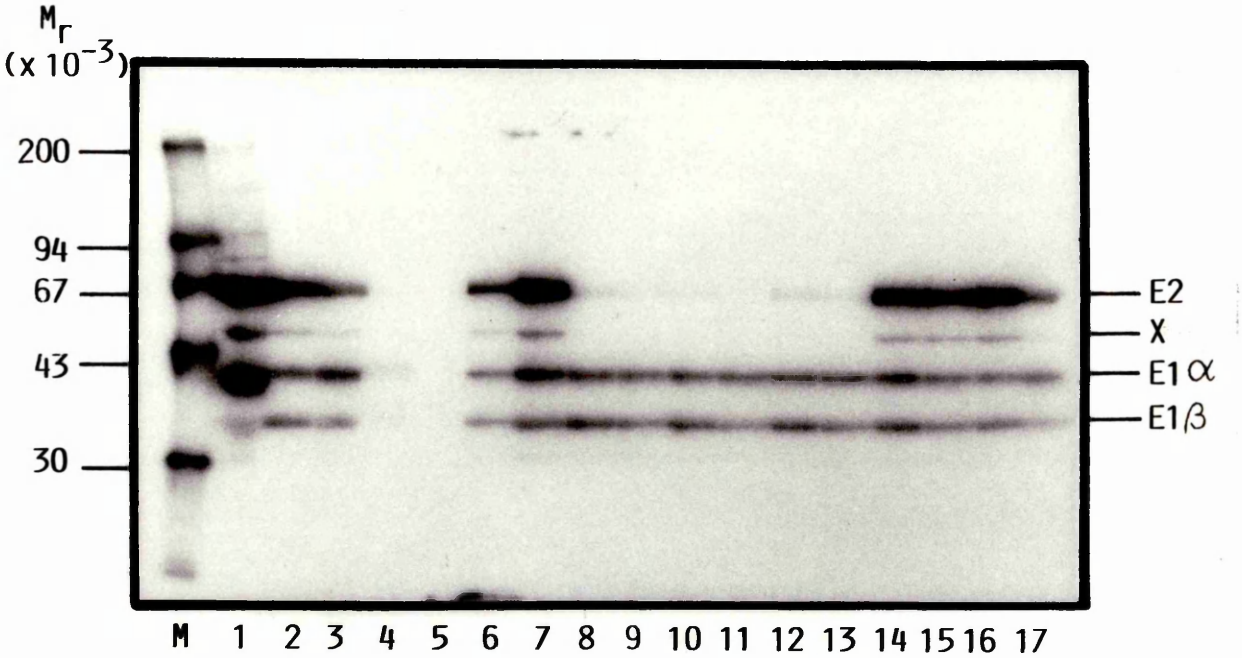
Figure 3.6 Substrate analogue protection of E2 and component X from NEM incorporation

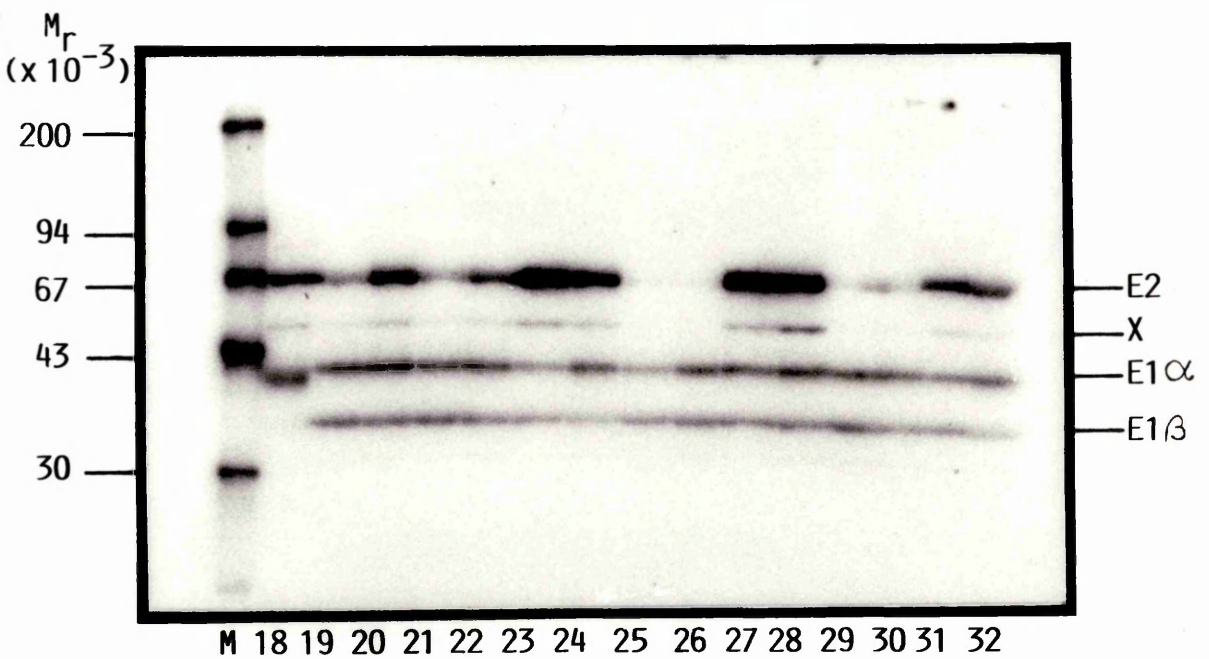
2mg of PDC was incubated in 20mM potassium phosphate pH7.4 containing 2.5mM NAD^+, 1mM $MgCl_2$ on 0.1mM TPP. NEM was added to a final concentration of 0.5mM. After 120 min excess NEM was removed by centrifugal gel filtration through a 1ml Sephadex G25 column equilibrated in the buffer detailed above (Penefsky, 1977).

60µg of NEM modified PDC was aliquoted into 30 tubes and the tubes separated into 15 pairs. A different substrate was added to one of each pair of tubes to a final concentration of 4mM. After incubation of substrate for 120 min at RT 1µCi of N-ethyl [2,3-^{14}C] maleimide was added to each of the 30 tubes followed immediately by the appropriate substrate to the second tube. After 20 min the reaction was quenched by the addition of 2-mercaptoethanol to 45mm. The samples were then added to Laemmli sample buffer boiled briefly before resolving on a 10% SDS/polyacrylamide gel and processed for fluorography (Section 2.2.4.5).

(lane M) ^{125}I M_r Markers
(lanes 1, 18) Simultaneous addition of luCi N-ethyl [2,3-^{14}C] maleimide and pyruvate (final concentration 4mM) to 30ug of PDC. (lanes 2, 3) pyruvic acid, (lanes 4, 5); Bromopyruvate, (lanes 6, 7) oxamate, (lanes 8, 9) glyoxylate, (lanes 10, 11) fluoropyruvate, (lanes 12, 13) acetyl carnitine, (lane 14, 15) Keto isovalerate, (lanes 16, 17) oxaloacetic acid, (lanes 19, 20) Keto-β-methylvalerate, (lanes 21, 22) α Keto isocaproic acid, (lanes 23, 24) α- ketobutyrate, (lanes 25, 26) 2-ketoglutarate, (lanes 27, 28) Ketovalerate, (lanes 29, 30) ρ-hydroxyphenylpyruvic acid, (lanes 31, 32) β-hydroxypyruvic acid. The first lane of each pair is the simultaneous addition of substrate analogue and radiolabel. The second lane of each pair is the addition of radiolabel after 120 min incubation in substrate analogue.

*Each substrate was prepared immediately before use.


Mr
(x 10-3)
200
94
67
43
30
E2
X
E1α
E1β
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17


Mr
(x 10-3)
200
94
67
43
30
E2
X
E1α
E1β
M 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32

incorporation with time in substrate indicating that this group of substrate analogues are transferred onto E2 and X but subsequently are unable to incorporate substrate induced modification of the second reduced sulphydryl group. This result suggests that these analogues are possible substrates for E1 but are subsequently may be trapped on E2 and component X. Of the remaining substrates analogues (lanes 6,7; 19,20; 21,22) α-oxamate, α-keto β-methyl-n-valerate, α-ketoisocaproate reveal increased NEM incorporation with time in substrate indicating that this group of substrate analogues are transferred very slowly onto E2 and X.

An interesting observation was that β-hydroxypyruvic acid as well as the standard substrates pyruvate and α-ketobutyrate were observed to protect E2 and component X after prolonged incubation in substrate. In all cases, both E2 and X were labelled in parallel with no preferential labelling of either E2 or X (lanes 2,3; 23, 24; 31 and 32). Unloading of the substrate analogues in the presence of CoASH was not attempted

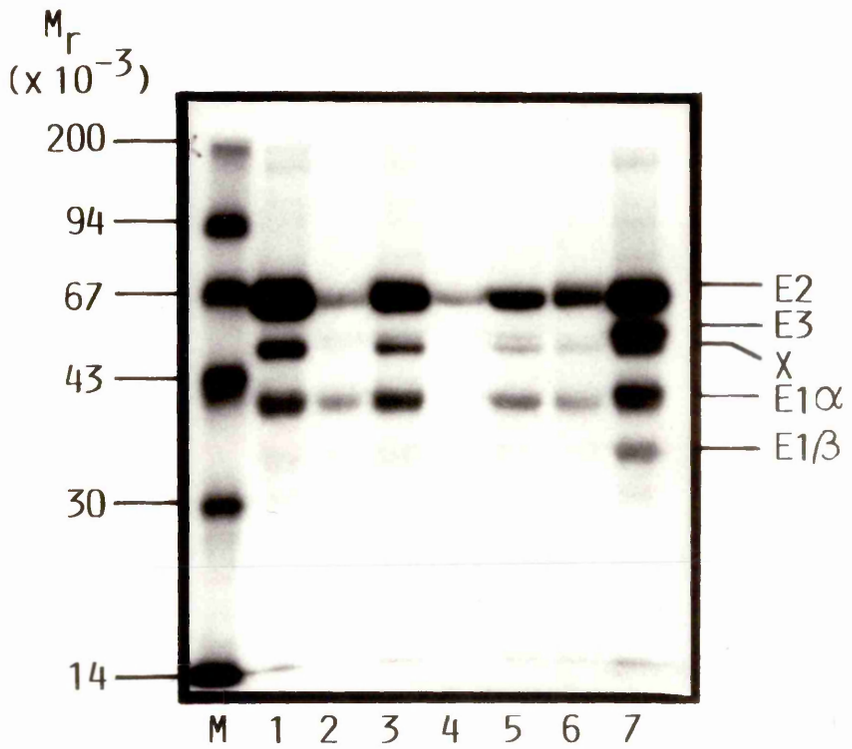
3.2.6 N-ethyl [2,3-^{14}C] maleimide incorporation into NADH reduced PDC incubated in various acyl CoAs

The rate limiting step in pyruvate dehydrogenase complex is the reductive acetylation of the core components of the complex (Cate and Roche, 1980). Failure to disrupt any interaction between E2 and X in the forward reaction may possibly be a result of E2 and component X being equally effective as substrate for E1. An alternative approach which does not rely on E1, is to incubate NADH reduced PDC with acetyl CoA this protects E2 and component X from NEM incorporation. This acetylation may be observed in the reverse reaction which depends on the specificity of the E2 component. Therefore it may be possible to preferentially label E2 or component X with a suitable CoASH derivative.

Figure 3.7 N-ethyl [2,3-^{14}C] maleimide incorporation into NADH reduced PDC

250µg of PDC was made to 100µl in K_2HPO_4 buffer pH7.0. NADH was added to a final concentration of 0.5mM. After 10min, to 30ug of NADH reduced PDC, the appropriate acyl CoA derivative was added to a final concentration of 0.4mM. After a further 20 min 1µCi of NEM was added to each tube. The reaction was stopped by the addition of 2-mercaptoethanol to a final concentration of 45mM.

(M) ^{125}I Marker, (lane 1) malonyl CoA, (lane 2) acetyl CoA, (lane 3) Succinyl CoA, (lane 5) acetoacetyl CoA, (lane 6) S-palmitoyl CoA, (lane 7) NADH reduced PDC.


Mr
(x 10-3)
200
94
67
43
30
14
M 1 2 3 4 5 6 7
E2
E3
X
E1α
E1β

A limited number of CoASH derivatives were employed in this study due to expense and availability. The acyl CoA derivatives were incubated in NADH reduced PDC for 20 min before the addition of [^{14}C] NEM.

The reaction was stopped on the addition of 2-mercaptoethanol and the samples were added to Laemmli sample buffer before resolving by SDS-PAGE and processing for fluorography (Section 2.2.4f).

As expected (lane 2) acetyl groups protect the complex from NEM incorporation (Fig. 3.7). Malonyl and succinyl CoA (lanes 1 and 3) revealed little protection of E2 and X indicating that these groups were probably not transferred onto E2 and X (lanes 1 and 3). Acetoacetyl CoA and S-palmitoyl CoA (lanes 5 and 6) offered partial protection of E2 and X. The results obtained from acetoacetyl CoA and s-palmitoyl CoA need to be treated with caution. Reduced labelling of the E1 α subunit in both cases is observed and this may be a consequence of variable loading of the gel sample.

3.3 Discussion

In this chapter we have shown that in the presence of CoASH, component X is involved in acetyl group transfer reactions during the normal catalytic cycle. If in the absence of CoASH the [^{14}C] acetylation of component X was a side reaction promoted by conditions required to trap [^{14}C] acetyl groups as acetyl dihydrolipoamide, incorporation of [^{14}C] acetyl groups into component X would not be expected to occur during normal turnover of the enzyme.

As normal turnover number of PDC is approximately 1800 per s, the normal loading occurred in the millisecond time scale.

Although we were successful in reducing the turnover of pyruvate dehydrogenase complex by low temperature labelling we were unable to observe preferential loading of E2 or component X under these conditions. The rate limiting step in pyruvate dehydrogenase complex is the reductive acetylation of E2 and component X by E1 (Cate and Roche, 1980) and from the data in Figure 3.3 E2 and component X undergo acetylation almost immediately under these conditions. It has been demonstrated that purified E1 from bovine kidney and heart catalysed the incorporation of ^{14}C-labelled acetyl groups into isolated E2 component (Linn et al., 1972; Barrera et al., 1972). Similar results were obtained with isolated lipoyl domains (Bliele et al., 1981). Recently Perham demonstrated that E1 of PDC in E. coli can incorporate ^{14}C-labelled acetyl groups, although poorly, into synthetic lipoyl peptides of E2 and into free lipoate and has also demonstrated that E1 will label free E2 domains rapidly. It is therefore unlikely considering the ability of E1 to reductively acetylate such poor substrates of E2 that slowing the turnover of the intact enzyme would disrupt sufficiently the transfer of acetyl group from E1 to E2 and component X. For transfer of acetyl groups between E2 and component X to be identified the reductive acetylation of E2 and component X would need to be disrupted in such a way that it was no longer the rate limiting step. (Perham and Packman, 1989)

An interesting observation is, in the presence of CoASH, ketobutyrate could act as a substrate for pyruvate dehydrogenase activity. Investigation of the properties of α-ketobutyrate revealed no contaminating pyruvate. It was also recognised that on

prolonged incubation of ∝ ketobutyrate in enzyme complex resulted in the protection of lipoic acid residues on E2 and component X from NEM incorporation. A similar result was observed with hydroxypyruvate. This phenomenon, first recognised after prolonged incubation with acetylating substrate, was thought to produce $\underline{S}^6\ \underline{S}^8$ (diacetyl) lipoyl groups, The formation of $\underline{S}^6\ \underline{S}^8$ (diacetyl) lipoamide has been detected previously by ^{13}C-NMR (O'Connor et al., 1982). The structure of ∝ ketobutyrate is analogous to pyruvate differing in length by one carbon unit, the close structural similarity to pyruvate is the probable cause of ketobutyrate effectiveness as a substrate for overall enzyme activity.

Rigorous kinetic analysis on the types of inhibition of complex activity was not attempted. However, it has been reported that modification of the methyl group in pyruvate produces compounds that behave initially as strong competitive inhibitors of pyruvate dehydrogenase complex of E. coli. (Bisswanger, 1981). Bromopyruvate, fluoropyruvate, hydroxypyruvate and hydroxyphenylpyruvate were found to be ineffective as substrates although incubation of these substrate analogues with pyruvate resulted in a slow inactivation of the complex. The mode of action of these substrate analogues on enzyme activity in E. coli is known to be complex (Lowe and Perham, 1984). Using bromopyruvate as an example they demonstrated that it was an active site inhibitor of free El component and, in the presence of TPP, the substrate analogue acts as a competitive inhibitor with pyruvate. It was also observed that El catalysed the decomposition of bromopyruvate rendering itself inactive. Bromopyruvate is also known to inactivate the intact complex in a TPP dependent process probably by reductively bromoacetylating the lipoic acid residues in the lipoate acetyltransferase.

Enzyme incubated in substrate analogue and pyruvate results in a slow inactivation of the complex. This is probably due to the presence of TPP in the NAD^+ reduction assay. With bromopyruvate, fluoropyruvate and hydroxyphenylpyruvate, the slow inactivation of the complex was probably related to a substrate analogue event on E1 or TPP as no incorporation of labelled NEM is observed on E2 and component X. The inactivation of the complex with hydroxypyruvate was a result of lipoic acid residues on the lipoate acetyltransferase becoming reductively hydroxyacetylated as demonstrated by protection from NEM incorporation.

Observation of the remaining substrate analogues reveals greater incorporation of labelled NEM into E2 and X after prolonged incubation in substrate. As no overall enzyme activity or inactivation in the presence of pyruvate was recorded the substrate analogues are obviously poor substrates loading onto E2 and X slowly before becoming trapped.

Using the reverse reaction which utilises E2 rather than E1, with a limited set of acyl CoA derivatives, no preferential labelling of E2 or component X was observed.

The activation of PDC kinase activity by a variety of CoA esters has been reported (Rahmatullah and Roche, 1985). Activation of the kinase occurs as a result of aceylation of specific sites on E2 and X. Several short chain CoA esters enhanced kinase activity including malonyl CoA, acetoacetyl CoA and propionyl CoA with methylmalonyl-CoA and butyryl-CoA inhibiting kinase activity. These results are in general agreement with the reductive acetylation of E2 observed with α ketobutyrate (propionyl CoA in the reverse reaction) and acetoacetyl CoA loading onto E2 and X observed in our study. Only the lack of loading utilising malonyl CoA is in disagreement with the findings of Rahmatullah and Roche (1985).

CHAPTER FOUR

SELECTIVE PROTEOLYSIS OF MAMMALIAN PYRUVATE DEHYDROGENASE COMPLEX

Part A : Effects of Trypsin Treatment

4. Selective proteolysis of mammalian pyruvate dehydrogenase complex - Part A

4.1 Introduction

Selective proteolysis of oligomeric enzymes or multienzyme complexes in which specific degradation of proteolytically-sensitive subunits can be achieved has proved a valuable approach in elucidating structural, organisational and functional relationships in a variety of complexes e.g. arom complex, PDC kinase and E2 core assembly (Coggins et al., 1985; Stepp et al., 1983; Kresze and Steber, 1979).

Previous studies have indicated that mammalian PDC contains an oligomeric core structure of defined geometry, consisting of the dihydrolipoamide acetyltransferase (E2) to which are bound 20-30 copies of pyruvate dehydrogenase (E1) and 6 copies of dihydrolipoamide dehydrogenase component(E3). The oligomeric E2 assembly of mammalian PDC has 60 E2 polypeptides arranged with 532 (icosahedral) symmetry. The appearance of mammalian PDC-E2 under an electron microscope is that of a pentagonal dodecahedron (Reed and Oliver, 1986). The OGDC and BCOADC have 24 copies of E2 arranged with 432 (octahedral) symmetry.

The acetyltransferase of the different complexes consists of several functional domains (Perham, 1979; Bliele et al., 1979, 1981; Kresze et al., 1980; Hu et al., 1986). Studies using limited proteolysis have shown that E2 consists of an inner domain which, in addition to containing the active site, interacts with the other E2 polypeptides, thereby maintaining the integrity of the central core assembly. It is also responsible for binding the E1 component of the complexes. A second outer domain containing the lipoic acid moieties interdigitates between the E1 and E3 components acting as a substrate for both these enzymes. The E3 component is apparently bound to a distinct conserved region of polypeptide on a folded domain between the lipoyl and the catalytic domains of E2.

In the core assembly of mammalian PDC there is an additional polypeptide, component X which co-purifies with, and is tightly-bound to, the assembled E2 core. One dimensional peptide maps of the ^{14}C-labelled enzyme, cleaved with a variety of specific and non specific proteases, indicates that the M_r 51000 polypeptide of mammalian PDC is not a fragment of the E2 subunit M_r 74000 (De Marcucci et al., 1986). It has also been shown that each component X, M_r 51000 contains a single lipoyl moiety which can be reductively acetylated (Hodgson et al., 1988).

Proteolysis of PDC from bovine kidney with papain or a leupeptin sensitive inactivase obtained from rat liver lysosomes, results in the rapid loss of complex activity (Kresze and Steber, 1979). However, none of the constituent activities of the complex is destroyed during inactivation of the overall reaction. It has been demonstrated that treatment of the complex with inactivase promotes the dissociation of the complex into its individual enzymes which, though being enzymatically active when assayed separately, are unable to catalyse the co-ordinated sequence of reactions required for pyruvate oxidation. The dissociation occurs as a consequence of limited proteolysis of the lipoate acetyltransferase core with the appearance of two fragments of M_r 29000 and M_r 26000 (Kresze and Steber, 1979). Limited proteolysis of the complex by papain results in a similar inactivation of the complex activity. In both situations fragmented lipoate acetyltransferase retains its intrinsic acetyltransferase activity although it is unable to bind the other component enzymes.

Mammalian OGDC and BCOADC have a single lipoyl domain and although there is some evidence from early isotope dilution studies suggesting that mammalian PDC also has a single lipoate residue per E2 (Reed et al., 1980), a more recent report suggests that the E2

polypeptide has two functional lipoate residues (Hodgson et al., 1988). It appears, however, that the general organisation of mammalian E2 resembles that of the equivalent enzyme from PDC of E. coli which contains three highly conserved lipoyl domains, located in tandem repeat at the N-terminus of the polypeptide (Stephens et al., 1983b). The lipoylated region of each E2 chain is released from the PDC complex by limited tryptic cleavage at the flexible hinge regions, which are rich in alanine, proline and charged amino acids. These hinge regions connect the lipoyl segments to each other and to the inner catalytic E2 core and are associated with the conformational mobility expected of the lipoyl segments (Graham et al., 1986).

An interesting recent development is the observation that limited proteolysis of the purified dihydrolipoyl acetyltransferase core by protease argC selectively converts component X into an inner domain fragment M_r 35500 and an outer lipoyl bearing fragment M_r 15500. The E2 subunit is converted more slowly into an inner domain fragment M_r 31000 and an outer domain fragment M_r 49000 which have M_r values at least 3000 and 10000 larger, respectively than the corresponding E2 fragments generated by trypsin (Rahmatullah et al., 1989). Addition of E3 to the argC-treated core results in little re-association of dihydrolipoamide dehydrogenase (E3) and consequently little or no restoration of overall complex activity. Also, addition of E3 to the E2/X core prior to treatment with argC resulted in partial protection of component X and overall loss of approximately 50% of complex activity which closely paralleled the extent of proteolysis of protein X under these conditions (Gopalakrishnan et al., 1989). From these observations, Gopalakrishnan and co-workers claim that the role of component X in mammalian PDC is to bind the E3 component to the core assembly (Gopalakrishnan et al., 1989).

This chapter deals with a similar approach. E2 and X are known to be more proteolytically sensitive than the other major component enzymes, namely E1 and E3. Therefore the effects of selective proteolysis of the E2/X core of PDC with trypsin and of component X with protease argC are investigated in detail in order to gain a greater insight into the involvement of these subunits in the structural and catalytic properties of the complex.

4.1.1 Selective proteolysis of PDC with trypsin

To equate the appearance of proteolytic fragments of E2 and/or X with loss of PDC activity, samples were withdrawn at different times from an incubation mixture containing [^{14}C] acetylated PDC and trypsin. The samples were added immediately to soya bean trypsin inhibitor (SBTI), analysed by SDS-PAGE and processed for fluorography (Section 2.2.4f). It is evident from Figure 4.1A that the E2 polypeptide M_r 74000 and component X M_r 51000 are rapidly degraded during incubation in trypsin. Three major peptides are observed: a transient M_r 45000 and stable M_r 38000 and M_r 15000 species. (The origins of these peptides will be discussed in Chapter 5.)

No effect on either E1 or E3 subunits or the individual activities was observed, although on prolonged incubation in trypsin the E1α subunit is slowly degraded (data not shown).

In a parallel study, PDC was incubated with trypsin and at the times specified (Fig. 4.1B), samples were removed and assayed immediately as described in Section 2.2.11a. Rapid inactivation of the complex was observed. After 10 min incubation in trypsin, enzyme activity had been reduced to approx. 20% of control values and after 40 min no enzyme activity could be detected. PDC was also incubated in SBTI prior to the addition of trypsin. No loss of enzyme activity was detected throughout the time course.

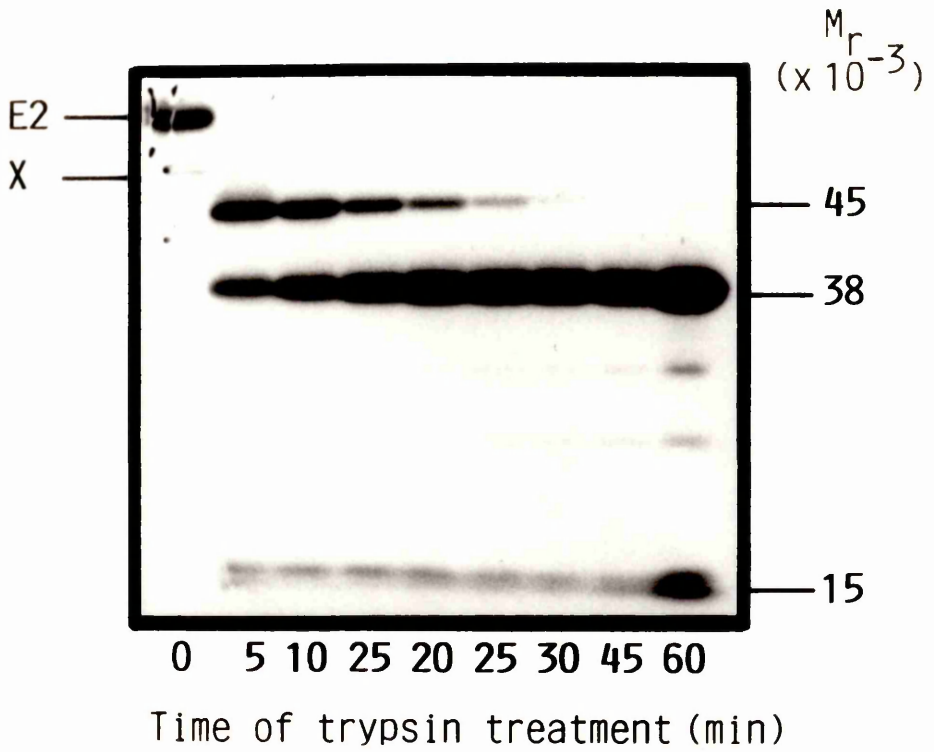
Figure 4.1A Selective release of [^{14}C] acetylated lipoyl domains from E2 and X polypeptides of native bovine heart PDC

Purified bovine heart PDC (500µg) was preincubated for 15 min with [2-^{14}C] pyruvate (0.2mM) in 20mM potassium phosphate buffer pH7.5 containing 1mM $MgCl_2$, 0.2mM TPP and 2.5mM NAD^+ before treatment with 1% (w/w) trypsin at 25°C for the indicated times. Samples (30µg) were removed, immediately treated with Laemmli sample buffer and subjected to fluorography following resolution on a 12.5% (w/v) SDS/polyacrylamide gel(Section 2.2.4f).

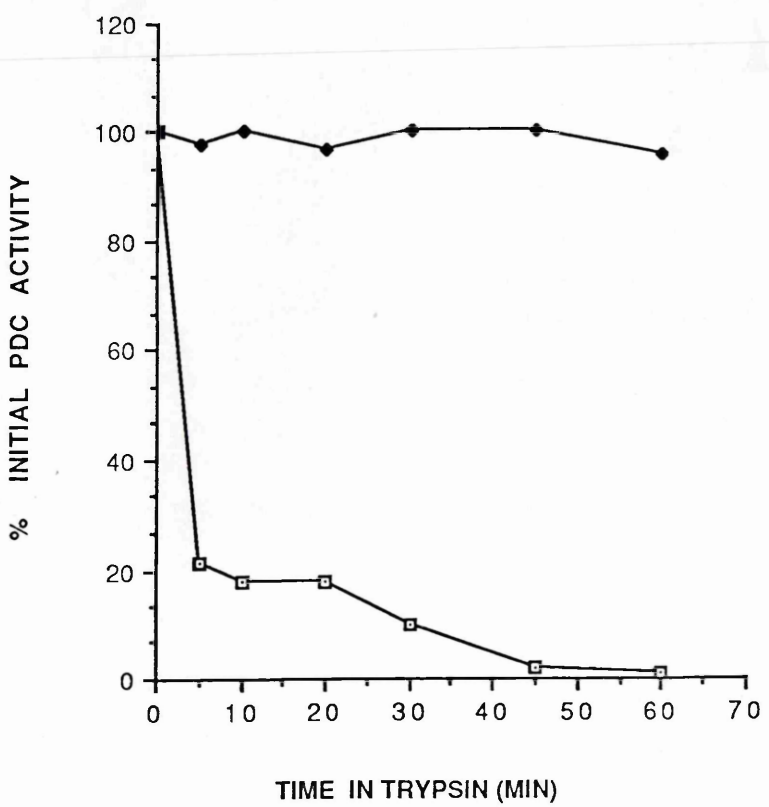
Figure 4.1B Effect of trypsin on overall PDC activity

PDC (100µg) was incubated in 20mM potassium phosphate buffer pH7.5 containing 1mM $MgCl_2$, 0.2mM TPP and 2.5mM NAD^+ at 25°C. Digestion was initiated by the addition of 1% (w/w) trypsin. At the times indicated samples (5µg) were removed and assayed for PDC activity (——⊡——). Similarly, PDC (100ug) was incubated with 2% (w/v) SBTI prior to incubation with 1% (w/v) trypsin (——◆——). Results are the mean of two determinations differing by less than 10%.

A


M_r (x 10^{-3})
E2
X
45
38
15
0 5 10 25 20 25 30 45 60
Time of trypsin treatment (min)

B


% INITIAL PDC ACTIVITY
120
100
80
60
40
20
0
0 10 20 30 40 50 60 70
TIME IN TRYPSIN (MIN)

4.1.2 The effect of tryptic digestion on the acetylation of PDC

In PDC S-acetylation in the presence of pyruvate generates a free thiol on the adjacent sulphur atom in the dithiolane ring which may be specifically modified by NEM (Fig. 3.1). In the absence of NEM, a slow second phase of acetylation protects against NEM modification. The best interpretation of this result is that the diacetylation reaction protects against NEM modification (Fig. 3.1).

To investigate if the peptides generated by tryptic digestion of PDC can still be acetylated by E1 and are still capable of diacetylation, the incorporation of [^{14}C] pyruvate into intact PDC and tryptic peptides was measured. Figure 4.2 compares the extent of [^{14}C] acetyl group incorporation into intact E2/X and tryptic peptides derived from them in the presence and absence of NEM. It is apparent that NEM pretreatment of the intact complex diminishes the incorporation from [2-^{14}C] pyruvate by approximately 50%. However, in the case of trypsin treated PDC, acetyl group incorporation was also reduced by approximately 50%. In this case, the presence of NEM had little effect on the profile of incorporation of acetyl groups into tryptic peptides.

In the presence of NEM only half of the sites are apparently available for acetylation; therefore the most probable interpretation of the result (Fig. 4.2) is that release of E2/X peptides from the core leads to loss of the ability to form the S^6S^8 (diacetyl) lipoamide intermediate.

4.1.3 Fluorographic detection of E2/X derived tryptic peptides after incubation with [^{14}C] pyruvate

It is possible that after digestion with trypsin certain of the lipoyl peptides generated from E2 and component X are not active substrates for acetylation with [^{14}C] pyrvuate and this results in the lower levels of incorporation observed with trypsin treated PDC

Figure 4.2 Time course of acetylation of native or trypsin treated PDC

Native PDC (200ug), incubated in the presence (—●—) or absence (—○—) of NEM (0.5mM), or trypsin treated PDC, incubated in the presence (—■—) or absence (—□—) of NEM, was acetylated by the addition of [2-^{14}C] pyruvate (0.2mM). On the addition of (2-^{14}C) pyruvate (0.2mM), aliquots (10ul) were withdrawn at the indicated times and spotted onto Whatman No. 1 filter paper discs to estimate the radioactivity incorporated into proteins (section 2.2.3a). Results are the mean of two determinations differing by less than 10%.

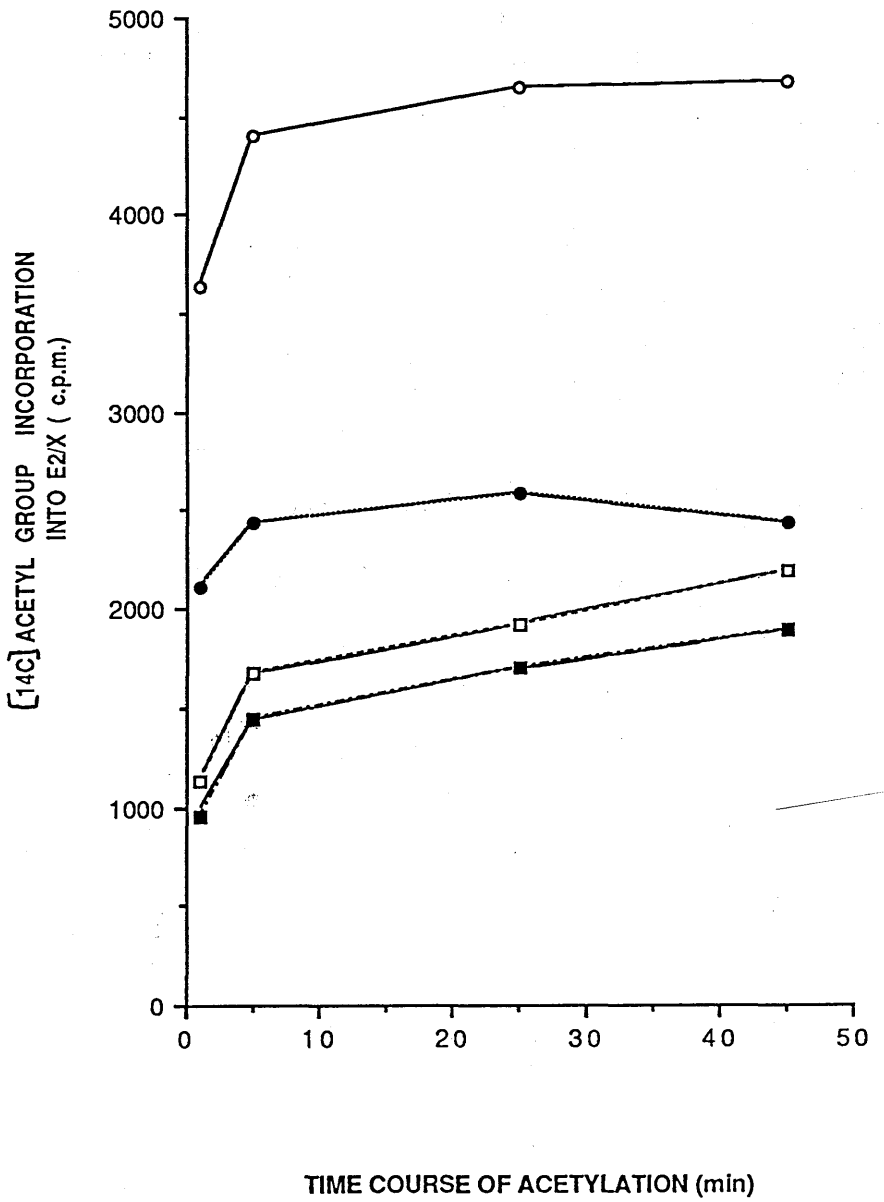

5000
4000
3000
2000
1000
0
0
10
20
30
40
50
[14C] ACETYL GROUP INCORPORATION
INTO E2/X (c.p.m.)
TIME COURSE OF ACETYLATION (min)

Figure 4.3 Fluorographic detection of E2/X derived tryptic peptide

Intact PDC (100μg) was incubated in 20mM potassium phosphate buffer containing 1mM $MgCl_2$, 0.2mM TPP and 2.5mM NAD^+ for 30 min in the presence of [2-^{14}C] pyruvate. After 30 min half of the [^{14}C] acetylated PDC sample was withdrawn and incubated in trypsin for 30 min, before the addition of 2% (w/v) SBTI to terminate the reaction.

Similarly trypsin treated PDC (50μg) was incubated with [2-^{14}C] under the conditions detailed above. Samples were added to Laemmli sample buffer before resolution by SDS-PAGE and processing for fluorography.

Lane M, ^{125}I Markers; lane 1, [^{14}C] acetylated intact PDC; lane 2, trypsin-treated, [^{14}-C] acetylated PDC; lane 3 [^{14}C] acetylated, trypsin-treated PDC.

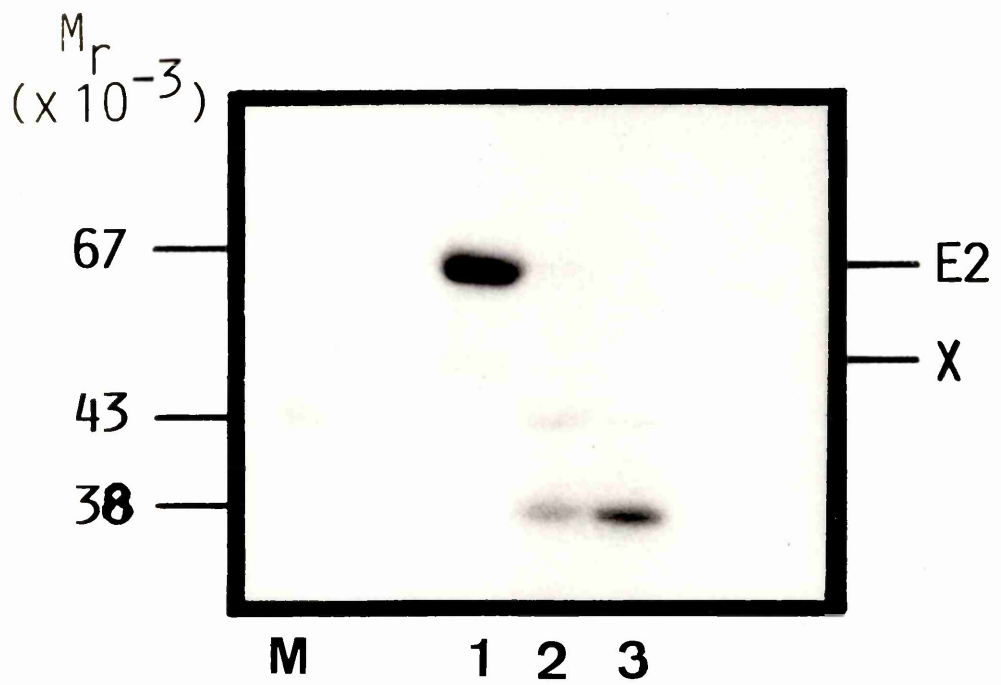

Mr
(x 10-3)
67
43
38
E2
X
M
1
2
3

Fig. 4.2). In order to establish if released peptides could be acetylated, PDC was incubated with trypsin, after 30 min the reaction was terminated by addition of SBTI and the trypsin treated sample was incubated with [2-^{14}C] pyruvate (lane 3). Similarly, PDC preincubated in [2-^{14}C] pyruvate was treated with trypsin as above (lane 2). In Figure 4.3, a similar profile of radiolabelled peptides is observed for both sets of conditions i.e. the presence of the M_r 45000, M_r 38000 species. The M_r 15000 peptide is not detectable on this occasion, but subsequent studies (see Fig. 4.4C) reveal that it is acetylated also.

4.1.4 Deacetylation of [2-^{14}C] pyruvate labelled intact and trypsin-treated PDC

Rapid deacetylation of both E2 and component X occurs on the addition of CoASH, the products being acetyl CoA and NADH. PDC modified by NEM is not deacetylated in the presence of CoASH, the acetyl group remaining trapped on the dithiolane ring (Fig. 3.1). To establish if the ^{14}C-labelled peptides generated from a tryptic digest could be deacetylated on the addition of CoASH and in the presence of NEM, PDC was incubated in trypsin to produce the recognised tryptic peptides and the reaction stopped by addition of SBTI. The digest was divided into two and one half incubated in [2-^{14}C] pyruvate while the other was incubated in NEM prior to the addition of radiolabelled substrate. This procedure was repeated for untreated PDC and NEM modified PDC and the extent of [^{14}C] acetylation incorporated into protein determined (Fig. 4.4A) (Section 2.2.3). After 30 min CoASH was added and again at the time points specified the release of [^{14}C] acetyl groups was monitored (Fig. 4.4B). At various times samples were also removed from each of the incubation mixtures, resolved by SDS-PAGE and processed for fluorography (Section 2.2.10f) (Fig. 4.4C).

Figure 4.4 Deacetylation of [2-^{14}C] pyruvate labelled intact PDC and trypsin-treated PDC

Native PDC (400ug), incubated in the presence (—O—) or absence (—●—) of NEM (0.5mM), or trypsin treated PDC, incubated in the presence (—◆—) or absence (—◇—) of NEM was acetylated by the addition of [2-^{14}C] pyruvate (0.2mM). Duplicate aliquots (10µl) were withdrawn at the times indicated and spotted onto Whatman No. 1 filter paper discs (Fig. 4.4A).

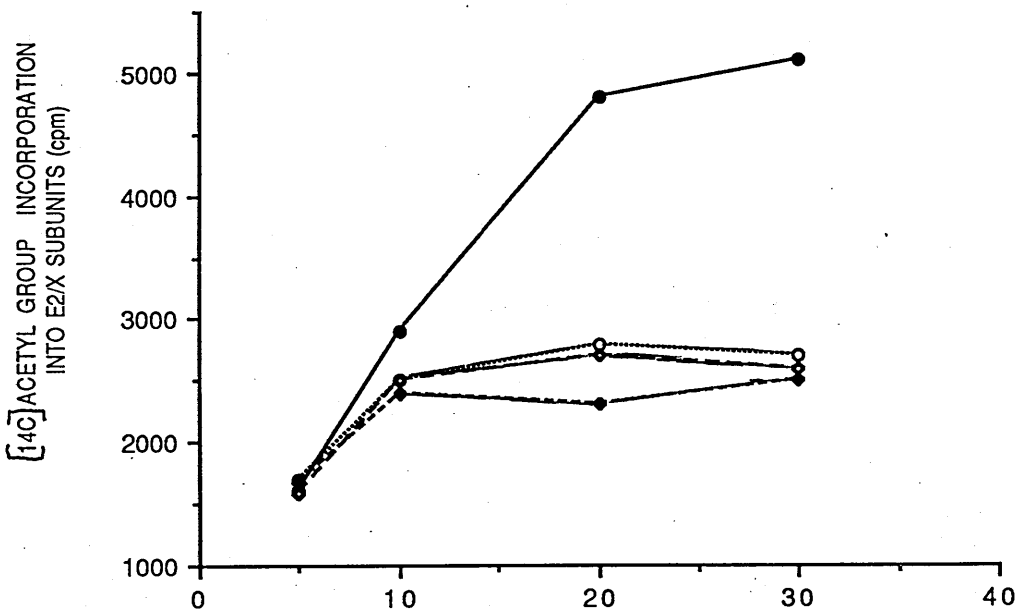
After 30 min excess N-ethylmaleimide was removed from the incubation mixtures by passing the samples through a Sephadex G25 column equilibrated in potassium phosphate buffer pH7.2.

CoASH (1mM) was added to the incubation mixture and at the time indicated samples (10µl) were withdrawn and treated as detailed above (Fig. 4.4.B). The data points are the average of duplicate determinations differing by less than 10%.

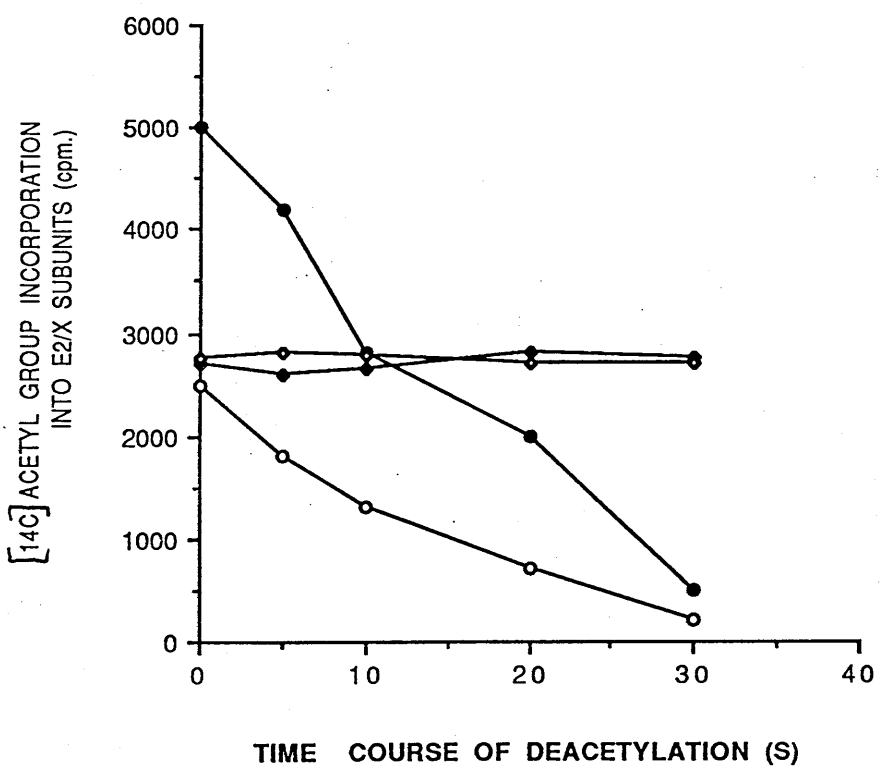
Samples (25µg) from each stage of the experiment detailed above were withdrawn, mixed with Laemmli sample buffer and resolved on a 10% (w/v) SDS/polyacrylamide gel and processed for fluorography (Section 2.2.4f) (Fig. 4.4.C).

Lane M, ^{125}I Markers; lanes 1, 2, [^{14}C] acetylated PDC in the absence and presence of CoASH; lanes 3,4 [^{14}C] acetylated PDC in the absence or presence of NEM; lanes 5,6 trypsin treated [^{14}C] acetylated PDC in the absence or presence of CoASH; lane 7 [^{14}C] acetylated trypsin treated PDC incubated in NEM; lane 8, as lane 7 plus the addition of CoASH.

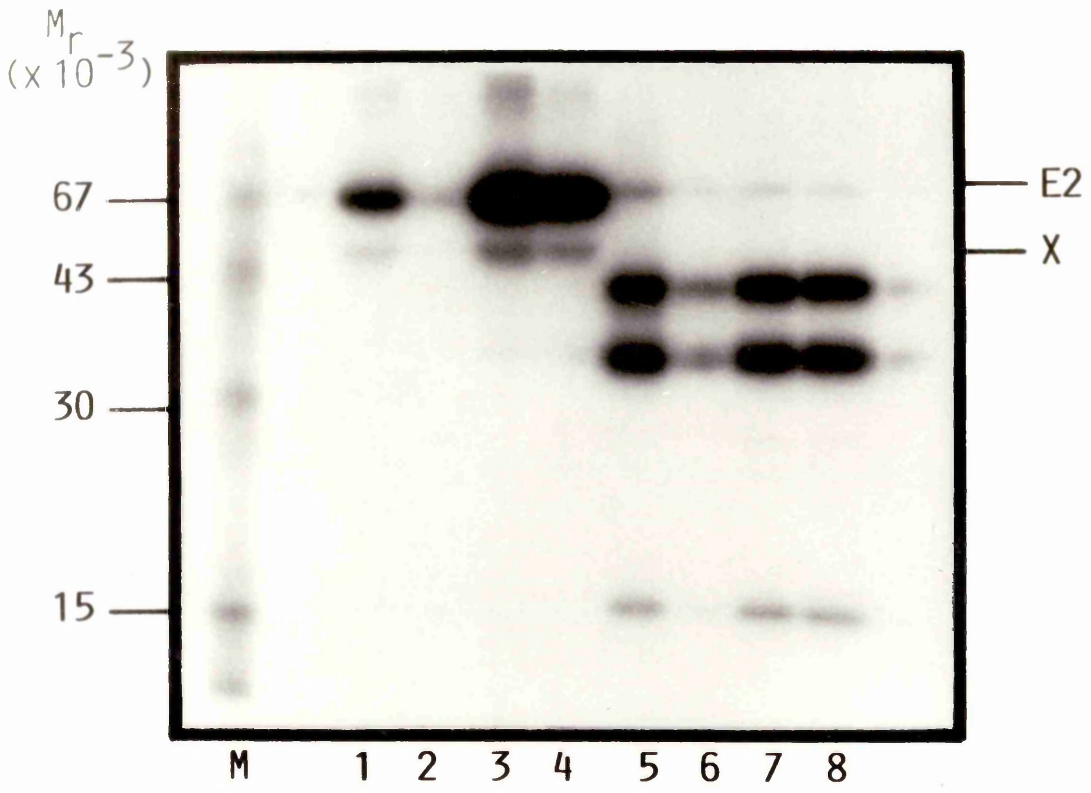
A


[14C] ACETYL GROUP INCORPORATION INTO E2/X SUBUNITS (cpm)
5000
4000
3000
2000
1000
0
10
20
30
40
TIME COURSE OF ACETYLATION (min)

B


[14C] ACETYL GROUP INCORPORATION INTO E2/X SUBUNITS (cpm.)
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
TIME COURSE OF DEACETYLATION (S)

C



These results confirm that, in comparison to untreated PDC, the amount of radiolabel incorporated into the tryptic peptides is equivalent to that observed for NEM modified PDC. This suggests that the tryptic peptides are no longer able to incorporate a second unit of acetylating substrate into the dithiolane ring which are therefore not protected against NEM modification. On incubation in CoASH, both untreated PDC and [^{14}C] acetylated peptides are rapidly deacetylated, as observed by the rapid decline in acid-precipitable radiolabelled material (Fig. 4.4B). Both NEM modified peptides and PDC remain acetylated on incubation in CoASH (Fig. 4.4). Confirmation of the results can be observed directly in Figure 4.4C. A reduced level of incorporation of ^{14}C-label is observed in E2 and X and tryptic peptides after incubation in CoASH (Fig. 4.4C). In this case the rapid deacetylation of the M_r 15,000 peptide can also be readily observed.

4.1.5 Association of tryptic peptides with E2 core assembly

Since it is now evident that the lipoyl peptides derived from E2 and X are capable of participation in the overall catalytic cycle of the complex, the association of the peptides with the E2 core is investigated.

After trypsin treatment, ^{14}C-acetylated PDC high M_r core structure are pelleted by ultracentrifugation so that both pellet and supernatant fractions can be investigated for ^{14}C labelled E2/X peptides as shown in Figure 4.5. In controls [^{14}C] acetylated E2/X are observed only in the pellet fraction. In contrast the major tryptic cleavage products M_r 38000 and M_r 15500 are visualised exclusively in the supernatant fractions (Fig. 4.5). Therefore the peptides generated from tryptic digestion of PDC are not tightly-associated with the core.

Figure 4.5 Association of tryptic peptides with E2 core assembly

PDC (500µg) was incubated in the presence or absence of NEM prior to the addition of [2-^{14}C] pyruvate. After 30 min, half of the intact PDC and NEM treated PDC was removed and incubated with trypsin for 45 min. The digestion was stopped by the addition of 2% (w/v) SBTI.

Intact PDC and the products of proteolysis were subjected to ultracentrifugation (150000 x g for 150 min) in a Beckmann Ti70 rotor. The supernatant fractions were precipitated by addition of 10% (w/v) TCA and the resultant pellet solubilised in Laemmli sample buffer (Section 2.2.4a). The pellets from the ultracentrifuge spin were solubilised in 20mM potassium phosphate buffer pH7.4 containing 1mM $MgCl_2$, 0.2mM TPP and 2.5mM NAD^+. Samples were resolved on a 10% (w/v) SDS/polyacrylamide gel and processed for fluorography.

Lane M, ^{125}I Markers; lane 1,2, supernatant fractions from intact PDC and NEM treated PDC respectively; lane 3,4, pellet fraction from intact PDC and NEM treated PDC; lane 5,6, supernatant fraction from trypsin treated PDC in the absence or presence of NEM; lane 7,8, pellet fraction from trypsin treated PDC in the absence or presence of NEM respectively.

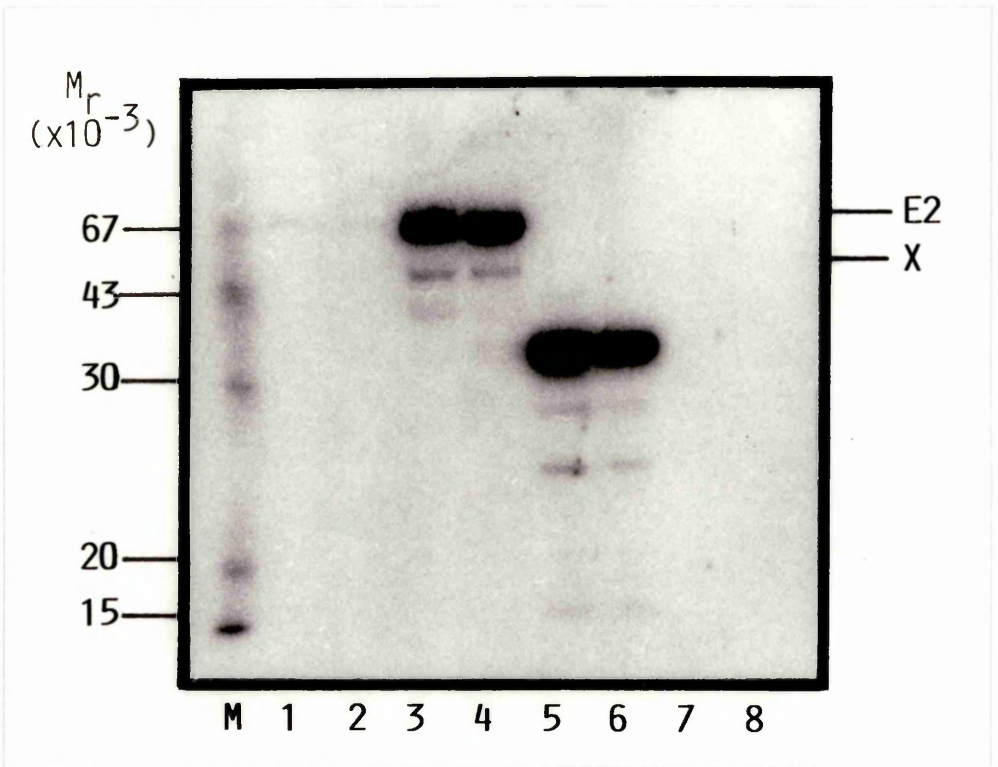
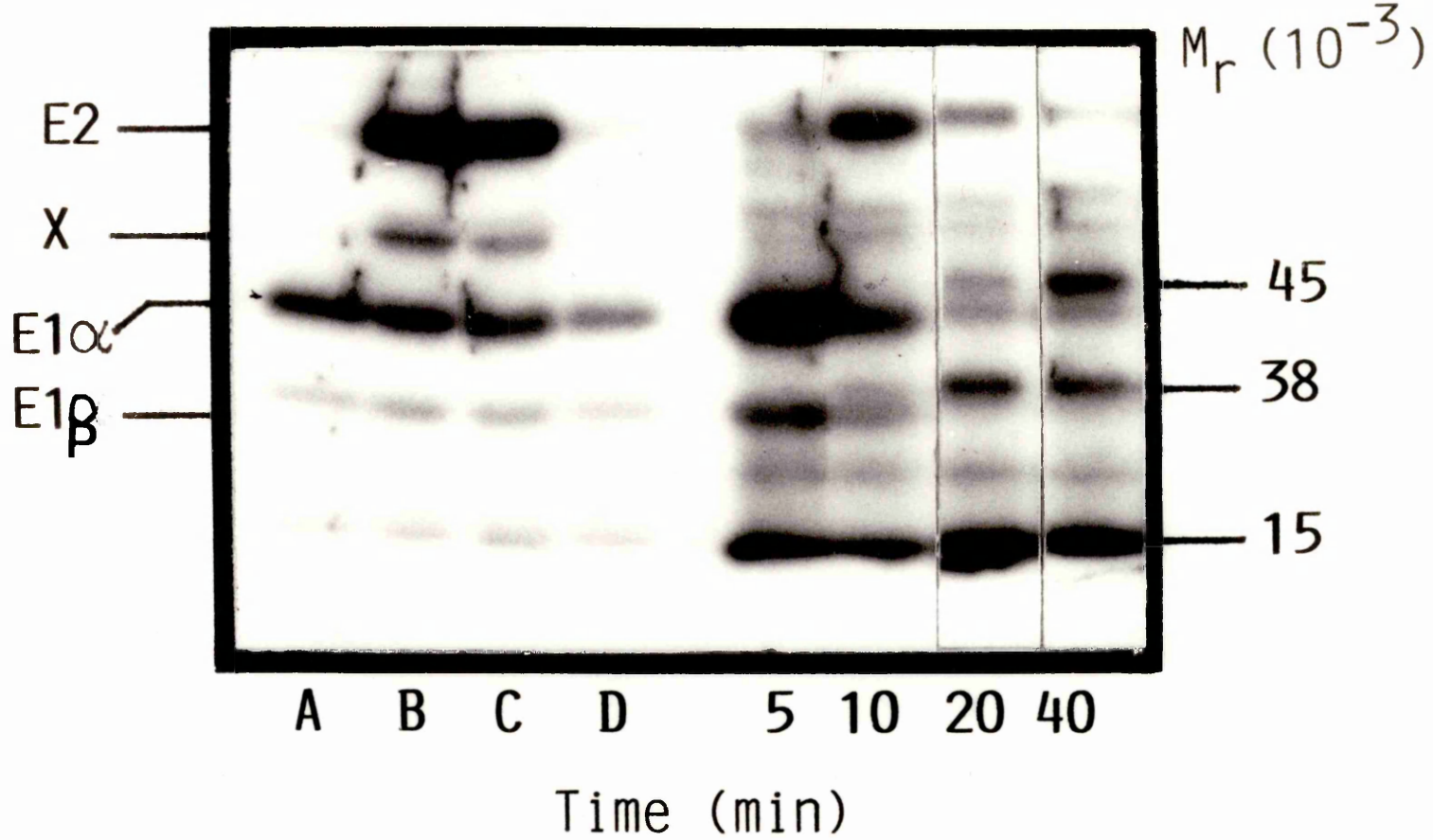

Mr
(x10-3)
67
43
30
20
15
E2
X
M 1 2 3 4 5 6 7 8

Figure 4.6 Incorporation of N-ethyl [2,3-^{14}C] maleimide into tryptic peptides of PDC

Native PDC (250µg) in 20mM phosphate buffer pH7.4 containing 1mM $MgCl_2$, 0.2mM TPP and 2.5mM NAD^+ was incubated in 1% (w/v) trypsin. At the times indicated aliquots (30µg) were removed and added to 2% (w/v) soya bean trypsin inhibitor. The samples were reduced by addition of NADH (0.5mM) and after 10 min the samples were acetylated in the presence of 0.5mM acetyl CoA. After a further 15 min, the samples were radiolabelled for 10 min on the addition of N,-ethyl[2,3-^{14}C] maleimide and excess N-ethyl maleimide was reacted with 45mM 2-mercaptoethanol. Laemmli sample buffer was added to the samples which were then resolved on a 10% (w/v) SDS/polyacrylamide gel and processed for fluorography (Section 2.2.4f).

As control, lane A, native PDC (30µg) was incubated in [^{14}C] NEM, lane B, incubated in NADH prior to the addition of [^{14}C] NEM; lane C, incubated in NADH prior to the simultaneous addition of [^{14}C] NEM and acetyl CoA; lane D, and NADH reduced PDC was incubated in acetyl CoA for 15 min prior to the addition of [^{14}C] NEM.

Time course: PDC treated with 1% (w/v) trypsin for 5, 10, 20, 40 min. Samples from the time course were processed as lane 4 and can be compared directly with lane D.


E2
X
E1α
E1β
M_r (10^{-3})
45
38
15
A
B
C
D
5
10
20
40
Time (min)

4.1.6 Incorporation of N-ethyl [2,3-^{14}C] maleimide into tryptic peptides of PDC

In intact PDC the rate limiting step is the reductive acetylation of E2 by E1. An alternative procedure for loading onto lipoyl groups using NADH and acetyl CoA involves the interaction of E3 and acetyl group transfer onto E2 or X which is completely independent of E1.

Preincubation of PDC in NADH followed by acetyl CoA results in a time dependent diminution of incorporation of radiolabelled NEM into component X and E2. Similar to PDC preincubated in pyruvate, PDC is protected from NEM modification (Cate et al., 1980).

To visualise the peptides generated from a tryptic digest, samples were removed and reduced by NADH. Acetyl CoA was added prior to the addition of [^{14}C] NEM and the samples resolved by SDS-PAGE. Proteolysis of the E2, X and the E1 αsubunit is observed (Fig. 4.6).

After 20 min the normal tryptic cleavage pattern is observed with the formation of two peptides M_r 45000 on M_r 38000 which are not protected by acetyl groups from NEM incorporation. Therefore with the formation of peptides from E2 and component X, PDC loses the ability to diacetylate the lipoic acid sites on E2 and consequently, on prolonged incubation in acetylating substrate, to protect from NEM modification.

Discussion

The loss of enzyme activity on incubation with trypsin parallels the release of lipoyl peptides from the E2 core. This is in contrast to the findings of Packman and Perham (1989), where similar treatment of PDC from E. coli released one or two lipoyl domains (of three) per E2 chain without affecting catalytic

activity. Recent evidence from our laboratory confirms the finding of Kresze, 1980, that, on incubation with trypsin, El and E3 are released from the core. Cleavage of the lipoyl peptides apparently occurs within the domains responsible for binding El and E3 and thus leads to dissociation of the complex. However, prolonged incubation with trypsin can destroy El activity (Kresze et al., 1980).

A consequence of the removal of the lipoyl peptides from E2/X was the inability of the enzyme to protect against NEM modification. This observation is best explained by the loss of the diacetylation reaction. E2 and X peptides generated from trypsin treatment are observed to incorporate greater levels of [^{14}C] NEM than similarly protected intact E2 and X which indicates lack of protection on the lipoyl peptides. It seems likely, as the peptides are no longer associated with the core, the interaction between E2/X peptides or/between the peptides and the high M_r core assembly has been reduced sufficiently to prevent diacetylation of the peptides. Although the peptides can participate in the overall catalytic cycle of the enzyme, their release from the core effectively decreases the effective concentration of the peptides by several orders of magnitude. A single turnover of the enzyme now occurs so slowly that enzyme activity cannot be recorded.

As preferential digestion of component X was not observed after trypsin treatment, possible functions of component X including its participation in diacetylation could not be elucidated in these studies.

SELECTIVE PROTEOLYSIS OF MAMMALIAN PYRUVATE DEHYDROGENASE COMPLEX

Part B : Effects of ArgC Treatment

4.2 Part B

During the course of this work Roche and co-workers reported their findings on limited proteolysis of PDC with protease argC (Rahmatullah et al., 1989;*a* Gopalakrishnan et al., 1989; Powers-Greenwood et al., 1989). Their main conclusions are as follows:

a) With isolated E2/X core assembly, component X is preferentially degraded by argC

b) The E3 component fails to bind effectively to argC treated E2 core

c) The E3 component protects component X from proteolysis

d) A marked reduction of PDC activity is observed on re-addition of the E1 and E3 components to argC treated core in comparison to untreated controls.

As detailed in the previous section, similar experiments using trypsin were being carried out, and as Roche and co-workers reported that argC exhibited a high specificity for component X, it was decided to investigate further the claims of this group using protease argC and to elucidate a possible role for component X in the complex.

4.2.1 Limited proteolysis of [^{14}C] acetylated PDC by argC and its effect on overall complex activity

As with trypsin, it was initially decided to determine the effect of protease argC on structure, organisation and catalytic function of PDC. Samples were withdrawn from an incubation mixture containing PDC preincubated with [2-^{14}C] pyruvate prior to degradation with protease argC. Figure 4.7A illustrates the slow degradation of component X while E2 remains virtually intact.

In a parallel experiment, PDC was incubated with protease argC, samples withdrawn at the times indicated (Fig. 4.7B) and

Figure 4.7A Limited proteolysis of [^{14}C] acetylated PDC with argC

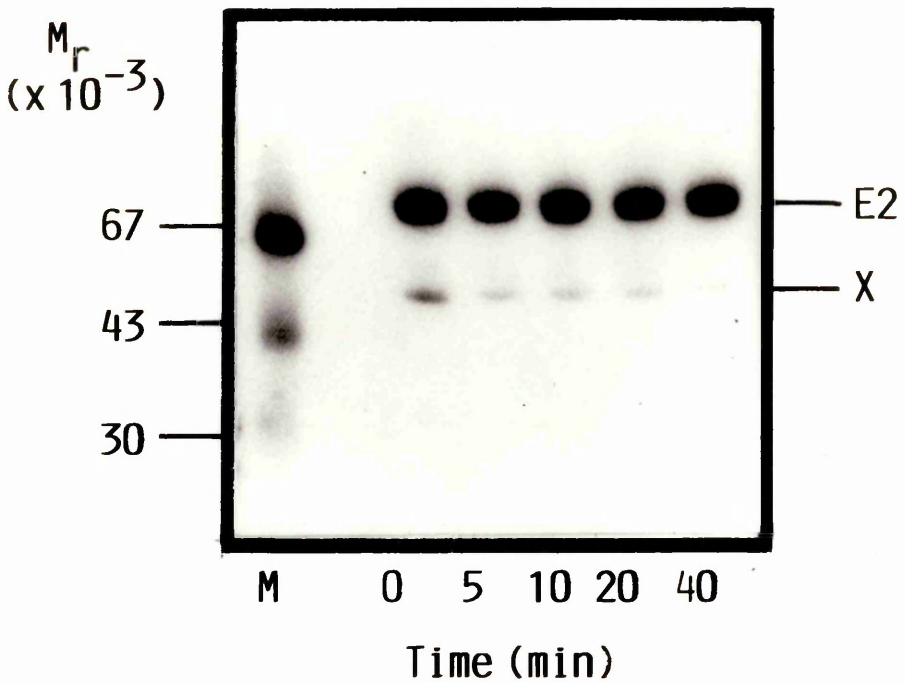
Purified bovine heart PDC (200µg) was incubated with [2-^{14}C] pyruvate (0.2mM) in 20mM potassium phosphate buffer pH7.4 containing 1mM $MgCl_2$ 0.2mM TPP, 2.5mM NAD^+. After 30 min 3% (w/v) argC was added and at the times indicated samples (30µg) were removed, added to Laemmli sample buffer and subjected to fluorography following resolution on a 10% (w/v) SDS/polyacrylamide gel.

Figure 4.7B Effect of protease argC on overall complex activity

PDC (100ug) was incubated with 3% (w/v) argC in 20mM potassium phosphate buffer containing 1mM $MgCl_2$, 0.2mM TPP and 2.5 NAD^+. At the times indicated samples (5µg) were removed and assayed immediately for PDC activity. The data observed in the figure are the average of two determinations of activity differing by less than 10%.

A

Limited proteolysis of [^{14}C] acetylated PDC with ARG C.



B

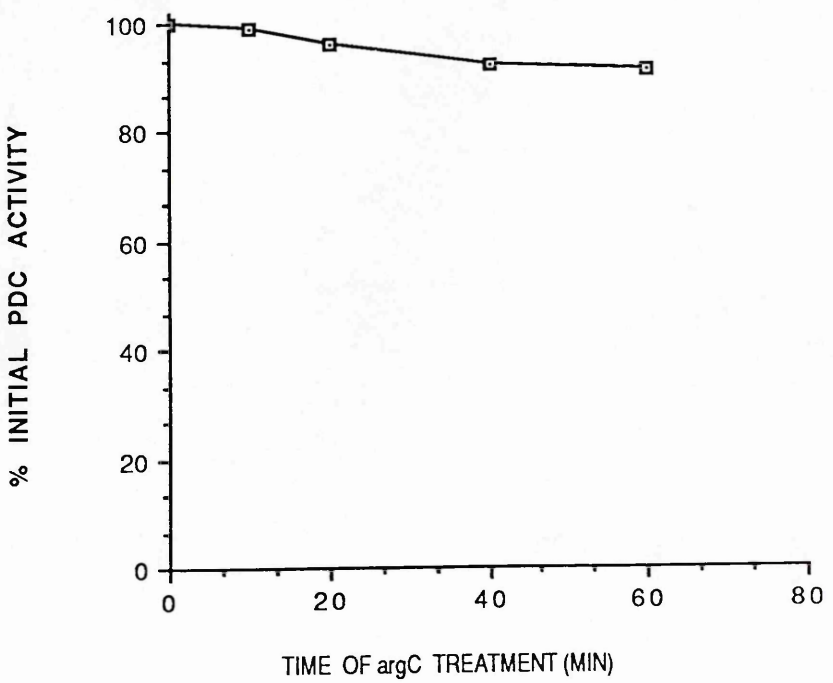
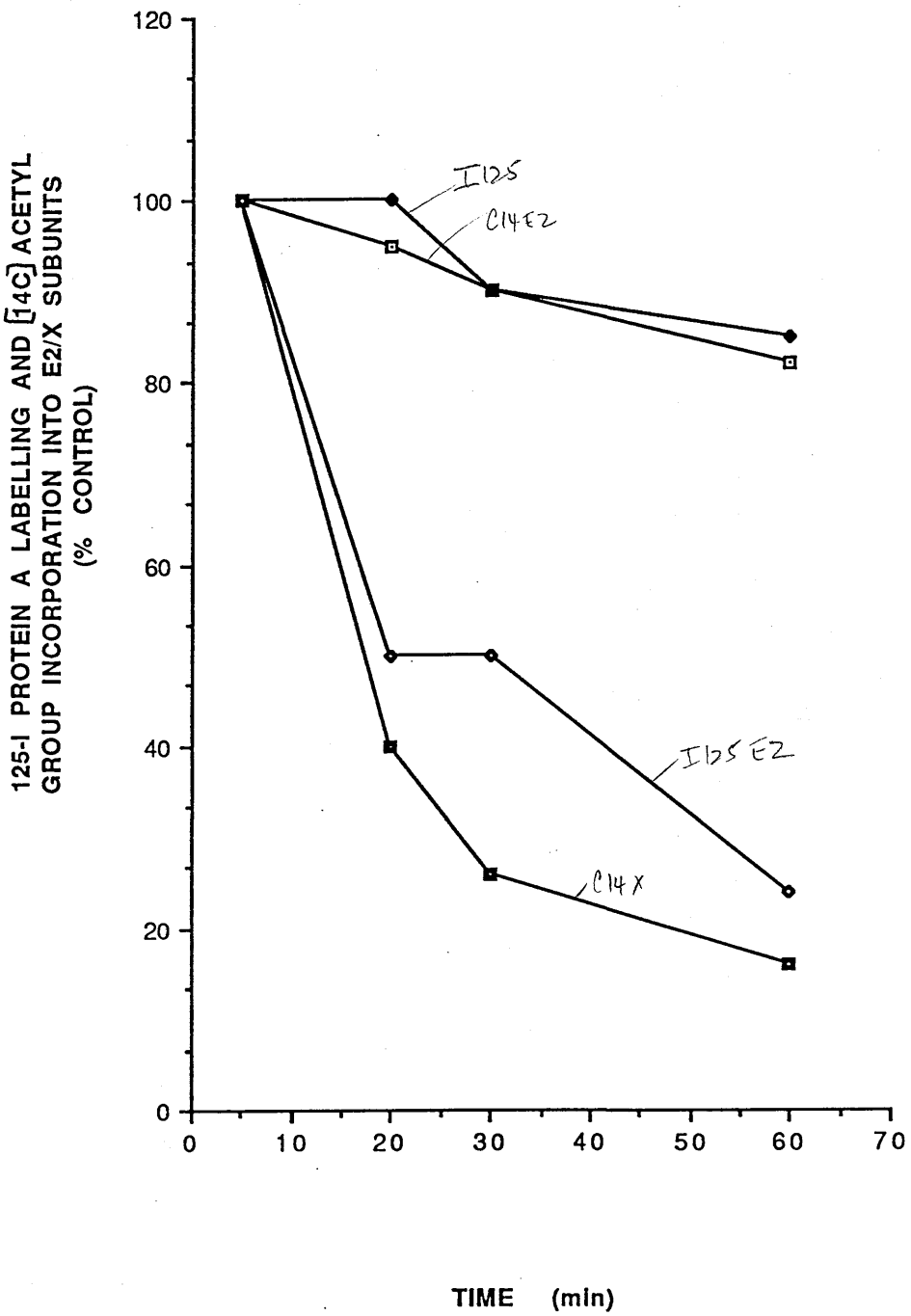


Figure 4.7C Extent of degradation of E2 and component X after limited proteolysis with argC

As detailed previoulsy [^{14}C] acetylated PDC (500µg) in 20mM potassium phosphate buffer pH7.4 containing 1mM $MgCl_2$, 0.2mM TPP and 2.5mM NAD^+, in 3% (w/v) was incubated in argC and at the times indicated duplicate samples (30µg) were removed, added to Laemmli sample buffer and resolved on a 12.5% (w/v) SDS/polyacrylamide gel. The proteins were stained with Coomassie blue before the gel was dried. The radiolabelled bands corresponding to E2 and X were excised from the gel and counted on a liquid scintillation counter (Section 2.2.3b). [^{14}C] acetylated E2 (—⊡—) [^{14}C] acetylated protein X (—■—)

Similarly PDC (50µg) was incubated with 3% (w/v) argC and at the indicated times samples (5µg) were removed and added immediately to Laemmli sample buffer. Samples (1µg) were resolved on an 12.5% (w/v) SDS polyacrylamide gel and subjected to immunoblotting analysis with anti-E2 and anti-X sera. The bands corresponding to E2 and X were cut from the nitrocellulose and added to 5ml liquid scintillant before counting. ^{125}I-labelled E2 (—◆—) ^{125}I-labelled protein X (—◇—).


125-I PROTEIN A LABELLING AND [14C] ACETYL GROUP INCORPORATION INTO E2/X SUBUNITS (% CONTROL)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
I125
C14E2
I125 E2
C14X
TIME (min)

assayed for PDC activity (Section 2.2.7a). A small 10-25% decrease in complex activity is observed after 60 min incubation with protease argC.

The extent of degradation of E2 and X was determined by two separate approaches:- (a) the determination of radioactivity in gel slices from Figure 4.7A was performed as described in Section 2.2.3b and (b) the polypeptides from a similar incubation of non-radiolabelled PDC with argC were electrophoretically transferred onto nitrocellulose and challenged with the appropriate antisera followed by ^{125}I-labelled protein A. The polypeptides, which were located by autoradiography, were cut from the nitrocellulose for estimation of radioactivity by scintillation counting. The results indicate that component X is slowly degraded to approximately 10% of its original value with the PDC activity remaining at approximately 80% of its original value. The slight inactivation (10-20%) of PDC closely parallels the minor degradation of E2 detected under these conditions.

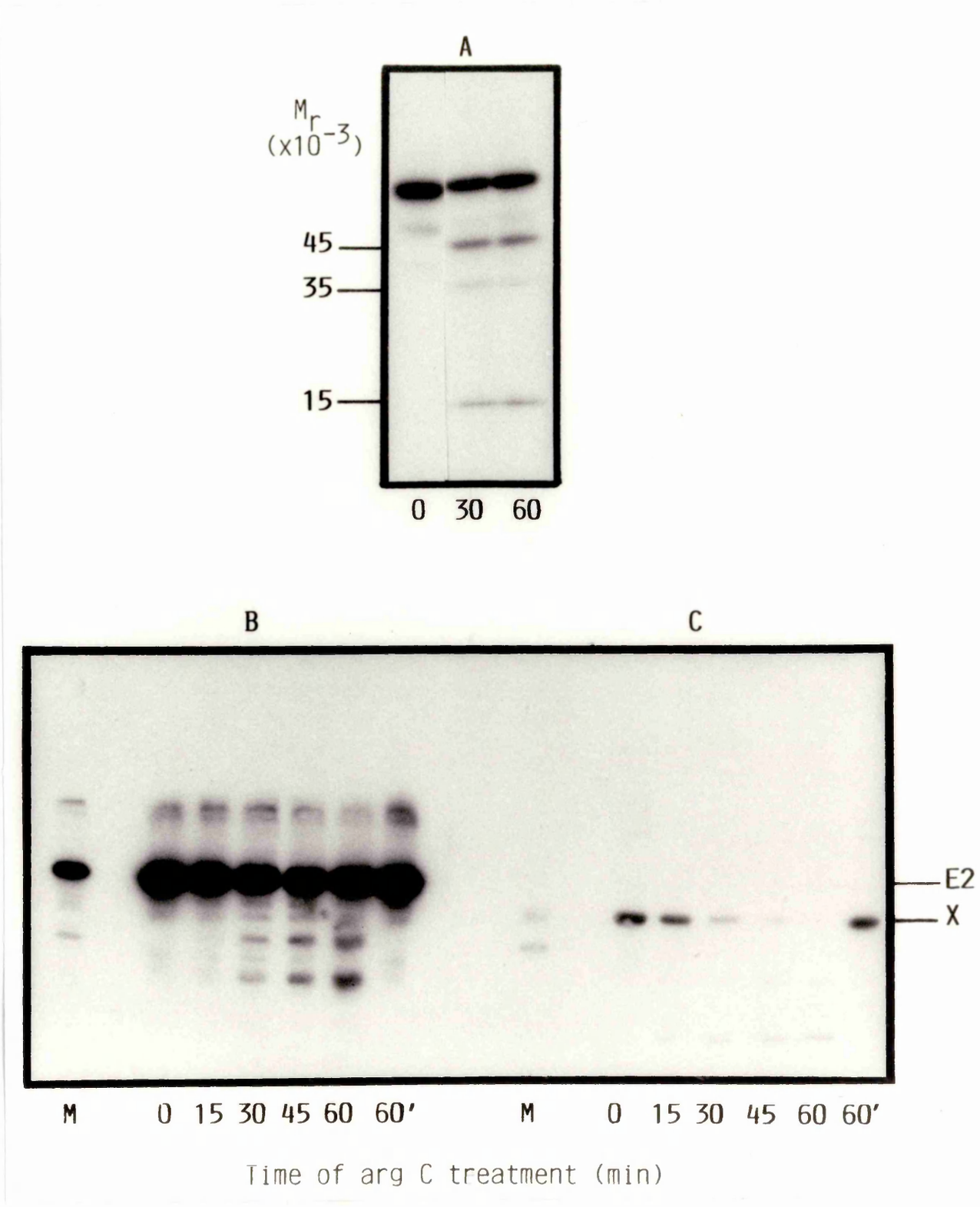
4.2.2 Immunological detection of proteolytic fragments from E2 and component X on incubation with protease argC

Digestion of [^{14}C] acetylated PDC with argC generates three major lipoyl peptides which can be detected by fluorography (Fig. 4.8A). Identification of those peptides derived from E2 and X respectively was achieved by immunoblotting with subunit specific antisera with samples obtained from a parallel digestion of non-radioactive labelled PDC (Fig. 4.8B and C). The pattern of E2 and X cleavage indicates that component X is preferentially digested with protease argC although limited proteolysis of E2 is also apparent. A single immunoreactive peptide from component X M_r 15500 is observed while two peptides are generated from E2 with M_r values 45000 and 35000.

Figure 4.8 Immunological detection of proteolytic fragments from E2 and component X on incubation with protease argC

Native PDC (100µg) was incubated with [2-^{14}C] pyruvate (0.2mM) prior to the addition of 3% (w/v) argC. At the times indicated, samples (30ug) were removed and added to Laemmli sample buffer and subjected to fluorography after resolution on a 12.5% SDS/polyacrylamide gel (Panel A).

Similarly native PDC (50µg) was incubated with 3% (w/v) argC and at the times indicated samples (5µg) removed and added directly to Laemmli sample buffer. The digested proteins (1ug) were resolved on a 12.5% (w/v) SDS/polyacrylamide gel. Gels were used for immunoblotting analysis (Section 2.2.9a) with antibody raised against E2 subunit (Panel B) and protein X (Panel C). PDC was also incubated in the absence of argC for 60 min (lane 60') as a control.


A
M_r (x10^{-3})
45
35
15
0 30 60
B
C
E2
X
M 0 15 30 45 60 60′ M 0 15 30 45 60 60′
Time of arg C treatment (min)

4.2.3 The effect of high salt concentration on PDC activity

Treatment of intact PDC with argC results in preferential (approx. 90%) removal of the lipoyl domains of component X with minimal release (10-20%) of lipoyl peptides derived from E2. Gopalakrishnan et al. (1989) reported that removal of E3 from the core assembly is necessary for preferential digestion of component X by protease argC. These conclusions were based on examination of silver stained gels and no effort was made to quantitate the extent of degradation of E2 in relation to component X. This group also observed that re-addition of E3 and E1 to argC digested core assembly failed to restore overall enzyme activity. They do not discuss the possibility of limited proteolysis of the other subunits or detail the percentage recovery of activity on reconstitution of the E1 and E3 subunits with the intact E2 core assembly.

An alternative method of releasing E3 and E1 from the core assembly without the necessity of preparing E2/X core and employing reconstitution analysis is to incubate PDC in 0.25M $MgCl_2$ or 1M NaCl (Kresze and Steber, 1979).

The effects of high salt concentrations on PDC structure and activity were therefore examined. PDC preincubated in 0.25M $MgCl_2$ or 1M NaCl was resolved on a gel filtration column and the products observed, as shown in Figure 4.9A. On incubation in high salt E1 and E3 are readily dissociated from the high M_r core structure.

Surprisingly, however, the data in Figure 4.9B also indicate that treatment of the enzyme with 0.25M $MgCl_2$ or 1M NaCl does not result in rapid inactivation of the complex.

Although high salt treatment of PDC appears to lower the affinity of E1 and E3 for the core (Fig. 4.9A) resulting in separation during gel filtration, interactions between E1, E3 and the core assembly must be maintained to a sufficient degree to promote normal complex activity.

Figure 4.9A SDS-PAGE analysis of constituent enzymes of PDC, after treatment in high salt followed by resolution on a gel filtration column

Native PDC (250ug) was incubated in 20mM potassium phosphate buffer, pH7.4 containing 2.5mM NAD^+, 1mM $MgCl_2$ 0.2mM TPP. Solid $MgCl_2$ (0.25 M) was added and the sample incubated for 30 min at room temperature.

The PDC sample was resolved on a Superose 12 column (10 x 300mm) and peak fractions were precipitated on the addition of 10% (w/v) TCA.. The resultant pellets were solubilised in Laemmli sample buffer, resolved on 12.5% SDS/polyacrylamide gel and stained with Coomassie blue.

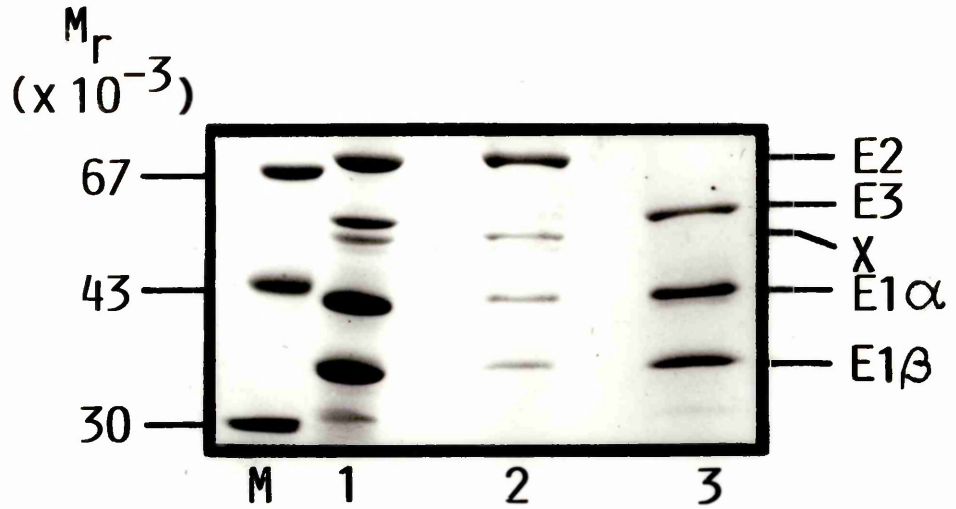
Lane M, M_r standards; lane 1, untreated PDC (10ug) resolved by gel filtration; lane 2, void volume peak from high salt treated PDC (10ug) resolved by gel filtration; lane 3, 2nd peak obtained from gel filtration column (10ug) after high salt treatment.

Figure 4.9B The effect of high salt concentration on PDC activity

PDC (100ug) was incubated in the presence (——O——) or absence (——●——) of solid $MgCl_2$ (0.25M) or NaCl and incubated in the buffer detailed above. At the time points indicated, samples (5ug) were removed and assayed for PDC activity. Results are the average of duplicate determinations differing less than 10%.

A similar result was obtained with PDC (100ug) incubated in 1M NaCl. Data not shown.

A


Mr
(x 10-3)
67
43
30
M
1
2
3
E2
E3
X
E1α
E1β

B

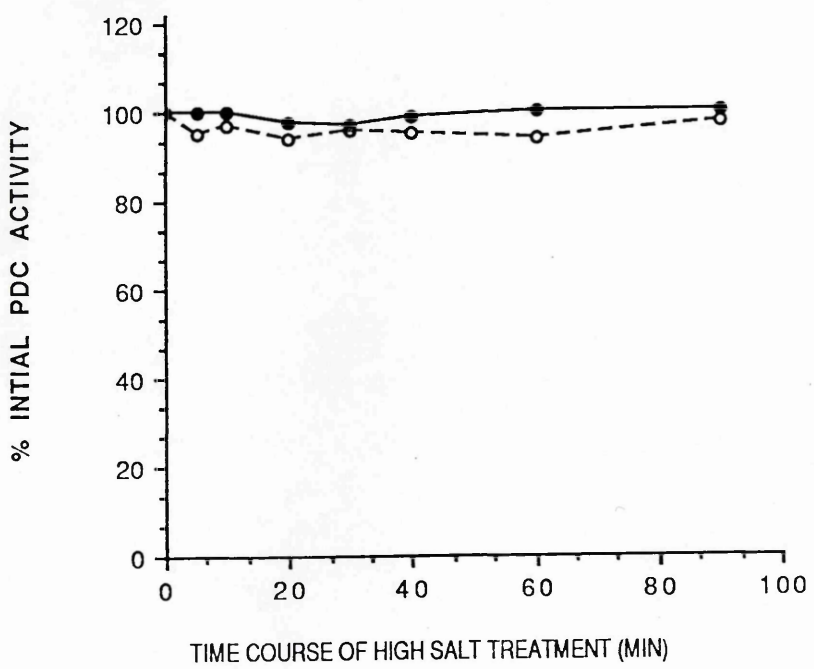
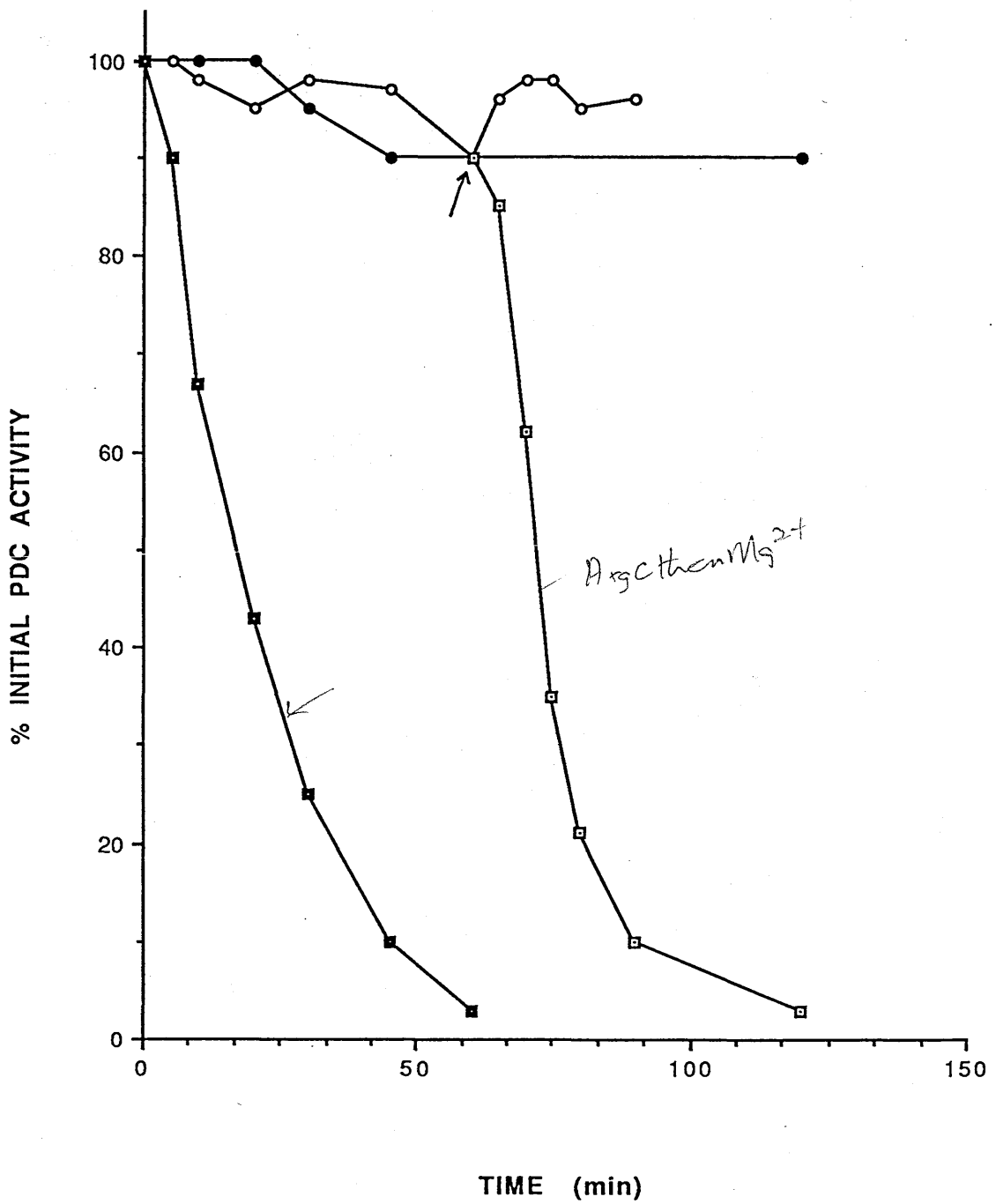

% INTIAL PDC ACTIVITY
120
100
80
60
40
20
0
0
20
40
60
80
100
TIME COURSE OF HIGH SALT TREATMENT (MIN)

Figure 4.10 The effect of high salt and argC on PDC activity

Native PDC (100ug) was incubated in 20mM potassium phosphate buffer, pH7.4 containing 2.5mM NAD^+, 1mM $MgCl_2$ and 0.2mM TPP. On the addition of 3% (w/v) argC samples (5ug) were removed and assayed immediately for PDC activity. At the times indicated by the arrow $MgCl_2$ was added to a final concentration of 0.25M and samples (5ug) removed at the times indicated and assayed for activity (——⊡——).

As controls PDC was incubated in 3% (w/v) argC (——●——) and 0.25M $MgCl_2$ (——○——) respectively for the duration of the experiment and at the times indicated samples (5ug) removed and assayed.

PDC (100µg) incubated in the buffer detailed above before the addition of solid 0.25M $MgCl_2$. After 60 min 3% (w/v) argC was added and at the indicated times samples assayed for activity (——■——). Each value represents the means of two separate determinations differing by less than ± 10%.


100
80
60
40
20
0
0
50
100
150
% INITIAL PDC ACTIVITY
TIME (min)
Arg thenMg2+

4.2.4 The effect of high salt and argC on PDC activity

The surprising observation that $MgCl_2$ or NaCl, although known to dissociate PDC complex into its constituent enzymes, had little effect on enzyme activity was investigated further.

As component X is preferentially degraded by argC, it was decided to examine the effect of high salt on argC-treated complex. PDC was incubated with argC under conditions which were known to digest approx. 90% of component X. This was achieved by monitoring overall complex activity until it had reached 80-90% of its original value (Fig. 4.10). On the addition of $MgCl_2$ samples were withdrawn and assayed for PDC activity (Fig. 4.10). Similarly, PDC preincubated in 0.25M $MgCl_2$ or 1M NaCl was treated with protease argC and again samples were removed and assayed at various time intervals thereafter.

On the addition of high salt to argC treated complex, a rapid decline in PDC activity was observed. This result was also seen on addition of argC to high salt treated complex. Thus a combination of high salt and argC treatment leads to rapid and complete inactivation of the complex. In contrast, PDC activity is not markedly affected by individual treatment with either high salt or argC as shown in control incubations.

4.2.5 Relationship between decline in PDC activity and extent of degradation of component X in PDC treated with argC in the presence or absence of high salt

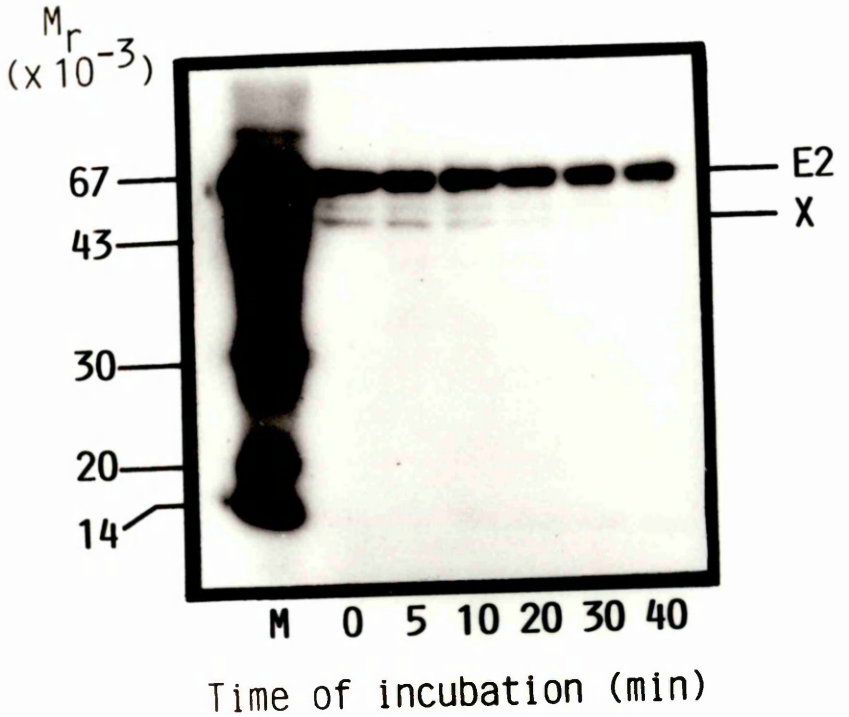
In an experiment designed to examine the relationship between the activity of the complex and the levels of proteolysis undergone by component X in the absence or presence of high salt, samples were removed from incubation mixtures containing [^{14}C]

Figure 4.11 Relationship between enzymatic activity of PDC and degradation of component X in PDC treated with argC in the presence or absence of high salt

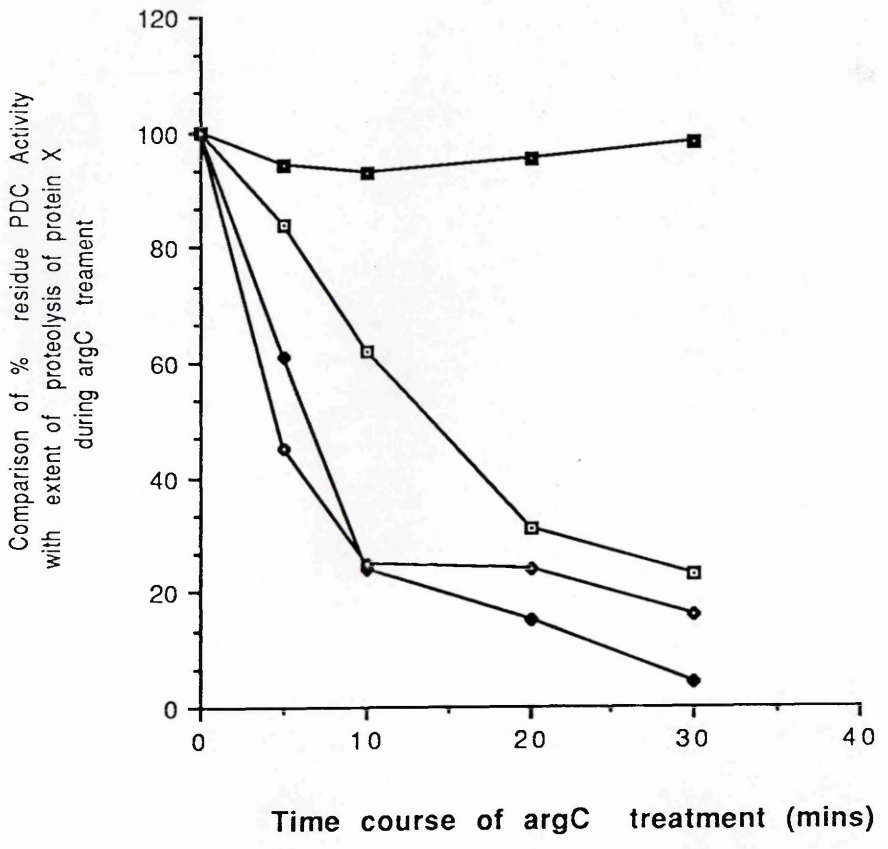
Native PDC (200µg) was incubated with [2-^{14}C] pyruvate in 20mM potassium phosphate buffer, pH7.4 containing 0.25M $MgCl_2$, 2.5mM NAD^+ and 0.2mM TPP. On the addition of 3% (w/v), argC in the presence (——◇——) or absence (——◆——) of $MgCl_2$ (0.25M), samples (30µg) were removed at the indicated times and added immediately to Laemmli sample buffer resolved in a 10% (w/v) SDS/polyacrylamide gel and processed for fluorography (Panel A). E2 and X bands were then densiometrically scanned. The photograph of proteolysis of component X in the absence of $MgCl_2$ is not shown.

In a parallel experiment PDC (100µg) was incubated in 20mM potassium phosphate buffer, pH7.4 containing 0.25M $MgCl_2$, 2.5mM NAD^+ and 0.2mM TPP. On addition of 3% (w/v) argC samples (5µg) were removed and immediately assayed for PDC activity (Panel B). (——□——) As a control untreated PDC was assayed for the duration of the time course (——■- -—).

A


Mr
(x 10-3)
67
43
30
20
14
E2
X
M 0 5 10 20 30 40
Time of incubation (min)

B


Comparison of % residue PDC Activity with extent of proteolysis of protein X during argC treament
120
100
80
60
40
20
0
0
10
20
30
40
Time course of argC treatment (mins)

acetylated PDC and protease argC, in the absence or presence of high salt, and added to Laemmli sample buffer (Figs. 4.11A; 4.7). In a parallel experiment the inactivation of PDC with argC and high salt was monitored (Fig. 4.11B) where rapid and complete inactivation of the complex is observed after 45 min.

The rates of degradation of component X by argC in the presence or absence of $MgCl_2$ proceeds at similar rates to each other. However, the rate of degradation of protein X is more rapid than the inactivation of the complex when incubated in argC and high salt concentrations. As high salt treatment of argC treated complex does not result in a more rapid degradation of protein X, inactivation of the complex under these conditions may be a result of lowering the affinity of E1 and E3 for the core assembly.

4.2.6 Release of E3 from E2/X core on incubation with argC

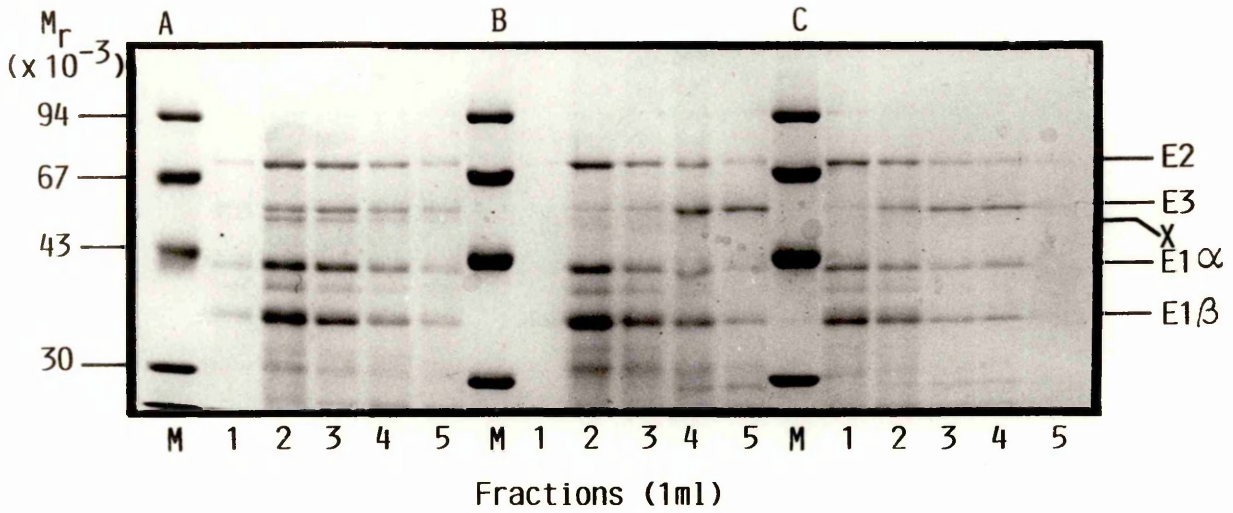
It has been observed that limited proteolysis of E2/X core by protease argC preferentially digested component X and as a consequence of the removal of the lipoyl domains of component X the E3 component failed to bind effectively to argC-treated E2 core (Gopalakrishnan et al., 1989).

In an attempt to study the release of E3 from argC treated PDC, intact complex was incubated in argC prior to resolution on a gel filtration column. Untreated PDC and high salt/argC-treated PDC samples were resolved in a similar manner. Samples were removed from the column fractions, assayed for complex activity (Fig.4.12C') and the remainder of the column fractions were analysed by SDS-PAGE. The finding that argC promotes release of E3 from the complex was confirmed both by Coomassie staining (Fig. 4.12A') and immunoblotting analysis (Fig. 4.12B') using anti-E3 specific serum. The separation of E3 from E2/X core assembly results in

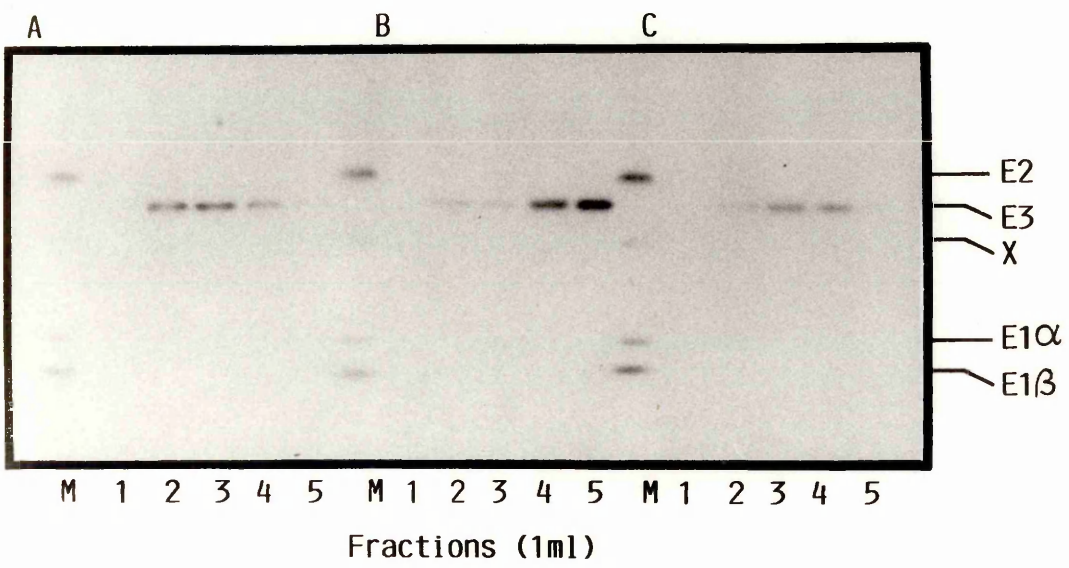
Figure 4.12 Release of E3 from E2/X core on incubation with argC

Native PDC (200µg) was incubated in 20mM potassium phosphate buffer, pH7.4 containing 1mM $MgCl_2$, 2.5mM TPP and 0.2mM TPP (A) plus 3% (w/v) argC (B) or plus 0.25M $MgCl_2$ and 3% (w/v) argC (C) and after 60 min each sample was passed through a 5ml Sepharose CL 4B column equilibrated in 20mM potassium phosphate buffer, pH7.4. 1ml fractions were collected and samples (100ul) removed and assayed for PDC activity (C'). The remaining samples (900ul) were precipitated on the addition of TCA [10% (w/v)]. The resultant pellets were solubilised in Laemmli sample buffer and resolved on a 10% (w/v) SDS/polyacrylamide gel and either stained with Coomassie blue (Fig. 4.12A') or immediately subjected to immunoblotting analysis with anti E3 serum (Fig. 4.12B'). Untreated PDC (—o—) argC treated PDC (—●—) and MgCl and argC treated PDC (—□—).

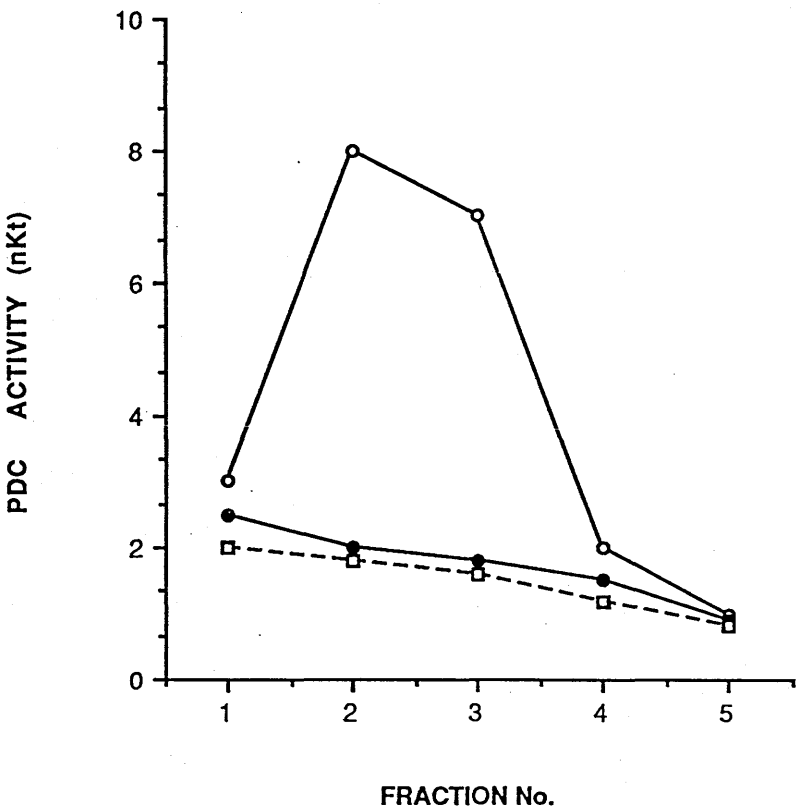
A'


Mr
(x 10-3)
94
67
43
30
A
B
C
E2
E3
X
E1α
E1β
M 1 2 3 4 5 M 1 2 3 4 5 M 1 2 3 4 5
Fractions (1ml)

B'


A
B
C
E2
E3
X
E1α
E1β
M 1 2 3 4 5 M 1 2 3 4 5 M 1 2 3 4 5
Fractions (1ml)

C'



a diminished yield of active complex compared to control enzyme. As discussed previously argC preferentially degrades component X in intact PDC, indicating that the lowered affinity of E3 is probably facilitated by proteolysis of component X

No release of El α or β subunits from the core was observed. under these conditions.

4.2.7 Study of the integrity of the E2/X core assembly after inactivation of the complex with argC and high salt

The rapid inactivation of PDC by a combination of high salt and protease argC treatment may occur as a result of disruption of the E2/X core assembly. Alternatively, as on incubation of PDC with papain (Kresze and Steber, 1979), inactivation may occur as a result of dissociation of the component enzymes, El and/or E3. The release of El α, M_r 42000, El β M_r 36000 and E3 M_r 55000 from the intact core allows separation of the high M_r intact core assembly, from the released polypeptides, by ultracentrifugation.

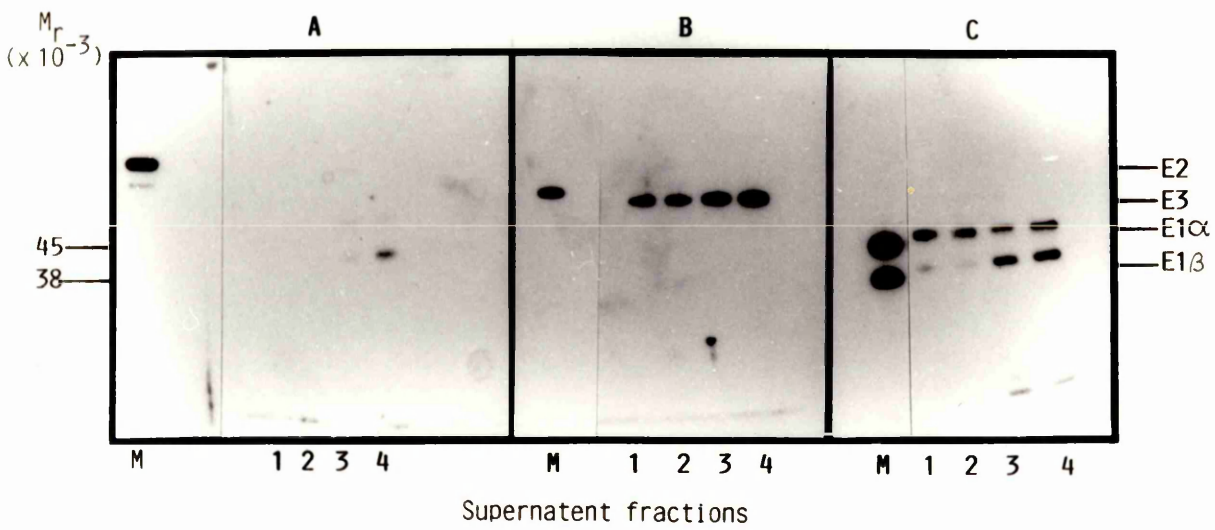
In an attempt to understand the inactivation of the complex on treatment with argC and high salt, PDC was incubated under the conditions described in Figure 4.13 and the E2/X core assembly pelleted by ultracentrifugation. Identification of the subunits was by immune replica analysis using subunit specific antisera (Fig. 4.13).

It is apparent from Figure 4.13 that E3 is released into the supernatant after treatment with argC or argC/high salt. Also, E2 is not observed in the supernatant of these samples but is detected in the pellet, which indicates that the integrity of the core is preserved after the release of E3 from both protease treated samples. Only minor degradation products of E2 are detected in the supernatant samples. El α and El β subunits are present in supernatant and pellet fractions from both intact and argC treated PDC samples.

Figure 4.13 Study of the integrity of the E2/X core assembly after inactivation of the complex with argC and high salt

Native PDC (500µg) was incubated in 20mM potassium phosphate buffer, pH7.4, 1mM $MgCl_2$, 0.2mM TPP and 2.5mM NAD^+ (lanes 1A, 1B and 1C); in the presence of 0.25M $MgCl_2$ (lanes 2A, 2B and 2C); with 3% (w/v) argC (lanes 3A, 3B and 3C); and with 0.25M $MgCl_2$ and 3% (w/v) argC (lanes 4A, 4B and 4C).

After 60 min benzamidine (1mM) was added and each sample diluted to 1ml with 20mM potassium phosphate buffer pH7.4. The samples were centrifuged at 150,000 x g for 2.5 hr. The supernatant fractions were precipitated by addition of TCA (Section 2.2.3a) and the resultant pellets solubilised in Laemmli sample buffer. Samples (1µg) were resolved on a 10% (w/v) SDS/polyacrylamide gel and immediately subjected to immunoblotting analysis with anti-E2 serum (Panel A), anti-E3 serum (Panel B) or anti-E1 serum (Panel C). Lane M, intact PDC (0.5ug)


M_r (x 10^{-3})
A
B
C
45
38
E2
E3
E1α
E1β
M 1 2 3 4
M 1 2 3 4
M 1 2 3 4
Supernatent fractions

As the E2 core is pelleted after proteolysis and high salt treatment, it indicates that a gross disruption of the core assembly is not responsible for the loss of enzymatic activity although a structural re-arrangement of the core resulting in overall loss of complex activity cannot be ruled out at this stage.

4.2.8 Labelling of PDC with [^{14}C] NEM after proteolysis with argC

On prolonged incubation in acetylating substrate E2 and component X become increasingly resistant to N-ethyl [2,3-^{14}C] maleimide (NEM) modification of the reduced thiols on E2 and X. This offers an alternative method of visualising the products of proteolysis of E2 and component X and the ability of argC-treated PDC on prolonged incubation in acetylating substrate to protect against NEM modification.

To examine the peptides generated by proteolysis with argC and their ability to be protected against NEM modification, samples were removed at various times from an incubation mixture containing PDC and argC and processed as described in Figure 4.14.

Under the conditions described in Figure 4.14, component X cannot be visualised. Reduced incorporation of radiolabel into E2 is observed over the time course which can be compared to the protection of the E2/X subunits shown in lane C. This indicates that E2 can still be protected from NEM modification. No peptides from E2 or component X are observed suggesting that they are also protected.

An interesting observation is that the M_r 55000 polypeptide (E3) incorporates NEM after proteolysis with argC (Fig. 4.14). This observation was repeated on incubation of PDC with high salt prior to the addition of NADH and [^{14}C] NEM (data not shown).

Figure 4.14 Enhanced susceptibility of E3 to [^{14}C] NEM modification after limited proteolysis of PDC with argC

Native PDC (500µg) was incubated in 20mM potassium phosphate buffer pH7.4 containing 1mM $MgCl_2$, 0.2mM TPP and 2.5mM NAD^+. On the addition of 3% (w/v) argC samples (50µg) were removed and at the times indicated added to benzamidine. NADH (0.5mM) was added and after 10 min samples were acetylated in the presence of acetyl CoA (0.5mM). The proteins were labelled by the addition of N-ethyl [2,3-^{14}C] maleimide (approximate final concentration of 1mM) and excess NEM removed by the addition of 2-mercaptoethanol. Laemmli sample buffer (X2) was added to each of the samples which were (50µg) resolved on a 10% (w/v) SDS/polyacrylamide gel and processed for fluorography.

Lane M ^{125}I-labelled M_r standards

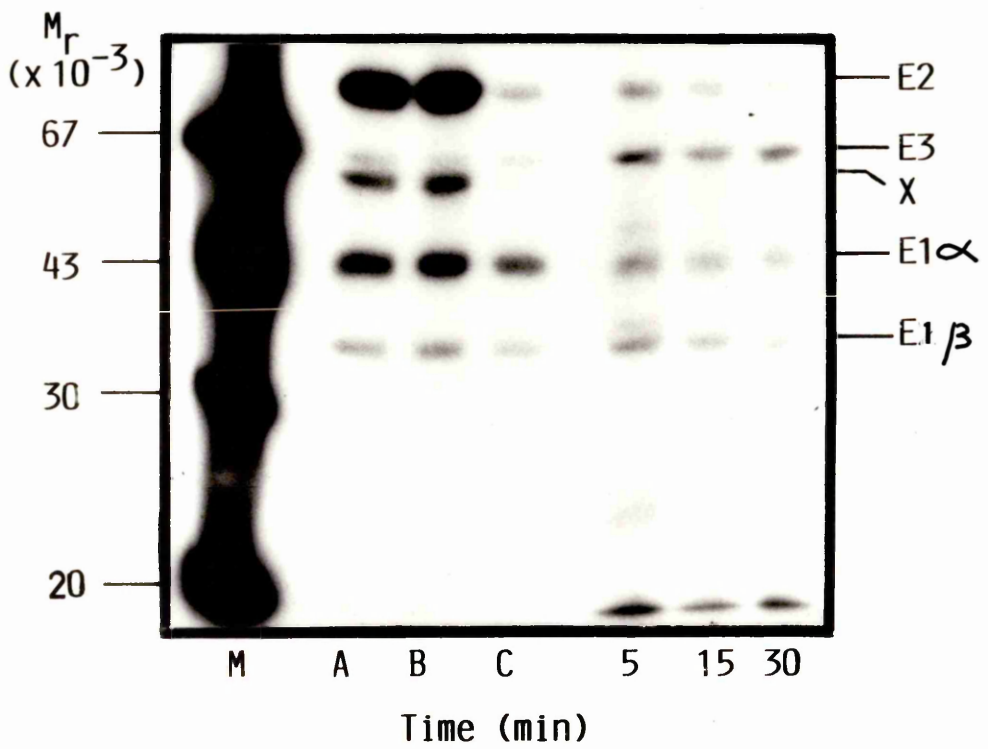
Lane A NADH reduced PDC (50ug) labelled with [^{14}C] NEM

Lane B Simultaneous addition of acetyl CoA and [^{14}C] NEM to NADH reduced PDC

Lane C NADH reduced PDC incubated with [^{14}C] NEM, after incubation in acetyl CoA for 15 min.

Samples from the time course 5, 10, 30 min can be compared directly with lane C.

Enhanced susceptibility of E3 to [^{14}C] NEM modification after limited proteolysis of PDC with ARG C.



4.2.9 Enhanced labelling of E3 may be at the active site

As discussed previously, proteolysis of PDC by argC and argC/high salt results in dissociation of the E3 component from the E2/X core. Also, under these conditions, an increased incorporation of [^{14}C] NEM into E3 is observed. The E3 component may be shielded in the intact complex and only rendered susceptible to NEM modification after release from the core assembly and treatment with NADH. The active site on E3 consists of a reactive cysteine disulphide which functions in catalysis. Substitution of the active site cysteine, by a serine residue using oligonucleotide-directed mutagenesis, abolished E3 activity, confirming the critical role of the redox-active disulphide in catalysis (Russell et al., 1989).

To test the hypothesis that modification of dissociated E3 is at the active site and is NADH dependent, PDC was incubated in argC and at various time points samples removed, processed as described in Figure 4.15 and the E3 activity of each determined. Similarly, PDC incubated in high salt was treated as above. Excess NADH was removed by dilution in centricon tubes (Section 2.2.2) prior to measurement of E3 activity (Fig. 4.15).

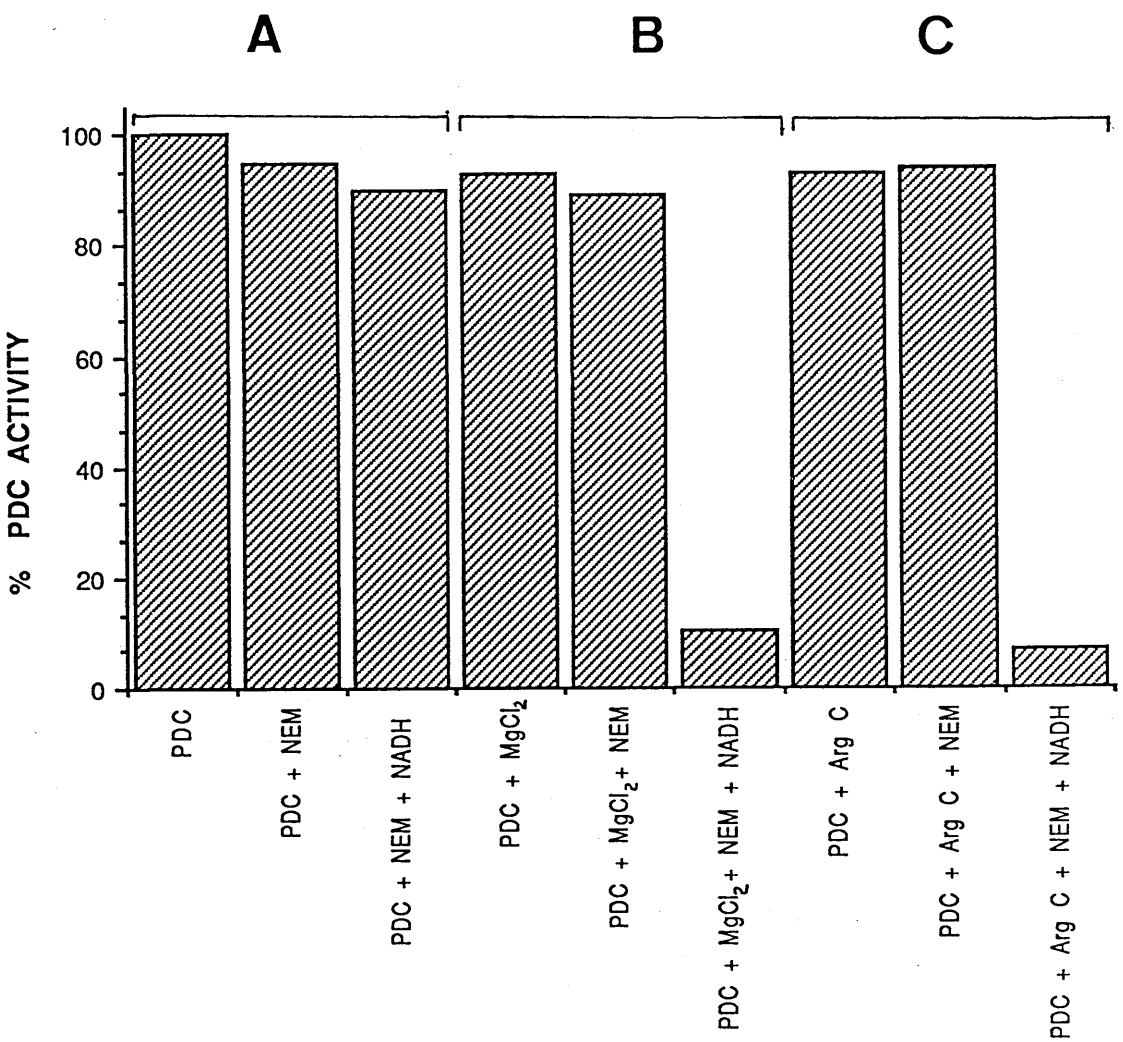
As E3 inactivation is not observed in intact PDC the inactivation seen in argC or high salt treated complex appears to result from the release of E3 from the core assembly. Furthermore, the inactivation following release of E3 is NADH dependent. On release the active site may be exposed making it, in the presence of NADH, susceptible to NEM incorporation and inactivation. The NADH dependent inactivation was confirmed by substituting NEM with other sulphydryl group reagents (oxidised glutathione and oxidised DTT) and a similar profile as in Figure 4.15 was obtained (data not shown). In intact PDC component X may be involved in protection of the active site on E3.

Figure 4.15 NADH dependent inactivation of E3 by NEM

Native PDC (100µg) was incubated in 20mM potassium phosphate buffer pH7.4 (Panel A). Similarly native PDC (100µg) was incubated in the above buffer plus 3% (w/v) argC (Panel C) and PDC (100µg) was incubated in potassium phosphate buffer plus 0.25M $MgCl_2$ (Panel B). After 60 min, NADH was added to a final concentration of 0.5mM followed by incubation for 10 min in 0.5mM N-ethylmaleimide. The reaction was stopped by the addition of 2-mercaptoethanol to 45mM.

Excess NADH was removed by dilution (1:100) with potassium phosphate buffer pH7.4 followed by a (5000 x g) centrifuge spin for 15 min in a centricon 30 tube (Section 2.2.2). The above process was repeated once more.

The samples (10µg) were assayed for E3 activity (Section 2.2.7a). Results are means of duplicate determinations which varied by less than 10%.


A
B
C
% PDC ACTIVITY
100
80
60
40
20
0
PDC
PDC + NEM
PDC + NEM + NADH
PDC + MgCl2
PDC + MgCl2+ NEM
PDC + MgCl2+ NEM + NADH
PDC + Arg C
PDC + Arg C + NEM
PDC + Arg C + NEM + NADH

4.2.10 Effect of protease argC on PDC kinase

It has been argued that the catalytic reduction and acetylation of protein X is sufficient to stimulate the intrinsic kinase activity of pyruvate dehydrogenase and therefore component X has a regulatory role in mediating the activation of pyruvate dehydrogenase kinase by changes in the state of acetylation of the complex (Rahmatullah and Roche, 1987).

Rapid and complete inactivation of PDC as measured by the NAD^+ reduction assay, occurs on incubation of the enzyme in ATP. To study the effect of component X on the regulation of the kinase and subsequently the rate of inactivation of the complex PDC was digested with argC under conditions which were known to degrade the lipoyl domains of component X. On the addition of ATP, to the argC treated complex, samples were removed and assayed for PDC activity (Fig. 4.16A). Untreated PDC was inactivated by ATP in a similar manner (Fig. 4.16A). From the data it is apparent that a more rapid rate of inactivation of protease treated PDC occurs on the addition of ATP. This result is confirmed from the measurement of incorporation of ^{32}P into the E1 α subunit of PDC (Fig. 4.16B).

4.2.11 Proteolysis of PDC by argC in various buffer systems

During this study on PDC kinase, it was observed that the E1α subunit was susceptible to proteolysis by argC in Tris-HCl buffer pH8.0. Therefore in an attempt to protect E1α from proteolysis by argC the incubation conditions were varied.

Proteolysis of PDC by argC in 50mM Tris/HCl, pH8.0 containing TPP and Mg^{2+} resulted in protection of the E1α subunit from degradation (Fig. 4.17D). The effect of TPP and Mg^{2+} on the E1α subunit is confirmed (Fig. 4.17) from the

Figure 4.16A Effect of protease argC on PDC kinase

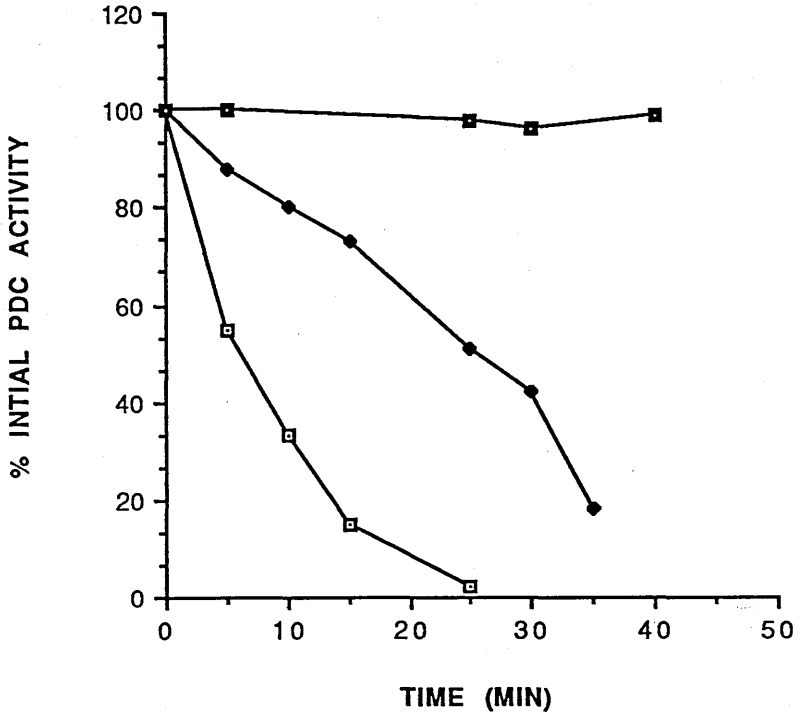
PDC (100µg) was incubated with 3% (w/v) argC in 50mM Tris HCl pH8.0 for 60 min. At the times indicated samples (5µg) were removed and assayed for PDC activity. The reaction was stopped by the addition of benzamidine to 1mM. After 60 min untreated PDC (—◆—) and argC treated (—□—) were incubated in 0.5mM ATP and at the times indicated samples (5µg) removed and assayed for PDC activity. As a control non-phosphorylated PDC (—■—) was also assayed. Results are means of duplicate determinations which varied by less than ± 5%.

The values of 100% activity for argC treated and untreated samples represent the maximum activity recorded before the addition of ATP.

Figure 4.16B Incorporation of ^{32}P into E1α subunit of PDC

PDC (100µg) was incubated in 50mM Tris HCl pH8.0. Reactions were initiated by the addition of [γ-^{32}P]ATP (0.2mM; 100000dpm/nmol). Aliquots of 10ul were applied to filter papers to estimate the time course of incorporation into TCA-precipitable material. Similarly PDC incubated with 3% (w/v) argC for 60 min was treated as above and the time course of incorporation of [γ-^{32}P] ATP into TCA-precipitable material determined for PDC (—□—) and argC treated PDC (—◆—) (Section 2.2.3a).

A


120
100
80
60
40
20
0
% INTIAL PDC ACTIVITY
0
10
20
30
40
50
TIME (MIN)

B

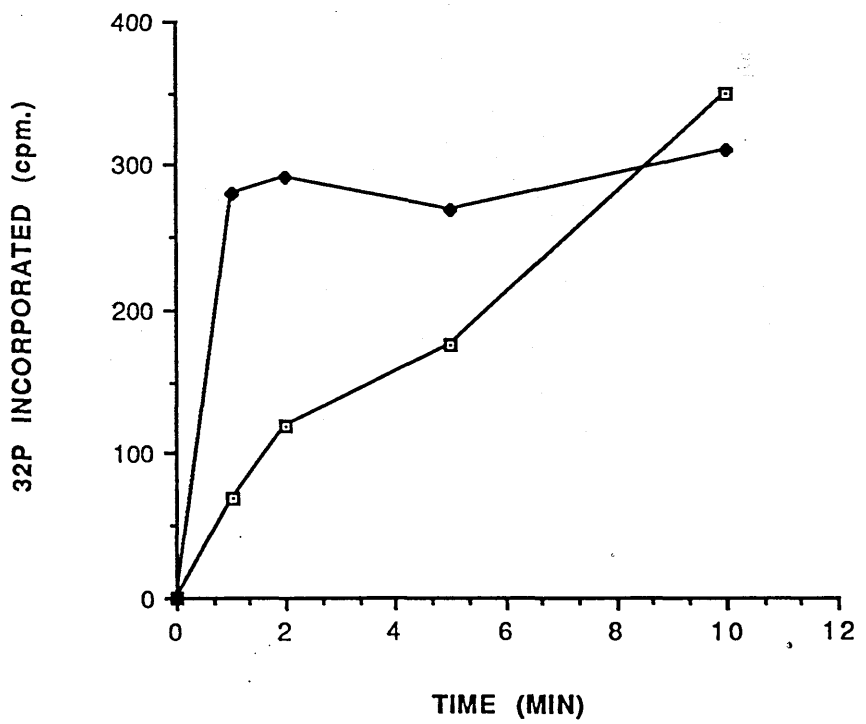
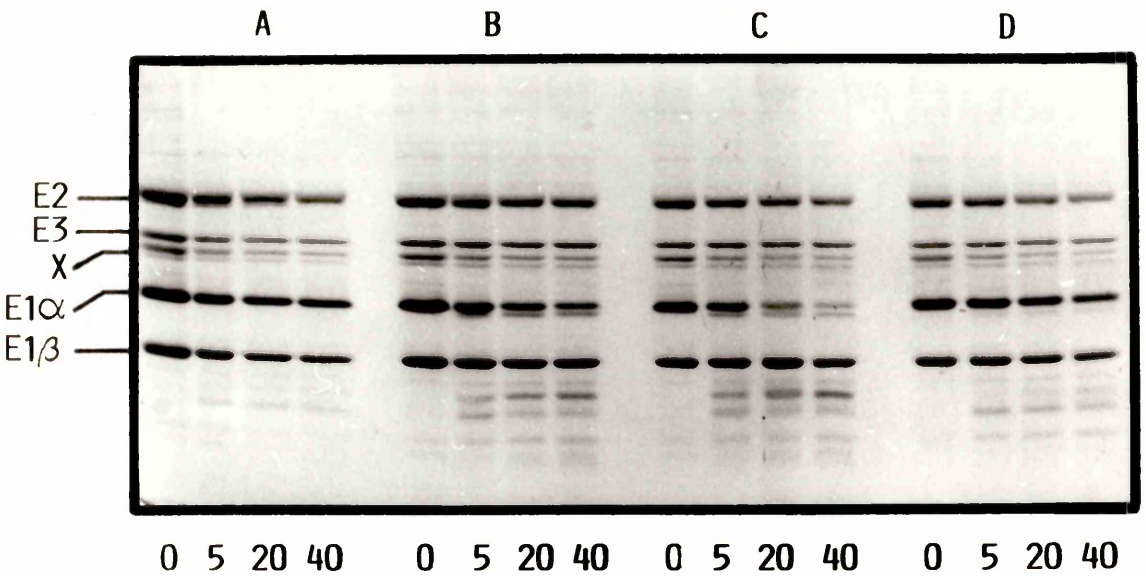

400
300
200
100
0
32P INCORPORATED (cpm.)
0
2
4
6
8
10
12
TIME (MIN)

Figure 4.17 Proteolysis of PDC by argC in various buffer systems

Native PDC (50ug) incubated with 3% (w/v) argC in 20mM potassium phosphate buffer containing 1mM $MgCl_2$, 0.2mM TPP (Panel A). ATP-inactivated PDC (50µg) incubated with 3% (w/v) argC in 20mM potassium phosphate buffer (Panel B). Native PDC incubated with 3% (w/v) argC in 50mM Tris-HCl pH8.0 (Panel C). Native PDC incubated with 3% (w/v) argC in 50mM-Tris HCl pH8.0 containing 1mM $MgCl_2$, 0.2mM TPP (Panel D).

At the times indicated, samples (10µg) were removed and added to Laemmli sample buffer and resolved on a 10% (w/v) SDS/polyacrylamide gel. Proteins were visualised by staining in Coomassie blue.


A
B
C
D
E2
E3
X
E1α
E1β
0 5 20 40 0 5 20 40 0 5 20 40 0 5 20 40
Time of incubation (min)

observation of PDC incubated in 20mM phosphate buffer pH7.4 containing TPP and Mg^{2+} (Fig. 4.17A). PDC incubated in Tris buffer pH8.0 and in the absence of TPP and Mg^{2+} results in significant degradation of the E1 α subunit by protease argC (Panel C).

An interesting observation is the phosphorylated E1 α subunit may also be partially protected against degradation by argC (Fig. 4.17, Panel B)

Further investigation into the effect of argC on the kinase confirmed the observations in Figure 4.16. Limited proteolysis of PDC with argC [1% (w/v)] in the presence of Mg^{2+} and TPP resulted in little degradation of component X or the E1 α subunit. A more rapid rate of incorporation of ^{32}P and inactivation of the complex compared to untreated PDC was observed. During the course of this work, Rahmatullah et al. (1989) confirmed the effect of argC treatment on the kinase.

Discussion

Summary

The reports of Roche and his group in relation to proteolysis of PDC with argC (Gopalakrishnan et al., 1989) have been evaluated in detail. Their findings, based essentially on visual inspection of silver stained gels and enzymatic assays following reconstitution of PDC have indicated that E3 protects component X from proteolysis by argC and that the argC treated core, on the addition of E1 and E3, can no longer interact with the E3 subunit. These claims need to be treated with caution.

In our study, it has been demonstrated quantitatively and immunologically that it is possible to degrade 90% of component X in intact PDC with argC with no significant effects on overall complex

activity. In support of our findings that E3 does not protect component X, it has been possible to release E3 from the intact core after limited proteolysis with argC. In addition, Roche and co-workers have not demonstrated the effect of argC on E1α or considered the consequences for overall enzyme activity.

A major new discovery described in this chapter is that release of E3 from the core assembly exposes the active site on E3, which, in the presence of NADH, is susceptible to inactivation by NEM, oxidised DTT or glutathione. Therefore a possible function for component X is to protect the active site of lipoamide dehydrogenase.

Discussion

Incubation of PDC with protease argC results in the release of most of the lipoyl domains from component X without affecting the overall enzyme activity of the complex. This observation is at variance with Roche's group who found that limited proteolysis of PDC with argC resulted in a loss of activity which paralleled the release of the lipoyl domains from component X (Gopalakrishnan *et al.*, 1989). However, complete release of the lipoyl domains of component X cannot be achieved without minor (10-20%) cleavage of E2 lipoyl domains, causing a slight reduction in overall complex activity. Proteolysis of PDC by argC releases two peptides from E2, M_r 35000 and M_r 45000, as confirmed by immunoblotting with monospecific E2 antiserum and a single immunoreactive peptide from component X, M_r 15000. Under certain conditions the E1α subunit of PDC also undergoes limited proteolysis.

A surprising observation was that incubation of complex in high salt, under conditions which were known to dissociate E1 and E3 from the E2/X core, did not affect the overall enzyme activity.

However, the complex was more susceptible to inactivation by high salt after protease argC treatment. Inactivation of the complex incubated in high salt and argC did not parallel the degradation of component X. In both the absence or presence of high salt, cleavage of component X proceeds at a more rapid rate than the loss of enzyme activity, suggesting that if intact protein X is essential for complex activity it does not become rate limiting until less than 10% is remaining although it may have an important function related to the acetylation of E2. Support for these findings has been obtained by analysis of PDC activity from patients lacking protein X. These patients show significant levels of PDC activity (see general discussion for greater detail).

A possible explanation for the inactivation of the complex with argC and high salt is that in the presence of either argC, or high salt, the affinity of E3 for the core has been lowered without affecting overall enzyme activity, whereas a combination of high salt and argC decreases the affinity of E3 for the core to a level where the complex is rendered inactive.

Examination of pellet and supernatant fractions following ultracentrifugation of argC treated PDC reveals that the pelleted core assembly remains intact while E3 and small peptides from the E2 core are released into the supernatant fractions. This theory is in agreement with the observation of Kresze _et al_. (1980) who show that complex incubated with papain dissociates with loss of overall enzyme activity while each subunit maintains its own activity.

Release of the E3 component from the intact complex after proteolysis with argC has been demonstrated. Proteolysis of the lipoyl domains of component X results in the E3 component dissociating from the core. This result is in contrast to the

findings of Roche and co-workers who report that the E3 component protects component X selectively (Gopalakrishnan et al., 1989). An E3 binding site at the C-terminus of E2 has been identified (Thekkumkara et al., 1988) and recent investigations have revealed an E3 binding site on component X from S. cerevisiae (Behal et al., 1990). Direct interaction between component X and E3 has also been claimed (Powers-Greenwood et al., 1989). An E2 oligomer (lacking protein X and fully retaining acetyltransferase activity) exhibited significantly reduced binding of the E3 component and a component X fraction (lacking E2 subunits) bound a significant amount of E3. Therefore it is probable that component X participates in the binding of E3 to the core assembly.

Release of E3 from the core assembly either by proteolysis with argC or treatment with high salt exposes a sulphydryl group on E3 which in the presence of NADH is modified with [^{14}C]NEM. Inactivation studies on argC treated complex indicated that modification of E3 with oxidised DTT, oxidised glutathione or NEM, was NADH dependent and resulted in inactivation of E3. The active site on E3 is a short 4 residue sequence containing a disulphide bond which functions in catalysis. This disulphide bond is readily reduced in the presence of NADH and consequently can be inactivated by an appropriate inhibitor (Williams et al., 1989). In the intact complex E3 remains tightly associated with the core and is fully active, therefore, component X may interact with the active site of E3 rendering inaccessible to modification by a variety sulphydryl group reagents.

Incubation of PDC in trypsin resulted in the loss of overall enzyme activity, and loss of the ability of the complex on prolonged incubation in acetylating substrate to protect the

S-acetyltransferase from NEM incorporation. Removal of 90% of the lipoyl domains of component X from the complex, with protease argC, did not effect the overall enzyme activity or the ability of the complex on prolonged incubation in acetylating substrate to protect from NEM modification and inactivation. Unfortunately the remaining 10% of the lipoyl domains of component X still associated with the core may be sufficient for component X to function in the diacetylation and protection of the complex from NEM modification. It has been reported that removal of over 90% of the E1 component of E. coli PDC does not affect the ability of E1 component to reductively acetylate E2 (Packman and Perham, 1986). A similar situation may apply with component X.

An interesting observation was that in the absence of TPP and Mg^{2+} argC promoted significant degradation the E1 α subunit of PDC. The effect of TPP and Mg^{2+} in protecting the E1 α subunit is unclear. Either binding of TPP to E1 α subunit occurs at a proteolytically susceptible site, thus preventing proteolysis by argC or TPP and Mg^{2+} induce about a conformational change on the E1 subunit with similar results. A similar effect may be observed with ATP inactivated complex where phosphorylated E1 α may be partially protected from protease degradation. Garland and Nimmo (1984) have reported that isocitrate dehydrogenase in the presence of $NADP^{+}$ can be protected against proteolysis. The binding of $NADP^{+}$ to, or phosphorylation of active isocitrate dehydrogenase induces a major conformational change, thus preventing proteolytic cleavage of the enzyme.

Roche and co-workers have reported that binding of E3 and E1 to argC degraded core assembly results in little restoration of the original complex activity and they conclude that the loss of

activity is caused by reduced E3 binding in the absence of component X. Any preparation of core assembly will have significant levels of E1 associated with the core [however, the data presented by this group (Gopalakrishnan et al., 1989) does not show the endogenous level of E1 α and β present in the E2 core assembly preparation] and therefore limited proteolysis of the core assembly will degrade the endogenous E1 α present. Therefore subsequent reconstitution studies performed on argC treated core assembly may result in reduced levels of activity due to the degradation of E1α present in the core assembly.

In reconstitution studies, in the absence of TPP and Mg^{2+}, Gopalikrishnan et al., 1989, observed with reconstituted PDC that the loss of activity, 50%, closely parallels the degradation of component X. Their finding did not consider the effect of argC on the E1 α subunit.

Recent studies, in the absence of TPP and Mg^{2+}, have shown that the loss of activity also parallels the degradation of the E1 α subunit and therefore this effect has to be considered when assessing the involvement of protein X in overall activity of the complex (data not shown).

In preliminary reconstitution studies, recovery of activity after high salt or argC treatment has not occurred. It is possible that the effect on PDC of high salt or argC causes a conformational change on the E2 core with resultant loss of E3 binding. If this is the case then our findings are in general agreement with Roches' group.

A more rapid rate of inactivation of protease treated complex by ATP was recorded. In the presence of TPP and Mg^{2+} the greater rates of inactivation and incorporation of γ ATP into the

complex is probably due to a protease effect on the kinase. The kinase consists of two subunits α and β M_r 48000 and M_r 450000 respectively. It has been reported that kinase activity resides in the α subunit and proteolysis of the small subunit with trypsin resulted in a slight increase in kinase activity (Stepp et al., 1983). It is thought the β subunit plays a structural role through favourable topographical positioning of the catalytic α subunit. Similarly limited proteolysis of the complex by argC may result in a more rapid inactivation and incorporation of ^{32}P into the complex which may be due to limited proteolysis of the kinase β subunit allowing the catalytic subunit to operate without regulation.

CHAPTER FIVE

STRUCTURAL AND FUNCTIONAL RELATIONSHIP OF COMPONENT X TO THE LIPOATE ACETYLTRANSFERASES (E2) COMPONENT

5. Structural and functional relationship of component X to the lipoate acetyltransferases (E2) component

5.1 Introduction

The lipoate acetyltransferase and protein X subunits of the bovine PDC core assembly are antigenically distinct polypeptides (De Marcucci and Lindsay, 1985; Jilka et al., 1986).

Recent cloning and sequence analysis of the human lipoate acetyltransferase (E2) gene (Coppel et al., 1988; Thekkumkara et al., 1988) in conjunction with earlier protein-chemical studies (Hodgson et al., 1988) have demonstrated the presence of two tandemly-repeated lipoyl domains of approximately 125 amino acids in length, located at the N-terminus of the polypeptide. A short, highly conserved E3 binding sequence is found distal to the second lipoyl domain followed by a C-terminal inter-subunit binding domain which also houses the acetyltransferase activity. The general structure for E2 was first elucidated for PDC from E. coli (Guest et al., 1985; Graham et al., 1986) which, however, contains 3 homologous lipoyl domains while the equivalent S. cerevisiae enzyme has only a single lipoyl domain (Niu et al., 1988). The exact number of lipoyl groups on component X is unknown although recent evidence suggests that it contains only one. Specific cross-linking studies on E2 and X with phenylene-o-bismaleimide via substrate generated thiols or lipoic acid groups has provided evidence for the presence of two lipoyl groups per E2 chain and only a single lipoate domain per protein X (Hodgson et al., 1988).

This chapter describes the immunological approach employed to investigate the possible number of lipoyl domains on component X. The monospecificity of high titre antisera raised against gel purified lipoate acetyltransferase and component X is demonstrated and the location of component X and E2 in various cell extracts is determined.

Figure 5.1 Immunoblotting analysis of lipoamide acetyltransferase (E2) and protein X in various cellular extracts

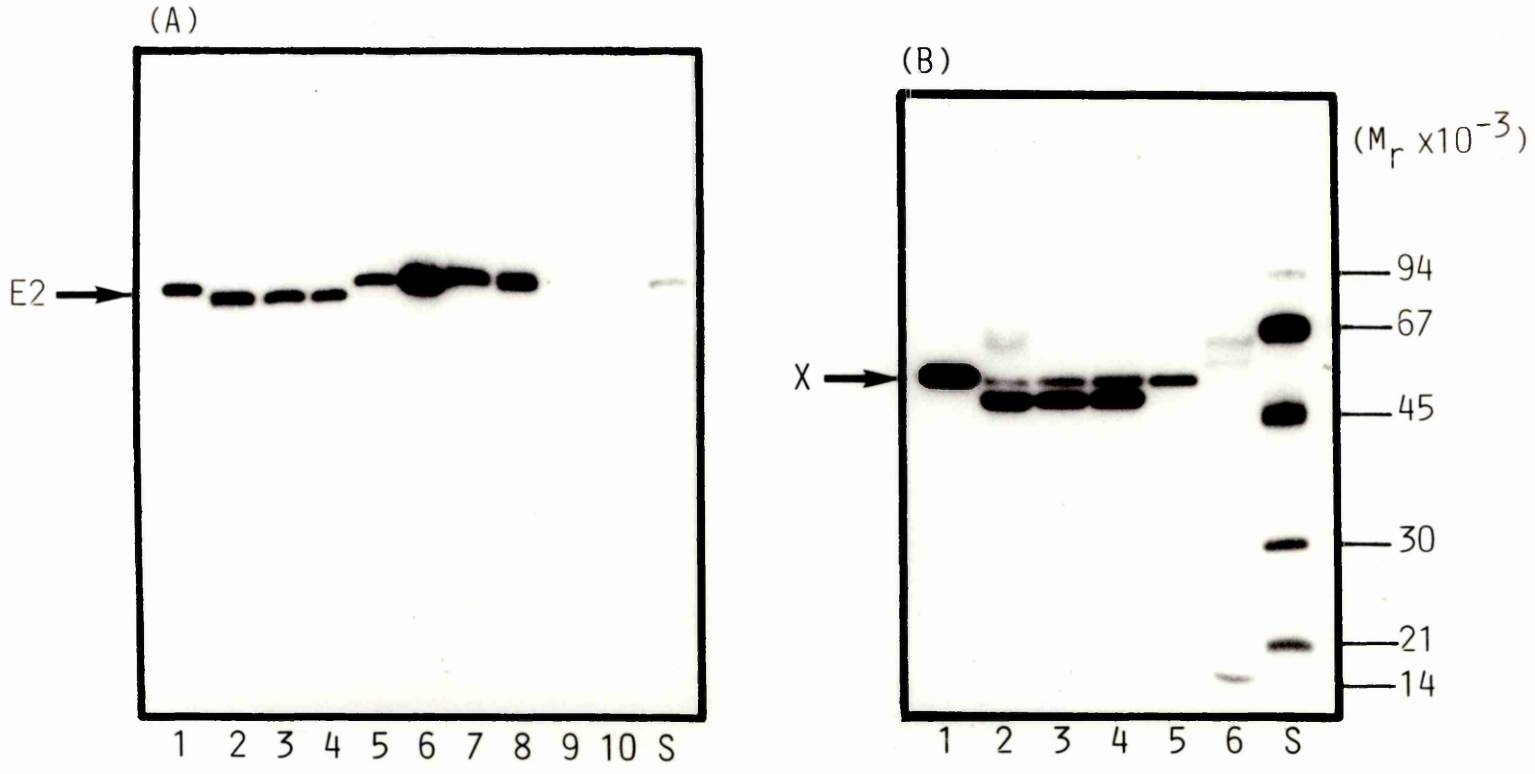
Purified PDC and various cellular extracts were resolved by electrophoresis on 10% (w/v) SDS/polyacrylamide gels. Gels were used for immunoblotting analysis (see Methods section for details) with antibody raised against the E2 subunit (Panel A) or protein X subunit (Panel B).

Panel A:

Lane 1 (and 5) 0.1μg PDC; lane 2, SDS extract of BRL cells, 80 μg; lane 3, BRL mitochondria, 40μg; lane 4, rat liver mitochondria, 40μg; lane 6, ox heart mitochondria, 15μg; lane 7, SDS extract of NBL-1 cells, 80μg; lane 8, SDS-extract of PK-15 cells, 80μg; lane 9, PK-15 mitochondria, 20μg; lane 10, PK-15 post-mitochondrial supernate, 60μg; lane S, ^{125}I-labelled M_r marker, bovine serum albumin.

Panel B:

Lane 1, 0.5μg purified PDC; lane 2, SDS extract of BRL cells; lanes 3 and 4, post-nuclear supernate, 50 and 60μg; lane 5, BRL mitohondria, 40μg; lane 6, nuclear fraction, 40μg; lane S, ^{125}I-labelled M_r markers.


(A)
E2
1 2 3 4 5 6 7 8 9 10 S
(B)
(Mr x10-3)
X
94
67
45
30
21
14
1 2 3 4 5 6 S

Conclusive evidence that component X is different, but homologous to E2 is presented and the location of the lipoyl domain on component X determined.

5.2 Results

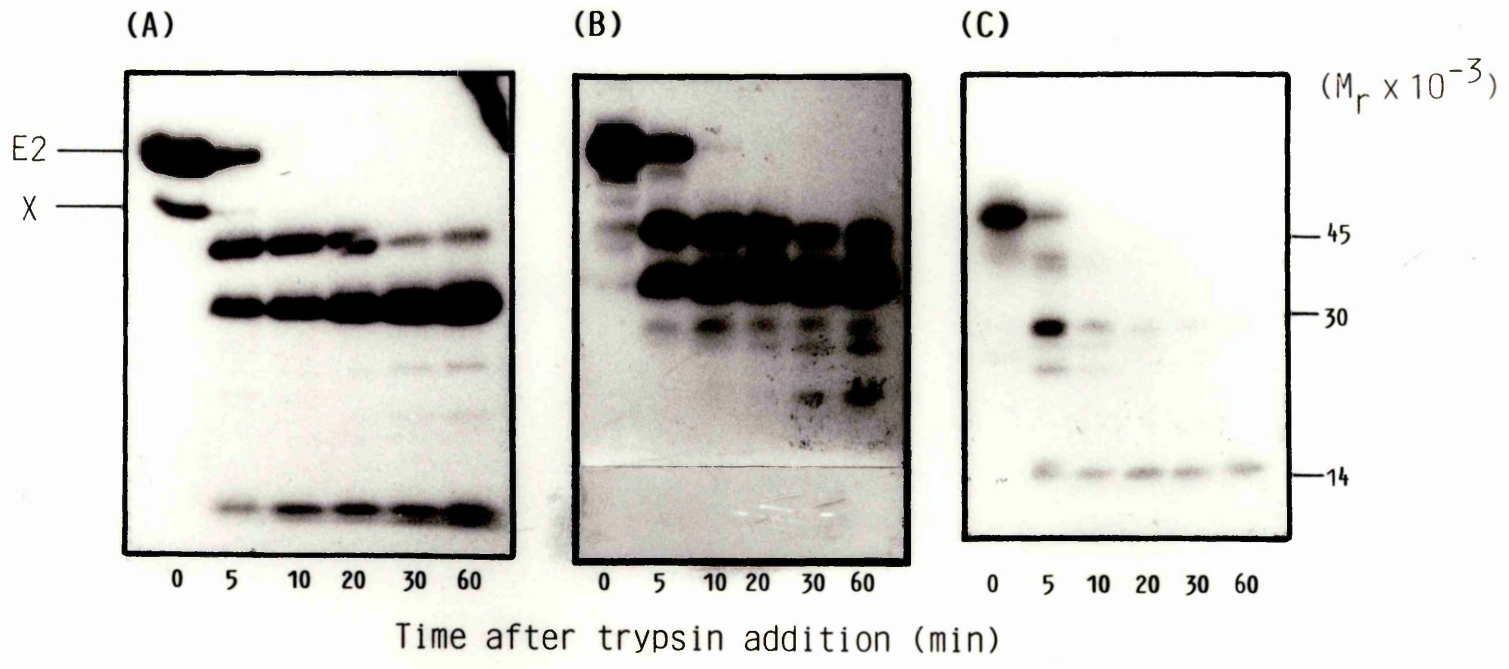
The monospecificity and high titre of antiserum raised to the gel purified lipoate acetyltransferase (E2) subunit of bovine heart PDC is demonstrated in Figure 5.1, where immune replica analysis (Panel A) is conducted against purified PDC and several cellular and mitochondrial extracts. Variations in the M_r values of the E2 subunit are evident in different cell lines. In particular, the E2 polypeptide in BRL cells (lanes 2-4) has a lower apparent M_r value of 68000 compared to 70000-72000 in bovine and porcine tissues. This finding is in agreement with the subunit molecular masses reported for lipoate acetyltransferase in purified rat liver (Matuda *et al.*, 1983), and bovine heart PDC (De Marcucci and Lindsay, 1985). No cross reaction of the E2 antiserum with protein X was observed.

In Figure 5.1, Panel B, the reactivity of X antiserum is illustrated against bovine heart PDC (lane 1), whole cell (lane 2), mitochondrial (lane 5), post nuclear supernatant (lane 3 and 4) and nuclear fractions (lane 6) derived from BRL cells. It is evident that the antibody to component X shows a strong positive cross reaction with an unidentified 48kDa species which is not present in either nuclear or mitochondrial fractions. Its exact subcellular location has not been determined. In mitochondrial extracts, however, this antibody reacts exclusively with the 52kDa component corresponding to protein X. Once again, anti X-IgG failed to recognise the E2 component in these cell cultures.

Figure 5.2 Selective tryptic release of ^{14}C acetylated lipoyl domains from E2 and X polypeptides of native bovine heart PDC

Purified PDC (500ug) was preincubated for 15 min with non-radioactive (panels B and C) or [2-^{14}C] pyruvate (panel A) in the presence of NEM (0.5mM). Excess NEM was reacted with 2-mercaptoethanol (45mM) before treatment of radiolabelled enzyme with 1% (w/w) trypsin at 25^{o}C for the indicated times. Samples (30ug) were removed, immediately treated with Laemmli sample buffer (2x) and subjected to fluorography following resolution on 12.5% (w/v) SDS-polyacrylamide gels.

(panel A) [^{14}C] acetylated peptide; panels (B and C) immunoblots of parallel samples with anti E2 and anti X sera, respectively.


(A)
(B)
(C)
E2
X
(Mr x 10-3)
45
30
14
0 5 10 20 30 60
0 5 10 20 30 60
0 5 10 20 30 60
Time after trypsin addition (min)

In Figure 5.2, Panel A, pure bovine heart PDC, preincubated with [2-^{14}C] pyruvate in the absence of CoASH to promote [^{14}C] acetylation of lipoyl groups on the proteolytically- sensitive E2 and X subunits, was then subjected to tryptic digestion for the indicated times to promote selective release of ^{14}C-labelled lipoyl domains. Specific proteolysis of the [^{14}C] acetylated domains is observed with protein X degrading rapidly to produce a unique, stable, radiolabelled 15kDa peptide (Panel B) as confirmed by immune replica analysis (Panel C).

In contrast, E2 is cleaved more slowly via a transient ^{14}C-labelled 45kDa species, finally generating a stable 38kDa lipoyl peptide (Panel A). Verification that these polypeptides were tryptic fragments of E2 and X, respectively, was again achieved by immunoblotting with subunit specific antisera (Panel B). A similar pattern of E2 and X-derived cleavage products could be obtained with several specific (e.g. V-8 protease) or non specific (e.g. papain, elastase) proteases (data not shown).

N-terminal amino acid sequence and quantitation of recovery obtained from bovine heart E2 and component X is detailed in Table 5.1.

Table 5.2 compares the N-terminal amino acid sequences of bovine heart E2 and protein X polypeptides with the equivalent primary structures of the human (Coppel *et al.*, 1988; Thekkumkara *et al.*, 1988) rat (Gershwin *et al.*, 1987) and *S. cerevisiae* (Niu *et al.*, 1988), lipoate acetyltransferases derived from determinations of the nucleotide sequences of the cloned genes. Protein X exhibits significant homology with all eukaryotic lipoate acetyltransferases examined to date, particularly evident in a highly conserved PS/ALSPTM sequence commencing at residues 9-12 from the N-terminus or at equivalent positions in the internally repeated

Table 5.1 N-terminal amino acid sequence of ox heart E2 and component X

Sequence No.	PTH X Amino Acid	umoles recovered	E2 amino acid	u moles recovered
1	ALA	28.1	SER	25
2	ASP	11.4	LEU	47.5
3	PRO	13.6	PRO	46.3
4	ILE	13.5	PRO	44.0
5	LYS	11.0	HIS	8.5
6	ILE	13.7	GLN	28.3
7	LEU	12.1	LYS	29.3
8	MET	11.45	VAL	36.2
9	PRO	10.3	PRO	30.0
10	SER	4.0	LEU	31.7
11	LEU	9.5	PRO	28.3
12	SER	3.8	SER	x 6.7
13	GLY/PRO	3.0/4.5	LEU	24.0
14	THR	3.7	SER	4.1
15	MET	3.2	PRO	17.0
16	GLU	2.8	THR	5.0
17	GLU	3.1	MET	9.0
18	GLY	6.2	GLN	10.5
19	ASN	1.8	ALA	7.5
20	ILE	1.3	GLY	5.0
21	(VAL)	x 1.0	THR	x 3.1
22	(LYS)	1.0	ILE	4.0
23			ALA	4.0
24			-	
25			-	
26			-	
27			LYS	4.2
28			LYS	4.8
29			-	
30			GLU	1.5

lipoyl domains of the human and rat enzymes. There are two additional positions in which complete identity is retained in all cases involving an invariant proline near the N-terminus and a glycine located in the C-terminal region of this peptide sequence. Interestingly, a conserved histidine at positions 3 or 4, present in all these lipoate acetyltransferases including the yeast enzyme is absent in protein X as is a conserved N-terminal serine (residue 1 or 2). The N-terminal sequence from protein X was subsequently confirmed by sequencing of peptides generated from limited proteolysis.

The corresponding amino acid sequence from the N-terminal and internally repeated lipoyl domains of human E2 reveals sequence identity in 15 out of 24 residues while protein X exhibits lower homology (10 out of 22 residues) with the equivalent N-terminal region from bovine heart E2. The N-terminal primary structures of human and bovine heart E2 are identical over the first 22 residues. Heterogeneity was also observed at residue 13 in the protein X sequence where both proline and glycine were found in comparable yields. During the course of this work, a similar, but not identical sequence was published for the N-terminal region of protein X from bovine kidney (Powers-Greenwood et al., 1989). Considerable sequence variability was noted amongst this group of enzymes in areas outwith the highly conserved regions as evidenced by alterations in sequence length and the presence of many conservative and non-conservative substitutions, notably in protein X which has several unique features e.g. two consecutive glutamic acid residues at positions 16 and 17.

Internal amino acid sequence from protein X was also obtained (Table 5.3). Limited proteolysis of gel purified protein X with protease V8 generated three major peptides of M_r 48000,

Table 5.2 Comparison of N-terminal amino acid sequences of protein X and various lipoate acetyltransferase (E2) from eukaryotic sources

Bovine heart E2 and protein X amino acid sequences were determined directly while the N-terminal human liver, E2(a), rat liver and yeast sequences were derived from the corresponding nucleotide sequences in the indicated publications. Primary structures of bovine heart E2 and protein X are continuous with blank positions present only to optimise sequence alignment. For human and rat liver enzymes the corresponding internal repeat sequences E2(b), at the start of the second lipoyl domain are included for comparison. Regions of complete or near complete sequence identity are highlighted as shown. Bracketed amino acids have been identified only tentatively.

Source of Enzyme	Amino Acid Sequence																								
Bovine heart E2		S	L	P	P	H	Q	K	V	P	L	P	S	L	S	P	T	M	Q	A	G	T	I	A	-
Bovine heart protein X	A	D	P	I	-	-	K	I	L	M	P	S	L	S	P/G	T	M	E	E	G	N	I	(V)	(K)	
Human liver E2 (a) (7,8)		S	L	P	P	H	Q	K	V	P	L	P	S	L	S	P	T	M	Q	A	G	T	I	A	R
E2 (b)		S	Y	P	P	H	M	Q	V	L	L	P	A	L	S	P	T	M	T	M	G	T	V	Q	R
Rat liver (19) E2 (b)		S	Y	P	V	H	M	Q	I	V	L	P	A	L	S	P	T	M	T	M	G	T	V	Q	R
S.cerevisiae (12) E2	A	S	Y	P	E	H	T	I	I	G	M	P	A	L	S	P	T	M	T	Q	G	M	L	A	A

Table 5.3 Internal amino acid sequence of component X

Gel purified protein X (20ug) (Section 2.2.4c) was incubated in 0.01% (w/v) V8 proteinase for 30 min. On the addition of Laemmli sample buffer the sample was resolved on a 10% SDS/polyacrlymade gel and then electrophoretically transferred to nitrocellulose. The nitrocellulose was stained with Coomassie blue and a band M_r 22000 cut out and sequenced.

Table 5.3: Internal Amino Acid Sequences of Component X

20kDa Component X Peptide

Sequence No.	PTH amino acid	pmoles recovered
1	D,G,A,P,V,F,I	4.2
2		
3	ALA	4.2
4	ASN	2.4
5	ILE	7.6
6	-	
7	LEU	3.0
8	ALA	3.3
9	-	
10	LEU	1.5
11	ILE	4.0
12	GLY	1.9
13	LEU	1.0
14	(LEU)	1.0
15	VAL	2.7
16	GLU	0.6
17	TYR	0.6
18	-	
19	ILE	3.2
20	-	
21	THR	0.2
22	ASP	0.6

M_r 35000 and M_r 30000. Amino acid sequencing of each of these peptides revealed that they were N-terminal fragments confirming the sequence of component X in Table 5.1 and indicating that the carboxy terminus end of protein X is more proteolytically sensitive. Internal amino acid sequence was determined from a fragment M_r 20000 (Table 5.3) from a digest with V8 protease on gel purified protein. Comparison of internal component X sequence revealed little homology with the primary structures of lipoate acetyltransferases derived from nucleotide sequence listed.

5.3 Discussion

Immunology of protein X and lipoate acetyltransferase (E2)

In view of the striking homology (45% identity) in the N-terminal sequences of the bovine heart E2 and X polypeptides, it may seem surprising that no immunological cross-reactivity is apparent employing antisera raised to the individual subunits. In one case, an anti-X serum has been reported to recognise the E2 enzyme also (Jilka *et al.*, 1986). Conflicting observations occur frequently with polyclonal sera where the characteristics of the IgG population depend on multiple factors, e.g. the method of antigen presentation the immunisation regime and individual animal responses.

Immunological studies have been widely used to demonstrate homologies within families of isoenzymes where a linear relationship has been established between the degree of cross-reaction and the extent of sequence divergence. However, systematic analysis of a selection of purified lysozymes revealed that enzymes exhibiting less than 60% homology did not usually show an immunological cross-reaction in precipitation assays or by microcomplement fixation (Prager and Wilson, 1971a & b).

Antibodies to native proteins are primarily against non-contiguous epitopes (three dimensional determinants) on the surface of the polypeptide while, for denatured antigen, linear elements of sequence (5-8 amino acids) are responsible for eliciting the major antibody response. If the common sequences are highly conserved e.g. the PS/ALSPTM sequence in E2 and X they may be very weak immunogens accounting for the lack of cross-reactivity between these two homologous proteins (De Marcucci et al., 1985). The absence of cross reaction may suggest that the E2 and X polypeptides are encoded by separate genes rather than products arising from alternate exon splicing of a single genomic copy. In the latter case the presence of long identical sequences in common exon segments should lead to immunological cross recognition by anti-E2 or anti-X sera. This argument has been confirmed recently (Behal et al., 1989), where extensive homology observed between E2 and X indicates that they evolved from a common ancestor.

Organisation, origins and functions of protein X

Limited amino acid sequence data on bovine heart protein X indicates that this protein contains a lipoyl domain located at its N-terminus and belongs to a family of homologous enzymes, all the other members of which possess lipoate acetyltransferase activity. The heterogeneity at position 13 in the protein X sequence where both proline and glycine were present, probably represents separate allelic forms of the enzyme as the PDC was purified from a single beef heart.

There are several reasons for suggesting that, in contrast to bovine E2, protein X contains only a single lipoyl domain:
(i) selective release of lipoyl domains from E2 and X by limited proteolysis with a variety of specific and non specific proteases

producing stable peptides, containing all the [^{14}C] acetylation sites, in the 35-42kDa range for E2 and 15-20kDA range for protein X (ii) the subunit molecular mass of protein X, 51kDa as opposed to 70kDa for E2 is best accounted for by the absence of a second lipoyl domain on protein X. These domains are known to migrate anomalously on SDS/polyacrylamide gels owing to the presence of Ala-Pro-rich linker regions (Graham et al., 1986) and (iii) specific cross-linking studies on E2 and X with phenylene-o-bismaleimide via substrate-generated thiols on lipoic acid groups has provided evidence for the presence of two lipoyl groups per E2 chain and only a single lipoate on protein X (Hodgson et al., 1988).

Both E2 and protein X participate in the acetylation reactions of the complex (De Marcucci et al., 1986) and apparently acts as independent substrates for reductive acetylation by pyruvate dehydrogenase (E1) (Rahmatullah and Roche, 1987). The inability to dissociate protein X from the E2 core assembly except under denaturing conditions also highlights the close physical and functional integration of these two components (De Marcucci and Lindsay, 1985). Indeed protein X contains a C-terminal domain, equivalent to the intersubunit binding domain of E2 and may be an effective replacement for E2 within the core structure. A recent report (Powers-Greenwood et al., 1989) also claims that protein X is involved in binding dihydrolipoyl dehydrogenase (E3) and thus may also possess an E3 binding region which is highly conserved in both prokaryotic and eukaryotic PDCs (Thekkumkara et al., 1988). An intriguing question, therefore, currently under investigation, relates to

the precise role of component X: is it a variant of E2 performing an essential structural or mechanistic role within the multimeric core assembly? Cloning and sequence analysis of the protein X gene will be an important first step in endeavouring to answer these questions (see Chapter 6 for details).

CHAPTER SIX

CLONING AND SEQUENCE ANALYSIS OF A cDNA ENCODING PROTEIN X

6. Cloning and sequence analysis of acDNA encoding protein X

6.1 Introduction

In all eukaryotic pyruvate dehydrogenase complexes studied to date, a structural core of high M_r is formed by 60 E2 subunits organised into a pentagonal dodecahedron to which El and E3 polypeptides are bound to produce a large multienzyme array of M_r 8.5 x 10^6. The cellular location of the pyruvate dehydrogenase complex is considered to be the mitochondrial matrix although there are indications that in plants the complex exists in both mitochondria and chloroplasts (Mierynk et al., 1988).

Eukaryotic PDC preparations also contain a prominent M_r 51000 species (M_r 48000 in yeast), component X, which is associated with the E2 core (De Marcucci and Lindsay, 1985). The presence of component X in mammalian PDC has been confirmed (Jilka et al., 1986; Behal et al., 1989) and like E2, this component is reductively acetylated in the absence of CoASH, with [2-^{14}C] pyruvate.

On incubation with CoASH, component X and E2 are rapidly deacetylated. The site of acetylation on the component X polypeptide, like E2 is reported to be lipoic acid (Hodgson et al., 1986). In earlier studies component X was considered to be a proteolytic fragment of E2; however no common peptides were produced when isolated E2 or component X were digested with a variety of specific and non-specific proteinases (De Marcucci et al., 1986).

Deoxyribonucleotide sequencing of a cDNA for the dihydrolipoamide acetyltransferase (E2) of human pyruvate dehydrogenase complex and examination of the deduced amino acid sequence (Thekkumkara et al., 1988) revealed that E2 shows the

presence of the amino terminal lipoyl bearing domain and the carboxy terminal catalytic domain. Both domains had been demonstrated to be present in E2 of PDC based on proteolytic cleavage of mammalian E2 (Bleile et al., 1979) and the sequencing of E. coli E2 (PDC) (Stephens et al., 1983b). The deduced amino acid sequence of human PDC-E2 also indicated the presence of two lipoyl binding sites in the lipoyl bearing domain. The second lipoyl-binding site shows approximately 80% homology with the first and the differences that exist between the two lipoyl-binding sites are conservative.

A dihydrolipoamide dehydrogenase (E3) binding site has been identified from E2 based on sequence homology between the E2 components of the three 2-oxo acid dehydrogenase complexes (Hummel et al., 1988). A sequence of 32 amino acids which is highly homologous to the E3-binding site, is also present in human PDC E2. Overall, there is approximately 90% homology in the E3-binding region between human and rat liver PDC E2 (Gershwin et al., 1987) and 53% homology between human and E. coli PDC E2 (Stephens et al., 1983). Further comparison of the amino acid sequences of the catalytic domains of human liver E2 and E. coli E2 revealed a segment of 14 amino acids near the carboxy-terminal with approximately 79% homology. A histidine residue is conserved in both sequences which may serve as the active site residue for catalysis.

Sequence analysis of a cDNA encoding component X would, hopefully, allow us to identify possible subunit binding sites (e.g. E3-binding site) on component X and identify through deduced amino acid sequence comparison with eukaryotic and prokaryotic acetyl transferase any region of sequence which may have a specific function (e.g. acetyltransferase activity).

There are several strategies which could be employed to clone the desired gene.

6.1.1 Nucleic acid hybridisation

This is the most commonly used method of screening cDNA libraries for clones of interest.

6.1.2 Homologous probes

Homologous probes contain at least part of the exact nucleic acid sequence of the desired cDNA clone. They are used in a variety of circumstances, for example, when a partial clone of an existing cDNA is used to isolate a full length clone from a cDNA library. Usually, a fragment derived from the existing clone is isolated, radiolabelled *in vitro* and used to probe the new library. Hybridisation with a homologous probe is always carried out under stringent conditions.

6.1.3 Partially homologous probes

Partially homologous probes are used to detect cDNA clones that are related, but not identical, to the probe sequences. If the same gene has already been cloned from another species or if a related gene has been cloned from the same species, it is possible that there is sufficient conservation of nucleic acid sequence to allow the screening of a cDNA library by hybridisation.

6.1.4 Synthetic oligonucleotide probes

Three types of oligonucleotide probes are in common use:

a) Single oligonucleotides of defined sequence

b) Pools of short oligonucleotides whose sequences are highly degenerate ($<$ 17 bases long)

c) Pools of longer oligonucleotide of lesser degeneracy ($>$ 17 bases long).

Degenerate oligonucleotide probes are used to screen libraries by hybridisation to take account of the degeneracy of the genetic code. There are 64 possible codons for only 20 amino acids; most amino acids are represented by more than one codon. Thus a certain sequence of amino acids can be encoded by a number of different oligonucleotides. A completely degenerate pool of oligonucleotides contains all DNA sequences that can code for a given sequence of amino acids. A pool of limited degeneracy consists of a selected subset of oligonucleotides that can code for a given sequence of amino acids.

a) Single oligonucleotides of defined sequence

Probes consisting of a single oligonucleotide of defined sequence usually correspond to part of the sequence of a previously cloned segment of DNA. In general, such oligonucleotides match with their target sequence perfectly, or nearly perfectly, and they are sufficiently long (19-40 nucleotides) to allow the use of hybridisation conditions which can guarantee discrimination between the target sequence and other closely related sequences. Single oligonucleotides of defined sequence are used to screen cDNA, genomic DNA libraries, or subclones derived from them by employing Southern, Northern and dot blot hybridisation to identify and detect the sequence of specific genes. Oligonucleotide probes used for these purposes are usually labelled by phosphorylation of 5(or 3)' termini with [α-^{32}P]ATP.

b) Pools of short oligonucleotides whose sequences are highly degenerate

Degenerate pools of short oligonucleotides containing all possible sequences that can code for a given tract of amino acids have been used as hybridisation probes to isolate both cDNA and genomic DNA clones (Maniatas et al., 1989). The sequences of these

oligonucleotides are typically chosen, using the genetic code, from short tracts of amino acid sequence determined by the sequencing of small quantities of highly purified proteins (Kent et al., 1987; Maniatas et al., 1989). As a consequence of the degeneracy of the genetic code several oligonucleotides must usually be synthesised to represent all possible combinations to code for a given short sequence of amino acids. Thus, if hybridisation conditions can be found under which only perfectly matched sequences form stable duplexes, cloned copies of the gene of interest can readily be isolated from cDNA or genomic DNA libraries. However, it is important to realise that not all strongly hybridising clones will necessarily contain sequences of the gene of interest.

There may be other oligonucleotides in the pool that, by chance, match perfectly with the sequence of other cloned genes. Furthermore hybridisation conditions cannot always be established that allow only perfectly matched hybrids to form, which leads to the isolation of other clones containing sequences which are closely related. Pools of degenerate oligonucleotides are used not to identify target clones unambiguously, but to select a series of candidate clones that can be tested further.

6.1.5 In vitro amplification of DNA by the polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is used to amplify a segment of DNA that lies between two regions of known sequence. Two oligonucleotides are used as primers for a series of synthetic reactions that are catalysed by DNA polymerase. These oligonucleotides lie on the opposite strands of the template DNA and flank the segment of DNA that is to be amplified. The template DNA is first denatured by heating in the presence of a large molar

excess of each of the two oligonucleotides and the four dNTPs. The reaction mixture is then cooled to a temperature that allows the oligonucleotide primers to anneal to their target sequences, after which the annealed primers are extended with DNA polymerase. The cycle of denaturation, annealing and DNA synthesis is then repeated many times. Because the products of one round of amplification serve as templates for the next, each successive cycle essentially doubles the amount of desired DNA product.

Although a relatively new technique, PCR amplification has already found extensive application in the diagnosis of genetic disorders, the detection of nucleic acid sequences of pathogenic organisms in clinical samples and the genetic identification of forensic samples from individual hairs or single sperm. In addition PCR is being used for the generation of specific sequences of cloned double stranded DNA, generation of probes specific for uncloned genes by selective amplication of particular segments of cDNA and generation of large amounts of DNA for sequencing.

6.1.6 Antibody screening

A cDNA copy of a eukaryotic transcript can be expressed as a fusion protein with a large proportion of the β-galactosidase enzyme (in vector λgtll). The eukaryotic portion of the fusion protein may be sufficiently antigenic to be detected by appropriate antibody probes (Young and Davis, 1983). Expression of the recombinant gene from the vector gal Z promotor is by treatment of actively replicating phage in a suitable *E. coli* host with isopropyl-β-D-thiogalactopyranoside followed by adsorption of proteins, from plaques, onto nitrocellulose paper. The method of antigen visualisation involves using specific IgG followed either by a second antibody directed against the species-specific epitopes of

the first antibody and colour development with 4-chloro-naphthol plus H_2O_2 (De Wet, 1984) or by ^{125}I-radiolabelled protein A. An equivalent approach has already been successful in isolating gene sequences. For example, the E2 component of human BCOADC was isolated and sequenced from a human cDNA library housed in λgt11 (Litwer and Danner, 1985) and similarly the full cDNA clone from human E1α of PDC has been isolated (De Meirleir et al., 1988).

As subunit specific antisera to each component of mammalian PDC was available it was decided to screen a human hepatoma cDNA library housed in the gene expression vector λgt11 (De Wet, 1984) using subunit specific antisera raised against the individual components of denatured and intact PDC. Recombinant λgt11 phage isolated by immunological screening would be investigated to confirm that sequences coding for PDC subunits were present.

6.2 Results

6.2.1 Reactivity of antisera with denatured PDC on a Western blot

The key to success with antibody screening lies in the quality of the antisera. It is essential that the antibody specifically recognises the denatured protein. Therefore to test the antibody signal produced from a Western blot, ox heart PDC was purified as described in Section 2.2.5a, resolved by SDS-PAGE on a 10% (w/v) slab gel and electrophoretically transferred to nitrocellulose paper (Section 2.2.9a), before challenging with purified IgG or antisera. Small amounts, 1ug and 0.5ug, of PDC subunits are readily detected by antisera to E2 and component X (Fig. 6.1). 1μg and 0.5μg of PDC is also detected by anti E2 IgG and component X IgG but at reduced intensities. Similar observations were made for PDC IgG and PDC antisera.

Figure 6.1 Immunological detection of PDC and PDC subunits with IgG prepared from antisera raised against PDC, E2 and component X

Samples of purified pyrvuate dehydrogenase complex were electrophoresed on a 10% (w/v) polyacrylamide slab gel and analysed by immunoblotting with antiserum and IgG raised against E2 (A). A similar blot was processed with antisera and IgG to component X (B) and antisera to PDC and IgG (C).

(A, B and C) lanes 1, 3 1μg of PDC lanes 2,4 0.5μg of PDC

(A) lanes 1,2 challenged with 1:100 dilution of E2 antiserum

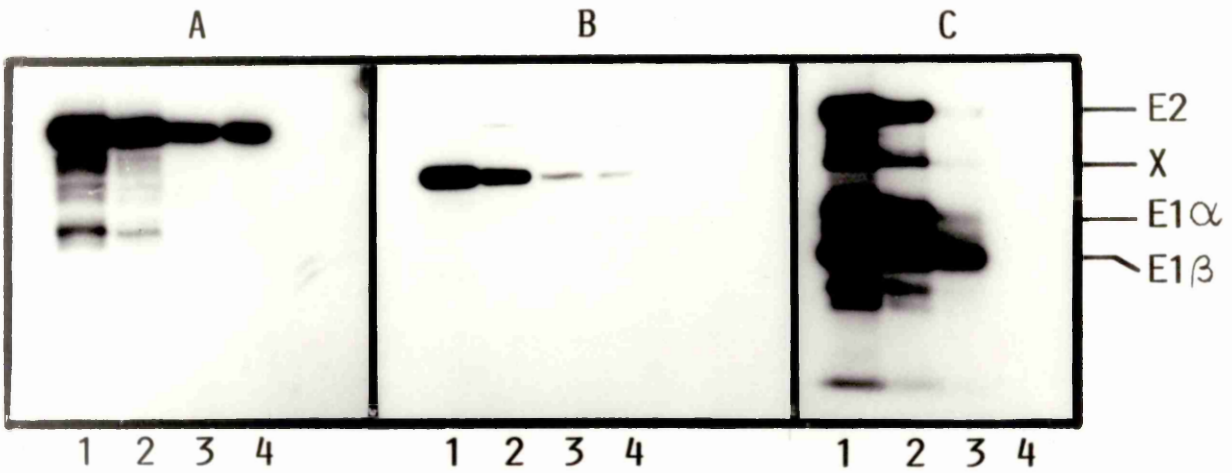
lanes 3,4 challenged with1:100 dilution of E2 IgG

(B) lanes 1,2 challenged and 1:100 dilution of component X antiserum

lanes 3,4 challenged with 1:100 dilution of component X IgG

(C) lanes 1,2 challenged with 1:100 dilution of pyruvate dehydrogenase antiserum

lanes 3,4 challenged with 1:100 dilution of pyruvate dehydrogenase IgG


A
B
C
E2
X
E1α
E1β
1 2 3 4
1 2 3 4
1 2 3 4

Denatured proteins transferred from an SDS-polyacrylamide gel are likely to display many of the same epitopes as the cognate fusion protein synthesised in bacteria. The foreign polypeptide sequences of most fusion proteins are unlikely to fold into completely native structures and after transfer to nitrocellulose, as in the screening process, may undergo further denaturation. Therefore it is likely that proteins detected by Western blot analysis (Fig. 6.1) will be recognised by the appropriate antisera, IgG when synthesised in bacteria as a fusion protein.

6.2.2 Dot blot analysis (Levels of antigen detected by antisera)

Ideally, the antibody should be capable of detecting as little as 50-100pg of antigen in an area the size of a small bacterial colony. The amount of antigen present in colonies and plaques expressing fusion proteins varies widely depending on the toxicity of the fusion protein, its rate of degradation and physical state. However, an antibody capable of detecting as little as 100pg of denatured antigen should allow all but the most labile of fusion proteins to be recognised.

In order to determine the levels of antigen which could be detected by antisera, native PDC (0.1ng to 50ng) was spotted onto nitrocellulose and challenged with the appropriate IgG or antiserum (Section 2.2.9c) followed by incubation with donkey-anti rabbit IgG conjugated with peroxidase. Colour development was initiated on the addition of 4-chloronaphthol plus H_2O_2 (Fig. 6.2).

Levels of 0.1ng of antigen were detected by E2 antiserum, component X antiserum and PDC antiserum. Only E2 IgG could detect levels of antigen approaching 0.1ng, component X IgG and PDC IgG could detect levels of 5ng of antigen (Fig. 6.2). As the most sensitive limits of detection possible was necessary it was decided to screen the human hepatoma cDNA λgt11 gene expression library with E2 IgG and antisera to component X and antisera to PDC.

Figure 6.2 Dot blot analysis

Native PDC, (50ng, 10ng,, 5ng, 1ng and 0.1ng) were spotted onto strips of nitrocellulose.

Panel A lane 1 PDC challenged with 1:100 dilution of X IgG

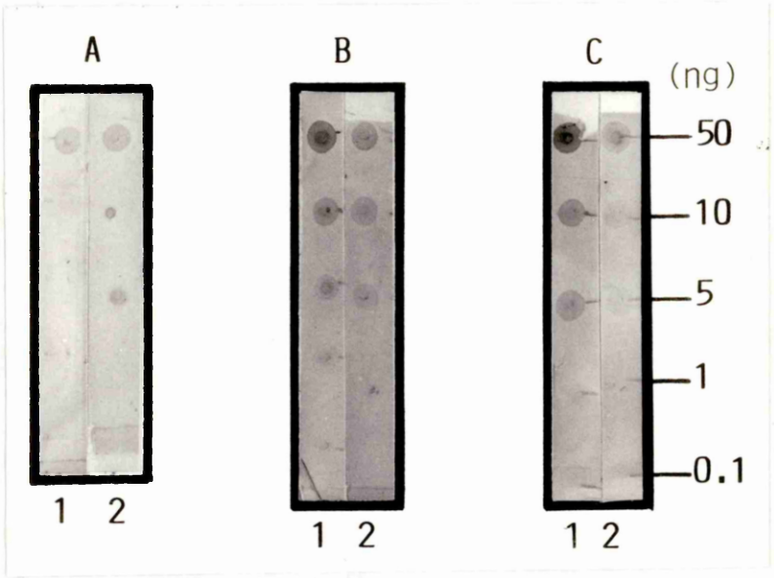
lane 2 PDC challenged with 1:100 dilution of X antiserum

Panel B lane 1 PDC challenged with 1:100 dilution of E2

lane 2 PDC challenged with 1:100 dilution of E2 antiserum

Panel C lane 1 PDC challenged with 1:100 dilution of PDC antiserum

lane 2 PDC challenged with 1:100 dilution of PDC IgG


A
B
C
(ng)
50
10
5
1
0.1
1 2
1 2
1 2

6.2.3 Screening of λgtll gene expression library

In simple terms the screening of a λgtll gene expression library involves the detection of a fusion protein by an antibody followed by the isolation and purification of the plaque expressing the appropriate fusion protein. λgtll libraries are screened with antibody probes as plaques on a confluent plate of E. coli Y1090 (Section 2.2.12a). Strain Y1090 contains several important features which make it a suitable host. It contains a lac fusion repressor, lac I^q, which prevents the lac Z-directed gene expression i.e. prevents the formation of fusion protein. This repression is overcome by the addition of IPTG when required.

Fusion proteins are alien to host cells and therefore often toxic to the host cell. To overcome this toxicity proteases will act to prevent formation of a fusion protein. In this situation two problems have to be overcome; firstly the stability of the fusion protein must be ensured and secondly, it must not adversely affect its host cell. Host Y1090 has a deficiency in lon protease, which increase the stability of the recombinant fusion protein and the formation of the fusion protein will not occur until the addition of IPTG which supresses the function of the lac repressor. Y1090 also has a supF gene to suppress the phage mutation causing defective lysis (S100).

As described in Materials and Methods (Section 2.2.12) 5 x 10 million phage were screened at 500,000 per 150mm plate. Each plate was overlaid with up to three different IPTG impregnated nitrocellulose filters and each filter processed and challenged with either anti E2 IgG, anti PDC or antisera to component X (Section 2.2.12d). Several positive clones were detected by all three probes after the primary screening. Subsequent rescreening of the isolated "positives" from E2 or PDC antisera probes failed to

Figure 6.3 Specificity of component X antisera for recombinant phage

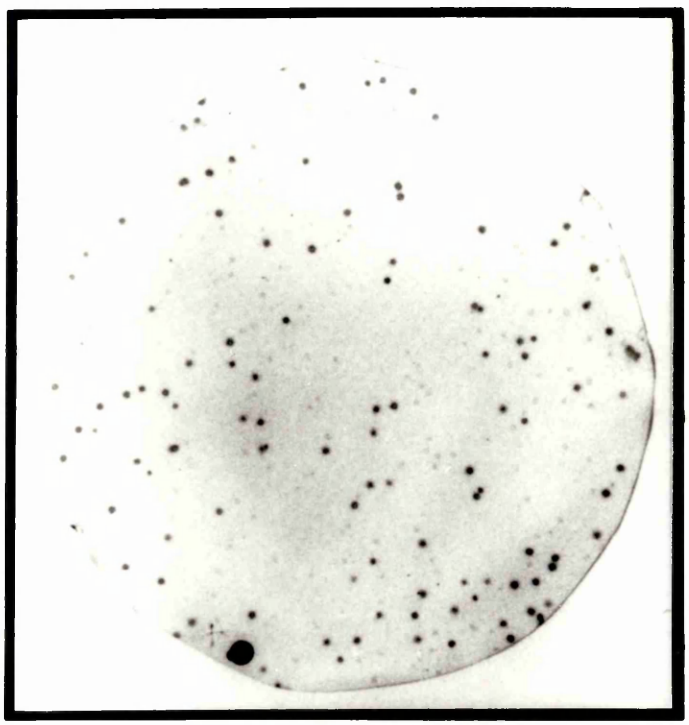
An equal number of non recombinant phage were mixed with

(A) approximately 100Pfu from the recombinant clone and plated onto an LB plate and transferred to nitrocellulose. The nitrocellulose was challenged with 1:100 dilution of antisera to component X and processed as described in Section 2.2.12d.

(B) approximately 1000Pfu from the recombinant clone plated onto an LB plate and transferred to nitrocellulose. The nitrocellulose was cut into three pieces and (1) challenged with 1:100 dilution of antisera to component X, (2) challenged with 1:100 dilution of E2 antisera and (3) challenged with 1:100 dilution of PDC antisera and processed as described in Section 2.2.12d.

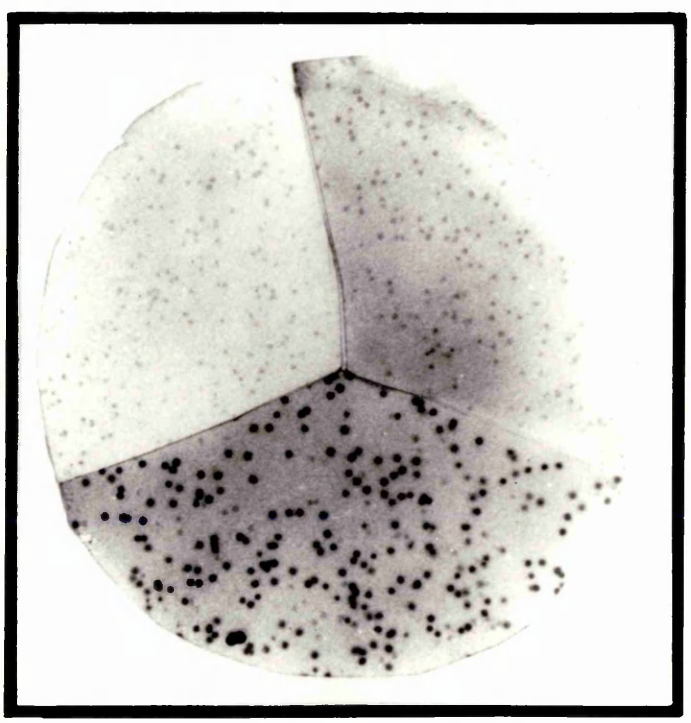
Specificity of component X antiserum
for purified recombinant phage.

A



B

Anti E2



Anti PDC

Anti X

confirm the original identification elicited from the plaque. Only a positive plaque to component X antisera survived the isolation and purification process until 100% positives to component X antiserum were identified.

The positive nature of the clone was confirmed in two ways: firstly, by rescreening with a 50:50 mixture of non-recombinant and recombinant component X phage (Fig. 6.3A), approximately 50% of the phage show a greater immune response to component X antisera confirming that the fusion protein of the recombinant phage is recognised by antibody to component X.

Secondly, 100% recombinant X phage were rescreened and challenged with component X antisera, E2 antisera and PDC antisera (Fig. 6.3B). A strong signal is elicited from component X antiserum and a weaker signal from PDC antisersum (an immune response to PDC antiserum is to be expected as component X is a component enzyme of PDC). E2 antiserum failed to detect the antigen produced by the phage. Therefore the specificity of the phage for component X antiserum was confirmed.

6.2.4 Preparation of lysogens

It is often useful to have sufficient amounts of polypeptide specified by the cloned segment of DNA. A crude lysate containing a particular recombinant antigen can be prepared by expressing a λgt11 recombinant as a lysogen in *E. coli* Y1089. Host Y1089 like Y1090 contains a repressor and a deficiency in lon protease. Where it differs from Y1090 is that it contains a mutation which enhances the frequency of phage lysogeny (Hfla 150). For production of a recombinant fusion protein, Y1089 is lysogenised with recombinant λgt11 containing the putative protein X DNA, the lysogens are grown to a high cell density, and the *lac* Z-directed

fusion protein induced by the addition of IPTG. Using replica plating, colonies which had grown at 32°C and not 42°C were isolated and purified. At 32°C the temperature-sensitive phage repressor (c1857) is functional. Four single colonies from an LB plate which had grown at 32°C and not at 42°C were used to inoculate 10ml LB and the phage grown overnight at 30°C. Occasionally the Y1089 lysogen will lyse even though Y1089 does not suppress the mutation causing defective lysis (S100) in λgt11. This is a consequence of the "leakiness" of the (S100) amber mutation, and secondly the accumulation of foreign protein in E. coli often renders it susceptible to lysis.

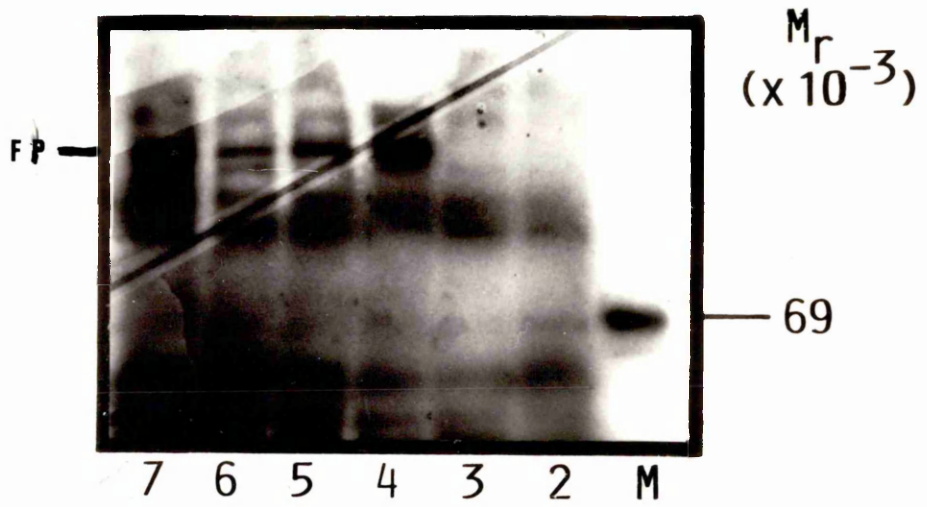
The overnight culture (100µl) was used to inoculate prewarmed (30°C) LB medium and the incubation continued until the OD_{600}=0.45 (approx. 3 h). The cultures were transferred for 15 min to a water bath at 44°C followed by the addition of IPTG to a final concentration of 10mM. After 1 h the induced lysogenic cultures were pelleted and the pellets resuspended in 500µl of lysate buffer and 30µl of each of the positive lysogens resolved on a 10% (w/v) SDS polyacrylamide gel, transferred electrophoretically to nitrocellulose and the nitrocellulose challenged with antiserum to component X. The nitrocellulose was processed as described in Section 2.2.9a.

An immune response to component X antiserum is observed from the fusion protein of four recombinant lysogens selected, appearing above the 69K marker (Fig. 6.4). No immune response is observed from non recombinant Y1089 lysogens prepared in a similar manner. The recombinant lysogens were also challenged with PDC and a weak immune response was detected from all but one of the recombinant lysogens. This recombinant was not processed further.

Figure 6.4 Identification of fusion protein of component X by immunoblotting

Crude lysates were prepared as described in Section 2.2.12.k and resolved on a 10% (w/v) SDS/polyacrylamide slab gel. Polypeptides were transferred onto nitrocellulose and subsequently analysed by immunoblotting technique and challenged with 1:100 dilution of antisera to component X. lanes 7-4 30µl of positive lysogens. lanes 3,2 30µl of non recombinant lysogens. lane M, M_r marker 69000.

Identification of a β- galactosidase fusion protein with component X antiserum



6.2.5 Preparation of DNA that encodes for protein X

In order to study the structure and function of the gene encoding the fusion protein recognised by component X antiserum DNA was prepared from 3 lysogens which gave a positive immune response (Section 2.2.13a). From the yields of DNA of the three recombinant lysogens only one recombinant X1A, produced amounts of DNA equivalent to that produced by non recombinant λgt11 (Fig. 6.5.). In view of the difficulties in preparing sufficient amounts of DNA from all three lysogens our work focussed on the DNA prepared from recombinant X1A.

6.2.6 Restriction endonuclease digestion of DNA

DNA (1μg) prepared from each of the three recombinants and the non-recombinant control were digested with the restriction enzyme EcoRI. As discussed in Section 6.2.5 the relative amounts of DNA prepared can be compared and only DNA from X1A approaches the levels of non-recombinant DNA (Fig. 6.5). On one side of the EcoRI insertion site (Fig. 2.1) there is an SstI and on the other a KpnI site, and the recombinant DNA was digested with SstI amd KpnI restriction enzymes. DNA fragments from the restriction enzyme digests were electrophoresed on a 1.5% (w/v) agarose and the results are visualised in Figure 6.2.6. EcoRI digestion of X1A releases a fragment of approximately 300 base pairs. The insert is confirmed in the double digest involving SstI and KpnI which produces a fragment of approximately 2.3kb. A 2kb size fragment is released from non recombinant DNA confirming the size of the insert (Fig. 6.5).

Figure 6.5 Restriction endonuclease digestion of recombinant λgt11

Agarose gel analysis.

lane 1 EcoRI digest of recombinant λgt11 X1

lane 2 EcoRI digest of recombinant λgt11 X1A

lane 3 EcoRI digest of recombinant λgt11 X16

lane 4 EcoRI digest of non recombinant λgt11

lane 5 SstI + KpnI digest of non recombinant λgt11

lane 6 SstI + KpnI digest of recombinant λgt11 X1

lane 7 SstI + KpnI digest of recombinant λgt11 X1A

lane 8 SstI + KpnI digest of recombinant λgt11 X6

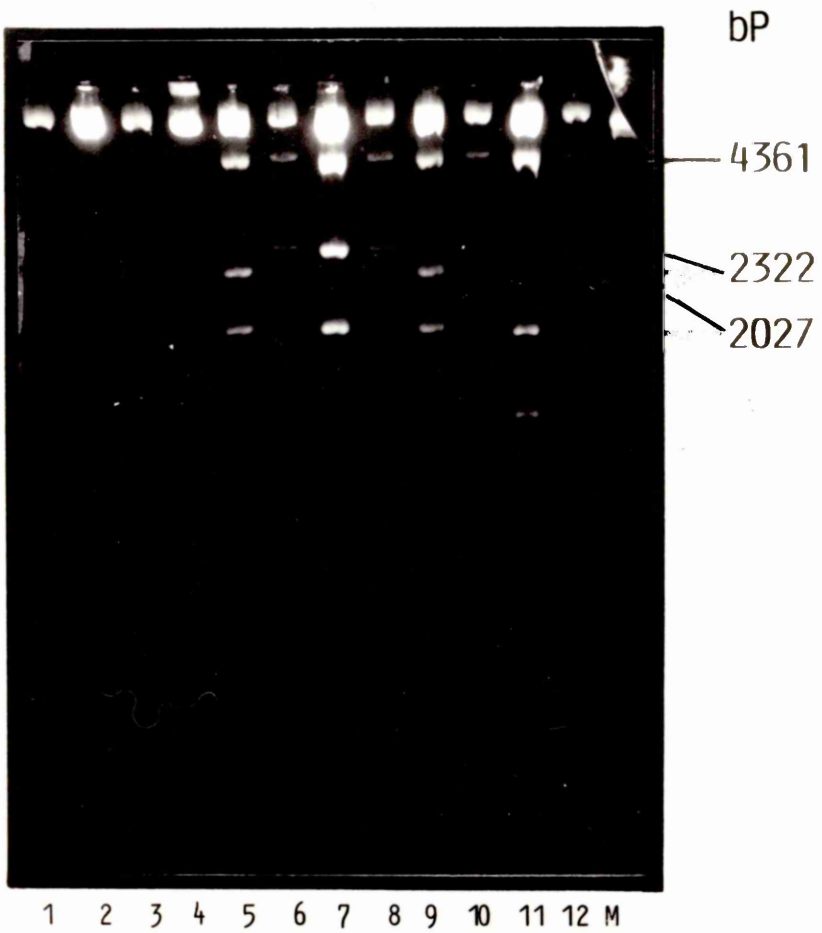
lane 9 EcoRI + SstI + KpnI digest of non recombinant λgt11

lane 10 EcoRI + SstI + KpnI digest of recombinant λgt11 X1

lane 11 EcoRI + SstI + KpnI digest of recombinant λgt11 X 1A

lane 12 EcoRI + Sst + KpnI digest of recombinant λgt11 X6

lane M HindIII digest of DNA


bP
4361
2322
2027
1 2 3 4 5 6 7 8 9 10 11 12 M

6.2.7 DNA sequencing

M13 is a single stranded filamentous phage. The phage enters a suitable host cell by way of the F pilus. On entering the cell, the virus is stripped of its protein coat and the single stranded viral DNA is converted to a double-stranded replicative form (RF). This stage is followed by DNA replication to give 100 + progeny RF molecules from which new single stranded viral DNA is synthesised. This is then packaged into viral coat proteins and extruded from the host cell without lysis. In this way, some 100 phage particles are produced per cell, per generation. The M13 life cycle can be exploited for the preparation of pure single stranded recombinant DNA by using the double stranded RF DNA as a cloning vector. Fragments of foreign DNA can be inserted into a suitable restriction enzyme site on the vector. M13 RF DNA carrying such a double stranded insert can be introduced into a suitable host by a transformation step. The resultant phage growth will lead to production of the hybrid molecules in both double stranded (RF) and single stranded (mature virus) forms, thus offering both an amplification step and a means of producing the insert DNA in single stranded form.

Although M13 does not lyse its host cell, the growth of the cell is retarded as a consequence of supporting phage growth. Thus when plated out those cells infected with M13 will show up as areas of slower growth, which look like turbid plaques on a lawn of uninfected cells.

Alternatively, under appropriate conditions the cells containing the M13 vector will grow to form a blue plaque, but this process is disrupted by the introduction of a fragment of foreign DNA within the M13 insertion site. As a result cells containing

recombinant phage will give colourless opaque plaques. This discrimination is based on the presence or absence of the enzyme β-galactosidase. E. coli host cells infected with M13 vector strains will, in the presence of the lac operon-inducer IPTG, produce a functional β-galactosidase. Such cells will hydrolyse the substrate X-gal to give a blue dye. Insertion of foreign DNA into an appropriate site in the DNA vector interferes with the production of β-galactosidase. Thus cells containing recombinant M13 give colourless plaques on an E. coli lawn in contrast to the blue plaques formed by cells containing the intact vector.

6.2.8 Preparation of competent cells

In order to introduce vector DNA into host E. coli cells the host cells are treated with $CaCl_2$ at $4^{o}C$ (Section 2.2.14f). It is necessary to use these competent cells for the transformation of E. coli strains by recombinant M13.

Host E. coli K12 (E. coli JM103) were grown overnight in 10ml of 2 x TY medium at $37^{o}C$. 2ml of the overnight culture was then used to inoculate fresh 2 x TY medium (40ml) and the cells were processed as described in Section 2.2.14f.

6.2.9 Transformation of competent cells

The ability to transfer plasmids into E. coli cells is brought about by the interaction of DNA and divalent cations at low temperatures ($4^{o}C$). The frequency of transformation may be improved by treating the cell/DNA mixture at elevated temperatures for a short period of time (heat shock) ($42^{o}C$).

Half of the ligated DNA prepared was added to 0.3ml of competent cells at $4^{o}C$. After 40 min the cells were heat shocked

at 42°C for 3 min and returned to ice. Fresh cells, IPTG and X-gal were added to the heat shocked cells followed by 3ml of molten H top agar at 42°C. The mixture was then poured onto H plates and incubated overnight at 37°C (Section 2.2.14f).

The following morning ten colourless plaques were selected.

6.2.10 Preparation of single stranded DNA

After overnight growth, transformed cells will have formed plaques. Cells infected with recombinant DNA form colourless plaques and cells infected with non recombinant DNA should form blue plaques. Infected cells from recombinant, colourless plaques are grown up to produce single stranded template for the sequencing reaction. Recombinants should not be stored as plaques but may be stored as purified single stranded DNA. Single stranded DNA was prepared from 10 colourless plaques as described in Materials and Methods (Section 2.2.14g).

6.2.11 Nucleotide sequencing of insert DNA

Single stranded DNA prepared from colourless plaques was primed with Amersham Universal primer. The M13 primer is a single stranded synthetic oligonucleotide complementary to a region of vector DNA which flanks the insertion site.

The sequence reaction was performed as described in the Amersham M13 cloning and sequencing handbook and the samples resolved by gel electrophoresis (Section 2.2.14k).

The gel autoradiograph from DNA for 2 colourless plaques is shown in Figure 6.6. The sequence from the recombinant DNA is different from M13 vector run as a control therefore it has been assumed that the insert has been successfully ligated into M13

Figure 6.6 Example of a polyacrylamide sequencing gel

DNA fragments synthesised in the presence of dideoxynucleotides were separated on a high resolution thin polyacrylamide gel (buffer gradient). Chain elongation was carried out with Klenow polymerase and the electrophoresis was terminated when the bromophenol blue had reached the bottom of the gel (2.5 h). Better resolution of larger fragments was obtained by performing electrophoresis for up to 5 h.


Clone
2
4
A C G T A C G T

Figure 6.7 Nucleotide sequence and predicted amino acid sequence determined from cDNA encoding protein X

Figure 6.8 Comparison of nucleotide sequence coding for mammalian protein X gene with the nucleotide sequence coding for yeast protein X gene

The nucleotide sequence for the mammalian protein X gene is indicated by upper case letters and that encoding for yeast protein X gene in the lower case letters. In regions of similarity the nucleotides encoding mammalian protein X gene which are identical to the encoding yeast protein X gene sequence are indicated by vertical lines.

```
1   GGTTACTTTCTTGGTGTAACGAACTAATAGCGCATTTCTGGAATTTGTACTCTCCAAGCT  60
      V  T  F  L  V  Y  R  T  N  S  A  F  L  E  F  V  L  S  K  L  -

61  GCTCAAAAAGCTCACAATTTTGTTTGATGAAATTCTGAGGCTCTTCCACAAGAAGTTTAA 120
      L  K  K  L  T  I  L  F  D  R  I  L  R  L  F  H  K  R  F  K  -

121 ATTCATCGAACACTTTGGCATAGCATTCATGAGGATCTGCAGCGGCACAGCACTTCTCTA 180
      F  I  E  H  F  G  I  A  F  M  R  I  C  S  G  T  A  L  L  Y  -

181 CAGTGGTTTCATATGTCTTGGCAAGTCTCAGCAGCAGCACGACAGAGTATCAGGTGCCTT 240
      S  G  F  I  C  L  G  K  S  Q  Q  Q  H  D  R  V  S  G  A  F

241 CTTGG 245
      L
```

```
     62 CTCAAAAAGCTCACAATTTTGTTTGATTGAATTCTGAGGCTCTTCCACAA 111
        || |||||    |  || |  |    ||||||  | ||  ||  | ||||
   1467 ctgaaaaaaacgaatatatactagcgttgaat.gttagcgtcaacaacaa 1515

    112 GAAGTTTAAATTCATCGAACACTTTGGCATAGCATTCAT 150
        |||||||||   |   ||   |  | | |  |||| |
   1516 gaagtttaatgacgcggaggccaaggcaaaaagattcct 1553
```

vector. Another noticeable feature seen in Figure 6.6 is that clone 2 appears to be different from clone 4 and the other 8 clones (data not shown). It is possible that, during ligation, the DNA fragment has been inserted into the vector in the opposite orientation. This allowed the reading of the sequence in the opposite direction and thus confirm the sequence. The nucleotide sequence and the predicted amino acid is determined in Figure 6.7.

6.2.12 Ligation of insert into vector PTZ 18

RF recombinant M13 vector was digested with EcoRI and ligated into EcoRI digested PTZ (Section 2.2.14e). After transformation into a suitable host, TG1, selection for ligated insert into PTZ 18 was by identification of colourless colonies on an IPTG, X-gal impregnated H + ampicillin plates.

6.2.13 Preparation of recombinant plasmid PTZ 18

Clear colonies from H plates were selected and spread onto H + ampicillin plates and grown overnight at 37°C. The colonies were further purified by replica plating and grown overnight at 37°C. No blue colonies were observed. Colonies were then selected and used to inoculate 10ul culture of L-broth containing ampicillin (50µg/ml) and recombinant plasmid prepared as in Section 2.2.15a.

6.2.14 Restriction digest of recombinant plasmid PTZ

Recombinant plasmid prepared (Section 2.2.15a) was digested with EcoRI releasing an approximate 300 base pair fragment (Fig. 6.9.).

Figure 6.9 EcoRI digest of recombinant PTZ 18

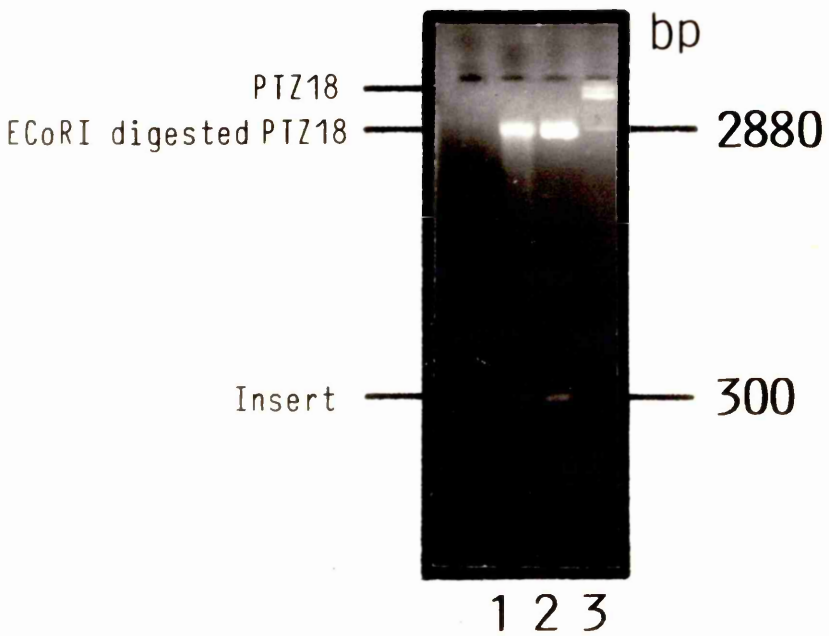
Agarose gel analysis of EcoRI digested plasmid PTZ 18R containing ligated insert from recombinant DNA X1A

lane 1 EcoRI digested vector PTZ containing inert X1A

lane 2 EcoRI digested vector PTZ containing insert X1A

lane 3 uncut vector PTZ 18R

The samples were electrophoresed in 1.5% (w/v) agarose gel.


bp
PTZ18
ECoRI digested PTZ18
2880
Insert
300
1 2 3

6.3 Discussion

Most strategies used to isolate genes from recombinant DNA libraries have employed nucleic acid probes (Davis et al., 1980; Maniatas et al., 1989). Genes can also be identified and isolated through the use of antibody probes of the antigen expressed by specific genes (Young and Davis, 1983). The phage expression vector was constructed to permit insertion of foreign DNA into the β-galactosidase structural gene lacZ under the control of the lac operator (Fig. 2.1). The recombinant DNA can be propagated lytically, and the expression of the foreign DNA is repressed by the lacI gene product. Production of the foreign antigen fused to β-galactosidase can rapidly be induced by the addition of IPTG to the culture medium. The presence of the lon mutation appears to permit accumulation of otherwise unstable novel proteins to levels which facilitate detection by immunological analysis.

In our studies we found that using the "dot blot" antigen detection method (Fig. 6.2) E2 IgG could detect levels of 0.1ng of PDC, component X IgG and PDC IgG could detect levels of 5ng of PDC. Antisera to component X and PDC could detect levels of 0.1ng of PDC. These values were compatible with the levels suggested by Young and Davis (1983) for cloning genes.

Although other methods of detection of antigen may be as sensitive as the "dot blot" (Section 2.2.9c) e.g. ^{125}I-protein A. The dot blot method has the distinct advantage of producing signals directly onto nitrocellulose filters, thereby exactly reproducing the pattern of plaques on the plate. This allows the precise location of a single plaque producing the signal to be determined X. Moreover, the frequency of false positives is very low (Young and Davis, 1983). While the ^{125}I-protein A protocol has the advantage of being sensitive and allowing controlled films exposure to maximise signal to noise ratio, it is useful only in the

detection of IgG and is prone to producing false positives. It was also felt the use of radiolabelled isotopes should be avoided wherever possible.

In order to maximise the detection of fusion proteins the most sensitive antibody/antigen combination was selected i.e. the library was screened with E2 IgG, antisera to component X and antisera to PDC.

Screening of the library

With each screen of the library several positives were detected by each of the antibodies. Subsequent rescreening of the initial positives failed to show any replication of the positive plaque. The maximum size of insert which can successful ligated into a λgt11 gene expression library is approximately 7kb. The complete E2 gene and PDC genes are approximately 1.8kb and 4.5kb respectively therefore they should not be unstable in the λgt11 vector. Another problem is that unusual polypeptides are efficiently degraded in *E. coli* (Edman *et al.*, 1981) and it may be that the fusion product of β-galactosidase and eukaryotic antigen is degraded by certain proteases. A third major problem with foreign antigen synthesis in *E. coli* is the presence of these unusual proteins is occasionally harmful to the cells. One of these factors or a combination could be responsible for the failure to reproduce the initial positive to E2 IgG and PDC antiserum.

Identification of component X clone

A plaque positive to component X antiserum survived the isolation and purification process, 100% positives being obtained (Fig. 6.3). It was also observed that preparative amounts of polypeptide specified by the component X clone, when subjected to immunoblotting (Section 6.4) gave an immune response to component X antiserum and a weaker response to PDC antiserum. These results confirmed that a clone of component X had been isolated and purified.

Restriction digest of DNA encoding protein X

Restriction digest of recombinant DNA revealed a 250-300 base pair fragment. The complete cDNA for protein X is expected to be approximately 1500 base pairs. Therefore in view of the strong immune response elicited by the fusion protein to component X antiserum the particular region of cDNA must encode for a highly immunogenic region protein X.

Sequence analysis of the DNA encoding protein X

The nucleotide sequence and the deduced amino acid of the protein X clone are shown in Figure 6.7. The open reading frame of 243 nucleotides encodes for 81 amino acids residues. It was expected as component X and E2 have a close relationship both physically and functionally a degree of homology would exist between the nucleotide sequences. Comparison of the mammalian E2 nucleotide sequence (Gershwin et al., 1987) and the nucleotide sequence obtained from the component X clone revealed little homology. Assuming that the component X clone is genuine this may suggest that component X has a unique function in mammalian pyruvate dehydrogenase.

During the course of this work the nucleotide sequence of component X from Saccharomyces cerevisiae was published (Behal et al., 1989). Comparison of the nucleotide sequence from yeast component X and the nucleotide sequence from mammalian component X clone revealed 70% homology between the two sequences at the carboxy terminal of yeast protein X. An area of high homology extending over 30 nucleotides is observed between base pairs(81-110) of the mammalian component X clone. A further region of homology exists between base pairs(62-70) this homology was achieved without any sequence addition or deletion to optimise the alignment (Fig. 6.8).

Comparison of the deduced amino acid sequences of yeast protein X and yeast E2 (Nui et al., 1988 and Behal et al., 1989) reveals that the amino terminal part of protein X resembles E2, but the carboxy-terminal part is quite different. The two proteins exhibit 50% sequence identity in the amino terminal that corresponds to the putative lipoyl bearing domain on E2. This extensive homology indicates that protein X and E2 evolved from a common ancestor. Protein X also lacks the carboxy terminal segment of E2 that contains the highly conserved sequence which is thought to be part of the putative catalytic site of all dihydrolipoamide acyltransferases (Guest, 1987). This finding suggests that yeast protein X, in contrast to E2, is not able to catalyse the transfer between the protein bound S-acetyldihydrolipoyl moiety and coenzyme A.

Evidence has been presented that in mammalian pyruvate dehydrogenase complex from bovine kidney, protein X contributes to the binding of the E3 component, perhaps by facilitating the transfer of reducing equivalents to E3 (Powers-Greenwood et al., 1989; Gopalakrishnan et al., 1989). We have observed, by studying the predicted amino acid sequence of yeast protein X, a possible E3 binding domain between residues (145-176) (Behal et al., 1989) shown below.

The amino acid sequence of the 32 residue E3 binding domain of yeast protein X.

-SLLLAENNI	153
SKQKALKEIAPSGSNGRLLK	173
GDV-	176

CHAPTER SEVEN

GENERAL DISCUSSION

7. General Discussion

Mammalian PDC has been studied extensively over the last twenty years. In particular its enzymatic and physiochemical characteristics have been the subject of detailed examination following its isolation from several tissues (Koike and Koike, 1976).

The regulation of this multienzyme complex by phosphorylation/dephosphorylation has also received considerable attention, as a result of its importance in the conservation of glucose in starvation and diabetes (Reed et al., 1980; Denton et al., 1981; Weiland, 1983; Reed and Yeaman, 1987).

The discovery of an extra polypeptide of the complex, M_r 51000 of unknown function (component X) has resulted in intensive investigation into the properties and function of this unique protein of mammalian PDC (De Marcucci and Lindsay, 1985; Hodgson et al., 1986; Jilka et al., 1986; Gopalakrishnan et al., 1989; Behal et al., 1989). These authors have shown that component X undergoes reductive acetylation on a lipoic acid site and in the presence of CoASH is also rapidly deacetylated. In addition, component X preferentially binds the E3 component to the core assembly. Consequently component X depleted core is unable to bind the E3 component or recover overall complex activity on reconstitution of the enzyme and this suggests that this polypeptide is essential for overall complex activity. It was also observed that component X does not contain a recognised acetyltransferase site. This putative catalytic site is thought to include the highly conserved His-Xaa-Xaa-Xaa-Asp-Gly segment near the carboxyl terminus (Guest, 1987).

7.1 Role of component X

Component X has been shown to participate in the normal turnover of the enzyme. It was demonstrated the component X was

involved in the catalytic cycle of the enzyme and was not reductively acetylated as a consequence of a side reaction promoted by conditions which trap acetyl groups on E2.

Inactivation of the complex by phosphorylation by the intrinsic protein kinase, causes a parallel decrease in the acetylation on E2 and X indicating that the latter protein can participate in acetylation/deacetylation reactions of the complex. This result suggests that loading of protein X must proceed through the formation of a TPP intermediate associated with E1.

Although the turnover of the enzyme was successfully slowed, as observed from low temperature radiolabelling of the complex elucidating the sequence of acetyl group transfer from E1 or between E2 and X, under these conditions, was unsuccessful.

In a separate approach to determine the sequence of acetyl group transfer within the complex, various substrate analogues were employed as potential substrates for PDC. Only α-ketobutyrate could substitute as a substrate for PDC and it was also recognised that 2-oxobutyrate could protect lipoic acid residues on E2 and X from NEM incorporation. This phenomenon, unique to eukaryotic PDC, was first observed with pyruvate as substrate and may offer an explanation as to the role of component X in the complex. The physiological basis for this phenomenon is unknown and it is possible it may be promoted by component X. In cases of starvation and diabetes not all the acetyl CoA generated can be metabolised by the tricarboxylic acid cycle and alternatively excess acetyl CoA is accumulated and converted to produce ketone bodies etc. By assuming that there are two active lipoyl domains per E2 chain and 60 E2 subunits, then it is possible the E2 core assembly can act as a reservoir for acetyl groups by incorporating 240 acetyl groups, thus preventing production of excess acetyl CoA. Investigation of the

levels of acetylation on E2 and X of the ratio of $NADH/NAD^+$ in patients with diabetes or malnutrition could provide a clue to the possible role of protein X in the complex.

7.2 Limited proteolysis of component X

Incubation of PDC with trypsin resulted in limited proteolysis of E2 and X with resultant loss of overall enzyme activity. The loss of activity was a result of cleavage of lipoyl domains from the E2 core assembly. As a consequence of the cleavage of the lipoyl domains the enzyme complex lost its ability to promote diacetylation of the lipoic acid residues on E2 and X and therefore its ability to protect against NEM modification. As E2 and X undergo similar degrees of proteolysis with trypsin it was not possible to determine the role of component X in the complex. An interesting observation was that limited protelysis of PDC with argC preferentially digested component X and released the E3 component from the E2 core assembly, supporting the observations of Roche's group that X was involved in E3 binding (Gopalakrishnan et al., 1989; Powers-Greenwood et al., 1989). However our evidence differs in several important respects from the observations reported by the above authors. E3 does not protect X from proteolysis by argC and extensive proteolysis of component X does not result in loss of overall complex activity although it could be argued 10% was sufficient to promote activity. On incubation of complex in high salt, the activity of the complex remained unaltered although, as with argC treatment the E3 component was released from the core assembly on resolution on a gel filtration column.

Incubation of PDC in argC and high salt leads to rapid inactivation of the complex. This result is best explained by incubation of PDC in argC or high salt lowers the affinity of E3 for

the core assembly without affecting overall enzymatic activity. It is only a combination of high salt and argC which lowers the affinity of the E3 compared for the core assembly sufficiently to effect the enzyme activity. These observations are supported by Kresze (1980) who observed that limited proteolysis of PDC with papain released the E1 and E3 components from the core assembly rendering the complex inactive although each of the components maintained their individual catalytic properties.

The binding of E3 to component X is further confirmed by binding studies on an E2 oligomer prepared free from component X (Powers-Greenwood et al., 1989). These authors observed reduced binding of E3 to the E2 oligomer and significant binding of E3 to the component X fragment although they fail to define the term significant.

The structural role of component X in the complex has not been fully considered. Component X may not directly bind E3 to the core assembly. Instead, it may position E3 in the correct orientation to ensure efficient binding to the core assembly (E2), or, component X may position E2 to effect efficient binding of E3. Therefore, limited proteolysis, and inactivation of the complex as reported by Roche and co-workers and lack of activity recorded by a yeast mutant lacking component X (Reed et al., 1990) could be caused by disruption to the core assembly. In support of the argument, removal of the subunit 3 of cytochrome c oxidase does not render the complex inactive. However, investigation into a yeast mutant lacking subunit 3 reveals that proper assembly of the complex is not completed when this subunit is missing and therefore the complex lacks overall enzymatic activity.

In recent weeks, investigation of cells taken from two French children (brothers) were characterised by a complete absence of protein X subunit on examination of the polypeptide profile of their PDC by immune replica analysis of mitochondrial and cellular extracts. Measurement of overall PDC activity revealed 20% to 30% of normal activity. However, preparation of cellular extracts was carried out in 0.15M KCl and 0.1M Tris/HCl pH8.0 and the level of activity recorded may be caused by the enzymes sensitivity to high salt concentration. Repeating the experiment in low levels of salt may give an accurate level of enzymatic activity in the absence of protein X. Hopefully, by examining the enzymology and regulation of the mutant PDCs we will determine how their properties differ from wild-type enzyme.

Several authors have reported that E2 of mammalian PDC possess an E3 binding domain located near the inner catalytic domain on E2 (Thekummkara et al., 1988; Coppel et al., 1989). Therefore, why should eukaryotic PDC possess an additional polypeptide which serves this function? It may simply be that PDC, as the most important enzyme of the 2-oxo acid dehydrogenase complexes, is ensuring priority in binding E3 over OGDC and BCOADC or it may be that component X serves an additional function.

Radiolabelling of argC or high salt treated NADH reduced complex with [^{14}C] NEM results in the enhanced labelling of the E3 component. Subsequent studies on the E3 enzyme revealed that inactivation by NEM on treated enzyme was NADH dependent leading to the conclusion that component X binds the E3 component at its active site, possibly protecting the active site on E3 from inactivation. Protecting the active site on E3 has physiological significance. In the cell glutathione is a potential inhibitor of E3 function and protection of the active site by component X may protect the complex

from the sulphydryl group inhibitor. Confirmation that the incorporation of NEM into the reduced active site of E3 may be achieved by limited proteolysis of radiolabelled E3 followed by amino acid sequencing of the radiolabelled fragment to determine if it has been incorporated into the active site. This would confirm cooperation between components of multienzyme complexes in substrate channelling and organisation.

E2 of E. coli has three tandomly repeated lipoyl domains per E2 chain while mammalian E2 has two lipoyl domains and a third lipoyl domain if component X is considered. Now that various constructs of E2 from E. coli are available lacking one or more lipoyl domains (Graham et al., 1986) an interesting investigation would be to determine the efficiency of E3 binding to each of the constructs and possibly assign a role to one or more of the lipoyl domains of E2 of E. coli.

7.3 Structural and functional relationship of component X to the E2 core assembly

Component X is associated with the other components of the complex in reproducible amounts. Based on quantitative scanning of Coomassie blue-stained bands of the complex in SDS-PAGE gels, it appears that 6% of the total absorbance is represented by this component. On the other hand, component X retains 14-16% of the total radioactivity associated with the complex after labelling in the presence of [2-^{14}C] pyruvate (De Marcucci et al., 1986). Assuming one "acetylatable" group per molecule of X, an estimated 8-10 molecules of polypeptide per core is obtained which agrees with the value calculated on the basis of densitometric scan of 8-12 molecules of component x per core. This provisional conclusion requires confirmation by employing more reliable stoichiometric analysis.

Selective release of the lipoyl domains from E2 and X by limited proteolysis with a variety of specific and non specific proteases produced stable peptides M_r 32000-42000 range for E2 and M_r 15000-20000 range for component X. This is best accounted for by the absence of a lipoyl domain from protein X and E2. Cross-linking studies revealed a species of M_r 100000 suggesting that component X could only form a dimer which is in agreement with a single lipoyl domain on component X.

Limited amino acid sequence data confirmed that protein X contains a lipoyl domain located at its N-terminus and belongs to a family of enzymes which possess acetyltransferase activity although the high degree of homology established at the N-terminal between E2 and X (45%) is not enough for cross reactivity to be observed.

7.4 Cloning and sequence analysis of protein X

Sequence data on component X from Saccharomyces cerevisiae has been reported (Behal et al., 1989). Two major observations were reported from the nucleotide and predicted amino acid sequence data: a) component X from yeast lacks the region which codes for acetyltransferase activity and b) little homology exists between E2 and component X in yeast outwith the N-terminal regions of the proteins. Further investigation in our laboratory into the component X sequence has revealed a possible E3 binding domain on component X supporting the conclusion component X binds the E3 component. A 250 base pair cDNA clone reveals approximately 70% homology with component X from yeast in the region of the carboxy terminus confirming that a mammalian clone of component X has been isolated. This fragment can now be employed as a probe to isolate the full-length cDNA clone. In conjunction with the N-terminal sequence and the 250 base pair fragment, PCR could be employed to create a larger fragment with the view to rapid sequence analysis and possible identification of active sites in the gene.

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